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Toll-like receptor expression and signalling in rat trigeminal neurons: Consequences for oral infection and mechanisms of orofacial pain.

Helley, Martin

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Plymouth University

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Toll-like receptor expression and signalling in rat trigeminal neurons: Consequences for oral infection and mechanisms of orofacial pain.

Martin Paul Helley

School of Dentistry
Plymouth University
Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Supervisors: Dr. Stephen W. N. Thompson
Dr. Jon H. Bennett
Prof. Simon K. Jackson
Abstract

Toll-like receptor expression and signalling in rat trigeminal neurons: Consequences for oral infection and mechanisms of orofacial pain.

Martin Paul Helley

The activation of Toll-like receptors (TLRs), expressed by peripheral sensory neurons, represents an innate surveillance function of sensory neurons with important consequences for neuronal function. Despite recent advances, many properties of neuronal TLRs remain poorly understood, particularly within the trigeminal system. The main objectives of this thesis, therefore, were to better characterise the expression and functional consequences of activation of neuronal TLRs in the setting of orofacial pain. A detailed, quantitative description of TLR4, TLR2 and TLR7 expression within neurochemically-identified sub-populations of trigeminal ganglion (TG) sensory neurons suggests that these receptors are primarily expressed by nociceptor sub-populations. Acute activation of these TLRs, in TG neurons, induced an increased gene expression of the pro-inflammatory cytokines, TNFα and IL-1β. In an attempt to identify further novel co-modulators of neuronal TLR activation, the expression of lysophosphatidylcholine acyltransferase (LPCAT) isoforms, a phospholipid remodelling enzyme that is known to mediate TLR4 activation in macrophages, are described within TG neurons. Under naïve conditions, LPCAT1 was expressed by a range of sensory neuron sub-types whereas LPCAT2 expression was confined to non-neuronal cells. Following nerve injury, the expression of LPCAT2 was induced in a small proportion of TG neurons in vitro whilst the expression of LPCAT1 remained unaltered. These results may support a role for LPCAT2 in neuronal TLR activation following a priming stimulus, such as nerve injury. Taken together, the results presented in this thesis support the hypothesis that trigeminal nociceptors can directly detect and respond to pathogenic challenge and tissue damage. The acute activation of TLRs, expressed by trigeminal nociceptors, results in the up-regulation of pro-inflammatory cytokines which are known to activate and sensitise neurons. The activation of neuronal TLRs may therefore contribute to the increased neuronal excitability and pain that accompanies common orofacial disorders.
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<td>Serotonin</td>
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<td>ACTB</td>
<td>β-actin</td>
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<td>AGPAT</td>
<td>1-acylglycerol-3-phosphate O-acyltransferase</td>
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<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>ATF</td>
<td>Activating transcription factor</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CamKII</td>
<td>Calcium/calmodulin dependent protein kinase II</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
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<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<tr>
<td>CIP</td>
<td>Congenital insensitivity to pain</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CoA</td>
<td>Coenzyme A</td>
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<td>Cyclooxygenase-2</td>
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<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
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<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
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<td>CT</td>
<td>Copy threshold</td>
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<td>CXCL1</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>dPBS</td>
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<td>DRG</td>
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<td>dsRNA</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Glial-derived neurotrophic factor</td>
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<td>GRP</td>
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<td>H$_2$SO$_4$</td>
<td>Sulphuric acid</td>
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<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<td>HE</td>
<td>High expressors</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HMGB1</td>
<td>High mobility group box 1</td>
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<td>Horseradish peroxidase</td>
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<td>Heat shock protein</td>
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<td>IASP</td>
<td>International Association for the Study of Pain</td>
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<td>Lysophosphatidylcholine acyltransferase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adapter-like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBOAT</td>
<td>Membrane-bound O-acyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MD</td>
<td>Myeloid differentiation factor</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallopeptidase</td>
</tr>
<tr>
<td>MMV</td>
<td>Mouse Minute Virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Na$_V$</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>NBA</td>
<td>Neurobasal-A</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-$\kappa$B essential modulator</td>
</tr>
<tr>
<td>NF-$\kappa$B</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament 200 kDa heavy molecular weight subunit</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>P2X3</td>
<td>Purinergic receptor P2X, ligand gated ion channel, 3</td>
</tr>
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<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAFR</td>
<td>Platelet activating factor receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostaglandin I$_2$</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor-interacting serine-threonine kinase 1</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RP105</td>
<td>Radioprotective 105</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>S100A8</td>
<td>S100 calcium-binding protein A8</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α and TIR motif-containing protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β activated kinase</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TG</td>
<td>Trigeminal ganglion</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R homology</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR-domain-containing adapter protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMD</td>
<td>Temporomandibular joint disorder</td>
</tr>
<tr>
<td>TMJ</td>
<td>Temporomandibular joint</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll-receptor-associated activator of interferon</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factors alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin receptor kinase A</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPA1</td>
<td>cation channel, subfamily A, member 1</td>
</tr>
<tr>
<td>TRPM8</td>
<td>TRP cation channel, subfamily M, member 8</td>
</tr>
<tr>
<td>TRPV1</td>
<td>TRP cation channel subfamily V member 1</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>Ubc</td>
<td>Ubiquitin-conjugating protein</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-----------</td>
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</tr>
<tr>
<td>VPL</td>
<td>Ventral posterior lateral</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventral posterior medial</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range</td>
</tr>
<tr>
<td></td>
<td>End of List</td>
</tr>
</tbody>
</table>
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This thesis could not have been completed without the support of colleagues, friends and family. I would like to take this opportunity to thank all of those who have contributed and to apologise for any omissions from the following list.

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Author’s Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

Relevant scientific seminars and conferences were regularly attended at which work was often presented. 1 paper has been published in a peer-reviewed journal.

A list of publications and attended conferences can be found in Appendices A and B, respectively.

Signed: ____________________

Date: ____________________

Word count for the main body of this thesis: 34,034
Chapter 1

Introduction
1. Introduction

1.1 Nociception and acute pain

The current International Association for the Study of Pain (IASP) definition of pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." (Merskey et al. 1994). The sensation of pain itself is a combination of nociception and the affective input from centres in the brain to add the emotive aspect of the sensation. Nociception is defined as "the neural process of encoding noxious stimuli" and is purely a cellular process (Merskey et al. 1994). These noxious stimuli are exclusively detected by specialised high threshold primary sensory neurons termed nociceptors. Under normal circumstances the acute activation of nociceptors causes a brief, sharp pain that is coupled to the activatory stimulus. Acute physiological or nociceptive pain is essential for normal development and identifying actual or potential danger to prevent harm. The requirement for acute physiological pain is demonstrated in individuals with mutations in the SCN9A gene coding for the voltage-gated sodium channel Na\textsubscript{v}1.7 that cause a condition known as congenital insensitivity to pain (CIP) (Cox et al. 2006). CIP often leads to repeated injury such as physical trauma and unintentional self injury during development and can cause severe disability and death.

1.1.1 The detection of noxious stimuli

Most nociceptors are polymodal in nature meaning that a single cell can be activated by noxious stimuli of thermal, mechanical and chemical origins. This is due to the unique arrangement of transduction channels on the peripheral terminals of nociceptors that initiate the process of action potential generation in response to specific stimuli (Basbaum et al. 2009). Whereas A\textbeta fibers, that respond to innocuous low-threshold stimuli, are myelinated and large in diameter (6-12 \(\mu\)m),
1. Introduction

nociceptors have relatively small diameters and are either thinly myelinated or un-
myelinated. Nociceptors themselves can be further divided into several sub-classes
each with distinct anatomical and physiological properties. Aδ fibers are medium
diameter (1-5 µm), thinly myelinated afferents that conduct signals at a velocity
of 5-30 m/s to transmit well localised, fast signals that manifest as an acute, first
pain. These Aδ fibers can be further split into two sub-types - type I or type II Aδ
nociceptors. Type I Aδ nociceptors primarily respond to mechanical and chemical
stimuli and are heavily implicated in pain following tissue injury. Type II Aδ noxi-
ceptors have a lower heat threshold than type I Aδ fibers and a higher mechanical
threshold. Therefore type II Aδ fibers primarily respond to noxious heat. The
other major class of nociceptors are small diameter (0.2-1.5 µm) unmyelinated C
fibers that transmit signals at a slower rate of 0.5-2 m/s manifesting as a poorly
localised, second pain. C fibers can be divided into peptidergic, non-peptidergic
and silent sub-types (Snider & McMahon 1998). The peptidergic/non-peptidergic
divide is based on the constitutive expression of particular neuropeptides as well as
further distinctive phenotypic markers. Peptidergic C fibers express Tropomyosin
receptor kinase A (TrkA) that responds to the neurotrophin nerve growth factor
(NGF) whereas non-peptidergic C fibers express c-Ret which responds to glial-
derived neurotrophic factor (GDNF) (see Woolf & Ma 2007). Non-peptidergic C
fibers also express high levels of the purinergic receptor P2X, ligand gated ion
channel, 3 (P2X3) and bind to the plant lectin isolectin B4 (IB4) (see Bradbury
et al. 1998. It is important to note that the above 'classical' characterisation of
nociceptors primarily relates to those innervating the skin (cutaneous nocicep-
tors). Other types of nociceptors exist, such as those innervating joints or viscera,
that display slight differences to those described above. Many nociceptors innerv-
vating joints and visceral tissues are classed as silent nociceptors (Grigg et al.
1986; Feng & Gebhart 2011). Under normal conditions these nociceptors are me-
chanically insensitive however they become responsive to mechanical stimulation
following a priming stimulus such as inflammation or tissue damage (Michaelis et al. 1996).

The transformation of thermal, chemical and mechanical stimuli into an electrical signal is performed by a range of specific transduction channels expressed on the peripheral terminals of the neuron. Transduction of heat is regulated by a range of transient receptor potential (TRP) channels. TRP cation channel subfamily V member 1 (TRPV1) is activated by noxious temperatures above 43 °C as well as low pH and capsaicin which is a compound found in chilli peppers (Caterina et al. 1997). TRPV1 was initially suggested to be the main thermal transduction channels however other TRP channels also act as thermal transducers. For example, TRPV2 is activated by temperatures over 52 °C whereas TRPV3 and TRPV4 respond to innocuous temperatures between 25°C and 35 °C (Guler et al. 2002; Leffler et al. 2007; Lumpkin & Caterina 2007; Rau et al. 2007). This allows the host to discretely respond to a range of different temperatures. Recently multiple TRP-independent thermosensation mechanisms have been suggested involving the chlorine channel anoctamin 1 and Orai channels (Xiao et al. 2011; Cho et al. 2012). The transduction of noxious cold stimuli is not as well understood when compared to noxious heat. TRP cation channel, subfamily M, member 8 (TRPM8) is widely accepted to mediate the detection of cool stimuli (below 25 °C) and can also respond to temperatures in the noxious range although TRPM8 K.O. animals retain the ability to detect noxious cold (below 15 °C) due to a very small percentage of neurons that remain responsive to cold (Bautista et al. 2007). It has been suggested that TRP cation channel, subfamily A, member 1 (TRPA1) responds to noxious cold as it is responsive to chemical cooling compounds such as menthol however conflicting results have been reported from two independent TRPA1 K.O. models (Bautista et al. 2006; Karashima et al. 2009).

A range of TRP channels are also implicated in chemical signal transduction.
Collectively TRP channels may respond to environment chemicals in foods such as chilli, garlic, mustard, horseradish, peppermint, oregano, sage and thyme (Macpherson et al. 2005; Xu et al. 2006). TRPA1, for example, is becoming increasingly prominent in the transduction of chemical signals as it can respond to a wide range of different compounds found in food such as mustard oil, wasabi and garlic (Bandell et al. 2004). It can also respond to a broad range of inflammation-related chemicals (see Bautista et al. 2013) and more recently has been shown to respond to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria which is a strong trigger of innate immunity (Meseguer et al. 2014). Further, it is well accepted that nociceptors can detect a range of inflammatory mediators including bradykinin, histamine, cytokines, chemokines, prostaglandins, protons, neuropeptides and neurotrophins through the expression of specific surface receptors (see Hucho & Levine 2007). The detection of these factors can induce spontaneous firing and action potential generation as well as the activation of intracellular signalling pathways, including p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK), that are associated with the generation of inflammatory pain (described in detail in section 1.2).

Noxious mechanical signal transduction remains a major uncertainty within the field of sensory neuroscience. Certain candidate receptors have been identified that are suggested to contribute towards noxious mechanical sensation such as pinching and pressure (see Hu et al. 2006; Basbaum et al. 2009). These include TRPV2 and TRPV4 which respond to osmotic stretch (Guler et al. 2002; Muraki et al. 2003; Mochizuki et al. 2009), TRPA1 (Kwan et al. 2009) and two pore domain, subfamily K (KCNK) potassium channels 2, 4 and 18 (Bautista et al. 2008). Recently, two transmembrane proteins known as Piezo 1 and 2 (alternatively Fam38A and B) have also been identified as possible candidate mechanical signal
transducers. Evidence has been presented to suggest a role for these proteins in innocuous mechanosensation however their involvement in the transduction of noxious mechanical stimuli is unclear (see Wood & Eijkelkamp 2012).

1.1.2 Central processing of pain

The central branches of primary nociceptive afferents of the somatosensory system enter the dorsal horn of the spinal cord and terminate mainly in laminae I, II and V whereas low threshold afferents terminate in deeper laminae III, IV and V (Todd et al. 2002). More specifically, $A\delta$ fibers terminate in laminae I and V whereas peptidergic C fibers terminate in lamina I and the dorsal region of lamina II and non-peptidergic C fibers in lamina II (see Ribeiro-da Silva & De Koninck 2009). Primary afferents terminating in lamina I synapse directly on to second order sensory neurons whereas those that terminate in lamina II synapse indirectly to second order neurons in the deeper laminae IV-VI via small interneurons. In deeper laminae such as lamina V both noxious and innocuous input can therefore synapse onto the same second order spinal neuron. These neurons are referred to as wide dynamic range (WDR) neurons. WDR neurons respond to stimuli in a graded manner and summate repetitive inputs to induce a short-term state of sensitivity known as wind-up (Mendell & Wall 1965; Wall & Woolf 1986; Woolf & Wall 1986).

Noxious stimuli detected by nociceptors that innervate cephalic tissues are processed by the trigeminal system. Trigeminal primary sensory neurons, contained within the trigeminal nerve (cranial nerve V), utilise slightly different ascending pathways when compared to somatic afferents due to the proximity of the tissue to the brain. Central branches of trigeminal primary afferents enter the central nervous system (CNS) through the pons region of the mid-brain (Sessle 2000). These
primary neurons descend along the spinal trigeminal tract to the trigeminal nucleus within the medulla where they synapse with second order neurons. Second order sensory neurons cross the mid-line and transmit information to somatotopically appropriate brain regions through various ascending pathways.

The most characterised ascending pathways of nociception are the spinothalamic and trigemino-thalamic pathways associated with the somatosensory and trigeminal systems respectively. Other pathways have also been identified that terminate in various other regions within the CNS such as the spinocervical, spinobulbar, spinopontine, spinomesencephalic, spinodiencephalic and spinothelencephalic pathways (see Lima 2009). As the name suggests, a major target of the spinothalamic and trigemino-thalamic pathways is the thalamus. The ventral posterior lateral (VPL) nucleus and the ventral posterior medial (VPM) nucleus of the thalamus receive information regarding sharp, fast pain from the somatosensory and trigeminal systems respectively. Dull, slow pain information from the deep laminae of the spinal cord is received by intralaminar nuclei of the thalamus and the parabrachial nucleus of the brainstem (Bourgeais et al. 2001). Signals received by thalamic nuclei are relayed onto various higher processing centres within the brain, such as the primary somatic sensory cortex, the cingulate gyrus and the insula allowing the stimulus to be perceived in multiple dimensions (Tracey & Mantyh 2007). Each area of the body is somatotopically represented by a particular group of neurons in the primary somatic sensory cortex to allow for accurate discrimination of sharp, fast pain in terms of location and intensity. Somatotopy is dependent upon the density of innervation in the peripheral tissue. For example the area of the somatic sensory cortex representing individual facial tissues are much larger than that of the forearm. Neurons of the intralaminar and the parabrachial nuclei relay information to the cingulate gyrus and insula within the cerebral cortex via the amygdala, in the case of the parabrachial nucleus,
to process the poorly localised sensations of slow pain such as dull burning and
deeper aching pain. The cingulate gyrus and insula regions are also partly re-
sponsible for adding emotional features to the perceived sensation (Bushnell et al.
2013). Ascending pathways also terminate in the periacqueductal gray and the
rostral ventral medulla in the brainstem to provide rapid feedback to the local
area where the initial stimulus was detected (Fields & Heinricher 1985; Fields
et al. 2005). These various ascending pathways and processing centres allow pain
to be perceived in multiple dimensions and allows for the generation of a complex
integrated signal to brings about multiple affector responses.

1.2 Chronic pain

Pain associated with an infection or injury is protective in the short term as it
alerts the host to the infection/injury and often limits the use of the affected tissue
to promote the healing process. In certain situations this acute pain does not re-
solve with the infection/injury and transitions into a state of chronic pain which
is maladaptive and significantly impacts on a patients quality of life. Whereas
acute physiological pain reflects the presence, intensity and duration of a stimulus,
chronic pain conditions cause an un-coupling of this stimulus-response relation-
ship so that the pain experienced is no longer a true reflection of the physiological
situation (see Woolf & Salter 2000). Chronic pain is an important co-morbidity
associated with many chronic disorders however it can also persist beyond the res-
olution of an initial insult and present as a pathology in itself. Approximately 20%
of the European population and 30% of the U.S. population suffer with chronic
pain (Breivik et al. 2006; Johannes et al. 2010). The total financial impact of
pain on society is estimated at $560-635 billion in the U.S.A. (Gaskin & Richard
2012). Apart from this financial impact, the physical and emotional toll on pa-
tients is a significant burden that greatly impacts on quality of life as indicated by the prevalence of psychological co-morbidities in chronic pain patients (Breivik et al. 2006). Although much is known about individual mechanisms associated with chronic pain the key mechanisms that mediate the transition to and maintenance of chronic pain states remain unknown. Importantly, the efficacy of current chronic pain treatments such as opioid analgesics are often sub-standard and ineffective in the short term and potentially harmful and addictive in the long term (Rapp et al. 1995). Therefore the development of safe and effective analgesics targeted towards chronic pain states is a focus of the pain research community.

Chronic pain is often categorised as either inflammatory or neuropathic in origin. Pain (dolor) is one of the cardinal signs of inflammation and is associated with infection and tissue injury. Inflammatory pain serves a protective purpose during the period of time where the initial infection is cleared and injured tissue is repaired. However, in the case of chronic inflammatory pathologies, inflammatory pain persists as long as the inflammatory response does and often increases in intensity. A characteristic of inflammatory pain is an exaggerated response to noxious stimuli, known as hyperalgesia. Hyperalgesia may occur across a range of stimulus modalities with mechanical and thermal hyperalgesia being commonly associated with inflammation. Inflammatory pain is initiated by the chemical milieu that develops at the local site of inflammation. Neuropathic pain is associated with trauma/disease of the nervous system such as autoimmune disease, diabetes, viral infection, channelopathies and nerve compression/trauma. This infection/injury is often accompanied by inflammation however it is a sterile inflammation due to the absence of an infection in the cause of physical trauma. Acute damage to nerve fibres causes a large degree of spontaneous firing and ectopic action potential generation that manifests as a sharp, pin-prick pain. Neuropathic pain involves various peripheral mechanisms that are also associated with inflammatory pain
but specifically induces a distinct set of central changes to the circuits of ‘pain pathways’ to alter long-term neuronal excitability.

1.2.1 Peripheral mechanisms of chronic pain.

Neuronal plasticity is a critical survival mechanism that functions to tailor neuronal function to environmental exposure. Peripheral sensitisation develops as a result of the transcriptional/translational up-regulation of pro-algesic factors such as cytokines and the post-translational sensitisation of multiple receptors and channels in peripheral sensory neurons in response to changes in the local micro-environment (see Bhave & Gereau 2004). Such a change in the local environment is commonly associated with inflammation and peripheral nerve injury. A broad range of chemical factors including, but not limited to, tumour necrosis factors alpha (TNFα), interleukin (IL)-1β, IL-6, chemokines, NGF, histamine, prostaglandin E₂ (PGE₂), adenosine triphosphate (ATP) and protons are produced by immune cells, non-neuronal cells within the nerve and damaged tissue which activate specific receptors expressed by sensory neurons (see Hucho & Levine 2007; Gold & Caterina n.d.). This ligand-receptor interaction is often sufficient to directly activate the nociceptor but can also induce a long lasting peripheral sensitisation of nociceptors through various transcriptional, translational and post-translational changes that affect protein production and receptor/channel expression (see Moalem & Tracey 2006). Sensitised nociceptors release factors from their peripheral and central terminals as well as from their soma in the sensory ganglia that can signal through autocrine and paracrine mechanisms to further sensitise themselves and surrounding neurons. Multiple specific mechanisms are involved in peripheral sensitisation which ultimately manifests as hyperalgesia and a reduced activation threshold of nociceptors. Under normal circumstances the plasticity associated with peripheral sensitisation would gradually return towards
the physiological state as the inflammatory response resolves however in the case of a chronic injury/inflammation peripheral sensitisation often persists. Specific examples of inflammatory mediator-induced peripheral sensitisation are described in the following paragraphs (see Fig. 1.1 for summary).

NGF is an inflammation- and tissue damage-associated neurotrophin that is released from keratinocytes, non-neuronal cells and invading immune cells. NGF activates its receptor TrkA on peptidergic C fibres and signals through MAPK, phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC) pathways to induce rapid changes in channel sensitivity in peripheral terminals in a protein kinase C (PKC)-dependent manner (Chuang et al. 2001). NGF is also internalised and is retrogradely transported to the nucleus of the nociceptor in the sensory ganglion to induce the transcriptional up-regulation of pro-nociceptive factors such as calcitonin gene-related peptide (CGRP), substance P (SP), TRP channels and the voltage gated sodium channel \( \text{Na}_v1.8 \) (Bennett et al. 1998; Bennett 2001; Ji et al. 2002). CGRP and SP are released by nociceptors both peripherally and centrally and signal to neurons and non-neuronal cells through paracrine mechanisms. Both are potent vasodilators and promote immune cell infiltration to the local area (Brain et al. 1985; Bossaller et al. 1992) and can directly activate p38 MAPK, ERK and protein kinase A (PKA) signalling in non-neuronal cells within sensory ganglia leading to the production of pro-inflammatory mediators (Cady et al. 2011). Neuropeptides also directly activate and sensitise nociceptors in the periphery and can also act centrally to modulate spinal mechanisms of nociception (see Seybold 2009).

Exogenous application of the inflammatory cytokines TNF\( \alpha \) and IL-1\( \beta \) are sufficient to cause spontaneous firing of nociceptors (Fukuoka et al. 1994; Sorkin et al. 1997; Schafers et al. 2008) and induce hyperalgesia through direct and indirect mechanisms (Junger & Sorkin 2000; Ozaktay et al. 2006). Neurons can directly de-
tect these cytokines through the expression of the receptors interleukin-1 receptor 1 (IL1-R1), Tumour necrosis factor receptor (TNFR)1 and TNFR2. Acute activation of neuronal IL1-R1 and TNFR1 by IL-1β and TNFα, respectively, causes a rapid facilitation of SP and CGRP release (Fukuoka et al. 1994; Opree & Kress 2000; Hou et al. 2003), induces prostaglandin production (Cunha et al. 1992), sensitisation of TRPV1-dependent currents (Nicol et al. 1997; Obreja et al. 2002) and also reduces the slow inactivation of tetrodotoxin (TTX)-resistant NaV channels in a p38 MAPK-dependent manner to increase neuronal excitability (Jin & Gereau 2006; Binshtok et al. 2008; Czeschik et al. 2008). TNFR2 is not normally activated in neurons and only contributes to neuronal sensitisation following nerve injury (Schafers et al. 2008). Transcriptional changes are mediated by the activation of signalling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 MAPK, ERK, JNK and PI3K (Pollock et al. 2002; Wei et al. 2007) whereas post-translational changes are suggested to be mediated by PKA- and PKC-dependent phosphorylation of receptors/channels (see Uceyler et al. 2007). TNFα and IL-1β can also indirectly sensitise neurons by activating positive feedback pathways in immune and non-neuronal cells to further promote the production of pro-inflammatory and pro-nociceptive mediators. For example, TNFα and IL-1β promote the production of NGF, prostaglandins, bradykinin and further promote their own synthesis by immune cells which can directly sensitise neurons (see Moalem & Tracey 2006). TNFα may also be produced by satellite cells in the sensory ganglion to promote a more general inflammation in the nervous system (Dubovy et al. 2006). This can then lead to a more diffuse systemic hyperalgesia as observed with many systemic infections.
Figure 1.1 – Peripheral sensitisation of nociceptors following inflammation. Inflammation and tissue damage leads to an increased production of pro-inflammatory mediators such as NGF, ATP, cytokines, bradykinin, histamine and prostaglandins by immune and non-neuronal cells. Specific receptors are expressed by peripheral nociceptors that recognise many of these factors and their activation initiates intracellular signalling cascades, including p38 MAPK, ERK1/2, JNK, PI3K and PLC pathways. The activation of these pathways promotes a transcriptional up-regulation of multiple channels, receptors and neuropeptides as well as a re-distribution of pre-existing channels to the peripheral terminals. Further, the activation of these receptors is often sufficient to induce the spontaneous firing of nociceptors. An increase in intracellular calcium leads to the activation of kinases, such as PKA, PKC and CamKII, and results in the phosphorylation of channels/receptors which ultimately reduces the activation threshold of the nociceptor. Multiple pro-inflammatory factors, such as CGRP and SP, are also released from the nociceptor terminal which can further promote the inflammatory response. Collectively these mechanisms reduce the activation threshold and therefore increase the excitability of the nociceptor both in the short term and long term to initiate/maintain a state of increased pain sensitivity.
The transcriptional and translational changes induced by pro-inflammatory mediators, described above, can cause the up-regulation of channel/receptor expression in peripheral neurons. This includes, but is not limited to, TRPV1 (Ji et al. 2002), TRPA1 (Diogenes et al. 2007), P2X3 (Fabbretti et al. 2006) and Na$_V$1.8 (Jin & Gereau 2006) channels and is often accompanied by a redistribution of existing channels towards the peripheral terminal of the neuron. The activation thresholds of these channels can also be reduced by protein kinase-dependent post-translational modifications which are activated by calcium signalling. Certain lipid mediators and protons can also directly alter channel/receptor kinetics by acting as allosteric modulators (Jordt et al. 2000; Cao et al. 2013). The specific pathways and kinases activated are receptor-specific however MAPK and PLC signalling and PKA, PKC and Calcium/calmodulin dependent protein kinase II (CamKII) activation are frequently implicated in channel up-regulation and sensitisation (Bhave et al. 2002; 2003; Jung et al. 2004; Jin & Gereau 2006; Varga et al. 2006).

Positive feedback mechanisms are key to the development of peripheral sensitisation and many current treatments for inflammatory pain reflect this. Non-steroidal anti-inflammatory drugs (NSAIDs) targeting cyclooxygenase-2 (COX-2) and also TNF$\alpha$ and NGF signalling-specific inhibitors are often prescribed in an attempt to interrupt this vicious cycle thereby reducing the degree of inflammation and associated pain (Sommer et al. 2001; Atzeni et al. 2005; Hefti et al. 2006). However the modulation of nociceptor plasticity to confer pain hypersensitivity is not limited to peripheral primary afferents. The activation of sensitised peripheral nociceptors not only produces changes in cellular processes at the peripheral terminal but also at the central synapse with second order sensory neurons. Activation of sensitised peripheral nociceptors can promote the release of neurotransmitters (e.g. glutamate) and neuropeptides (e.g. SP and CGRP) from central terminals to sensitise
second order neurons in the dorsal horn/trigeminal nucleus (see Latremoliere & Woolf 2009).

1.2.2 Central mechanisms of chronic pain.

The sensitisation of second order spinal neurons is known as central sensitisation. This confers long term alteration in pain hypersensitivity associated with inflammatory and neuropathic pain. The onset of central sensitisation alters the central circuitry responsible for spinal nociception to such an extent that innocuous stimuli that do not normally activate nociceptive pathways are now able to produce a painful response. This is a phenomenon known as allodynia and, along with hyperalgesia, are frequently associated with chronic inflammation, peripheral neuropathies and central pain pathologies (Jensen & Finnerup 2014). It should be noted that central sensitisation can occur without peripheral involvement in the case of injuries to the CNS. Although peripheral and central sensitisation share some common clinical symptoms the molecular mechanisms responsible for their manifestation are distinct. Whereas peripheral sensitisation is restricted to neurons innervating the site of inflammation/injury and resolves as inflammation is cleared, central sensitisation can also induce hypersensitivity referred to uninjured tissue. Central sensitisation often develops in an activity-dependent manner but can also persist after the initial insult has disappeared. Multiple mechanisms contribute to central sensitisation, however the sensitisation of N-methyl-D-aspartate receptors (NMDAR), activation of glial cells and the loss of inhibitory connections (disinhibition) are suggested to be most prominent (see Fig 1.2 for summary). Glutamate is the main neurotransmitter at the excitatory synapse between primary afferents and second order neurons within the CNS. Under normal conditions glutamate rapidly activates $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) to initiate the generation of action
potentials in post-synaptic spinal neurons. Repeated stimulation, associated with inflammatory and neuropathic pain, causes the activation of an additional glutamate channel called NMDAR which is silent under normal conditions due to the presence of a Mg$^{2+}$ block. A summation of excitatory input promotes the increased release of CGRP and SP by pre-synaptic neurons. This increased neuropeptide release causes a temporal summation of neuropeptide-induced potentials in the post-synaptic neuron. This, in turn, promotes the removal of the Mg$^{2+}$ block allowing NMDAR to be activated by subsequent glutamate (Mayer et al. 1984). This activation of NMDAR is critical in the development and maintenance of central sensitisation (Woolf & Thompson 1991). Once active, depolarisation of post-synaptic neurons through NMDA receptors causes an increase in intracellular calcium which can further modulate NMDAR sensitivity to ultimately strengthen synapses. The activation of calcium-dependent MAPK pathways, PKA, PKC, PI3K and Src kinase further alters NMDAR properties (see Basbaum et al. 2009; Latremoliere & Woolf 2009). The phosphorylation of AMPAR and NMDAR by these kinases causes an increase in channel density at the synaptic membrane and responsiveness to subsequent glutamate (Carvalho et al. 2000; Lau & Zukin 2007). The increased excitability of the second order spinal neuron means that it can now be activated by inputs that are normally sub-threshold (Ma & Woolf 1996).

Just as excitatory synapses are strengthened in chronic pain states, inhibitory synapses are often lost through a process known as disinhibition (Moore et al. 2002). Inhibitory interneurons within the dorsal horn or trigeminal nucleus release inhibitory neurotransmitters such as glycine and γ-aminobutyric acid (GABA) to tightly regulate spinal neuron activation. This regulatory signal is critical in order to couple the response to a given stimulus. Disinhibition occurs following peripheral and central neuropathies and has been shown to contribute towards the development of mechanical allodynia (Keller et al. 2007). It is not known whether
disinhibition involves the death or reduced activity of regulatory interneurons and whether the cellular mechanisms induced by nerve injury are primarily active in the interneuron itself or neighbouring neurons. Regardless of these unanswered questions, disinhibition remains a key component of central sensitisation.

The communication between glial cells and neurons is also altered in the setting of chronic inflammation and nerve injury. Astrocytes and microglia normally provide homeostatic support to neurons however an altered state of communication can promote glial activation and, in turn, contributes towards neuronal sensitisation (see Watkins et al. 2001). Microglia are tissue resident macrophages in the CNS and are central to the orchestration of a central neuroinflammatory response. Microglia are rapidly activated within the dorsal horn following nerve injury (Jin et al. 2003) and inflammation (Fu et al. 1999) and begin to release pro-inflammatory mediators such as TNFα and IL-1β (see Hanisch 2002), the actions of which have been previously described. Astrocytes are also activated in chronic pain states although their specific contributions to pain hypersensitivity are unknown. It is suggested that astrocytes may be more involved with the maintenance of chronic pain rather than initiation due to their delayed yet prolonged activation in persistent pain models (Zhuang et al. 2005). More recent focus has centred around the involvement of peripheral and central neuroimmune signalling in chronic pain states. Following the onset of inflammation, the communication between neurons, non-neuronal and immune cells is altered both in the peripheral area of inflammation as well as within the sensory ganglia and the CNS (see Marchand et al. 2005; Scholz & Woolf 2007). Inflammatory mediators such as cytokines, chemokines, neuropeptides, lipid mediators, ATP and neurotransmitters all act through specific receptors expressed by neurons, glial cells and immune cells to promote peripheral and central sensitisation through previously described mechanisms.
Figure 1.2 – Mechanisms of central sensitisation. (1) Repeated activation of primary afferents leads to the increased release of excitatory neurotransmitters such as glutamate, SP and CGRP at the central synapse within the dorsal horn or trigeminal nucleus. An increase in excitatory post-synaptic potentials promotes the removal of the Mg$^{2+}$ block and subsequent activation of the normally silent glutamate receptor NMDAR. Receptor activation results in an increased level of intracellular calcium and activation of calcium-dependent pathways including MAPKs, PI3K and PLC. These pathways ultimately induce a transcriptional up-regulation of multiple factors to promote increased neuronal excitability. The activation of kinases also leads to post-translational modifications to channels/receptors that further promote excitability. (2) The release of neurotransmitters from the pre-synaptic terminal is sufficient to stimulate and activate glial cells. Activated glial cells produce factors such as pro-inflammatory cytokines, chemokines, ATP, brain-derived neurotrophic factor (BDNF) and PGE$_2$ which can act through receptors on the pre- and post-synaptic terminals to promote an increase in synaptic strength and neuronal excitability. (3) Under normal circumstances, inhibitory interneurons release GABA and glycine to produce a descending inhibitory tone that regulates the pain response. Following injury, this descending inhibition can be lost, through a process known as disinhibition, resulting in an increased excitability of central neurons. Collectively these mechanisms act to induce a long-term sensitisation of central nociceptors so that the response to noxious stimuli is exaggerated (hyperalgesia) and previously innocuous stimuli are now able to induce a painful response (allodynia).
1.3 Orofacial pain

The studies that underpin the understanding of the above peripheral and central mechanisms of neuronal sensitisation were mainly performed on neurons from the somatosensory system. When discussing orofacial pain it is important to consider the potential differences in these mechanisms due to the slight anatomical and physiological differences between the trigeminal and somatosensory systems (summarised by Bereiter et al. 2009). Orofacial pain is classified as pain that localises to an area superior to the neck, anterior to the ears and inferior to the orbitomeatal line whereas craniofacial pain is a broader term that includes pain localised to the head, face and neck. According to the American Academy of Orofacial Pain, the current scope of orofacial pain includes temporomandibular joint (TMJ) disorders, masticatory musculoskeletal pain, cervical musculoskeletal pain, neurovascular pain, neuropathic pain, orofacial dystonias, headache and more systemic disorders that cause poorly localised orofacial pain (see Leeuw & Klasser 2013). The most common orofacial pain conditions are considered to be toothache (caries), TMJ disorders (TMDs) and trigeminal neuropathies however pain can also accompany late-stage periodontal disease. According to previous epidemiological studies it is estimated that approximately 22% of the U.S. general population had encountered some form of orofacial pain within 6 months prior to questioning (Hargreaves & Cohen 2010) and 7% of the U.K. population suffered with chronic orofacial pain of some nature (Aggarwal et al., 2006). As with any chronic pain condition, chronic orofacial pain patients often develop psychological co-morbidities and experience a significant reduction in their quality of life (Korszun 2002).

The current understanding and characterisation of the peripheral and central mechanisms of orofacial pain is very much dependent on the specific location
of the primary afferent. For example, primary afferents innervating the facial skin and tooth pulp are relatively well characterised when compared to those innervating the periodontal tissues. The pulp is densely innervated and is particularly sensitive to changes in the local environment, possibly due to it being contained within a thick, protective layer of dentine. Pulp inflammation, as is seen with carious tooth decay, can promote the peripheral sensitisation of primary afferents through well characterised mechanisms. Specialist techniques such as microdialysis and pulpal perfusion have been used in past studies to investigate the effects of multiple factors on pulpal afferents (see Khan & Hargreaves 2010). For example, the application of capsaicin to the pulp induces the release of CGRP (Bowles et al. 2003), SP (Khan & Hargreaves 2010), PGE$_2$ and NGF (Chidiac et al. 2001). Further, an infiltration and activation of immune cells within the pulp results in the production of various inflammatory mediators that can activate and sensitise nociceptors (see Jain et al. 2013). A role for various inflammatory mediators have also been demonstrated in models of deep orofacial pain. For example, increased levels of SP, CGRP, serotonin (5-HT), TNF$\alpha$, IL-1$\beta$ and PGE$_2$ have been identified in the arthritic TMJ and are associated with spontaneous pain, hyperalgesia and allodynia (see Kopp 2001). The peripheral sensitisation of neurons innervating the TMJ and lip/whisker pad, in the setting of chronic inflammation, results in an increased neuronal excitability and reduced threshold of activation. This has been shown to involve a potentiation of TTX-R sodium currents and alteration of potassium currents (Takeda et al. 2006; Morgan & Gebhart 2008). It should be noted however that no change in sodium currents were observed in TMJ inflammation (Flake & Gold 2005).

Peripheral injury to the trigeminal nerve is associated with increased spontaneous and mechanically-induced firing as well as an alteration in the expression of various receptors and channels including TRPV1 (Biggs et al. 2007) and multiple
Na\textsubscript{V} channels (Davies et al. 2006) at the injury site. Nerve injury is also sufficient to induce a range of changes in neuropeptide expression within the injured nerve (Bird et al. 2003). An increased rate of neuronal activation following injury has also been demonstrated to be further increased by the presence of the neuropeptides SP, CGRP and vasoactive intestinal peptide (VIP) (Loescher et al. 2001; Robinson et al. 2004). Interestingly some of the mechanisms associated with the development of pain following trigeminal nerve injury are distinct from those associated with spinal nerve injury. For example, differences in the time course of spontaneous firing, neuropeptide expression and degree of sympathetic sprouting within the sensory ganglion have been reported (see Fried et al. 2001).

The peripheral sensitisation of orofacial primary afferents following inflammation and nerve injury is also associated with alterations in channel expression (Biggs et al. 2007), neuropeptide production (Elcock et al. 2001) and intercellular communication within the trigeminal ganglion (TG). This can promote the activation of satellite cells and subsequent release of pro-inflammatory mediators (Freeman et al. 2008; Vause & Durham 2009). This is indeed the case in TMD pain where an increased level of activation of glia and immune cells within the trigeminal ganglion has been demonstrated (Villa et al. 2010). The production of pro-inflammatory mediators including IL-1\textbeta and SP as well as the activation of PKC within the trigeminal ganglia can promote the sensitisation of neighbouring neurons through paracrine mechanisms and can therefore contribute to the spread of orofacial pain (Safieh-Garabedian et al. 1995; Takeda et al. 2005; Freeman et al. 2008).

In addition to the above peripheral mechanisms, a range of central changes have been identified in multiple orofacial pain models. The acute activation of central neurons of the trigeminal system following peripheral exposure to inflammatory irritants and neuropeptides has been demonstrated by the increased expression of cFos (Hathaway et al. 1995; Worsley et al. 2007; Bowler et al. 2013). The central
sensitisation of nociceptors and WDR neurons within the subnucleus oralis and
ventrobasal thalamus following peripheral application of irritants has also been
reported (Kaneko et al. 2005; Zhang et al. 2006; Kawamura et al. 2010). This
central sensitisation also occurs in the case of chronic inflammation and nerve
injury and can last for prolonged periods (Dubner & Ren 2004; Yamazaki et al.
2008; Tsuboi et al. 2011). Various mechanisms have been shown to contribute to
the observed activation and sensitisation of central neurons including NMDAR,
nitric oxide (NO), protein kinase and P2XR signalling (Allen et al. 2011; Sessle
2011). Taking P2XR as a specific example, the application of P2XR agonists to
the tooth pulp and TMJ is sufficient to induce mechanical hyperalgesia, allodynia
and increase peripheral receptive fields (Hu et al. 2002; Chiang et al. 2005) and
these can be blocked by the application of receptor antagonists (Adachi et al.
2010; Watanabe et al. 2010). Alterations is descending regulatory mechanisms
are also altered in chronic orofacial pain. Specifically, TMDs have been associated
with a loss of descending inhibition (disinhibition) as well as an increased level of
descending facilitatory signals (Sarlani et al. 2004; King et al. 2009).

Glial cell activation within trigeminal central structures have also been shown to
play a major role in the development and maintenance of chronic orofacial pain
states. Peripheral inflammation and nerve injury are associated with the increased
activation of microglia and astrocytes (Yeo et al. 2001; Xie et al. 2007; Okada-
Ogawa et al. 2009; Villa et al. 2010). Blocking this activation of microglia and
astrocytes has been shown to partially reduce the development and maintenance
of hyperalgesia and allodynia (Xie et al. 2007; Shimizu et al. 2009; Itoh et al.
2011). A delayed inhibition of astrocyte activity, 7 days after the development
of pulpitis, is also sufficient to reduce central sensitisation and mechanical hy-
peralgesia (Tsuboi et al. 2011). The role of P2X4 and P2X7, expressed by glial
cells within the subnucleus caudalis, has also been demonstrated to mediate the
increased activity and production of ATP and pro-inflammatory cytokines associated with the development of central sensitisation (Chiang et al. 2005; Itoh et al. 2011).

Various chronic orofacial pain states therefore share common mechanisms with those associated with the development of peripheral and central sensitisation in the somatosensory system. However it is important to note that a number of pain states also display distinct differences when compared to those of the somatosensory system. As mentioned above, previous studies have mainly focussed on primary afferents innervating the pulp, TMJ, masseter muscles and cutaneous facial skin. These afferents are therefore relatively well characterised compared to those innervating other orofacial tissues such as the periodontal tissues. Gingivitis and periodontitis are prevalent conditions that are associated with inflammation within periodontal tissues. The presence of an inflammatory response is sufficient to induce a range of peripheral and central changes in neuronal function, as previously described. However the effect of inflammatory mediators on sensory neurons, specifically innervating periodontal tissues such as the gingiva and periodontal ligament, for example, have not been investigated.

1.4 Periodontal disease

The microbiome present within the oral cavity is second in complexity only to the colon. Over 700 prevalent species of commensal microbes have been identified within the healthy oral flora (Dewhirst et al. 2010; Ge et al. 2013). The majority of these are bacterial species, however viruses, protozoa, archaea and fungii are also present (see Wade 2013). The presence of this flora is central to normal homeostasis within the oral cavity as its mere presence normally prevents the invasion of and colonisation by pathogenic species, possibly due to the lack of
available binding sites in the cavity (Vollaard & Clasener 1994). This is reflected in the aetiology of periodontal disease. This initially establishes as gingivitis with plaque formation by opportunistic aerobic species and then develops into periodontal disease associated with a more complex plaque composition dominated by anaerobic species. This gradual formation of a complex pathological plaque is dependent upon the early opportunistic microbial species which provide binding sites for more pathogenic microbial species. Whilst many orofacial disorders are often considered less of a priority when compared to other human disease states, an association between dysregulation of the oral microbiome and increased risk of developing systemic disease such as cardiovascular disease, diabetes and rheumatoid arthritis has recently been uncovered (Genco & Van Dyke 2010; Lundberg et al. 2010; Lalla & Papapanou 2011).

Gingivitis is one of the most prevalent bacterial human disease with approximately 90% of adults affected (see Coventry et al. 2000). Dental plaque formation begins with the selective adsorption of salivary glycoproteins such as α-amylase, immunoglobulin (Ig) A, mucin, acidic proline-rich protein and cystatins onto the tooth surface to form the salivary pellicle (Murray et al. 1992; Ahn et al. 2002). This pellicle provides binding sites for primary colonising species such as *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Treponema* and *Synergistetes* species (Nyvad & Kilian 1987; Zijnge et al. 2010). Interactions between early colonising species can produce an environment that promotes the additional recruitment and attachment of other species to the immature plaque (see Kolenbrander et al. 2006).

Early, immature plaque can induce a low grade immune response that produces an inflammatory exudate contained within the gingival crevicular fluid (GCF). The GCF also promotes plaque development and the induction of gingivitis (Peters et al. 2012). Plaque formation in gingivitis is supra- gingival and is relatively easy to control. Good oral hygiene is normally sufficient to break down the plaque and
maintain it in an immature state. However, if good oral hygiene is not practised, in susceptible individuals the plaque is allowed to develop; numbers of Gram-negative anaerobic species increase in proportion and produce virulence factors that result in tissue damage and an the activation of a local immune response.

The continued presence of a complex plaque causes a chronic low grade inflammation and subsequent damage to local tissue. Interestingly it is the host response that primarily causes the irreversible tissue damage (Eskan et al. 2012). The gingival pocket, the space between the gingiva and the tooth structures, increases in depth and the plaque is therefore allowed to migrate to sub-gingival structures. This environment is highly supportive of anaerobic species which dominate sub-gingival plaques from periodontitis patients (Ximenez-Fyvie et al. 2000). Continued protease production by plaque bacteria causes a gradual progression of tissue damage from the gingiva to the supporting tooth structures such as the periodontal ligament and the alveolar bone. Resorption of the alveolar bone causes the tooth to loosen and become increasingly mobile until it is eventually lost (see Pihlstrom et al. 2005).

Periodontitis associated with dental plaque can be classified as either chronic or aggressive with the latter being associated with a more rapid rate of progression, increased severity and earlier onset (see Armitage 1999). Both forms of the disease can be further categorised as generalised or localised with the latter being specific to the tissues surrounding individual teeth rather than the whole mouth or a large proportion of the gingiva. Chronic periodontal disease presents with inflamed gums that are tender and often bleed, receding gums, loose and sensitive teeth, halitosis and also painful mastication. Systemic manifestations may also accompany both forms of the disease that are mainly related to mental well-being (Page et al. 1983). These symptoms also present with aggressive forms of the disease however severity and disease progression are greatly elevated. The chronic
disease is most prevalent in middle aged adults whereas the aggressive form is often seen in juveniles (Armitage 1999). Both chronic and aggressive periodontitis present with phases of active tissue damage and periods of remission that can last for years. Factors that mediate this switch to and from an active state are currently unknown. During remission periods, symptoms of the disease do not present even though deep gingival pockets are maintained. Currently the disease is managed by improved dental hygiene including the use of specific toothpaste or mouthwash, mechanical scaling of plaque, the use of anti-bacterial or anti-inflammatory medication and surgical intervention to promote bone regeneration however ultimately it is an irreversible condition that will lead to tooth loss (see Roshna & Nandakumar 2012).

A recent oral microbiome sequencing study showed that the levels of approximately 200 species were significantly altered in sub-gingival plaques (Ge et al. 2013). Early studies into the microbiome associated with chronic periodontitis grouped various bacterial species into different colour-assigned groups (Socransky et al. 1998). These were characterised based on the similarity between species and also their correlation with clinical symptoms of the disease. Three bacterial species, *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, are identified as key pathogenic species associated with the disease and are frequently identified in the majority of chronic periodontitis patients. These are classified as ‘red complex’ bacteria and are thought to play a key orchestrating role in tissue destruction and disease progression due to their correlation with increased bleeding upon probing (Socransky et al. 1998). This is primarily elicited by their ability to activate host responses but also through multiple virulence factors including proteinases, cell wall expressed polysaccharides, hemin-binding proteins and fimbriae (Holt & Ebersole 2005). Other secondary colonising species are primarily located within the green and orange complexes whereas primary
colonisers are mainly located in the yellow and purple complexes. Aggressive periodontitis however is primarily associated with the overwhelming presence of *Aggregatibacter actinomycetemcomitans*, an invasive Gram-negative facultative rod as well as *Porphyromonas gingivalis* (Zambon et al. 1988; Schacher et al. 2007). Current theories regarding the development of periodontal disease centre around the ‘keystone pathogen’ theory (Hajishengallis et al. 2012). It is suggested that *P. gingivalis* triggers a dysbiotic relationship between the host and the oral flora that allows the plaque to develop and it is this overall shift in microbial activity that promotes the pathology associated with the disease. This theory is supported by the fact that the presence of *P. gingivalis* alone is not sufficient to cause periodontitis in a germ-free mouse model (Hajishengallis et al. 2011).

The change in composition of the oral flora associated with periodontitis is sufficient to activate a host immune response. The activation of macrophages and neutrophils promote bacterial clearance through the production of cytokines, phagocytosis and oxidative killing mechanisms (see Cekici et al. 2014). The production of cytokines promotes blood vessel dilation, increased permeability and the subsequent infiltration of immune cells into the local tissues. It is this inflammatory response that causes the majority of host tissue damage that is observed in chronic periodontitis (see Taubman et al. 2005). The immune response generated in periodontitis is often insufficient for full clearance of the infection. Therefore, in susceptible individuals, a chronic, relapsing and remitting pathology can develop that is associated with a progressive destruction of periodontal tissues. This is, in part, due to the multiple host evasion mechanisms employed by pathogenic bacterial species such as *P. gingivalis* to promote its survival and the subsequent development of a mature plaque (see Hajishengallis & Lamont 2014 for a detailed review). The increased production of pro-inflammatory mediators, including TNFα, IL-1β, IL-6 and PGE₂ (see Yucel-Lindberg & Bage 2013 for detailed review), in chronic
periodontitis patients would predict that a peripheral sensitisation of sensory neurons innervating periodontal tissue may also develop. These inflammatory mediators are known to contribute towards the peripheral and central sensitisation of sensory neurons however this has not been demonstrated within the setting of periodontitis.

1.5 Toll-like receptors

As previously mentioned, the presence of a mature plaque is sufficient to activate the host immune response via multiple innate receptor families. One major class of receptors that are implicated in the pathogenesis of periodontitis are the Toll-like receptors (TLRs). TLRs are categorised as a class of pattern recognition receptors (PRRs) that detect specific pathogen-associated (PAMPs) and damage-associated molecular patterns (DAMPs) released by damaged/stressed host cells to mediate the activation of an innate immune response (Takeda & Akira 2005). The Toll protein and its signalling pathway were first identified in Drosophila melanogaster and were demonstrated to be involved in the immune response to fungal infection (Lemaitre et al. 1996). A mammalian homolog of the Toll receptor, subsequently named the Toll-like Receptor, was discovered (Medzhitov et al. 1997) and to date 13 TLRs have been identified, 11 of which are confirmed to be present in humans (Bowie & O’Neill 2000; Akira et al. 2006). TLRs are expressed on various innate and adaptive immune cells, such as macrophages, dendritic cells, regulatory T cells, B cells, mast cells, natural killer cells, neutrophils, basophils and utilise receptor dimerisation to obtain pathogen specificity allowing a tailored, pathogen-specific response (Takeda & Akira 2005). Activation of TLRs typically results in the production of multiple pro-inflammatory mediators, such as cytokines and chemokines, which exert a wide range of functions to orchestrate the innate and
adaptive immune response.

1.5.1 Ligand binding

TLRs are type I transmembrane receptors that contain an extracellular leucine-rich-repeat (LRR) domain for ligand binding. As previously mentioned each TLR recognises a specific set of PAMPs and DAMPs (Table 1.1). This receptor specificity is essential in order to ascertain what type of pathogen has invaded the host system and to generate an efficient innate response to facilitate clearance of the infection. If a host cell has become damaged through infection or injury its contents can spill out into the extracellular matrix and elicit effects on neighbouring cells many of which can promote further tissue damage. It is therefore important to alert the immune system to these damaged cells and one mechanism by which this can be achieved is through TLR activation. The activation of TLRs by endogenous ligands has a different functional outcome when compared to activation by exogenous ligands. Activation by exogenous ligands primarily drives a pro-inflammatory response whereas endogenous ligands do not induce such a strong response and there is more emphasis on tissue repair (Miyake 2007). The discovery that TLRs can sense tissue damage is a significant development as it highlights a role for TLRs in pathophysiological states other than infection (Okun et al. 2011).

The sub-cellular location of TLRs reflect the location at which they will encounter their specific ligands; TLR1, 2, 4, 5, 6 and 10 are located on the cell membrane whilst TLR3, 7, 8 and 9 are present on the intracellular endosomal membranes as they recognise nucleic acids of both bacterial, viral and host origin (see Kawai & Akira 2007). TLR activation upon ligand recognition relies on receptor dimerisation. TLR3, 4, 5, 7, 8 and 9 form homodimers whereas TLR2 forms a heterodimer.
1. Introduction

Table 1.1. Summary of TLR ligands (* denotes TLRs identified in mice only.)

<table>
<thead>
<tr>
<th>TLR</th>
<th>Exogenous ligand</th>
<th>Endogenous ligand</th>
<th>References</th>
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<tbody>
<tr>
<td>TLR2</td>
<td>Peptidoglycan</td>
<td>HMGB1</td>
<td>Schwandner et al. 1999</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Yu et al. 2006</td>
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<tr>
<td>Phospholipomannan</td>
<td>HSP70</td>
<td>Asea et al. 2002</td>
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<tr>
<td>tGPI-mucin</td>
<td>Hyaluronic acid</td>
<td>Coelho et al. 2002</td>
<td>Shimada et al. 2008</td>
</tr>
<tr>
<td>Haemagglutinin</td>
<td>α-synuclein</td>
<td>Bieback et al. 2002</td>
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<tr>
<td>Porins</td>
<td></td>
<td></td>
<td>Massari et al. 2002</td>
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<tr>
<td>Lipoarabinomannan</td>
<td></td>
<td>Means et al. 1999</td>
<td></td>
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<tr>
<td>Glocuronoxylomannan</td>
<td></td>
<td>Yauch et al. 2004</td>
<td></td>
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<tr>
<td>Atypical lipopolysaccharide</td>
<td></td>
<td>Darveau et al. 2004</td>
<td></td>
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<tr>
<td>TLR2/1</td>
<td>Triacyl lipoproteins</td>
<td></td>
<td>Omueti et al. 2005</td>
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<tr>
<td>TLR2/6</td>
<td>Diacyl lipoprotein</td>
<td></td>
<td>Omueti et al. 2005</td>
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<td></td>
<td>lipoteichoic acid</td>
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<td>Kang et al. 2009</td>
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<td></td>
<td>zymosan</td>
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<td>Gantner et al. 2003</td>
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<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>dsRNA</td>
<td>Alexopoulou et al. 2001</td>
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<tr>
<td>TLR4</td>
<td>Lipopolysaccharide</td>
<td>HSP60</td>
<td>Poltorak et al. 1998</td>
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<td></td>
<td>Fusion protein (RSV)</td>
<td>HSP70</td>
<td>Ohashi et al. 2000</td>
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<td></td>
<td>Envelope protein (MMV)</td>
<td>Fibrinogen</td>
<td>Asea et al. 2002</td>
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<td></td>
<td>Glocuronoxylomannan</td>
<td></td>
<td>Rassa et al. 2002</td>
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<tr>
<td></td>
<td>Environmental Nickel</td>
<td></td>
<td>Smiley et al. 2001</td>
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<td>Glucuronoxylomannan</td>
<td></td>
<td>Yu et al. 2006</td>
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<td>Glycosylinositolphospholipids</td>
<td>Fibronecctin</td>
<td>Okamura et al. 2001</td>
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<td>Environmental Nickel</td>
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<td>Debierre-Grockiego et al. 2007</td>
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<td>TLR5</td>
<td>Flagellin</td>
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<tr>
<td>TLR7</td>
<td>ssRNA</td>
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<td>Fabbri et al. 2012</td>
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<td>ssRNA</td>
<td>microRNAs</td>
<td>Heil et al. 2004</td>
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<td></td>
<td></td>
<td>Fabbri et al. 2012</td>
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<tr>
<td>TLR9</td>
<td>Cpg-containing DNA</td>
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<td>Hemmi et al. 2000</td>
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<td></td>
<td>Haemozoin</td>
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<td>Coban et al. 2010</td>
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*Table continued on next page.*
with either TLR1 or 6 to distinguish between triacyl and diacyl lipoproteins, respectively (see Kawasaki & Kawai 2014). It should be noted however that previous studies have also demonstrated that TLR4 may form a heterodimer with TLR1, 2 and 5 (Spitzer et al. 2002; Mizel et al. 2003; Wang et al. 2014). TLR10 is thought to form a heterodimer with TLR1 or 2 although the specific ligands remain unknown (Akira et al. 2006). Certain TLRs also rely on co-receptors for ligand recognition. For example TLR4 and TLR2 associate with the co-receptor CD14 and TLR4 also recruits myeloid differentiation factor (MD)-2 in order to fully activate the receptor (Wright et al. 1990).

Certain TLRs are known to translocate to specific membrane micro-domains known as lipid rafts following activation (Triantafilou et al. 2002). Intracellular signalling pathways also cluster at these domains and can be activated following receptor translocation. The exact mechanisms that mediate the translocation of TLRs to lipid raft domains are poorly understood however it has previously been suggested that a lysophospholipid recycling enzyme, known as lysophosphatidylcholine acyltransferase (LPCAT), may be involved. LPCATs are known to regulate the lysophospholipid/phospholipid ratio of the cell membrane and can therefore alter certain membrane properties and functions such as fluidity and associated lateral movement of membrane proteins (Drecktrah et al. 2003). Indeed, Jackson et al. 2008 displayed how LPCAT inhibition in innate immune cells prevented the translocation of TLR4 into lipid raft domains and subsequently

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<tr>
<td>TLR10</td>
<td>Listeria</td>
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<td></td>
<td>Influenza A</td>
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<td>TLR11*</td>
<td>Uropathogenic bacteria</td>
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<td>Profilin-like protein</td>
<td>Yarovinsky et al. 2005</td>
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<td>TLR12*</td>
<td>Profilin-like protein</td>
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<tr>
<td>TLR13*</td>
<td>rRNA</td>
<td>Oldenburg et al. 2012</td>
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reduced the inflammatory response in response to LPS. A recent study has now shown that it is specifically the isoform LPCAT2, and not LPCAT1, that mediates the activation of TLR4 and TLR2 through a physical interaction with the receptors (Abate & Jackson 2015).

1.5.2 TLR signalling

TLRs contain an intracellular signalling domain termed the Toll/IL-1R homology (TIR) domain which undergoes conformational changes after ligand binding to promote the recruitment of adapter proteins (Bowie & O’Neill 2000). The adapter proteins which are recruited differ between each TLR dimer but include myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (MAL), TIR-domain-containing adapter protein (TIRAP), Toll-receptor-associated activator of interferon (TRIF), TRIF-related adapter molecule (TRAM) and sterile α and TIR motif-containing protein (SARM) (Akira & Takeda 2004). The different combination of adapter molecules determines which transcription factors are activated and what type of response is generated. There are two classic signalling pathways that can be activated following TLR activation; the MyD88-dependent and –independent (TRIF-dependent) pathways (Fig. 1.3). All TLRs, except TLR3 which exclusively signals through the TRIF-dependent pathway, utilise the MyD88 adapter molecule. TLR4 is unique as it is capable of signalling through both pathways (Kawai & Akira 2007b). TLR2 and 4 are reliant on an additional adaptor protein, TIRAP, in order to recruit MyD88 (Horng et al. 2001) and TLR4 also requires TRAM to activate the TRIF-dependent pathway (Kawai & Akira 2007b). The MyD88-dependent pathway primarily results in the rapid activation of NF-κB, a transcription factor that governs the expression of pro-inflammatory genes, whereas the TRIF-dependent pathway activates the transcription factor interferon regulatory factor (IRF) 3 responsible for the induction of type I interferon
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(IFN) genes to produce an anti-viral response (Kaisho & Akira 2006).

1.5.2.1 MyD88-dependent pathway

The binding of MyD88 to the TIR domain of the TLR induces conformational changes to promote the recruitment of interleukin-1 receptor-associated kinase (IRAK) family members such as IRAK1, IRAK2 and IRAK4 through death domain interactions. Phosphorylation causes the IRAKs to dissociate and interact with TNF receptor associated factor (TRAF) 6 which, in turn, forms a complex with ubiquitin-conjugating protein (Ubc) 13 and TGF-β activated kinase (TAK) 1 (Chen 2005). This complex is responsible for the activation of both the MAPK pathway and the IκB kinase (IKK) complex. The activation of the MAPK pathway leads to the activation of the transcription factor activator protein 1 (AP-1), composed of a group of proteins belonging to the c-Fos, c-Jun, activating transcription factor (ATF) and Jun dimerisation protein (JDP) families, which controls cellular processes such as apoptosis, differentiation and proliferation (Ame- yar et al. 2003) as well as the production of pro-inflammatory mediators. The activated IKK complex, consisting of subunits IKKα, IKKβ and IKKγ/NEMO, induces the degradation of IκB via phosphorylation subsequently allowing for the nuclear translocation of NF-κB to drive the production of inflammatory cytokines such as TNFα, IL-1β and IL-6 (see Kawai & Akira 2007a).

1.5.2.2 TRIF-dependent pathway

The discovery that MyD88-deficient mice display normal activation of IRF3 and IFNβ induction following exposure to TLR3 and TLR4 ligands led to the identification of TRIF and the MyD88-independent pathway (Yamamoto et al. 2002). TRIF is recruited to the TIR domain of TLR3 and TLR4, via TRAM in the
1. Introduction

Figure 1.3. Summary of TLR signalling pathways. TLR4, 5, 11 and 12 as well as TLR2/1 and TLR2/6 heterodimers are expressed at the cell membrane whereas TLR3, 7, 8 and 9 are expressed intracellularly on the endosomal membrane. TLR4 is unique in the fact that it can also localise to endosomal membranes. Following ligand-induced dimerisation and activation of receptors, the TIR domains of the receptors engage a range of adapter proteins including MyD88, TRIF, TRAM and TIRAP. Association of these adapter proteins to the TIR domains allows the activation of downstream signalling cascades through a series of interactions between IRAK and TRAF molecules. These ultimately lead to the activation of transcription factors such as NF-κB, IRFs and the AP-1 group (c-Fos, c-Jun, ATF). The majority of TLRs, with the exception of TLR3, signal through the MyD88-dependent pathway which primarily results in the production of pro-inflammatory cytokines. TLR3, and TLR4, signal through the TRIF-dependent pathway which induces the production of an anti-viral response through type I IFN production. It should be noted that TRIF-dependent signalling is able to induce a delayed production of pro-inflammatory cytokines through interactions involving TRAF6 and RIP1.
case of TLR4, and forms a complex with non-canonical IKKι/IKKe and TANK-binding kinase 1 (TBK1). This complex phosphorylates IRF3 allowing it to form a dimer which drives the expression of type I IFN genes and the subsequent anti-viral response after nuclear translocation (Sharma et al. 2003; Fitzgerald et al. 2003).

Interestingly, MyD88 deficient mice remain able to activate NF-κB albeit with delayed kinetics suggesting that the TRIF-dependent pathway is also capable of activating NF-κB (Kawai et al. 1999). The mechanism by which TRIF goes on to activate IRF3 or NF-κB is governed by which end of the molecule is phosphorylated. Both the N-terminal and C-terminal portions of the molecule activate the NF-κB promoter but only the N-terminal activates the IRF3 promoter (Yamamoto et al. 2002). The N-terminal portion of TRIF interacts with TRAF6 (Sato et al. 2003), which is involved in NF-κB activation. The C-terminal portion of TRIF associates with receptor-interacting serine-threonine kinase 1 (RIP1), a deficiency in which causes impaired NF-κB activation in response to TLR3 ligands (Meylan et al. 2004). Therefore both TRAF6 and RIP1 are responsible for the TRIF-dependent activation of NF-κB through interactions on the N-terminal and C-terminal portion of TRIF, respectively.

1.5.2.3 Negative regulation

TLR signalling forms an integral part of the inflammatory response by driving the production of many pro-inflammatory mediators. The uncontrolled production of these mediators can cause serious tissue damage and contribute towards chronic pathophysiologies such as rheumatoid arthritis (Feldmann et al. 2001). Therefore it is essential to tightly control the degree of TLR signalling and subsequent inflammatory response through multiple mechanisms of negative regulation. This in-
includes intracellular inhibitors that target specific tiers of TLR signalling pathways from TIR domain interactions to transcription factor activity (see Kawai & Akira 2007b). The activation of and communication between these regulators is key to coordinating a controlled pathogen-specific innate response. It should also be noted that each individual inflammatory and pain-associated molecule, produced downstream from TLR activation, possesses distinct positive and negative feedback mechanisms which act to further complicate the scene. The combination of positive and negative feedback signals for all factors involved ultimately converge to generate either a pro-inflammatory/pro-nociceptive or anti-inflammatory/anti-nociceptive response.

1.6 Toll-like receptor signalling in chronic pain

Prolonged inflammation at various sites within the nervous system contributes to the pathology associated with many neurological disorders including chronic pain (see Nicotra et al. 2012), neurodegeneration (Meraz-Rios et al. 2013), stroke (Jin et al. 2010), spinal cord injury and neuropsychiatric illness (Najjar et al. 2013). As TLRs generate a pro-inflammatory response in immune cells it was hypothesised that they may also mediate this inflammatory damage within the nervous system. Indeed, multiple lines of evidence have shown that TLR activation contributes to inflammation-induced damage associated with neurodegeneration (Walter et al. 2007; Richard et al. 2008), autoimmunity (see Fischer & Ehlers 2008) and stroke (Lehnardt et al. 2007). TLRs also play a key role in the induction and maintenance of chronic pain states (see Nicotra et al. 2012). The link between TLRs and chronic pain was first shown in a series of association studies investigating TLR4 expression levels in models of neuropathic and inflammatory pain. TLR4 messenger ribonucleic acid (mRNA) is elevated in the spinal cord, brainstem and
forebrain following nerve transection (Raghavendra et al. 2003) and the peripheral application of CFA (Raghavendra et al. 2004). As well as TLR4, CD14 mRNA is up-regulated in the spinal cord following nerve transection and this induction follows a time course that correlates with the development of allodynia, peaking at 14 days post-injury (DeLeo et al. 2004). TLR4 knock out (K.O.) mice, CD14 K.O. mice and rats that receive a TLR4 antisense oligodeoxynucleotide display a reduced behavioural sensitivity and decreased expression of spinal microglial markers and cytokines following L5 nerve transection (Tanga et al. 2005; Cao et al. 2009). Two similar studies also used small interfering RNA (siRNA) against TLR4 to demonstrate its involvement in the development of cancer pain and chronic constriction injury-induced pain (Lan et al. 2010; Wu et al. 2010). Pharmacological blocking of TLR4 is also able to prevent and reverse preclinical neuropathic pain models (Bettoni et al. 2008; Hutchinson et al. 2007; 2008; 2010).

These findings have been extended to demonstrate the involvement of other TLRs in chronic pain. TLR2 mRNA is up-regulated following nerve injury (Shi et al. 2011) and TLR2 activation is shown to partially mediate glial cell activation and production of inflammatory cytokines following nerve injury. TLR2 K.O. mice display reduced capacity to develop mechanical allodynia and thermal hyperalgesia (Kim et al. 2007; Shi et al. 2011). TLR3 has also been shown to activate spinal glia and promote the development of tactile allodynia following peripheral nerve damage (Obata et al. 2008). Although the involvement of TLR7 and TLR9 in chronic pain have not been investigated using animal models their activation on microglia and astrocytes causes an increase in multiple pro-inflammatory mediators that are known to affect neuronal excitability (Bowman et al. 2003; Butchi et al. 2010; El-Hage et al. 2011). Interestingly, peripheral blood mononuclear cells from chronic pain patients display increased responsiveness to TLR7 ligand
stimulation, as well as TLR2 and TLR4 (Kwok et al. 2012).

The focus for these prior studies was the involvement of TLRs expressed by immune and glial cells. Indeed, the activation of TLRs expressed by microglia and astrocytes induces the production of multiple pro-inflammatory mediators (Carpentier et al. 2005; Jack et al. 2005; El-Hage et al. 2011), many of which are known to affect neuronal excitability. However, there is now a growing body of evidence to suggest that TLRs are also expressed by neurons in multiple locations throughout the peripheral nervous system (PNS) and CNS. This implies that pathogens could activate or modulate the nociceptive pathway by a far more direct mechanism. It is suggested, therefore, that TLRs can have both a direct and indirect actions on nociceptive neurons to alter pain processing pathways although the specific mechanisms are largely unknown.

1.6.1 TLR expression in the nervous system

Various past studies have characterised the expression of TLRs in the different cell types of the CNS and PNS and it has become clear that each cell type displays a unique assortment of TLRs that display differing levels of expression. Microglia express a full compliment of TLRs (Bsibsi et al. 2002; Olson & Miller 2012; Jack et al. 2005). This is not surprising given the resident tissue macrophage activity of microglia. Comparatively, the expression of TLRs in astrocytes and oligodendrocytes is more restricted than that of microglia. The most prominent TLR expressed by astrocytes is TLR3 whereas TLR1-9 and 11 have also been detected, although there is some variation between studies (Bsibsi et al. 2002; Bowman et al. 2003; Farina et al. 2005; Jack et al. 2005; Mishra et al. 2006). Activation of TLRs in astrocytes is shown to mediate the production of pro-inflammatory and neuroprotective mediators (Bsibsi et al. 2006; Kim et al. 2008). Oligodendro-
cytes primarily express TLR2 and TLR3 and the activation of these mediates cell survival (Bsibsi et al. 2012).

Within the PNS, Schwann cells primarily express TLR2, 3 and 4 (Oliveira et al. 2003; Lee et al. 2007; Cheng et al. 2007; Goethals et al. 2010) however low level mRNA expression of all other TLRs (from 1-9) has recently been demonstrated (Goethals et al. 2010). Studies concerning satellite glial cells within peripheral ganglia however are distinctly lacking. Recently it has been shown that satellite cells express TLR2 (Kim et al. 2011) and TLR4 (Tse et al. 2014b; Wu et al. 2015) under certain circumstances and the activation of TLR4 by paclitaxel induces TNFα production (Wu et al. 2015). The expression of TLRs in PNS resident macrophages has not been studied although it is likely that they would express a full complement of TLRs given their importance in the innate immune response.

Prehaud et al., 2005 were the first to demonstrate TLR expression in CNS neurons by showing the human cell line NT2-N expressed TLR3 and activation resulted in the induction of an anti-viral response (Lafon et al. 2006). Cortical neurons have since been shown to express TLR2, 3, 4 and 8 (Ma et al. 2006; Cameron et al. 2007; Tang et al. 2007; 2013). TLR1-9 mRNA is also expressed in hippocampal neurons, with TLR4 displaying the greatest level of expression (Hu et al. 2013), and TLR4 activation induces an inflammatory response (Hu et al. 2013; Zhao et al. 2014).

Primary sensory neurons are perhaps the most characterised cell type within the PNS with respect to TLR expression. Two previous studies have utilised real-time PCR to detect the expression of a broad range of TLRs in sensory neurons, with varying results. Goethals et al., 2010 report that embryonic murine dorsal root ganglion (DRG) sensory neurons express TLR2-6 and 8 mRNA with TLR3-
5 displaying the highest levels of expression. Ochoa-Cortes et al., 2010 however detect TLR1-9 mRNA, with TLR1 and 4-6 displaying relatively high levels of expression, in murine colonic DRG neurons. The expression of TLR4 has been demonstrated in TG (Wadachi & Hargreaves 2006; Ferraz et al. 2011; Diogenes et al. 2011; Vindis et al. 2014; Lin et al. 2015) and DRG neurons (Acosta & Davies 2008; Goethals et al. 2010; Due et al. 2012; Tse et al. 2014a; 2014b). More specifically, TLR4 co-localises with markers of peptidergic and non-peptidergic populations within the rat DRG (Due et al. 2012) and the rat and human TG (Wadachi & Hargreaves 2006; Diogenes et al. 2011; Ferraz et al. 2011; Lin et al. 2015). TLR4 expression has also been identified in the peripheral terminals of human TG sensory neurons innervating the tooth pulp (Wadachi & Hargreaves 2006; Ferraz et al. 2011; Lin et al. 2015) and in various locations of the enteric nervous system (Barajon et al. 2009). Information regarding the expression of other cell surface TLRs on sensory neurons is limited. Human and mouse DRG neurons have also been shown to express TLR3, 7 and 9 (Qi et al. 2011). TLR3 and TLR7 co-localise with TRPV1 and gastrin-related peptide (GRP) populations, as well as TRPA1 for TLR7 only (Liu et al. 2010; Liu et al. 2012; Qi et al. 2011; Park et al. 2014). TLR3 and 7 are also expressed in neurons of the enteric nervous system (Barajon et al. 2009). Interestingly, a recent study has demonstrated that TLR5 is expressed by sensory neurons in the DRG (Xu et al. 2015). This expression occurs mainly in large diameter neurons that express neurofilament 200 kDa heavy molecular weight subunit (NF200) thus highlighting a need for more detailed characterisation of previously identified neuronal TLRs. Although mRNA expression of TLR1, 2, 6 and 8 has been reported (Goethals et al. 2010; Ochoa-Cortes et al. 2010) no subsequent study has identified protein expression.
1.6.2 TLR signalling in neurons

Whilst microglia (Jung et al. 2005) and astrocytes (Krasowska-Zoladek et al. 2007; Gorina et al. 2011) signal through both the MyD88-dependent and -independent pathways, the pathways activated downstream from neuronal TLR activation are largely unknown. A role for MyD88 signalling in CNS neuronal TLR signalling has only been demonstrated following TLR7 activation (Liu et al. 2013). Similarly a role for TRIF-dependent signalling has not been directly proven although it is suggested given that the neuronal cell line NT2-N produced IFNβ following TLR3 activation (Prehaud et al. 2005; Lafon et al. 2006). The signalling pathways implicated downstream from neuronal TLR activation within the CNS seem to be dependent on the nature of the activatory stimulus and the specific type of neuron. For example whilst hippocampal neurons utilise NFκB signalling following TLR4 activation to generate a pro-inflammatory response (Hu et al. 2013; Zhao et al. 2014) the activation of TLRs in cortical neurons, following oxygen deprivation and ischemic injury, mediates cell death independently of NFκB (Ma et al. 2006; Cameron et al. 2007; Tang et al. 2007; Leow-Dyke et al. 2012; Tang et al. 2013). Therefore it is suggested that cortical neurons may utilise non-canonical pathways following TLR activation.

The understanding of TLR-associated intracellular pathways activated in primary sensory neurons is limited. Although the presence of MyD88 has been identified in peptidergic DRG neurons (Ochoa-Cortes et al. 2010; Li et al. 2014) and it is known to play an instrumental role in IL-1R signalling (Davis et al. 2006), only one previous study has directly demonstrated that the MyD88-dependent pathway is activated following TLR activation. Following LPS-binding to TLR4 a rapid activation of NFκB, as well as ERK1/2 and p38 MAPK, drives the transcriptional up-regulation of multiple pro-inflammatory factors (Tse et al. 2014a).
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The presence of the TRIF has also been identified in both small, peptidergic and non-peptidergic DRG neurons (Li et al. 2014) although the functionality of the TRIF-dependent pathway has not yet been demonstrated and, following TLR4 activation, no IFNβ production is observed (Tse et al. 2014a). The intracellular signalling pathways utilised by other neuronal TLRs have not been investigated although a functional interaction between TLR7 and TRPA1, independent from MyD88, MAPK and protein kinase signalling, has been demonstrated suggesting that non-canonical pathways may also be implicated in peripheral sensory neurons (Park et al. 2014). It is also suggested that intracellular signalling may not always be required for neuronal TLRs to affect physiological processes as direct activation of sensory neurons has been identified following TLR3, 4 and 7 activation (Due et al. 2012; Liu et al. 2012; Park et al. 2014).

1.6.3 Biological roles of neuronal TLRs

Given the primary role of TLRs in immune cells there is also a probability that neuronal TLRs may be at least partly responsible for the direct detection of infection/injury within the nervous system. Indeed, the direct activation of neuronal TLRs within the CNS have been implicated in the induction of an inflammatory response, neuronal survival and also neuronal development/growth. For example, the activation of TLR4 in cortical and hippocampal neurons induces the transcriptional upregulation of pro-inflammatory mediators such as TNFα, IL-1β, IL-6, regulated on activation, normal T cell expressed and secreted (RANTES) and chemokine CXC motif ligand (CXCL) 1 (Hu et al. 2013; Leow-Dyke et al. 2012; Zhao et al. 2014). In addition, ischemic injury upregulates the expression of TLR2, 4 and 8 and the activation of these TLRs promotes apoptosis (Tang et al. 2007; 2013). A range of endogenous molecules are known to activate these receptors and it is thought that some of these may mediate the observed neuronal
cell death following energy deprivation and ischemic injury. Certain neuronal TLRs are also capable of regulating neuronal growth under physiological conditions. The activation of TLR3, 7 and 8 all reduce neurite outgrowth in cortical and hippocampal neurons suggesting that they negatively regulate neuron growth in the developing and adult brain (Ma et al. 2006; Cameron et al. 2007; Liu et al. 2013; 2015).

Direct activation of peripheral sensory neurons has been observed following the activation of TLR3, 4 and 7, as characterised by the presence of inward currents and an increase in intracellular calcium (Due et al. 2012; Liu et al. 2012; Park et al. 2014). The effects of TLR7 activation have also been shown to be dependent upon an association with TRPA1; removing either TLR7 or TRPA1 from the equation prevented the observed responses (Park et al. 2014). The majority of previous studies have demonstrated the ability of neuronal TLR activation to induce transcriptional and post-translational changes that are known to increase neuronal excitability through mechanisms of peripheral sensitisation. Activation of TLR3, 4, 7 and 9 induces the expression of pro-inflammatory and pro-algesic mediators such as cytokines (TNFα, IL-1α and IL-1β), chemokines (CC motif ligand (CCL) 5 and CXCL10), PGE₂, COX-2 and nociceptin (Acosta & Davies 2008; Ochoa-Cortes et al. 2010; Qi et al. 2011; Tse et al. 2014a). The activation of neuronal TLR4 by endogenous DAMPs has been shown to promote nociception associated with inflammation and tissue damage (Ohara et al. 2013; Miller et al. 2014; Lin et al. 2015). It has also been shown that the activation of neuronal TLR3, 4, 7/8 and 9 induces the transcriptional up-regulation and sensitisation of TRPV1 channels through post-translational modifications (Qi et al. 2011; Ferraz et al. 2011; Diogenes et al. 2011). LPS-dependent activation of TLR4 in TG neurons triggers an influx and intracellular accumulation of calcium ions as well as the sensitisation of TRPV1 and TRPV1-dependent release of CGRP (Diogenes et al. 2011).
2011; Ferraz et al. 2011). Therefore LPS, from odontogenic infections such as *P. gingivalis*, can directly sensitise nociceptors leading to a hypersensitivity to pain. DRG neuron stimulation with TLR3, TLR7/8 and TLR9 ligands also cause an up-regulation of TRPV1 as well as a redistribution of the receptor from neuronal cell bodies to sensory nerve endings (Qi et al. 2011); something which has been reported to be involved in the development of hyperalgesia *in vivo* (Ji et al. 2002). TRPV1 was also sensitised and an increased calcium influx occurred upon activation. The role of TRPV1 in neuronal plasticity is well documented and the above experimental evidence showing TRPV1 sensitisation following neuronal TLR activation provides a key insight into a possible mechanism of TLR-mediated neuronal plasticity that could also be relevant to other signal transduction molecules. The activation of TLR3, but not TLR7, is also necessary for eliciting mechanical, thermal, inflammatory or neuropathic pain in mice whereas both are shown to be important in the induction of an itch response (Liu et al. 2010; 2012). TLR3 K.O. mice display impaired spinal cord synaptic transmission and central sensitisation. Interestingly, the activation of TLR3 and TLR7 have also been shown to mediate pruritis (an itch response). The mechanisms of nociception and pruritis are strikingly similar and both share certain common signalling pathways and cellular receptors/channels.

## 1.7 Hypothesis and aims

The expression of neuronal TLRs on trigeminal sensory neurons represents a potential mechanism for the detection of pathogens that is independent from the innate immune response. However it is important to characterise the expression of TLRs within specific sub-populations of sensory neuron in order to better understand the potential consequences of receptor activation. For example, the
activation of TLRs expressed by nociceptors may have implications for the development of pain hypersensitivity and the orchestration of a local immune response. Therefore it is hypothesised that the activation of trigeminal neuronal TLRs by a range of exogenous ligands, including those associated with periodontitis, is sufficient to bring about alterations in neuronal function that may promote the development and maintenance of a heightened pain response. The specific aims of the thesis are as follows:

- To provide a detailed description of a range of TLR expression within neurochemically-identified sub-populations of sensory neurons within the trigeminal ganglion and to compare this with their distribution within somatic ganglia.

- To investigate TLR-dependent induction of cytokine gene expression in trigeminal ganglion sensory neurons.

- To investigate the potential role for the phospholipid-modifying enzyme LP-CAT in the activation of neuronal TLRs.
Chapter 2

Materials and methods
2. Materials and methods

2.1 Materials & methods

2.1.1 Materials

Table 2.1 details the supplier and catalogue/model number of the reagents, materials, equipment and analysis software that were used in the experiments performed in this thesis, with the exception of antibodies that are detailed in tables 2.3, 2.4, 2.5, 2.6 and 2.7.

2.2 Animal care

Male Sprague Dawley rats (Charles River, UK) were group-housed in a temperature and humidity controlled environment with a 12 h light/dark cycle (lights on at 8:00 A.M.) with food and water available ad libitum. Adult animals, >3 months of age, weighing 250-350 g were used in all experiments. All experiments adhered to guidelines described by Schedule 1 of the UK 1986 Animals (Scientific Procedures) Act. Death was confirmed by the permanent cessation of the heart beat before proceeding with dissection. Table 2.2 details the number of animals used in each experiment.
Table 2.1. Details of the reagents, materials, equipment and software used in the following methods.

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<td>B-27 supplement</td>
<td>ThermoFisher Scientific</td>
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<td>Biotinylated protein ladder</td>
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<td>Bovine serum albumin</td>
<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<td>CLI-095</td>
<td>InvivoGen</td>
<td>thrl-cli95</td>
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<td>DAPI</td>
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<td>Dispase Type II</td>
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<td>Euthatal (sodium pentobarbital)</td>
<td>Merial</td>
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<td>HEPES</td>
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*Table continued on next page.*
2. Materials and methods

*continued from previous page.*

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<td>L-glutamine</td>
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<td>Invitrogen</td>
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<td>Laminin</td>
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<tr>
<td>Luminata Crescendo HRP substrate</td>
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<td>Invitrogen</td>
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<td>NuPAGE LDS sample buffer</td>
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<td>NP00007</td>
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<td>NuPAGE MOPS SDS running buffer</td>
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<td>NP0321BOX</td>
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2. Materials and methods

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0.5 mL tubes                    | Fisher Scientific       | 11508232         |

*Table continued on next page.*
2. Materials and methods

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<td>Nuclease-free 1.5 mL tubes</td>
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2. Materials and methods

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<td>Nikon</td>
<td>Eclipse 80i</td>
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<td>Orbital shaker</td>
<td>Stuart Scientific</td>
<td>SO3</td>
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<td>Vortex</td>
<td>Velp Scientifica</td>
<td>ZXclassic</td>
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<td>PCR miner</td>
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<td>VisionWorks</td>
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End of table.
2. Materials and methods

Table 2.2. Breakdown of animal usage per experiment.

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<td><strong>TLR expression in sensory neurons.</strong></td>
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<td>TLR expression and co-expression with neurochemical markers</td>
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<tr>
<td>TLR-associated molecule gene expression profile</td>
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</tr>
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<td><strong>PAMP-induced cytokine production by sensory neurons.</strong></td>
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</tr>
<tr>
<td>TLR-associated molecule &amp; cytokine gene expression in vitro</td>
<td>6</td>
</tr>
<tr>
<td><em>E. coli</em> LPS-induced cytokine gene expression</td>
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<tr>
<td><em>P. gingivalis</em> LPS-induced cytokine gene expression</td>
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<tr>
<td>Pam3CSK4-induced cytokine gene expression</td>
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<tr>
<td>Imiquimod-induced cytokine gene expression</td>
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<tr>
<td><strong>LPCAT expression in sensory neurons.</strong></td>
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<tr>
<td>LPCAT expression and co-expression with neurochemical markers</td>
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<td>LPCAT gene expression in vitro</td>
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<td><strong>Total</strong></td>
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2.3 Immunohistochemistry (IHC)

2.3.1 Tissue processing

Age-matched animals were euthanized by intraperitoneal (I.P.) injection of a lethal dose of sodium pentobarbital and transcardially perfused with 0.1 M phosphate buffered saline (PBS) (pH 7.4) followed by 4% w/v paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). TG, L4/L5 DRG, maxillary nerve, incisors, lung and
2. Materials and methods

Spleen were dissected and post-fixed in 4% w/v paraformaldehyde in 0.1 M phosphate buffer for 2 h at 4 °C before cryoprotection in 20% w/v sucrose solution overnight at 4 °C. Rat incisors were cracked open to isolate tooth pulp prior to post-fixation. The following day, tissue samples were frozen in OCT compound, using liquid nitrogen, and stored at -80 °C until sectioning. Tissue samples were then cryo-sectioned at 9 µm, sequentially thaw-mounted onto SuperFrost Plus slides and stored at -20 °C in cryoprotection solution (30% w/v sucrose, 30% v/v ethylene glycol in 0.1 M PBS). Sequential sectioning using multiple slides ensured that sections across the whole ganglion were represented on each slide. For example, section 1-10 were mounted onto slides 1-10, respectively. Then section 11-20 were mounted onto slides 1-10 as before. This was repeated until 6-8 tissue sections were mounted onto each slide.

2.3.2 Staining procedure

For expression and co-expression experiments, tissue from 3 individual animals was used and 3 slides of tissue from each source, TG and DRG, were selected per animal. Therefore, for each target protein, roughly 54-72 tissue sections were stained per tissue type.

Slides were chosen at random, placed in a Coplin jar on an orbital shaker and washed 3 x 10 min in 0.1 M PBS. A PAP pen was used to create a hydrophobic barrier around the tissue sections to ensure that liquid did not wick off the slides. Permeabilisation and non-specific blocking stages were combined by incubating sections in 10% v/v donkey serum (in 0.1 M PBS, 0.2% v/v Triton-X-100 and 0.1% v/v sodium azide) for 1 h in a humidity chamber at room temperature. Following further 3 x 10 min washes in 0.1 M PBS, sections were incubated with primary antibodies in a humidity chamber for 24 h at 4 °C, with the exception of
the TLR4 primary antibody which was incubated for 48 h. For dual-labelled co-expression experiments, sections were incubated with primary antibodies against target antigens in conjunction with either TRPV1, P2X3, NF200 or β-III tubulin; see Table 2.3 for more information on primary antibodies used for IHC. Primary antibodies were pooled together before incubation to allow for an even distribution of the two primary antibodies. Following incubation, sections were washed 3 x 10 min in 0.1 M PBS and then incubated with appropriate combinations of species-matched secondary antibodies depending on the primary antibodies used; see Table 2.4 for more information on secondary antibodies used for IHC. Secondary antibodies were again pooled together before application and tissue sections were incubated for 3 h at room temperature in a dark humidity chamber. For all of the following steps, measures were taken to minimise the amount of light the sections were exposed to. Sections were washed 3 x 10 min in 0.1 M PBS and incubated with 4’, 6-diamidino-2-phenylindole (DAPI) (100 ng/mL, in 0.1 M PBS) for 1 h in a humidity chamber at room temperature to stain nuclear chromosomal DNA. One final 10 min wash in 0.1 M PBS was performed before sections were air-dried and a cover slip applied using FluorSave mounting reagent. Slides were left to dry overnight in the dark before being sealed with nail varnish and stored at 4 °C until required for imaging.

2.3.3 Image acquisition & analysis

Images were captured using a Nikon Eclipse 80i epifluorescence microscope equipped with a Nikon DS-Qi1Mc camera using NIS-Elements software. All images were taken using 20X or 60X objectives coupled with a 10X eyepiece. Neuronal soma were identified morphologically as well as by the presence of a DAPI stain; neuronal nuclei generally appear more rounded and slightly darker than those of non-neuronal cells. Only neuronal profiles with a visible nuclear DAPI counterstain
were included in the counting process. For expression and co-expression analysis, 3 images were taken from each tissue section on the slide to ensure that a realistic representation of expression throughout the tissue section as a whole was given. This was repeated on each tissue section (6-8 per slide) so that in total roughly 54-72 images were captured and quantified per ganglion. Image analysis and quantification was performed on monochrome images by a single investigator who was blinded to the slide identity. A threshold for positive counting was set using mean fluorescence intensity units, determined by two independent investigators for each antibody. Subjective visual criteria were first used to determine positive and negative immunoreactivity (IR) for each target protein. A threshold was then determined by considering the fluorescence intensity units from these positive and negative cells. This was performed for each independent experiment to account for variability in staining due to experimental conditions. Co-expression of targets with phenotypic markers was determined by positive identification of markers in individual images obtained by switching between fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) filters. Following identification of positive profiles in individual images, co-expression was confirmed in merged images. Co-expression was expressed as a percentage of target markers expressing phenotypic markers and vice-versa.

2.3.4 Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM), n=3. Comparisons between groups were made using a Student’s t-test, as appropriate, performed on IBM SPSS statistics software (1 animal = 1 unit for statistical analysis). Differences were considered statistically significant when p < 0.05.
2. Materials and methods

2.4 Immunocytochemistry (ICC)

Staining on cultured cells was performed as for immunohistochemistry except for the following variations. Culture medium was aspirated and cells, grown on glass coverslips, were washed briefly in 0.1 M Dulbecco’s PBS (dPBS). Cells were then fixed by incubation with 4% w/v paraformaldehyde in 0.1 M phosphate buffer for 1 h at room temperature. All staining was performed in 6 well plates. Once stained, coverslips were removed from wells, briefly air-dried and inverted onto a drop of FluorSave mounting medium on a SuperFrost microscope slide. Slides were dried, sealed and stored as previously described.

2.5 Antibody characterisation

Tables 2.3 and 2.4 detail the primary and secondary antibodies used for IHC experiments, respectively. A series of quality control experiments were performed to verify the specificity of the antibodies used. The antibodies/antisera used for the neurochemical markers have been categorised elsewhere (Vulchanova et al. 1997; Guo et al. 1999; Michael & Priestley 1999; Kiasalari et al. 2010). These antibodies/antisera performed as previously described when in our hands. Primary rat spleen tissue was used as a positive control for the TLR and LPCAT2 antibodies because of the high proportion of immune cells found in the spleen. Primary lung tissue was used as a positive control for the LPCAT1 antibody due to the high expression of LPCAT1 in lung alveolar cells (Nakanishi et al. 2006). To test the specificity of the TLR2 primary antibody pre-incubation with 10 µg of blocking peptide (Santa Cruz Biotechnology., sc-12504 P) for 2 h at room temperature was performed before staining tissue sections as described previously. No blocking peptides were available for the TLR4, TLR7, LPCAT1 and LPCAT2
2. Materials and methods

Table 2.3. Details of primary antibodies used for IHC/ICC.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Species</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Goat</td>
<td>Mouse, Rat</td>
<td>Santa Cruz Biotechnology (sc-12504)</td>
<td>1:200</td>
</tr>
<tr>
<td>TLR4</td>
<td>Rabbit</td>
<td>Human, Mouse, Rat</td>
<td>Abcam (ab13556)</td>
<td>1:200</td>
</tr>
<tr>
<td>TLR7</td>
<td>Rabbit</td>
<td>Human, Mouse, Rat</td>
<td>Imgenex (IMG-581A)</td>
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<tr>
<td>LPCAT1</td>
<td>Rabbit</td>
<td>Human, Mouse, Rat</td>
<td>ProteinTech (16112-1-AP)</td>
<td>1:200</td>
</tr>
<tr>
<td>LPCAT2</td>
<td>Rabbit</td>
<td>Human, Rat</td>
<td>Novus Biologicals (NBP1-88921)</td>
<td>1:200</td>
</tr>
</tbody>
</table>

**Neurochemical Marker**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Host</th>
<th>Species</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF200</td>
<td>Mouse</td>
<td>Wide species range</td>
<td>Sigma-Aldrich (N0142)</td>
<td>1:4000</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Guinea-Pig</td>
<td>Rat</td>
<td>Neuromics (GP14100)</td>
<td>1:200</td>
</tr>
<tr>
<td>P2X3</td>
<td>Guinea-Pig</td>
<td>Human, Mouse, Rat</td>
<td>Novus Biologicals (NB100-1658)</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-III Tubulin</td>
<td>Mouse</td>
<td>Wide species range</td>
<td>Abcam (ab7751)</td>
<td>1:500</td>
</tr>
</tbody>
</table>

primary antibodies used here. For the TLR4 and TLR7 antibodies we performed staining on tissue known to be negative for the antigen in question. Peripheral rat red blood cells were used as negative controls for both TLR antibodies. LPCAT expression has been identified in a wide range of tissue and cell types. Therefore, in the absence of a suitable negative control sample, the specificity of the LPCAT antibodies were tested by western blotting. Single, specific bands were observed for both antibodies at the expected molecular weights. Non-specific binding of the rabbit primary antibodies was also tested for by using a rabbit polyclonal IgG isotype control (abcam, ab27478). To test the specificity of the secondary antibodies TG sections were incubated with secondary antibodies in the absence of primary antibodies. Unstained TG sections were used to observe any auto-fluorescence within the tissue. See individual experimental chapters for the results of antibody characterisation experiments.
2. Materials and methods

<table>
<thead>
<tr>
<th>Host</th>
<th>Species</th>
<th>Reactivity</th>
<th>Fluorochrome</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey</td>
<td>Goat</td>
<td>Alexa Fluor 488</td>
<td>Abcam (ab150129)</td>
<td>1:500</td>
<td></td>
</tr>
<tr>
<td>Donkey</td>
<td>Rabbit</td>
<td>Alexa Fluor 488</td>
<td>Abcam (ab150073)</td>
<td>5μg/mL</td>
<td></td>
</tr>
<tr>
<td>Donkey</td>
<td>Mouse</td>
<td>Alexa Fluor 555</td>
<td>Life Technologies (A-31570)</td>
<td>5μg/mL</td>
<td></td>
</tr>
<tr>
<td>Donkey</td>
<td>Guinea-Pig</td>
<td>Alexa Fluor 594</td>
<td>Jackson Immuno Research (706-585-148)</td>
<td>1:500</td>
<td></td>
</tr>
</tbody>
</table>

2.6 Western blotting

2.6.1 Tissue processing

Age-matched animals were euthanised by I.P. injection of a lethal dose of sodium pentobarbital and transcardially perfused with 0.1 M PBS (pH 7.4) and TG, L4/5 DRG and cortex were rapidly removed. Tissue was cut up into small pieces and placed in a suitable volume of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM sodium chloride, 0.1% v/v SDS, 1% v/v Triton-X 100, 1% v/v protease inhibitor, 5% v/v phosphatase inhibitor) (50 μL per TG/DRG pair and 200 μL per piece of cortex tissue). Tissue was sonicated on ice (3 bursts of 11 s with 30 s breaks in between bursts) and incubated on ice for 30 min with regular trituration. Lysates were collected by centrifugation at 5,000 x g for 2 min at 4 °C and stored at -80 °C until required. RAW 264.7 lysates were harvested directly from the culture dish. Media was aspirated and cells were washed once with 0.1 M PBS and incubated with RIPA buffer on ice for 30 min. Lysates were collected as above.
2. Materials and methods

2.6.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The total protein content of lysates were quantified using the Micro BCA Protein Assay Kit, according to manufacturers instructions, in a 96 well plate format. Samples were prepared at a concentration of 1 $\mu$g/$\mu$L by adding 12.5 $\mu$L of NuPAGE LDS sample buffer, 5 $\mu$L of NuPAGE sample reducing agent and adjusting the final volume to 50 $\mu$L with MilliQ water. Samples were denatured by heating at 70 °C for 10 min and placed on ice until loading the gel. 10 $\mu$L of biotinylated protein ladder was heated at 90 °C for 4 min and then combined with 10 $\mu$L of SeeBlue and kept on ice ready for loading. NuPAGE Novex 4-12% bis-tris gels were rinsed with deionised water, the comb was gently removed and the wells were washed 3x with running buffer. The gel was inserted into the XCell SureLock Mini-cell and the outer chamber was filled with NuPAGE MOPS SDS running buffer (prepared from 20X stock using deionised water). A smaller volume (200 mL) of running buffer with 2.5% v/v NuPAGE sample reducing agent was used to fill the inner chamber of the mini-cell. 20 $\mu$g (20 $\mu$L) of each sample was loaded into the appropriate wells and 20 $\mu$L of biotinylated protein ladder/SeeBlue mix was loaded into the end well. Proteins were separated by running the gel at 200 V for approximately 50 min.

2.6.3 Transfer onto PVDF membrane

NuPAGE transfer buffer was prepared in deionised water from 20X stock and samples were transferred onto a polyvinylidene fluoride (PVDF) membrane using a wet transfer method performed on an XCell II Blot Module. Membranes were cut to size and activated by immersion in methanol for 15 s followed by 2 min in MilliQ water. Filter papers, membranes and blotting pads were then soaked in NuPAGE
transfer buffer (10% v/v methanol, 1% v/v NuPAGE antioxidant in deionised water). If two gels were being transferred together then the methanol percentage was increased to 20% v/v. The transfer assembly was created by placing a filter paper on the back of the gel and the activated membrane on the front followed by a filter paper on top of this. A roller was used to remove trapped air from the assembly. Two blotting pads were placed on either side of the assembly. The finished assembly was placed into the transfer module making sure of the correct orientation to ensure that proteins would be transferred onto the membrane. The transfer module was placed in the mini-cell and filled with transfer buffer. The outer chamber was also filled with transfer buffer and the transfer was run at 30 V for 90 min. Once the transfer was finished the membrane was placed protein-side up in MilliQ water. Transfer quality was assessed by observing the SeeBlue marker on the membrane and no longer in the gel.

2.6.4 Western blotting

Membranes were blocked with 5% w/v milk powder in PBS 0.1% v/v Tween-20 for 1 h at room temperature on an orbital shaker. Once blocked the membranes were transferred to a 50 mL Falcon tube with the protein side forming a lumen and incubated with primary antibody overnight at 4 °C on a rotator mixer (see table 2.5 for information on primary antibodies). The membranes were then washed 4 x 5 min with 0.1 M PBS, 0.1% v/v Tween-20 on an orbital shaker and incubated with horseradish peroxidase (HRP) linked secondary antibodies (pooled solution of antibodies targeting the primary antibodies of the protein of interest and the biotin marker, see table 2.6 for more details) for 30 minutes at room temperature on an orbital shaker. The membranes were again washed 4 x 5 min with 0.1 M PBS, 0.1% v/v Tween-20 before being developed by incubating with Luminata Crescendo HRP substrate for 5 minutes, protected from light.
2. Materials and methods

Table 2.5. Details of primary antibodies used for western blotting.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Species Reactivity</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPCAT1</td>
<td>Rabbit</td>
<td>Human, Mouse, Rat</td>
<td>ProteinTech (16112-1-AP)</td>
<td>1:1000</td>
</tr>
<tr>
<td>LPCAT2</td>
<td>Rabbit</td>
<td>Human, Rat</td>
<td>Novus Biologicals (NBP1-88921)</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-III Tubulin</td>
<td>Rabbit</td>
<td>Human, Mouse, Rat</td>
<td>Sigma-Aldrich (T2200)</td>
<td>1:1000</td>
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</table>

Table 2.6. Details of secondary antibodies used for western blotting.

<table>
<thead>
<tr>
<th>Host</th>
<th>Target</th>
<th>Tag</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Rabbit IgG</td>
<td>HRP-linked</td>
<td>Abcam (ab6721)</td>
<td>1:2500</td>
</tr>
<tr>
<td>Goat</td>
<td>biotin</td>
<td>HRP-linked</td>
<td>Abcam (ab19221)</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

2.6.5 Image acquisition

Imaging was performed on a ChemiDoc-it² imaging system using VisionWorksLS Acquisition and Analysis software.

2.6.6 Membrane stripping and re-probing

Membranes were stripped to allow for subsequent re-probing for other targets. Membranes were washed 2 x 5 min with 6 M guanidine hydrochloride, 0.1 M
2. Materials and methods

β-mercaptoethanol in 0.1 M PBS, 0.2% v/v Triton-X 100, 0.1% v/v Tween-20 followed by 4 x 5 min washes in 0.1 M PBS, 0.05% v/v Tween-20. Membranes were then re-probed following the same procedure as described above.

2.7 RAW 264.7 cell culture

The RAW 264.7 cell line is an adherent monocyte/macrophage cell line that is frequently used in immunological studies. As macrophages express a full complement of TLR associated molecules, RAW 264.7 cells were used as a positive control for western blotting and qPCR (see Appendix E). RAW 264.7 cells were plated out into 6 well plates at a seeding density of $5 \times 10^5$ cells per well and incubated overnight to allow for cells to adhere to the coverslips. Cells were cultured and maintained as an adherent monolayer in DMEM culture medium (10% foetal calf serum, 1mM L-glutamine) at 37 °C, 5% CO$_2$.

Cultured RAW 264.7 cells were a kindly supplied by Wondwossen Abate and Hanaa Alrammah.

2.8 Primary neuron cell culture

2.8.1 Trigeminal ganglion dissociation

The protocol used for TG dissociation in this study was adapted from Malin et. al. 2007 (more information on the adaptions and optimisation of neuronal culture methods can be found in Appendix C). Briefly, age-matched Sprague-Dawley rats were euthanized by exposure to rising concentrations of CO$_2$. Animals were transcardially perfused with chilled Hank’s buffered salt solution (HBSS, Calcium and
Magnesium-free), TG were rapidly removed and cut into 10-12 small pieces. Tissue was incubated with 1.5 mL of pre-warmed papain solution (60 units papain, 1 mg L-cysteine, 3 µL sodium bicarbonate (NaHCO₃) in HBSS) for 30 min at 37 °C with gentle mixing halfway. Tissue was pelleted by centrifugation at 800 x g for 5 min and the supernatant discarded. The tissue was resuspended in 3 mL of pre-warmed collagenase/dispase solution (12 mg collagenase type II and 14 mg dispase type II in HBSS) and incubated for 30 min at 37 °C with gentle mixing halfway. 3 mL of pre-warmed trypsin inhibitor (1 mg/mL in HBSS) was added to stop enzymatic digestion and tissue was then pelleted by centrifugation at 800 x g for 5 min. A single cell suspension was created by trituration (6-8 times) with a sterile fire-polished, silicon-coated glass pipette in pre-warmed L15 medium (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% v/v Penicillin/Streptomycin (Pen/Strep)) until the mixture appeared cloudy with small chunks of tissue still visible. This cell suspension was then passed through a 30/60% v/v Percoll gradient at 1800 x g for 10 min in order to remove the majority of non-neuronal cells, axonal and cellular debris. The neuron-enriched cell layer and the majority of the 30% Percoll layer (see Fig 2.1) were harvested and diluted in 10 mL of L15 medium. This cell suspension was then centrifuged at 1800 x g for 6 min, the supernatant discarded, and the pellet resuspended in 75 µL/coverslip pre-warmed Neurobasal-A (NBA) medium (2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep).

2.8.2 Cell plating

Glass coverslips were detergent-cleaned and hot-oven sterilised before use. The day before culture, coverslips were placed in a 6 well plate and coated in poly-D-lysine and laminin (both 20 µg/mL in HBSS) and incubated overnight at 4 °C. Before plating, coverslips were warmed to room temperature and washed 2x
2. Materials and methods

Figure 2.1. Cell suspension separation following Percoll spin. Following 10 min centrifugation at 1800 x g, a thin layer of axonal/cellular debris is located on top of the 30% Percoll layer. Neurons are primarily located at the 30%/60% Percoll interface but can also be located in the 30% Percoll layer.

with cell culture water. Cells were plated out onto a suitable number of coverslips depending on the experiment (2 coverslips per animal for ICC, 3 coverslips per animal for qPCR). Coverslips were air-dried and 75 µL of cell suspension was added to each coverslip in a doughnut shape. This prevented the majority of cells from grouping together in the middle of the coverslip. Cells were incubated for 2-3 hours in a 37 °C, 5% CO₂ incubator. Wells were then flooded with 3 mL of NBA medium (2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep) per well. The success of the cell culture was dependent upon the cell plating density. Cells plated at too high/low density would not display the expected level of neurite outgrowth.
2. Materials and methods

2.8.3 Cell culture conditions

Primary neurons were maintained in NBA medium (2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep) at 37°C, 5% CO₂ for a maximum of 2 days. No media changes were required during this period.

2.8.4 Treatment with TLR agonists

Following 48 h in vitro, cells were exposed to a range of TLR agonists for subsequent real-time polymerase chain reaction (qPCR) experiments. Cell culture media was removed and replaced with fresh, pre-warmed NBA media (2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep) containing either 1 µg/mL E. coli LPS, 1 µg/mL P. gingivalis LPS₁-six, 1 µg/mL P. gingivalis LPS₁-four, 500 ng/mL of the synthetic triacylated lipopeptide Pam3 CSK4 or 5 µg/mL of the imidazoquinoline amine analogue imiquimod. Control groups were exposed to NBA medium (2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep) alone. Cells were
2. Materials and methods

incubated for 2 h at 37 °C, 5% CO₂ prior to sample collection.

For TLR4 antagonism experiments cells were pre-treated with 1 µg/mL of the cyclohexene derivative CLI-095 in NBA medium (2% v/v B-27, 0.5 mM Gluta-MAX, 1% v/v Pen/Strep) for 2 h prior to exposure with *E. coli* LPS. The same concentration of CLI-095 was maintained during LPS exposure.

2.9 THP-1 cell culture and differentiation

THP-1 cells were used as a control cell line for *P. gingivalis* LPS isoform exposure. THP-1 cells were maintained as a suspension in Roswell Park Memorial Institute (RPMI)-1640 media (supplemented with 2 mM L-glutamine, 1% v/v Pen/Strep and 10% v/v foetal calf serum) at 37 °C, 5% CO₂. Cells were sub-cultured at a ratio of 1:5 every 3 days.

THP-1 cells were differentiated into adherent M1 and M2 macrophages by treatment with phorbol myristate acetate (PMA) and vitamin D₃, respectively. THP-1 cells were differentiated into pro-inflammatory M1-like macrophages over a period of 3 days. THP-1 cells were incubated with 25 ng/mL of PMA (in culture medium) for 2 days before being further maintained overnight and for more days in fresh media. THP-1 cells were differentiated into anti-inflammatory M2-like macrophages over a period of 7 days. THP-1 cells were incubated with 10 µM of vitamin D₃ (in culture medium) for 4 days, culture media was then replenished and cells were again incubated with 10 µM of vitamin D₃ for a further 3 days. Culture medium was then removed and cells exposed to 1 µg/mL of each individual *P. gingivalis* LPS isoform (in culture medium) for 4 h. Following treatment with *P. gingivalis* LPS isoforms, supernatants were harvested and stored at -20 °C until required for ELISA.
Differentiated M1 and M2 macrophages were kindly supplied by Alexander Strachan.

2. Materials and methods

2.10 Enzyme-linked immunosorbent assay (ELISA)

A 96 well plate was pre-coated with 4 µg/mL of capture antibody in 0.1 M PBS (mouse anti-human TNFα) overnight at 4 °C. Plates were blocked with 2% w/v bovine serum albumin (BSA) in 0.1 M PBS for 1 hour at room temperature. Meanwhile, a series of standards ranging from 13 - 10,000 pg/mL of human recombinant TNFα were prepared. Plates were washed 3x with 0.01% v/v Tween-20 in distilled water and 50 µL of either test sample or standard was added to each well. Plates were then incubated for at least 1.5 h at room temperature. Plates were washed 3x with 0.01% v/v Tween-20 in distilled water and incubated with 0.5 µg/mL of detection antibody (mouse anti-human TNFα) in 0.1 M PBS, 1% w/v BSA for 1 h at room temperature. Plates were again washed 3x with 0.01% v/v Tween-20 in distilled water and incubated with 50 µL biotinylated-streptavidin HRP in 0.1 M PBS, 1% w/v BSA for 1 h at room temperature. Plates were washed 3x with 0.01% v/v Tween-20 in distilled water and 100 µL/well of TMB substrate reagent was added to allow colour to develop at room temperature. The reaction was stopped by adding 50 µL of 1.8 M sulphuric acid (H₂SO₄) to each well. The absorbance at 450 nm was then read on a VersaMax plate reader. The concentration of unknown samples was extrapolated from a standard curve created on SoftMax Pro software.

ELISA was kindly performed by Alexander Strachan.
2. Materials and methods

Table 2.7. Details of antibodies used for ELISA.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Species</th>
<th>Reactivity</th>
<th>Conjugate</th>
<th>Supplier (Cat. Number)</th>
<th>Working Dilution</th>
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<tbody>
<tr>
<td>TNFα</td>
<td>Mouse</td>
<td>Human</td>
<td>None</td>
<td>None</td>
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<tr>
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<td>Human</td>
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<td>Biotin</td>
<td>BD biosciences (554511)</td>
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<td>HRP</td>
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</tr>
</tbody>
</table>

2.11 Real-time polymerase chain reaction (qPCR)

2.11.1 RNA isolation

All reagents used were of RNA grade and all plastics were RNase/DNase free. Total mRNA from dissociated TG neurons and spleen tissue was isolated using RNAqueous-Micro kit according to the manufacturer’s instructions. Cells, grown on glass coverslips, were lysed with 100 μL lysis buffer (4 M guanidium thiocyanate, 0.024 M sodium citrate, 0.49% w/v sarkosyl, 0.13% w/v β-mercaptoethanol in nuclease-free water) per coverslip. Coverslips were scraped and the lysates transferred to 0.5 mL tubes on ice. Typically 3 coverslips were pooled together at this stage to form one sample. Lysates were briefly vortexed and a half volume of 100% ethanol was added (e.g. 50 μL ethanol added to 100
µL lysate). Samples were briefly vortexed and kept on ice whilst loading onto the filter. 150 µL of sample at a time was loaded onto the filter by centrifuging at 16,000 x g for 10 s. Filters were then washed 1x with wash solution 1 and 2x with wash solution 2/3 by centrifugation at 16,000 x g for 10 s. The filter was dried by centrifugation at 16,000 x g for 1 min before the bound RNA was eluted by incubating the filter with 10 µL of pre-warmed (70 °C) elution solution for 1 min and centrifugation at 16,000 x g for 30 s. This elution step was performed twice to yield a final elute of 20 µL. Isolated RNA was treated with DNase I (1 µL DNase I and 2 µL DNase buffer per 20 µL of sample) for 20 min at 37 °C to remove any genomic deoxyribonucleic acid (DNA) contamination. The DNase I enzyme was inactivated by adding inactivation reagent (2 µL per 20 µL sample), incubating for 2 min with a brief vortex halfway and centrifugation at 16,000 x g for 1.5 min.

Total RNA concentration of samples was determined using a Qubit 2.0 Fluorometer with the Qubit microRNA Assay Kit. Typically, cultured neurons from one animal produced samples with a RNA concentration of approximately 10 ng/µL. Sample purity was assessed by measuring 260/280 and 260/230 absorbency ratios using a nanodrop 2000. Only samples with a 260/280 ratio of >1.8 were used for reverse transcription.

2.11.2 Reverse transcription

First-strand complementary DNA (cDNA) was synthesised from 100 ng RNA using the SuperScript VILO cDNA Synthesis Kit, which contained SuperScript III reverse transcriptase, performed on a Veriti thermal cycler. Reaction conditions: 25 °C for 10 min, 42 °C for 2 h, 85 °C for 5 min. cDNA was stored at -20 °C until used for qPCR.
2.11.3 qPCR

Relative quantification was achieved with qPCR using probe-based detection chemistry (see table 2.8 for specific probe information). For the gene expression profile (see Chapter 3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a sole endogenous control gene. For the PAMP-induced cytokine gene expression experiments (see Chapter 5) GAPDH, β-actin (ACTB) and 18s were all used as endogenous control genes. All qPCR experiments used TaqMan Fast Advanced Master Mix, containing AmpliTaq Fast DNA Polymerase, and were performed in triplicate on a QuantStudio 12k Flex system with a Fast 96-well Block using 2.5 ng (1 µL) of template cDNA in a total reaction volume of 10 µL per well (see Appendix D for optimisation experiments). Reaction conditions: 50 °C for 2 min, 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s. An initial no reverse transcription (RT) control for each sample type was performed to assess the effectiveness of the DNAse treatment used which returned an undetectable result. No template controls were also included in every run and returned undetectable results.

2.11.4 Data analysis

A gene expression profile from each tissue type was created using Δ copy threshold (C_T) values, with GAPDH set at 100. Analysis of gene expression data between tissue types was performed using the 2^−ΔΔC_T method (Livak & Schmittgen 2001) with target gene expression in each sample normalised against the endogenous control gene GAPDH.

For PAMP-induced cytokine gene expression experiments data were analysed using the Pfaffl method (Pfaffl 2001). Target gene expression in each sample was
2. Materials and methods

Table 2.8. Details of primer and probe sets used for qPCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Company</th>
<th>Reference/Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn00569848_m1</td>
</tr>
<tr>
<td>MD-1 (LY86)</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn01434815_m1</td>
</tr>
<tr>
<td>MD-2 (LY96)</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn01448830_m1</td>
</tr>
<tr>
<td>CD14</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn00572656_m1</td>
</tr>
<tr>
<td>MyD88</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn01640049_m1</td>
</tr>
<tr>
<td>TRAM</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn02082474_s1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn01525859_g1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn00580432_m1</td>
</tr>
<tr>
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<td>Life Technologies (TaqMan)</td>
<td>Rn01410330_m1</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Life Technologies (TaqMan)</td>
<td>Rn00569434_s1</td>
</tr>
<tr>
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<td>Life Technologies (TaqMan)</td>
<td>Rn01756070_m1</td>
</tr>
<tr>
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<td>Life Technologies (TaqMan)</td>
<td>Rn01531165_m1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Primer Design (geNORM)</td>
<td>ge-DD-12</td>
</tr>
<tr>
<td>ACTB</td>
<td>Primer Design (geNORM)</td>
<td>ge-DD-12</td>
</tr>
<tr>
<td>18s</td>
<td>Primer Design (geNORM)</td>
<td>ge-DD-12</td>
</tr>
</tbody>
</table>

normalised to the geometric mean of the endogenous control genes. Amplification efficiencies and C_T values were calculated on the basis of all fluorescent data points using real time PCR miner online software (Zhao & Fernald 2005).

2.11.5 Statistical analysis

Data are displayed as mean ± SEM, n=3. Comparisons between groups were made using a one-way analysis of variance (ANOVA) with either Tukey’s or Dunnett’s post hoc tests or Student’s t-test, as appropriate, using SPSS statistics software (1 animal/culture preparation = 1 unit for statistical analysis). Differences were considered statistically significant when p < 0.05.
Chapter 3

Toll-like Receptor 4, 2, 7 & associated receptor component expression in trigeminal & dorsal root ganglion sensory neurons.

Published in part as:

3. Introduction

Since the discovery by Wadachi & Hargreaves (2006) of TLR4 expression on trigeminal sensory neurons, a range of functional TLRs have been shown to be expressed by neurons in both the peripheral and central nervous system (see section 1.6.1 for a detailed description). TLR signalling has been identified as a critical component in the development of multiple pain states although this is thought to be mediated by non-neuronal cells such as microglia and infiltrating immune cells (see Nicotra et al. 2012). The discovery that sensory neurons express TLRs and subsequent research has uncovered a mechanism for direct interaction between pathogen and sensory neurons with implications for both nociception and the inflammatory response. Sensory neurons are now considered therefore to possess an innate surveillance function whereby they can directly detect and respond to PAMPs and DAMPs. Given the range of various TLR ligands, of both exogenous and endogenous origin, neuronal TLR signalling has the potential to play a major role in the development and maintenance of chronic inflammatory and neuropathic pain. Despite the identification of TLR expression in peripheral sensory neurons a detailed description of their expression within functional sub-populations is lacking, particularly within the trigeminal system.

Historically, sensory neurons have been divided into various sub-populations based upon their anatomy, physiology and neurochemistry in order to better understand their function and phenotype. It is now widely accepted that multiple types of sensory neuron exist within a sensory ganglion each with distinct properties that contribute to the phenotype of the cell (see Julius & Basbaum 2001). Given this heterogeneity in phenotype, it is important to carefully consider the methods used to characterise neuronal phenotype within the sensory neuron population as a whole. In the present study, TRPV1, P2X3 and NF200 are used as neurochem-
ical markers of neuronal sub-populations in order to characterise the expression patterns of TLR2, TLR4 and TLR7 within sensory ganglia. TRPV1 is a thermal transduction channel that is expressed by a large small-/medium-diameter C- and Aδ-fibres of both peptidergic and, to a lesser extent, non-peptidergic sub-populations within the DRG and TG (Caterina et al. 1997; Tominaga et al. 1998). TRPV1 sensitisation is a key mechanism for the development and maintenance of both inflammatory and neuropathic pain given that a range of pro-inflammatory factors can sensitise the channel (see Levine & Alessandri-Haber 2007 for an in depth review of TRPV1). P2X3 is an ATP-sensitive purinergic receptor that is expressed by small diameter, non-peptidergic C- and Aδ-fibres (Bradbury et al. 1998; Dunn et al. 2001). P2X3-mediated activation has been demonstrated in various models of inflammatory and neuropathic pain (Barclay et al. 2002; Jarvis et al. 2002; McGaraughty et al. 2003). NF200 is often used as a marker of myelinated neurons given that all A fibres display NF200-IR whereas C-fibres do not (Lawson & Waddell 1991).

TLR4 expression has previously been shown to co-localise to a proportion of peptidergic and non-peptidergic populations within the rat DRG (Due et al. 2012) and the rat and human TG (Wadachi & Hargreaves 2006; Diogenes et al. 2011; Lin et al. 2015). TLR4 mRNA has also been identified in sensory neurons of the rat and mouse DRG (Acosta & Davies 2008; Goethals et al. 2010; Tse et al. 2014a; 2014b). In the DRG, the reported percentage of TLR4-positive neurons in vivo is approximately 30% (28-34%, Due et al. 2012; Tse et al. 2014b) and in vitro ranges between 45-60% of total neurons (Acosta & Davies 2008; Tse et al. 2014a). Whilst it has been shown that 19% of neurons in the maxillary region of the TG and 29% of neurons innervating the gingivomucosa express TLR4, mainly in small- to medium-sized neurons (Vindis et al. 2014), there is currently no quantitative data for TLR4 expression within the TG as a whole. TLR4 expression
has also been identified in the peripheral terminals of human TG sensory neurons innervating the tooth pulp (Wadachi & Hargreaves 2006) and in various locations of the enteric nervous system (Barajon et al. 2009).

Previous studies have identified the expression of TLR7 in small/medium sized DRG neurons and co-expression with TRPV1, TRPA1 and gastrin-releasing peptide (GRP) has been observed (Barajon et al. 2009; Liu et al. 2010; Park et al. 2014) although no quantitative analysis has been performed. TLR7 has also been identified in the enteric nervous system (Barajon et al. 2009). TLR7 mRNA has been identified in DRG neurons (Liu et al. 2010) although a separate study did not replicate these findings (Goethals et al. 2010).

Information regarding TLR2 expression in the peripheral sensory neurons is severely limited. Murine DRG sensory and motor neurons reportedly express TLR2 mRNA (Goethals et al. 2010; Ochoa-Cortes et al. 2010) however a separate study suggests that TLR2 protein expression is absent in neurons and is limited to satellite cells within the murine DRG (Kim et al. 2011).

There is also some debate regarding the composition of the TLR4 receptor complex expressed by sensory neurons. In innate immune cells a functional TLR4-signalling complex consists of TLR4, CD14 and MD-2 (Akira & Takeda 2004). Both TG and DRG neurons express CD14 mRNA and protein (Wadachi & Hargreaves 2006; Acosta & Davies 2008; Ochoa-Cortes et al. 2010; Tse et al. 2014a; 2014b). DRG neurons reportedly express MD-1 mRNA and protein but little MD-2 and no RP105 mRNA or protein (Acosta & Davies 2008). Upon activation, neuronal TLR4 is reported to form an atypical co-receptor complex with CD14 and MD-1 (Acosta & Davies 2008). MD-1 classically interacts with the TLR4 homologue, radioprotective 105 (RP105) to regulate TLR4 signalling (Ohto et al. 2011). More recent studies however have shown that DRG neurons do indeed express MD-2
3. TLR expression in sensory neurons.

and RP105 mRNA and protein in addition to MD-1 mRNA and protein (Ochoa-Cortes et al. 2010; Tse et al. 2014a; 2014b). Additionally it was shown that the majority of TLR4-positive neurons co-localise with CD14 and MD-2, rather than MD-1 (Tse et al. 2014a).

Whilst it has been shown that nociceptors express a range of TLRs, a detailed quantitative analysis of TLR expression within multiple sensory neuron sub-populations has not been performed, particularly within the TG. Further describing the expression of TLR2, 4 and 7 within primary sensory neuron populations is a pre-requisite for further functional analysis of receptor activation and the role of these ligands within the trigeminal system as a whole. In the current set of experiments it is hypothesised that nociceptors possess the required molecular components to directly detect and respond to ligands of bacterial, viral and endogenous origin. Using well-defined neurochemical markers, a quantitative analysis of TLR4, TLR2 and TLR7 expression within sensory ganglion neuron sub-populations is performed. The expression of TLRs in peripheral nerve fibres within the tooth pulp and maxillary nerve tissue is also demonstrated. Finally, previous studies have provided conflicting results when describing TLR accessory molecule expression within sensory neurons. Therefore a gene expression profile of TLR signalling-associated components within the TG is described.

3.2 Results

3.2.1 Antibody characterisation

When performing any immunofluorescence analysis of protein expression it is essential to correctly characterise the antibodies used. Therefore a series of control experiments were performed to validate the antibodies used in this study (see
3. TLR expression in sensory neurons.

Multiple TLR2-IR, TLR4-IR and TLR7-IR cells were observed in spleen tissue (Fig. 3.1). No TLR4-, TLR2- or TLR7-specific staining was observed in red blood cells; only a low level of non-specific staining was observed (Fig. 3.3). When the TLR2 antibody was pre-incubated with a blocking peptide, no positive staining was observed in the TG; only a low level of non-specific staining was observed (Fig. 3.4). TG tissue stained with an IgG isotype control did not display any specific, positive staining (Fig. 3.5). The above results demonstrate the specificity of the TLR primary antibodies used in these experiments. No positive staining was observed for all secondary antibodies when primary antibodies were omitted from the staining procedure (Fig. 3.6). This demonstrates the specificity of the secondary antibodies that were used. Finally, a minimal level of autofluorescence was detected in the TG and DRG (Fig. 3.7). Taken together, these control experiments verify that any positive staining observed in subsequent experiments is a true reflection of antigen expression within the tissue and is not due to non-specificity of the antibody.
Figure 3.1. TLR expression in spleen tissue as a positive control. Images show fluorescent micrographs of spleen tissue stained with antibodies against TLR4 (A-B), TLR2 (C-D) and TLR7 (E-F). Multiple positively stained profiles were identified for TLR4 (A), TLR2 (C) and TLR7 (E). Images B, C and F show an overlap of the respective TLR-IR plus DAPI staining. Areas contained within the indicated borders (dashed lines) have been magnified in Fig. 3.2. Scale bar 100 µm.
Figure 3.2. Images show selected areas of Fig. 3.1 that have been magnified to give a more detailed representation of TLR staining within the spleen. Similarly to the previous figure, multiple positively stained profiles were identified for TLR4 (A), TLR2 (C) and TLR7 (E). Images B, C and F show an overlap of the respective TLR-IR plus DAPI staining. Scale bar 100 µm.
Figure 3.3. TLR expression in red blood cells as a negative control. Images show fluorescent micrographs of rat red blood cells stained with antibodies against TLR4 (B), TLR2 (C) and TLR7 (D). Phase contrast microscopy was used to identify red blood cells (A). No positive staining was observed in red blood cells for any of the TLR antibodies used (B-D). Scale bar 50 µm.
Figure 3.4. TLR2 staining in TG after pre-incubation with blocking peptide. Images show fluorescent micrographs of rat TG stained with a pre-adsorbed antibody against TLR2 (A). The TLR2 antibody was incubated with 10 µg of blocking peptide for 2 h at room temperature before staining tissue as previously described. Dual-labelling was performed with NF200 (B) to show a selection of neuronal profiles in more detail. An overlay of combined images with a DAPI counterstain is also included (C). No specific TLR2-IR and a minimal amount of non-specific background staining was observed (A). Scale bar 50 µm.

Figure 3.5. IgG isotype control staining in TG. Images show fluorescent micrographs of TG tissue stained with an IgG isotype control. A low level of non-specific background staining was observed (A). A DAPI counterstain was also included to identify neuronal profiles (B). Scale bar 50 µm.
Figure 3.6. Secondary antibody control staining in spleen tissue. Images show fluorescent micrographs of spleen tissue stained with secondary antibodies in the absence of primary antibodies in order to observe non-specificity of secondary antibodies. Minimal levels of non-specific background staining was observed for DoRb AF488 (A), DoM AF555 (B), DoG AF488 (D) and DoGP AF594 (E). Scale bar 50 µm.

Figure 3.7. Autofluorescence in rat sensory ganglia. Images show fluorescent micrographs of un-stained rat TG (A) and DRG (B) in order to determine the level of autofluorescence within the tissue. A minimal level of autofluorescence was observed under the TRITC channel for both tissues. Scale bar 50 µm.
Figure 3.8. Images show fluorescent micrographs of TG and DRG stained with antibodies specific for TLR4, TLR2 and TLR7. Multiple neuronal profiles display intracellular granular TLR4-IR within the TG (green, A) and DRG (green, B), arrows show selected examples of positive neurons. A proportion of non-neuronal cells that were morphologically identified as satellite cells also display TLR4-IR within both TG and DRG, selected examples indicated by arrowheads (A, B). Multiple neuronal profiles also display TLR2-IR within the TG (green, C) and DRG (green, D), arrows show selected examples of positive neurons. A proportion of non-neuronal cells that do not resemble satellite cells display strong TLR2-IR, selected examples indicated by arrowheads (C, D). TLR7-IR was also identified within the TG (green, E) and DRG (green, F), arrows show selected examples. All non-neuronal cells within the tissue were negative for TLR7. Areas contained within the indicated borders (dashed lines) have been magnified in Fig. 3.9. Scale bar 50 μm. Images representative of three biological replicates.
Figure 3.9. Images show selected areas of Fig. 3.8 that have been magnified to give a more detailed representation of TLR staining patterns within the TG (A, C, E) and DRG (B, D, F). Similarly to the previous figure, arrows show selected examples of neurons that display positive IR for TLR4 (A-B), TLR2 (C-D) and TLR7 (E-F). Arrowheads show selected examples of non-neuronal cells that display positive IR for TLR4 (A-B) and TLR2 (C-D). Scale bar 50 µm. Images representative of three biological replicates.
3. TLR expression in sensory neurons.

3.2.2 TLR4 expression & co-expression within the adult mammalian TG & DRG

Single labelling IHC was performed on rat TG and DRG to observe the cell-specific pattern of expression of TLR4. A quantitative analysis of TLR4 expression within the sensory neuron population as a whole was then performed by expressing TLR4-IR neurons as a percentage of total neuronal profiles. A positive intracellular, granular staining pattern for TLR4 was detected in multiple neuronal profiles within the TG and DRG (Fig. 3.8A-B). The majority of non-neuronal cells in the ganglia were negative for TLR4 although a small proportion of fusiform cells enveloping neuronal soma that were morphologically identified as satellite cells did display weak TLR4-IR (Fig. 3.8A-B). TLR4-IR was identified in 29.27 ± 3.39% and 32.04 ± 2.91% of total neuronal profiles in the TG and DRG, respectively. The difference in TLR4 expression between the TG and DRG was not statistically significant (Student’s t-test).

Dual-labelling IHC of TLR4 with the neurochemical markers TRPV1, P2X3 and NF200 was performed to provide an in-depth description of TLR4 expression with respect to different functional sensory neuron sub-populations. A quantitative analysis of co-localisation was performed by expressing co-labelled neurons as a percentage of marker positive neurons. In the TG, TLR4-IR was identified in 53.7 ± 0.9%, 76.6 ± 0.4% and 3.5 ± 0.7% of TRPV1-, P2X3- and NF200-IR neurons, respectively (Fig. 3.10). Within the DRG, TLR4-IR was identified in 56.1 ± 1.4%, 81.9 ± 0.6% and 4 ± 1.3% of TRPV1-, P2X3- and NF200-IR positive neurons, respectively (Fig. 3.11). The difference in TLR4 co-localisation with neurochemical markers between the TG and DRG was not statistically significant (Student’s t-test). However, TLR4 co-localised more frequently with P2X3-positive neurons compared to TRPV1-positive neurons (TLR4/TRPV1 co-expression vs. TLR4/P2X3 co-expression vs. TLR4/NF200 co-expression).
3. TLR expression in sensory neurons.

TLR4/P2X3 co-expression, $p < 0.0001$ for TG and DRG, Student’s $t$-test).

Alternatively, co-labelled neurons were expressed as a percentage of TLR4-IR neurons. This provides a description of neurochemical marker expression within the total TLR4-positive neuron population. In the TG, $57.8 \pm 0.9\%$, $58.2 \pm 2\%$ and $9.2 \pm 1.5\%$ of TLR4-IR neurons co-expressed the markers TRPV1, P2X3 and NF200, respectively. In the DRG, $68.2 \pm 4.7\%$, $76.5 \pm 2.5\%$ and $6.9 \pm 2.1\%$ of TLR4-IR neurons co-expressed TRPV1, P2X3 and NF200 respectively (see table 3.1 for summary).
Figure 3.10. Fluorescence micrograph of rat TG dual-labelled for TLR4-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR4-IR with neurochemical markers. A substantial proportion of TLR4-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all TRPV1-IR neurons express TLR4, examples indicated by open arrows (A, B, C). A substantial proportion of TLR4-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). Also however, not all P2X3-IR neurons express TLR4, examples indicated by open arrows (D, E, F). There was minimal co-localisation between TLR4-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR4-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
Figure 3.11. Fluorescence micrograph of rat DRG dual-labelled for TLR4-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR4-IR with neurochemical markers. A substantial proportion of TLR4-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all TRPV1-IR neurons express TLR4, examples indicated by open arrows (A, B, C). A substantial proportion of TLR4-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). There was minimal co-localisation between TLR4-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR4-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
3. TLR expression in sensory neurons.

3.2.3 TLR2 expression & co-expression within the adult mammalian TG & DRG

Single labelling IHC was performed on rat TG and DRG to observe the cell-specific pattern of expression of TLR2. A quantitative analysis of TLR2 expression within the sensory neuron population as a whole was then performed by expressing TLR2-IR neurons as a percentage of total neuronal profiles as defined using a DAPI counterstain. TLR2-IR was identified in multiple neuronal profiles within the TG and DRG (Fig. 3.8C-D). The majority of non-neuronal cells within the ganglia were negative for TLR2 although a small number of cells displayed very strong TLR2-IR (Fig. 3.8C-D). TLR2 was expressed by $32.6 \pm 3.5\%$ and $42.5 \pm 5.1\%$ of total neuronal profiles in the TG and DRG, respectively. The difference in TLR2 expression between the TG and DRG was not statistically significant (Student’s $t$-test).

Dual-labelling IHC of TLR2 with the neurochemical markers TRPV1, P2X3 and NF200 was performed to provide an in-depth description of TLR2 expression with respect to different functional sensory neuron sub-populations. A quantitative analysis of co-localisation was performed by expressing co-labelled neurons as a percentage of marker positive neurons. In the TG, TLR2 was expressed by $65.6 \pm 4.9\%$ and $66.1 \pm 4.5\%$ of TRPV1 and P2X3 positive neurons, respectively (Fig. 3.12A-F). TLR2 did not co-localise with NF200 positive neurons (Fig. 3.12G-I). In the DRG, TLR2 was expressed by $83.5 \pm 1.9\%$ and $87.2 \pm 1.5\%$ of TRPV1 and P2X3 positive neurons, respectively (3.13A-F). The difference in TLR2 co-localisation with each marker between the TG and DRG was statistically significant (TRPV1 TG vs. DRG, $p < 0.05$; P2X3 TG vs. DRG, $p < 0.05$, Student’s $t$-test). Again, in the DRG, TLR2 did not co-localise with NF200 (Fig. 3.13G-I).
Alternatively, co-labelled neurons were expressed as a percentage of TLR2-IR neurons. This provides a description of neurochemical marker expression within the total TLR2-positive neuron population. In the TG, $67.6 \pm 2.6\%$ and $49.8 \pm 1.9\%$ of TLR2-positive neurons co-expressed TRPV1 and P2X3, respectively. In the DRG, $79.1 \pm 2.9\%$ and $64.6 \pm 2.1\%$ of TLR2-positive neurons co-expressed TRPV1 and P2X3, respectively (see table 3.1 for summary).
Figure 3.12. Fluorescence micrograph of rat TG dual-labelled for TLR2-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR2-IR with neurochemical markers. A substantial proportion of TLR4-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all TRPV1-IR neurons express TLR2, examples indicated by open arrows (A, B, C). A substantial proportion of TLR2-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). There was minimal co-localisation between TLR2-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR2-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
Figure 3.13. Fluorescence micrograph of rat DRG dual-labelled for TLR2-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR2-IR with neurochemical markers. A substantial proportion of TLR4-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all TRPV1-IR neurons express TLR2, examples indicated by open arrows (A, B, C). A substantial proportion of TLR2-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). There was minimal co-localisation between TLR2-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR2-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
3.2.4 TLR7 expression & co-expression within the adult mammalian TG & DRG

Single labelling IHC was performed on rat TG and DRG to observe the cell-specific pattern of expression of TLR7. A quantitative analysis of TLR7 expression within the sensory neuron population as a whole was then performed by expressing TLR7-IR neurons as a percentage of total neuronal profiles as defined using a DAPI counterstain. Similarly to both TLR4 and TLR2, TLR7-IR was identified in multiple neuronal profiles within the TG and DRG (Fig. 3.8E-F). However, unlike TLR4 and TLR2, TLR7 staining was restricted to neurons and was excluded from all non-neuronal cells within the ganglia. TLR7 was expressed by 32.4 ± 1.8% and 35 ± 3.9% of total neuronal profiles in the TG and DRG, respectively. The difference in TLR7 expression between the TG and DRG is not considered statistically significant (Student’s *t*-test).

Dual-labelling IHC of TLR7 with the neurochemical markers TRPV1, P2X3 and NF200 was performed to provide an in-depth description of TLR7 expression with respect to different functional sensory neuron sub-populations. A quantitative analysis of co-localisation was performed by expressing co-labelled neurons as a percentage of marker positive neurons. In the TG, TLR7 was expressed by 75.7 ± 2.5% and 61.6 ± 2.1% of TRPV1 and P2X3 positive neurons respectively (Fig. 3.14A-F). TLR7 did not co-localise with NF200 positive neurons (Fig. 3.14G-I). Within the DRG, TLR7 was expressed by 78.6 ± 2.6% and 54.8 ± 9.4% of TRPV1 and P2X3 positive neurons respectively (Fig. 3.15A-F). Again, TLR7 and NF200 expression were mutually exclusive (Fig. 3.15G-I). The difference in co-expression of TLR7 with neurochemical markers between the TG and DRG is not considered statistically significant (Student’s *t*-test. However TLR7 co-localised more frequently with TRPV1-positive neurons compared to P2X3-positive neurons in
three tissues (TLR7/TRPV1 co-expression vs. TLR7/P2X3 co-expression, \( p < 0.05 \) for TG, Student’s \( t \)-test. The difference was not statistically significant in the DRG (\( p = 0.07 \)).

Alternatively, co-labelled neurons were expressed as a percentage of TLR7-IR neurons. This provides a description of neurochemical marker expression within the total TLR7-positive neuron population. In the TG, 43.4 \( \pm \) 8.1\% and 34.8 \( \pm \) 3.1\% of TLR7-positive neurons also expressed TRPV1 and P2X3, respectively. In the DRG, 43.2 \( \pm \) 10.5\% and 32.2 \( \pm \) 4.8\% of TLR7-positive neurons also expressed TRPV1 and P2X3, respectively.

Table 3.1 and Fig. 3.16 provide a full summary of TLR4, TLR2 and TLR7 co-expression with neurochemical markers within the TG and DRG.
Figure 3.14. Fluorescence micrograph of rat TG double labelled for TLR7-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR7-IR with neurochemical markers. A substantial proportion of TLR7-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Some neurons expressing low levels of TRPV1-IR neurons do not display TLR7-IR, examples indicated by open arrows (A, B, C). A substantial proportion of TLR7-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). Also however, not all P2X3-IR neurons display TLR7-IR, examples indicated by open arrows (D, E, F). There was minimal co-localisation between TLR7-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The vast majority of NF200-IR neurons did not express TLR7-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
Figure 3.15. Fluorescence micrograph of rat DRG double labelled for TLR7-IR (green, A, D, G) plus either TRPV1-IR (red, B), P2X3-IR (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of TLR7-IR with neurochemical markers. A substantial proportion of TLR7-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Some neurons expressing low levels of TRPV1-IR do not display TLR7-IR, examples indicated by open arrows (A, B, C). A substantial proportion of TLR7-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). There was minimal co-localisation between TLR7-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR7-IR, examples indicated by open arrows (G, H, I). Scale bar 50µm. Images representative of three biological replicates.
3. TLR expression in sensory neurons.

3.2.5 TLR expression in peripheral nerve fibres innervating orofacial tissues.

Dual-labelling IHC was performed in order to investigate the expression of TLRs along the peripheral maxillary nerve and nerve terminals of TG sensory neurons within maxillary incisor dental pulp, using β-III-tubulin as a marker of peripheral neuron terminals/axons. A high degree of non-specific binding of the TLR4 antibody was observed in peripheral tissues and it was therefore difficult to draw any valid conclusions about TLR4 expression in peripheral nerve fibres. Multiple peripheral nerve axons within the tooth pulp display both TLR2- and TLR7-IR (Fig. 3.17A-C and Fig. 3.17D-F, respectively). Multiple axons within the maxillary nerve also display TLR2- and TLR7-IR (Fig. 3.18A-C and Fig. 3.18D-F,
### Table 3.1. Summary of TLR4, TLR2 and TLR7 co-expression with neurochemical markers within the TG and DRG.

<table>
<thead>
<tr>
<th>Neurochemical label</th>
<th>% of labelled cells that express TLR4</th>
<th>% of TLR4-positive cells that express label</th>
<th>% of labelled cells that express TLR2</th>
<th>% of TLR2-positive cells that express label</th>
<th>% of labelled cells that express TLR7</th>
<th>% of TLR7-positive cells that express label</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV1</td>
<td>53.7 ± 0.9%</td>
<td>57.8 ± 0.9%</td>
<td>65.6 ± 4.9%</td>
<td>67.6 ± 2.6%</td>
<td>75.7 ± 2.5%</td>
<td>42.6 ± 8.1%</td>
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<tr>
<td>TRPV1</td>
<td>56.1 ± 1.4%</td>
<td>61.4 ± 1.4%</td>
<td>75.7 ± 2.5%</td>
<td>67.6 ± 2.6%</td>
<td>81.9 ± 0.6%</td>
<td>44.1 ± 3.3%</td>
</tr>
<tr>
<td>P2X3</td>
<td>76.6 ± 0.4%</td>
<td>58.2 ± 2.0%</td>
<td>66.1 ± 4.5%</td>
<td>49.8 ± 1.9%</td>
<td>61.6 ± 2.1%</td>
<td>33.4 ± 3.1%</td>
</tr>
<tr>
<td>P2X3</td>
<td>76.4 ± 0.4%</td>
<td>49.8 ± 1.9%</td>
<td>61.6 ± 2.1%</td>
<td>33.4 ± 3.1%</td>
<td>9.2 ± 1.3%</td>
<td>6.9 ± 1.3%</td>
</tr>
<tr>
<td>NF200</td>
<td>3.5 ± 0.7%</td>
<td>9.2 ± 1.3%</td>
<td>6.9 ± 1.3%</td>
<td>3.5 ± 0.7%</td>
<td>9.2 ± 1.3%</td>
<td>6.9 ± 1.3%</td>
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<tr>
<td>NF200</td>
<td>3.4 ± 3.1%</td>
<td>9.2 ± 1.3%</td>
<td>6.9 ± 1.3%</td>
<td>3.5 ± 0.7%</td>
<td>9.2 ± 1.3%</td>
<td>6.9 ± 1.3%</td>
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<tr>
<td>TG</td>
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<td>DRG</td>
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3. TLR expression in sensory neurons.
3. TLR expression in sensory neurons.

Figure 3.17. TLR2 (A-C) and TLR7 (D-F) expression in nerve fibres innervating the rat dental pulp. Images show fluorescent micrographs of rat incisor tooth pulp co-stained with antibodies against either TLR2 (A) or TLR7 (D) and β-III-tubulin (B, E). Combined images (C, F) also include a DAPI counterstain. Both TLR2-IR and TLR7-IR were detected within multiple nerve fibres. Scale bar 50 µm. Images representative of three biological replicates.

respectively). Collectively these images suggest that TLR2 and TLR7 are indeed expressed by trigeminal sensory neurons within peripheral tissues.

3.2.6 Expression of TLR4 signalling-associated molecules in TG sensory neurons

A gene expression profile for a selection of TLR signalling-associated components was compiled by performing qPCR on acutely dissociated TG sensory neurons. Specifically, the gene expression of TLR4 and three TLR4 accessory molecules, MD-1, MD-2 and CD14, as well as two intracellular signalling molecules that represent two pathways of the TLR intracellular signalling cascade, MyD88 and TRAM, was evaluated. The MyD88 pathway is active downstream from all other TLRs (including TLR2 and TLR7), with the exception of TLR3 which signals solely via the MyD88-independent pathway. TLR4 is unique in the fact that it
Figure 3.18. TLR2 (A-C) and TLR7 (D-F) expression in nerve fibres contained within the maxillary nerve. Images show fluorescent micrographs of rat maxillary nerve co-stained with antibodies against either TLR2 (A) or TLR7 (D) and β-III tubulin (B, E). Combined images (C, F) also include a DAPI counterstain. Both TLR2-IR and TLR7-IR were detected within multiple nerve fibres. Scale bar 50 µm. Images representative of three biological replicates.

can activate both pathways following receptor activation. For reference purposes, the pain-associated genes for TRPV1, P2X3 and TrkA were also included. The expression of these target genes relative to the housekeeping gene GAPDH (set at 100) is shown for TG sensory neurons (Fig. 3.19A) and control (spleen tissue, Fig. 3.19B), respectively. All target genes were expressed in TG sensory neurons ($C_T < 35$) albeit at lower levels than TRPV1, P2X3 and TRKA expression, respectively (Fig. 3.19A). Gene expression levels of the TLR-associated components within TG sensory neurons were also compared to that of primary spleen tissue which is known to contain a broad range of TLR-expressing immune cells. All genes were expressed at lower levels in TG sensory neurons than in spleen tissue (Fig. 3.19C). MD-1 was expressed $290.1 \pm 36.3$ fold lower in TG sensory neurons compared to primary spleen tissue although this was not surprising given the high B lymphocyte content of spleen tissue (Cesta 2006). TLR4 gene expression was the most comparable between the different tissue types with a $9.1 \pm 1.1$ fold lower
expression in TG sensory neurons. The remaining targets were expressed $9.8 \pm 2.1$ (TRAM), $13 \pm 2.1$ (MD-2), $24.5 \pm 3.1$ (MyD88) and $40 \pm 8.2$ (CD14) fold lower in TG sensory neurons than the spleen.

### 3.3 Discussion

Previous studies have shown both TLR4 and TLR7 to be expressed by putative nociceptive neurons within mammalian sensory ganglia (Wadachi & Hargreaves 2006; Liu et al. 2010; Qi et al. 2011; Due et al. 2012; Park et al. 2014; Lin et al. 2015) whereas TLR2 expression is restricted to non-neuronal cells (Kim et al. 2011). In the current study, it is demonstrated that TLR4, TLR2 and TLR7 are all expressed by sensory neurons within the TG and DRG. Further, the expression of these TLRs specifically localised to functional sub-populations of nociceptive neurons. Co-localisation of TLR4 and TLR7 with TRPV1 has been shown previously (Wadachi & Hargreaves 2006; Park et al. 2014; Lin et al. 2015), however this is the first quantitative description of their expression within adult mammalian sensory ganglia in relation to functional neuronal phenotype.

The degree of TLR4 expression for both TG and DRG falls within the range of expression of TLR4 previously observed in DRG sections (28-34% of total neurons) (Due et al. 2012; Tse et al. 2014b). Certain properties of sensory neurons, including the expression and co-expression between phenotypic markers, have been shown to vary between the TG and DRG (see Bereiter et al. 2009) however there was no significant difference in expression levels of TLR4 or TLR7 between the tissue types. TLR2 was however expressed by a larger proportion of neurons in the DRG compared to the TG (approximately 10%), although this was not considered statistically significant. Previous studies have reported that non-neuronal cells in peripheral ganglia do not express TLR4 (Due et al. 2012; Tse et al. 2014a)
3. TLR expression in sensory neurons.

Figure 3.19. TLR4 signalling-associated component gene expression in dissociated TG sensory neurons (A) and spleen tissue (B) as determined by qPCR. All genes are expressed relative to GAPDH levels (set at 100). Three pain-associated genes are also included in (A) for reference purposes. All targets were expressed in sensory neurons ($C_T < 35$). All TLR-associated genes were expressed at noticeably lower levels than each of the three pain-associated genes, respectively. The difference in gene expression between TG sensory neurons and spleen tissue was calculated using the $\Delta \Delta C_T$ method (C). All targets were expressed in TG sensory neurons at lower levels compared to spleen tissue. Data are displayed as mean ± S.E.M., n = 3 individual cell preparations.
however here we show that a proportion of satellite cells do indeed display TLR4-IR. This observation has been confirmed in a recent study that also identified TLR4-IR in satellite cells within the TG (Lin et al. 2015). Although Tse et al., 2014a, 2014b found that non-neuronal cells within the DRG did not express TLR4 under normal conditions they subsequently showed that they do indeed begin to express TLR4 once contact with neurons is removed in vitro. Therefore it seems as though non-neuronal cells express TLR4 in an inducible manner and therefore may play a role in neuroimmune signalling within the PNS.

TLR4, TLR2 and TLR7 are expressed by both heat- and ATP-responsive nociceptors in the TG and DRG due to a high degree of co-expression with TRPV1 and P2X3, respectively. Interestingly there was a noticeable difference in co-localisation levels of certain TLRs with the different markers. Co-expression data shows that TLR4 more readily co-localised with P2X3- compared to TRPV1-expressing neurons in the TG and DRG. Conversely, TLR7 more readily co-localised with TRPV1- compared to P2X3-expressing neurons in the TG and DRG. The amount of co-localisation of TLR2 with TRPV1- and P2X3-expressing neurons is comparable in the TG and DRG although the percentage of co-localisation with both markers is statistically higher in the DRG. The degree of co-localisation with either marker also varies when comparing between the three TLRs. In the TG, TLR7 displayed a greater percentage of co-localisation to TRPV1-positive neurons when compared to TLR2 and, similarly, TLR2 displayed a greater percentage when compared to TLR4 (TLR7>TLR2>TLR4). When comparing co-localisation of TLRs with P2X3 the observed pattern is TLR4>TLR2≈TLR7. In the DRG a different pattern is observed. With respect to co-localisation with TRPV1-positive neurons, TLR2 displayed a comparable amount of co-localisation to TLR7 and both of these TLRs displayed larger degree of co-localisation compared to TLR4 (TLR2≈TLR7>TLR4). When compar-
ing co-localisation with P2X3 the observed pattern is TLR2≈TLR4>TLR7. The functional significance of these observations are currently unknown although Park et al. (2014) have recently shown that TLR7 interacts with TRPA1, expressed by a proportion of TRPV1-positive neurons, to directly activate sensory neurons. With respect to TLR2, these comparisons correspond to the observation that TLR2 is expressed by a greater number of total neurons in the DRG. It is worth noting here that there is a degree of co-expression between TRPV1- and P2X3-expressing populations reported in previous literature (Guo et al. 1999; Ichikawa & Sugimoto 2004; Kim, Chung, Jo, Kim, Bae, Jung, Kim & Oh 2011) and therefore a degree of overlap in the current co-expression data must be considered. It has been shown that P2X3 activity can mediate subsequent TRPV1 sensitisation (Saloman et al. 2012). Therefore, given the high degree of co-expression with each marker described here, it is probable that a proportion of TLR-positive neurons co-express both TRPV1 and P2X3. We found minimal co-localisation between TLR4, TLR2 and TLR7 with NF200 suggesting that these TLRs selectively localise to C-fibre nociceptors, but not low-threshold non-nociceptive sensory neurons. Preferential and direct activation and/or sensitisation of nociceptors by pathogens and damage-released endogenous molecules via TLR4, TLR2 and TLR7 is therefore highly likely.

There is now extensive evidence for the involvement of neuroimmune signalling in the development and maintenance of chronic pain states (reviewed by Nicotra et al. 2012). TLRs are an important class of receptor in the complex intercellular signalling network that develops in the setting of chronic pain. TLR4 is the most widely studied member of the receptor family in this setting and it is rapidly up-regulated following the onset of inflammatory and neuropathic pain (DeLeo et al. 2004; Raghavendra et al. 2004). TLR4 deletion (Tanga et al. 2005; Agalave et al. 2015) and pharmacological inhibition are both able to prevent (Bettoni
3. TLR expression in sensory neurons.

et al. 2008) and reverse behavioural hypersensitivity (Hutchinson et al. 2007; 2008; 2010; Lan et al. 2010; Wu et al. 2010) and decrease the production of pro-inflammatory mediators in models of neuropathic pain (Tanga et al. 2005; Lan et al. 2010). Importantly, the involvement of TLR4 in the development and maintenance of chronic pain has also been demonstrated in a human model. A low intravenous dose of LPS sensitised nociceptors to a subsequent capsaicin challenge and participants experienced an increase in capsaicin-dependent flare, allodynia and hyperalgesia; although the effect of LPS on neurons in this study is likely to be mainly indirect (Hutchinson et al. 2013). Findings from past studies have been extended to include the contribution of TLR2 and TLR3 to central nervous system preclinical pain models (Kim et al. 2007; Obata et al. 2008; Mei et al. 2011). TLR3 (Liu et al. 2012) and TLR7 (Park et al. 2014) activation has also been shown to directly activate peripheral sensory neurons in the murine DRG. Early research in this field has focussed mainly on TLR4 however more TLRs are emerging as important mediators of pain hypersensitivity. Whilst the role, for example, of TLR7 in clinical pain models has yet to be studied, peripheral blood mononuclear cells from chronic pain patients display increased responsiveness to TLR7 ligand stimulation, as well as TLR2 and TLR4 (Kwok et al. 2012).

From the results presented in this chapter it can be inferred that, in several of these settings, TLR activation is likely to occur directly on sensory neurons and is not necessarily dependent on the activation of the innate immune system. The ability of neurons to directly detect and respond to pathogenic ligands independently from the innate immune system represents a rapid response mechanism that has significant consequences for nociceptor activation, sensitisation and altered neuronal-non-neuronal cell communication. Following TLR4 activation, for example, peripheral sensory neurons display increased excitability (Ochoa-Cortes et al. 2010; Due et al. 2012), sensitisation of TRPV1 and potentiation of
3. TLR expression in sensory neurons.

TRPV1-dependent neuropeptide production (Diogenes et al. 2011; Ferraz et al. 2011). Various pro-inflammatory mediators are also induced downstream from acute TLR4 activation (Ochoa-Cortes et al. 2010; Tse et al. 2014a). Neuronal TLR7 activation induces rapid inward currents and action potentials to increase neuronal excitability through mechanisms involving TRPA1 (Liu et al. 2010; 2012; Park et al. 2014). An up-regulation and sensitisation of TRPV1 and induction of pro-inflammatory mediators is also observed following neuronal TLR7 activation (Qi et al. 2011). It is likely that these pro-inflammatory mediators can signal through autocrine and/or paracrine mechanisms to activate nociceptors, further alter transcriptional/translational expression of various factors and contribute to the local inflammatory response (see Sommer & Kress 2004; Binshtok et al. 2008; Milligan & Watkins 2009; Üceyler et al. 2009 for focussed reviews). Therefore the activation of neuronal TLRs may also be relevant for the acute pain response. As previously mentioned, the expression of TLR2 has not been previously identified in peripheral sensory afferents and therefore the functional implications downstream from neuronal TLR2 activation is unknown. In chapter 4 of this thesis we demonstrate that neuronal TLR2 is functional and capable of generating a response following ligand exposure. Despite this, a significant amount of work is required to fully understand the properties of neuronal TLR2 and its ability to modulate nociceptor physiology.

The expression of TLRs in TG neurons within peripheral tissues has great implications for oral pathology. The various densely innervated, specialised sensory organs innervated by the trigeminal nerve combined with the plethora of invasive pathogens implicated in infections of the oral cavity would suggest that direct pathogen-neuron interactions are a frequent occurrence in multiple disease settings. TLR4 expression has been identified in tooth pulp previously (Wadachi & Hargreaves 2006) and here it is shown that TLR2 and TLR7 are also expressed
in neurons that innervate the rat tooth pulp, which are considered to be largely nociceptive (Vang et al. 2012). Therefore bacteria such as *P. gingivalis* (TLR4) and *S. mutans* (TLR2) as well as a range of enteroviruses (TLR7), e.g. coxsackievirus, may potentially interact with neuronal TLRs to directly activate or sensitise nociceptors and contribute towards disease pathology. A range of tissue-damage released endogenous molecules are now known to activate various TLRs (see Yu et al. 2010) and neuronal TLR activation via endogenous ligands has been demonstrated in the TG (Ohara et al. 2013) and DRG (Miller et al. 2014; Park et al. 2014). Acute and chronic tissue damage associated with oral diseases, such as later-stage gingivitis, may therefore also promote pain hypersensitivity directly via the activation of neuronal TLRs.

This study is the first to demonstrate a co-localisation of TLR4, TLR2 and TLR7 with P2X3-expressing neurons. P2X3 activation, by ATP, causes rapid nociceptor depolarisation and plays a prominent role in the sensitisation of nociceptors and alteration of neuronal/non-neuronal cell communication following the onset of inflammation (Oliveira et al. 2005; Fabbretti 2013) and nerve injury (Honore et al. 2002; Hsieh et al. 2012). LPS has been shown to evoke an upregulation and sensitisation of P2X3 receptors in primary trigeminal ganglion neuron cultures however it is not known whether this is through a direct action on neurons or secondary to the activation of non-neuronal cells and subsequent release of ATP and inflammatory mediators (Franceschini et al. 2013). Here it is demonstrated that a large proportion of P2X3-positive neurons co-express TLR4 suggesting that LPS could directly modulate P2X3 receptor function. A large proportion of P2X3-positive neurons co-express TLR4, TLR2 and TLR7 suggesting that other bacterial (both Gram-negative and Gram-positive) and viral infection could also directly modulate P2X3 receptor function. It is therefore reasonable to suggest that the activation of neuronal TLRs could be sufficient to sensitise P2X3 in a
3. TLR expression in sensory neurons.

similar manner to that of TLR4-dependent TRPV1 sensitisation.

The exact composition of the TLR4 co-receptor complex that is required for neuronal TLR4 activation remains unclear. The co-localisation of TLR4 with the co-receptor CD14 has been identified in capsaicin-responsive nociceptors within the TG (Wadachi & Hargreaves 2006) and the involvement of CD14 in neuropathic pain has been demonstrated (Cao et al. 2009). However there is conflicting evidence as to whether MD-1 or MD-2 are involved in neuronal TLR4 signalling. In innate immune cells the TLR4 receptor complex consists of TLR4, CD14 and MD-2 (Akashi-Takamura & Miyake 2008). MD-1, a MD-2 homologue, normally forms a complex with RP105, a TLR4 homologue that lacks an intracellular TIR homology domain (Medzhitov 2001). One study has shown that neuronal TLR4 interacts with MD-1 rather than the conventional co-receptor MD-2 (Acosta & Davies 2008). This latter study also showed a lack of MD-2 expression in DRG sensory neurons. However more recent studies have identified both MD-1 and MD-2 mRNA and protein expression in DRG nociceptors and shown a preferential co-localisation of TLR4 with MD-2 (Ochoa-Cortes et al. 2010; Tse et al. 2014a). Results presented in this chapter show that acutely dissociated TG sensory neuron preparations express TLR4, CD14, MD-1, MD-2, MyD88 and TRAM mRNA. This in agreement with Tse et al. (2014a) and Ochoa-Cortes et al. (2010) that sensory neurons express both MD-1 and MD-2. MyD88 and TRAM expression suggests that both the MyD88-dependent and MyD88-independent branches of the TLR4-signalling pathway are available following neuronal TLR4 activation. MyD88-dependent signalling downstream of neuronal TLR4 and IL-1β receptor activation has been shown (Davis et al. 2006; Qi et al. 2011; Tse et al. 2014a) however the role of MyD88-independent signalling in peripheral sensory neurons is yet to be demonstrated. The expression of all targets within the TG were much lower than three common pain-associated genes (TRPV1, P2X3 and TrkA) al-
though this was expected given the primary function of neurons. The expression of each target in the TG was lower than the corresponding expression in spleen tissue. Again this was expected given that TLR signalling is an essential component of innate immune cells that can be found in large numbers in the spleen. The acutely dissociated sensory ganglia cell preparations used in this study contained a proportion of non-neuronal cells and as we have shown that a small number of satellite cells display TLR4-IR it is therefore not possible to directly assign this gene expression purely to neurons. However, given the TLR expression patterns observed in this study as well as the techniques used when dissociating ganglia we believe that it is reasonable to suggest that the gene expression is largely neuronal. It is worth noting that co-receptor components, such as MD-2 and CD14, exist in soluble forms that are secreted from cells to actively participate in the LPS response (Bazil et al. 1989; Visintin et al. 2001). Therefore co-receptor components do not necessarily need to be expressed by neurons to mediate neuronal TLR4 signalling, a phenomenon which has been demonstrated in lung epithelial cells (Kennedy et al. 2004).

In summary, the results presented in this chapter demonstrate that a substantial proportion of nociceptors within sensory ganglia express TLR4, TLR2 and TLR7. It is also shown that TLR2 and TLR7 are expressed in peripheral nerve fibres within the dental pulp. Therefore it is likely that these TLRs may be activated by their respective agonists (see Table 1.1) under pathological conditions. The activation of TLRs may be sufficient to induce an alteration in nociceptor physiology and may contribute towards the initiation and maintenance of pain hypersensitivity. Collectively these results support the hypothesis that nociceptors possess the required molecular components to directly detect and respond to ligands of bacterial, viral and endogenous origin independently from the innate immune response.
Chapter 4

The TLR-dependent induction of cytokine gene expression in trigeminal sensory neurons \textit{in vitro}.
4. Cytokine gene expression.

4.1 Introduction

TLR signalling in innate immune cells represents a critical component of the first line of defence against invading pathogens. Following receptor activation, downstream signalling cascades ultimately result in the production of various inflammatory mediators, such as cytokines and chemokines, and the up-regulation of co-stimulatory molecules. TLR signalling is therefore a major driving factor for the initiation and orchestration of the localised inflammatory response and the subsequent development of a tailored adaptive immune response. An in-depth review of TLR signalling pathways can be found in Chapter 1 of this thesis. TLR signalling in non-neuronal cells, such as glial cells and immune cells within the CNS and in peripheral tissues, is a critical component of a neuroimmune axis that develops with inflammatory and neuropathic pain states. The activation of these TLRs ultimately results in the indirect sensitisation of neurons associated with a prolonged immune response (see Nicotra et al. 2012). Various pro-inflammatory cytokines, for example, that are produced by innate immune cells at the site of infection/injury can interact with specific receptors on peripheral sensory neurons to directly activate and/or sensitise the cell. TNFα, IL-1β and IL-6 are perhaps the most characterised cytokines that are known to induce neuronal plasticity (see Miller et al. 2009) however some other novel cytokines such as IL-17 (Segond von Banchet et al. 2013) and IL-33 (Han et al. 2013) have also been demonstrated to directly alter sensory neuron responses.

As mentioned in previous chapters, TLRs have been implicated in various models of chronic pain. The majority of these past studies, however, have suggested that the activation of TLRs is likely to occur on non-neuronal cells and infiltrating immune cells. More recently, it has been demonstrated that TLR activation may occur directly on sensory neurons. For example, the activation of TLR3 (Liu...
et al. 2012) and TLR7 (Park et al. 2014) activation has been shown to directly activate peripheral sensory neurons in the murine DRG. Further, the activation of neuronal TLRs has also been shown to induce transcriptional, translational and post-translational alterations to transduction channels and neuropeptide production. The majority of past studies have been performed using DRG neurons with only a limited number focussing on TG neurons. In the trigeminal system, the activation of neuronal TLR4 by *P. gingivalis* LPS sensitises and up-regulates TRPV1 and potentiates the capsaicin-induced release of the vasoactive neuropeptide CGRP (Diogenes et al. 2011; Ferraz et al. 2011). A role for neuronal TLR4 in mediating pain induced by endogenous compounds released following oral tissue damage has also been demonstrated (Ohara et al. 2013). This latter study showed that the administration of HSP70 or LPS to the rat tooth resulted in a TLR4-dependent increase in mechanical and thermal hyperalgesia. The activation of neuronal TLR4 has been shown to induce inward currents (Ochoa-Cortes et al. 2010; Due et al. 2012), nociceptin expression (Acosta & Davies 2008) and the MyD88-dependent production of pro-inflammatory mediators (Ochoa-Cortes et al. 2010; Tse et al. 2014a; Lin et al. 2015) in murine DRG neurons. Certain DAMPs are also capable of activating TLR4 on DRG neurons to promote the production of pro-inflammatory mediators (Miller et al. 2014). Activation of TLR3 and TLR7 directly activates DRG nociceptors and also induces an itch response (pruritis) (Liu et al. 2010; 2012). More recently the activation of TLR7, by endogenous microRNAs (miRNA), has been shown to rapidly activate DRG neurons through mechanisms that involve TRPA1 (Park et al. 2014). Activation of TLR3, 7 and 9 also induces the production of pro-inflammatory mediators, up-regulates TRPV1 expression and sensitises TRPV1 activation in DRG neurons (Qi et al. 2011). Given the unique location and developmental origin of the TG as well as the specialist sensory organs that are innervated by trigeminal sensory neurons it would be incorrect to assume that the properties of TLRs expressed on DRG
neurons translate to those of TG neurons. Indeed many differences between TG and DRG neurons have been documented (see Bereiter et al. 2009). Therefore further characterisation of neuronal TLRs within the trigeminal system is essential in order to understand their role in the acute pain response and also in the development of chronic pain following oral infection/injury.

A recent oral microbiome sequencing study showed that the oral flora associated with a healthy oral cavity consists of approximately 750 different microbial species and the levels of roughly 200 of these species were significantly altered in sub-gingival plaques found in periodontal disease (Ge et al. 2013). Three ‘red complex’ bacteria have been identified in chronic periodontal disease that are thought to play a key orchestrating role in tissue destruction and disease progression (Socransky et al. 1998). *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* can act in a synergistic fashion to cause the destruction of periodontal tissue. It is now widely accepted that *P. gingivalis* has a central role in the development of both chronic and aggressive periodontal disease. Although *P. gingivalis* is capable of initiating an innate immune response it has recently been suggested that the bacterium acts as a keystone pathogen to promote an overall shift in microbiome behaviour and create a dysbiotic relationship with the host (Hajishengallis et al. 2012). Multiple pathogenic species within the mature plaque then contribute toward the development of a chronic inflammatory response in sub-gingival tissue and the subsequent tissue damage that is associated with the chronic disease. Although the majority of bacterial species implicated in chronic periodontal disease are Gram-negative anaerobic bacteria a host of other microbial species have been associated with the disease such as Gram-positive bacteria and also certain viruses and fungi (see Wade 2013). The polymicrobial and chronic nature of periodontal disease means that there are an array of potential PAMPs and DAMPs contained within the localised area of infection that could
potentially interact with neuronal TLRs and other innate receptor families.

*P. gingivalis* possesses multiple virulence factors, including LPS, that promote immune activation and tissue damage associated with periodontal disease (Bainbridge & Darveau 2001). The bacterium releases copious amounts of LPS, often contained within vesicles coated in fimbriae, that are able to penetrate host tissue and actively promote tissue destruction (Grenier & Mayrand 1987). LPS consists of three major components; an outer polysaccharide, an oligosaccharide core and an inner lipid A region (Dixon and Darveau, 2005). Lipid A is a phosphorylated glucosamine disaccharide that has multiple attached fatty acids and is the crucial component of LPS that is responsible for the activation of TLR4 (Park et al. 2009). *P. gingivalis* LPS is known to contain multiple, structurally different, lipid A species with varying affinity for TLR4 and therefore a differing level of inflammatory potency (Kumada et al. 1995; Darveau et al. 2004; Al-Qutub et al. 2006; Herath et al. 2013). Whereas the immune response to canonical LPS from *E. coli* is well characterised, that of *P. gingivalis* LPS and other non-enterobacterial species has been the source of past debate. *E. coli* LPS is delivered to CD14, either in the soluble form or expressed on the host cell membrane, by LPS binding protein (LBP). The LPS is then transferred to a receptor complex consisting of TLR4 and MD-2. LPS binding causes receptor dimerisation and the subsequent activation of intracellular signalling cascades associated with host defence mechanisms. *P. gingivalis* LPS however has previously been shown to signal through both TLR2 (Bainbridge & Darveau 2001; Hirschfeld et al. 2001; Martin et al. 2001) and TLR4 (Tabeta et al. 2000; Ogawa et al. 2002) with varying downstream effects. The ability of *P. gingivalis* LPS to activate TLR2 has previously been attributed to lipopeptide contamination of isolated LPS however it is now understood that the bacterium is indeed able to signal through TLR2 and TLR4 due to the heterogeneity of *P. gingivalis* lipid A structures (Darveau
Specifically, *P. gingivalis* lipid A structures can vary in the composition of the attached fatty acids and number of phosphorylation sites within the molecule (Dixon & Darveau 2005). Di-, mono- and non-phosphorylated as well as penta- and tetra-acylated isoforms of *P. gingivalis* lipid A have been identified (Kumada et al. 1995). The bacterium is able to actively alter the phosphorylation and acylation status of lipid A molecules in response to changes in the local environment (Al-Qutub et al. 2006; Curtis et al. 2011). It is these variations in lipid A structure that ultimately determine the receptor affinity and inflammatory potency of the LPS structure. For example, synthetic di-phosphorylated, penta-acylated is particularly potent TLR4 agonist (Kumada et al. 2008; Coats et al. 2009). In addition, mono-phosphorylated, penta-acylated (LPS1690) also displays strong TLR4 activity (Reife et al. 2006) whereas mono-phosphorylated, tetra-acylated (LPS1435) is able to interact with both TLR2 and TLR4 although it does not generate a strong host response (Darveau et al. 2004). In human gingival fibroblasts *P. gingivalis* LPS1690 up-regulates the expression of IL-6 and IL-8, whereas *P. gingivalis* LPS1435 does not (Herath et al. 2013). Interestingly, the non-phosphorylated isoform of *P. gingivalis* lipid A is a particularly weak TLR4 agonist and both mono-phosphorylated and non-phosphorylated isoforms display TLR4 antagonist activity (Coats et al. 2009). Therefore when examining the biological roles of *P. gingivalis* LPS it is critically important to pay particular attention to the individual isoforms of lipid A present.

The microbiology of periodontal disease, as detailed above, demonstrates the complexity and multifactorial nature of many oral diseases. This, combined with the dense innervation of oral tissues, makes the direct interaction between neurons and the oral flora a likely, and regular, occurrence. In the case of orofacial diseases the presence of more potent and pathogenic PAMPs, as well as DAMPs released fol-
following tissue damage, is likely to be detected by neuronal TLRs. This interaction may contribute towards the initiation of a local inflammatory response and peripheral sensitisation of nociceptors. Although significant progress has been made over the past decade, since Wadachi and Hargreaves (2006) first demonstrated that TG sensory neurons express TLR4, our understanding of the functional consequences of neuronal TLR activation remains limited, particularly within the trigeminal system. As mentioned previously, the detection of \textit{P. gingivalis} LPS by TG neurons expressing TLR4 has been demonstrated (Diogenes et al. 2011; Ferraz et al. 2011) however the induction of pro-inflammatory cytokines following TLR activation has not been demonstrated within the TG. In the previous chapter we have demonstrated that trigeminal nociceptors express TLR4, TLR2 and TLR7 under normal conditions. In this chapter it is hypothesised that these TLRs are capable of detecting and responding to a range of pathogenic ligands. Using primary neuronal cultures it is demonstrated that TG sensory neurons are capable of directly responding to acute stimulation by agonists of the aforementioned TLRs resulting in the induction of pro-inflammatory cytokine gene expression. The cytokine response of TG sensory neurons to multiple isoforms of \textit{P. gingivalis} LPS is also a focus of this chapter.

\section*{4.2 Results}

\subsection*{4.2.1 Primary neuronal cell culture}

Cell cultures were closely monitored during culture and assessed for cell viability at 48 h \textit{in vitro}. Visual checks of cell appearance were made at 2 h, 12 h, 24 h and 48 h \textit{in vitro} with specific focus on the appearance of cytoplasm and membrane integrity. Furthermore, neurite outgrowth within three separate culture prepara-
4. Cytokine gene expression.

Figure 4.1. An example of neurite outgrowth of TG sensory neurons after 48 h in vitro, demonstrated by β-III-tubulin staining. Arrows show examples of neurons with extensive neurite outgrowth whereas open arrows show an example of a neuron that did not display any visible outgrowth. Image representative of 3 individual culture preparations. Scale bar 50 µm.

The expressions was assessed by ICC at 48 h in vitro, using β-III-tubulin as a neuron-specific stain. All neurons were counted manually and the percentage of cells that displayed neurite outgrowth was expressed as a percentage of total neurons (Fig. 4.1). The calculated neuronal viability at 48 h in vitro was 91.5 ± 3.3% (n=3 separate cultures).

4.2.2 TLR co-receptor gene expression in vitro

Cultured primary neurons are not in a native state in vitro as they have undergone axotomy during cell isolation. They may therefore demonstrate injury-induced transcriptional alterations that could influence their response to exogenous ligands. Prior to exposing cells to exogenous TLR ligands it was therefore important to measure the change in the gene expression of TLR4 and associated co-receptors/signalling molecules under normal culture conditions. TLR4, MD-1,
Table 4.1. Summary of qPCR data showing the changes in TLR4, MD-1, MD-2, CD14, MyD88, TRAM, TNFα, IL-1β, IL-6 and IFNβ mRNA over 48 h in vitro. All fold changes are relative to the endogenous control genes GAPDH, ACTB and 18s. Data presented as mean ± S.E.M. n = 3 individual culture preparations.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Relative fold change at 12 h in vitro</th>
<th>Relative fold change at 48 h in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>1.21 ± 0.27 fold decrease</td>
<td>1.63 ± 0.5 fold increase</td>
</tr>
<tr>
<td>MD-1</td>
<td>1.71 ± 0.22 fold decrease</td>
<td>6.13 ± 0.07 fold decrease</td>
</tr>
<tr>
<td>MD-2</td>
<td>1.42 ± 0.02 fold increase</td>
<td>2.42 ± 0.46 fold increase</td>
</tr>
<tr>
<td>CD14</td>
<td>7.41 ± 1.64 fold increase</td>
<td>5.7 ± 1.63 fold increase</td>
</tr>
<tr>
<td>MyD88</td>
<td>1.73 ± 0.27 fold increase</td>
<td>1.82 ± 0.54 fold increase</td>
</tr>
<tr>
<td>TRAM</td>
<td>1.05 ± 0.03 fold decrease</td>
<td>1.29 ± 0.15 fold increase</td>
</tr>
<tr>
<td>TNFα</td>
<td>2.25 ± 0.67 fold increase</td>
<td>2.55 ± 0.96 fold increase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.9 ± 0.57 fold increase</td>
<td>1.21 ± 0.25 fold decrease</td>
</tr>
<tr>
<td>IL-6</td>
<td>109.33 ± 50.82 fold increase</td>
<td>16.92 ± 5 fold increase</td>
</tr>
<tr>
<td>IFNβ</td>
<td>8.9 ± 2.77 fold increase</td>
<td>5.55 ± 1.89 fold increase</td>
</tr>
</tbody>
</table>

MD-2, CD14, MyD88 and TRAM mRNA levels were measured at 12 h and 48 h in vitro by qPCR, using acutely dissociated tissue (0 h) as a reference sample. TLR4, MD-2, MyD88 and TRAM mRNA levels did not display any significant changes in gene expression at either 12 h or 48 h. MD-1 mRNA expression was not significantly different to control at 12 h (1.71 ± 0.22 fold decrease) however at 48 h MD-1 mRNA was significantly down-regulated by 6.13 ± 0.07 fold \((p < 0.001, 48 \text{ h vs. 0 h})\). CD14 mRNA was significantly up-regulated by 7.41 ± 1.64 fold and 5.7 ± 1.63 fold at 12 h \((p < 0.001, 12 \text{ h vs. 0 h})\) and 48 h \((p < 0.01, 48 \text{ h vs. 0 h})\), respectively. All fold changes are relative to the endogenous control genes GAPDH, ACTB and 18s. See figure 4.2 and table 4.1 for summary data.

These data indicate that, at 12 h and 48 h in vitro, the gene expression of TLR4, MD-2, MyD88 and TRAM are not significantly affected by axotomy whereas MD-1 and CD14 are.
4. Cytokine gene expression.

4.2.3 Cytokine gene expression in vitro

Similar considerations were also taken into account with regard to the expression of pro-inflammatory cytokines in vitro. Therefore changes in TNFα, IL-1β, IL-6 and IFNβ mRNA levels were measured at 12 h and 48 h in vitro by qPCR, again using acutely dissociated tissue (0 h) as a reference sample (Fig. 4.3). TNFα and IFNβ mRNA did not significantly differ from control levels at 12 h and 48 h in vitro. IL-6 was significantly up-regulated by 109.33 ± 50.82 fold and 16.92 ± 5 fold at 12 h (p < 0.01, 12 h vs. 0 h) and 48 h (p < 0.01, 48 h vs. 0 h), respectively. IFNβ was significantly up-regulated by 8.9 ± 2.77 fold and 5.55 ± 1.89 fold at 12 h (p < 0.01, 12 h vs. 0 h) and 48 h (p < 0.01, 48 h vs. 0 h), respectively. All fold changes are relative to the endogenous control genes GAPDH, ACTB and 18s. See figure 4.3 and table 4.1 for summary data.
4. Cytokine gene expression.

Figure 4.3. Relative gene expression of TNFα, IL-1β, IL-6 and IFNβ in primary sensory neuron cultures over 48 h \textit{in vitro}. A; IL-6 mRNA is significantly elevated at 12 h (**, $p < 0.01$, 12 h vs. 0 h) and begins to return towards control levels at 48 h, although it remains significantly elevated compared to acutely dissociated tissue (**, $p < 0.01$, 48 h vs. 0 h). B; Replication of the same graph as in A with a smaller scale to allow the differences in gene expression of other cytokines to be examined in more detail. TNFα and IL-1β do not display any significant differences in gene expression at 12 h or 48 h \textit{in vitro}. IFNβ mRNA was significantly up-regulated at 12 h (**, $p < 0.01$, 12 h vs. 0 h) and 48 h (**, $p < 0.01$, 48 h vs. 0 h) \textit{in vitro}. All statistical comparisons were made using one-way ANOVA with a Dunnett’s \textit{post hoc} analysis. Data presented as mean ± S.E.M.
These data indicate that axotomy significantly induces the increased gene expression of IL-6 and IFNβ whilst the gene expression of TNFα and IL-1β remain unaltered at 12 h and 48 h in vitro.

### 4.2.4 PAMP-induced induction of cytokine gene expression in TG sensory neurons *in vitro*

#### 4.2.4.1 *E. coli* LPS-dependent induction of cytokine gene expression

In order to demonstrate that TG neurons express functional TLR4, cultured neurons were exposed to *E. coli* LPS (1 µg/mL for 2 h) after 48 h in vitro. TNFα and IL-1β mRNA were both significantly up-regulated by 8.08 ± 0.47 fold (*p* < 0.001, *E. coli* LPS treated vs. control) and 4.11 ± 0.45 fold (*p* < 0.01, *E. coli* LPS treated vs. control), respectively (Fig. 4.4A). IL-6 (1.05 ± 0.06 fold increase) and IFNβ (1.3 ± 0.22 fold increase) did not show significant change when compared to control.

Pre-treatment with the TLR4-specific inhibitor CLI-095 (1 µg/mL, 2 h pre-treatment) prior to LPS exposure significantly reduced the LPS-induced expression of both TNFα (*p* < 0.01, control+LPS vs. CLI-095+LPS) and IL-1β (*p* < 0.01 control+LPS vs. CLI-095+LPS)(Fig. 4.4B-C). TNFα remained significantly up-regulated by 3.37 ± 0.74 fold when exposed to LPS following pre-treatment with the inhibitor (*p* < 0.01, CLI-095+control vs. CLI-095+LPS). No up-regulation of IL-1β was observed in the presence of the inhibitor alone.

These data show that *E. coli* LPS is able to significantly induce the transcriptional up-regulation of TNFα and IL-1β in a TLR4-dependent manner but has no effect on IL-6 and IFNβ gene expression.
4. Cytokine gene expression.

Figure 4.4. *E. coli*-dependent induction of cytokine mRNA expression via TLR4. A; *E. coli* LPS exposure (1 µg/mL, 2 h) resulted in a significant up-regulation in TNFα (***, p < 0.001, *E. coli* LPS treated vs. control) and IL-1β mRNA (**, p < 0.01, *E. coli* LPS treated vs. control) whilst IL-6 and IFNβ mRNA were unaltered. B; Pre-treatment with CLI-095, a TLR4-specific inhibitor, significantly reduced the induction of TNFα by approximately 60% (**, p < 0.01, LPS vs. CLI-095+LPS). TNFα remained significantly up-regulated in the presence of the inhibitor (**, p < 0.01, CLI-095 vs. CLI-095+LPS). C; Pre-treatment with CLI-095 completely inhibited the induction of IL-1β (**, p < 0.01, LPS vs. CLI-095+LPS). All statistical comparisons were made using one-way ANOVA with a Tukey’s post hoc analysis. Data presented as mean ± S.E.M.

4.2.4.2 *P. gingivalis* LPS-dependent induction of cytokine gene expression

In a series of further experiments, TG neurons were treated with two separate isoforms of *P. gingivalis* LPS, LPS1690 and LPS1435. Changes in cytokine gene expression were again measured by qPCR (Fig. 4.5). *P. gingivalis* LPS1690 treatment did not have any significant effect upon the gene expression levels of either TNFα (1.16 ± 0.11 fold decrease), IL-1β (1.23 ± 1.19 fold decrease), IL-6 (1.19 ± 0.3 fold increase) or IFNβ (1.2 ± 0.14 fold decrease). Similarly, following exposure with *P. gingivalis* LPS1435, TNFα (1.25 ± 0.23 fold increase), IL-1β (1.04 ± 0.32 fold increase), IL-6 (1.25 ± 0.37 fold decrease) and IFNβ (1.42 ± 0.3 fold decrease) gene expression levels were not significantly altered compared to control.
4. Cytokine gene expression.

Figure 4.5. Relative gene expression of TNFα, IL-1β, IL-6 and IFNβ following exposure to \( P. \) gingivalis LPS \( 1690 \) and LPS \( 1435 \) (1 \( \mu \)g/mL, 2 h). Neither isoform of \( P. \) gingivalis LPS induced a significant alteration in expression of any target gene compared to control expression. All statistical comparisons were made using one-way ANOVA with a Tukey’s post hoc analysis. Data presented as mean ± S.E.M.

Figure 4.6. \( P. \) gingivalis LPS isoform-dependent (1 \( \mu \)g/mL, 4 h) induction of TNFα production by M1 and M2 macrophages differentiated from THP-1 cells. \( P. \) gingivalis LPS \( 1690 \) and LPS \( 1435 \) both significantly induced the increased production of TNFα by M1 and M2 macrophages compared to unstimulated cells (***, \( p < 0.001 \)). All statistical comparisons were made using one-way ANOVA with a Dunnett’s post hoc analysis. Data presented as mean ± S.E.M.
Bio-activity of the *P. gingivalis* LPS isoforms were confirmed by exposing M1 and M2 macrophages, differentiated from THP-1 cells, to each individual LPS isoform (1 µg/mL, 4 h). The production of TNFα was then measured by ELISA (Fig. 4.6). *P. gingivalis* LPS\textsubscript{1690} significantly induced the production and release of 8995.29 ± 971.18 pg/10\textsuperscript{6} cells (\(p < 0.001\), one-way ANOVA with Dunnett’s post hoc analysis) and 1764.48 ± 19.17 pg/10\textsuperscript{6} cells (\(p < 0.001\), one-way ANOVA with Dunnett’s post hoc analysis) by M1 and M2 macrophages, respectively. Similarly, *P. gingivalis* LPS\textsubscript{1435} significantly induced the production and release of 33545.9 ± 811.65 pg/10\textsuperscript{6} cells (\(p < 0.001\)) and 6566.6 ± 105.4 pg/10\textsuperscript{6} cells (\(p < 0.001\)) by M1 and M2 macrophages, respectively.

These data show that neither *P. gingivalis* LPS\textsubscript{1690} or LPS\textsubscript{1435} were able to significantly induce the transcriptional alteration in TNFα, IL-1β, IL-6 or IFNβ within TG neurons *in vitro*. Both isoforms however were able to induce an expected response in a macrophage cell line.

### 4.2.4.3 Pam3 CSK4-dependent induction of cytokine gene expression

*P. gingivalis* LPS has been previously shown to signal via both TLR4 and TLR2. In the previous chapter it was also shown that a significant proportion of nociceptors express TLR2. Therefore, in order to demonstrate the functionality of TLR2 in TG sensory neurons, cells were exposed to the TLR2/1 heterodimer-specific synthetic agonist Pam3 CSK4 (500 ng/mL, 2 h). The relative gene expression of pro-inflammatory cytokines were measured by qPCR (Fig. 4.7). TNFα mRNA was significantly up-regulated by 18.21 ± 1.53 fold (\(p < 0.001\), Pam3 CSK4 treated vs. control). IL-1β mRNA was also significantly up-regulated by 4.6 ± 0.34 fold (\(p < 0.001\), Pam3 CSK4 treated vs. control). IL-6 (1.68 ± 0.24 fold increase) and IFNβ (1.59 ± 0.43 fold increase) mRNA levels were not significantly altered
4. Cytokine gene expression.

Figure 4.7. Relative gene expression of TNFα, IL-1β, IL-6 and IFNβ following Pam3 CSK4 exposure (500 ng/mL, 2 h). Exposure to Pam3 CSK4 induced the significant up-regulation of TNFα (***, $p < 0.001$, Pam3 CSK4 treated vs. control) and IL-1β mRNA (***, $p < 0.001$, Pam3 CSK4 treated vs. control). IL-6 and IFNβ mRNA expression were not statistically different from the control group. All statistical comparisons were made using one-way ANOVA with a Tukey’s post hoc analysis. Data presented as mean ± S.E.M.

These data suggest that TG sensory neurons express functional TLR2, the activation of which significantly induces the transcriptional up-regulation of TNFα and IL-1β.

4.2.4.4 Imiquimod-dependent induction of cytokine gene expression

In addition to TLR4 and TLR2, the previous chapter identifies the expression of TLR7 in trigeminal nociceptors. To test the functionality of neuronal TLR7, cells were exposed to the synthetic TLR7 agonist imiquimod (5 µg/mL, 2 h). Relative gene expression of cytokines were measured by qPCR (Fig. 4.8). TNFα mRNA was significantly up-regulated by 12.34 ± 1.23 fold ($p < 0.001$, imiquimod treated vs. control). IL-1β mRNA was also significantly up-regulated by 3.66 ± 0.53 fold compared to control.

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4. Cytokine gene expression.

![Graph showing relative gene expression of TNFα, IL-1β, IL-6, and IFNβ following imiquimod exposure.](image)

**Figure 4.8.** Relative gene expression of TNFα, IL-1β, IL-6 and IFNβ following imiquimod exposure (5 µg/mL, 2 h). Exposure to imiquimod induced the significant up-regulation of TNFα (***, p < 0.001, imiquimod treated vs. control) and IL-1β mRNA (*, p < 0.05, imiquimod treated vs. control). IL-6 and IFNβ mRNA expression were not statistically different from the control group. All statistical comparisons were made using one-way ANOVA with a Tukey’s post hoc analysis. Data presented as mean ± S.E.M.

(p < 0.05, imiquimod treated vs. control). Again IL-6 (2.11 ± 0.55 fold increase) and IFNβ (1.47 ± 0.27 fold increase) mRNA levels were not significantly altered compared to control.

These data suggest that TG sensory neurons express functional TLR7 and receptor activation, similarly to TLR4 and TLR2, significantly induces the transcriptional up-regulation of TNFα and IL-1β.

### 4.3 Discussion

The functional activation of neuronal TLR4, TLR2 and TLR7 is demonstrated in this chapter through their capacity to induce the increased gene expression of pro-inflammatory cytokines following ligand-induced receptor activation. The activation of TLR4, TLR2 and TLR7 by well characterised agonists was suf-
4. Cytokine gene expression.

Sufficient to induce the transcriptional up-regulation of the pro-inflammatory and pro-nociceptive cytokines TNFα and IL-1β.

Although the use of primary neuronal cultures is a powerful tool to determine cellular signalling mechanisms, the technique has a number of limitations that must be considered when interpreting results. First, it is important to understand that neuronal cultures do not represent normal, uninjured neurons. The dissection and dissociation process exposes the cell preparation to multiple sources of potential stress and injury. The initial dissection and mechanical dissociation inflicts a complete axotomy to peripheral and central axons which is associated with a distinct set of transcriptional and translational changes. Additionally, the enzymatical dissociation process could potentially contribute to cellular stress. Therefore a range of transcriptional responses to such stress may alter cell physiology *in vitro*. Alterations in gene/protein expression would also be expected *in vitro* as the neurons initiate a regeneration programme. This is illustrated in the fact that neuronal cultures display extensive neurite outgrowth after 48 h (see Fig. 4.1). Results in the current study show however that the expression of the majority of receptor components are not significantly altered at either time point. MD-1 is significantly down-regulated at 48 h and CD14 is significantly up-regulated at both time points. CD14 mRNA expression peaks at 12 h and begins to return toward control levels at 48 h whereas MD-1 mRNA shows a progressive down-regulation over the 48 h.

The injury-dependent expression of TLR receptor components has not previously been investigated in cultured sensory neurons, however CD14 is shown to be up-regulated in spinal microglia following peripheral nerve injury (Tanga et al. 2004). It is therefore not surprising therefore that the expression of CD14 in neurons is also altered under the conditions encountered in the present study. Changes in MD-1 expression has not been previously observed following nerve injury and the functional consequences of this altered expression are currently unknown.
Furthermore the present study shows that, following neuronal injury, the gene expression of two of the cytokines that were central to this investigation (TNFα and IL-1β) remain unaltered, whilst a further two (IL-6 and IFNβ) were significantly up-regulated. The increase in IFNβ production is interesting as it is associated with TRIF-dependent (MyD88-independent) signalling and this pathway has yet to be demonstrated downstream from neuronal TLR activation. Although the majority of cellular debris is removed during the Percoll gradient stage of the dissociation process a small amount remains in culture. A range of tissue damage-associated mediators, associated with the presence of cellular debris, may therefore be able to interact with neurons in vitro and could potentially induce the up-regulation of IFNβ mRNA expression. IL-6 mRNA is substantially up-regulated at 12 h but, similarly to IFNβ, this induction is greatly reduced at 48 h. The induction of IL-6 gene expression is expected as it is well accepted that IL-6 production is induced following nerve injury (Murphy et al. 1995) and contributes toward the regeneration of injured neurons (Zhong et al. 1999) and the activation of non-neuronal cells (Klein et al. 1997). The time course of IL-6 mRNA expression in the present study is comparable to previous in vivo experiments (Murphy et al. 1995) as an increase in IL-6 gene expression was identified within 1 day of neuronal injury. This increase in IL-6 gene expression, combined with the extensive neurite outgrowth, also demonstrates the viability of the neuronal cultures used in the present study. Since there were no major changes in the gene expression of TLR receptor components or cytokines at 48 h in vitro, with the exception of IL-6, and any changes observed in gene expression levels that did vary from control levels were generally less pronounced at 48 h, this time point was chosen for exposure to TLR agonists.

The second major limitation of the use of primary neuronal cultures is the difficulty to identify the specific cellular origin of ligand-induced cytokine expression.
4. Cytokine gene expression.

The neuronal cultures used in the present study contain a small proportion of non-neuronal cells and cellular debris. The majority of non-neuronal cells and debris are removed by the Percoll gradient spin however a small proportion of each remain and are present in vitro. Further, the dissociation process does not favour one particular size or functional sub-type of neuron. Therefore both nociceptor and non-nociceptor populations of TG sensory neurons are represented. It cannot therefore be shown that the increased cytokine gene expression measured in this study is specifically occurring in nociceptors. In the previous chapter it was demonstrated that the expression of neuronal TLR4, 2 and 7 is exclusive to nociceptor populations of sensory neurons however the possibility that the expression patterns of these TLRs may be altered in vitro cannot be discounted. Indeed, satellite cells from the DRG have been shown to display increased expression of TLR4 following the loss of neuronal communication in vitro (Tse et al. 2014b). In the previous chapter the expression of TLR4 is identified in a small proportion of cells that morphologically resemble satellite cells under naïve conditions. Therefore it is possible that the increased expression of cytokine mRNA may be partly due to the activation of TLR4 on non-neuronal cells. However, given the percentage of nociceptors that express each individual TLR and the lack of an induction of TLR4 gene expression under normal culture conditions it is reasonable to suggest that the induction of gene expression following TLR activation is primarily occurring within neurons and more specifically nociceptors.

This is the first study, to our knowledge, to investigate the TLR activation-induction of pro-inflammatory cytokine gene expression in TG sensory neurons. The up-regulation of TNFα and IL-1β mRNA in DRG sensory neurons has previously been demonstrated following acute E. coli LPS-dependent TLR4 activation (Tse et al. 2014a). Results from this present study show that TG sensory neurons respond to acute E. coli LPS exposure in a similar manner. Both TNFα and
IL-1β mRNA are significantly up-regulated however the induction of IL-1β is not as pronounced as that observed in DRG neurons (Tse et al. 2014a). Using the TLR4-specific inhibitor, CLI-095, it is also shown that the induction of these cytokines is primarily dependent upon TLR4 activation. LPS has also been shown to activate certain non-canonical receptors including TRPA1 (Meseguer et al. 2014) and various caspases (Shi et al. 2014). Whilst IL-1β mRNA up-regulation was predominantly TLR4-dependent, the reduction of TNFα induction was limited to approximately 60%. This suggests that the *E. coli* LPS-induced gene expression of TNFα may also be mediated by receptors other than TLR4 on sensory neurons.

Previous studies have shown that *P. gingivalis* LPS interacts with neuronal TLRs to sensitize TRPV1 and facilitate the rapid release of CGRP in TG neurons (Dio-genes et al. 2011; Ferraz et al. 2011). In the present study, mono-phosphorylated, penta-acylated (LPS1690) and mono-phosphorylated, tetra-acylated (LPS1435) lipid A-containing LPS isoforms were able to induce an expected response in macrophages, differentiated from a monocyte cell line (THP-1), but in our hands did not alter cytokine gene expression in cultured TG neurons. It is somewhat expected that the mono-phosphorylated, tetra-acylated isoform did not induce a response as it has previously been shown to be a relatively weak TLR4 agonist and can also act as a TLR4 antagonist under certain circumstances (Darveau et al. 2004; Coats et al. 2009; Herath et al. 2013). The penta-acylated isoform however is considered relatively immunologically potent compared to other isoforms. The results from the present study, combined with those of past reports, would suggest that TG sensory neurons are able to detect the presence of *P. gingivalis* LPS via TLRs but do not mount a significant cytokine response. *P. gingivalis* LPS is generally considered to be less potent than that of enterobacterial species such as *E. coli* (Holden et al. 2014) and it has been suggested that this is one reason why the
activated host immune response is often insufficient for clearance of the infection. The inability of sensory neurons to mount a cytokine response to \( P. \text{gingivalis} \) LPS may also contribute to this as the release of cytokines in the periphery would have major implications for the innate immune response and subsequent bacterial clearance.

In addition to the involvement of TLR4, the present study also shows that the activation of TLR2 in TG sensory neurons induces the up-regulation of TNF\(\alpha\) and IL-1\(\beta\). In the previous chapter it was discussed that the expression of TLR2 in primary sensory neurons, prior to this study, had not been identified. The results presented in this chapter demonstrate the functional activation of TLR2 in TG sensory neurons suggesting that this receptor is present and able to detect the presence of Gram-positive bacteria as well as a wide range of endogenous ligands (see Table 1.1). The TLR2 agonist used in this study, Pam3 CSK4, is a specific agonist for the TLR2/1 heterodimer. Therefore results suggest that TG sensory neurons may also express TLR1. Gram-positive bacteria are frequently identified in the healthy oral flora (Dewhirst et al. 2010) however many are also associated with the diseased state. For example, a range of \textit{Streptococcus spp.} and \textit{Actinomyces spp.} within the oral cavity are commonly associated with carious tooth decay and pulpitis (Tanzer et al. 2001) and some less common oral pathologies such as osteomyelitis of the jaw (see Hudson 1993). Tissue damage associated with physical trauma or chronic inflammation within oral tissues causes the release of multiple endogenous factors that may also activate TLR2. TMJ disorders, carious tooth decay and chronic periodontal disease, for example, are all associated with a gradual and progressive tissue destruction. The expression and direct activation of TLR2 on TG sensory neurons by Gram-positive species and endogenous factors may therefore contribute towards the inflammation and pain associated with various orofacial disorders.
A previous study has shown that the activation of TLR3, 7/8 and 9 in DRG neurons results in the up-regulation of IL-1β, IL-1α, CCL5 and CXCL10 (Qi et al. 2011). Here it is shown that TLR7 activation in TG sensory neurons not only induces the up-regulation of IL-1β but also TNFα. The previous study used a dual agonist of TLR7 and TLR8 however, here, a TLR7 specific agonist is used to demonstrate the functionality of TLR7 in TG sensory neurons. TLR7 is commonly associated with ssRNA viral infections, a major class of which are enteroviral strains, with Cocksackie virus being particularly relevant to the oral cavity (see McCullough & Savage 2005). Also relevant to this study is the ability of TLR7 to recognise multiple miRNAs. miRNAs are endogenous factors that post-transcriptionally regulate gene expression (Bartel 2004) however recent studies have shown that certain microRNAs can activate TLR7 (Fabbri et al. 2012) and play a role in the regulation of neuronal growth (Liu et al. 2015), neurodegeneration (Lehmann et al. 2012) and pain (Zhao et al. 2010; Park et al. 2014). The direct activation of TLR7 by certain miRNAs is also sufficient to rapidly depolarise DRG neurons to elicit a pain response (Park et al. 2014). TLR7 activation on TG sensory neurons by viral ssRNA and/or endogenous microRNAs may therefore be sufficient to directly activate neurons in addition to the transcriptional alteration detailed in the present study.

Although the current study shows that the activation of multiple neuronal TLRs results in the transcriptional up-regulation of TNFα and IL-1β it does not demonstrate whether these changes were also replicated at the protein level. The major limitation of working with TG sensory neurons compared to those of the DRG is the sample size. Whilst there are numerous DRG located along the entire length of the spinal cord, only two TG are found in each rodent. This, combined with a loss of cells during tissue dissociation, results in a limited cell harvest. Assuming that the observed transcriptional alterations were matched at the translational
level, both TNFα and IL-1β are able to promote neuronal sensitisation both in the periphery and spinal cord through multi-cellular mechanisms.

Cytokine signalling is critical for the initiation and maintenance of pain hypersensitivity (Uceyler et al. 2007). In many conditions associated with chronic pain, an increase in pro-inflammatory cytokines is observed (see Uceyler et al. 2009) and treatment with anti-inflammatory cytokines or inhibitors of pro-inflammatory cytokines is often sufficient to reduce this pain (Sommer et al. 1999; 2001; Milligan et al. 2005a; 2005b). An increased expression of TNFα and IL-1β are associated with multiple models of inflammatory and neuropathic pain (see Uceyler et al. 2009). Both exhibit a broad range of effects on neurons, non-neuronal cells and immune cells that can alter the pain phenotype through multiple peripheral and central mechanisms (Junger & Sorkin 2000; Ozaktay et al. 2006). Both are able to directly activate peripheral neurons to produce acute, spontaneous firing which is associated with the development of hyperalgesia and allodynia (Fukuoka et al. 1994; Sorkin et al. 1997; Obreja et al. 2002; Murata et al. 2006; Ozaktay et al. 2006; Schafers et al. 2008). This rapid activation of neurons is also associated with a potentiation of CGRP and SP release in a manner that is independent from transcriptional alterations (Fukuoka et al. 1994; Opree & Kress 2000; Hou et al. 2003).

TNFα and IL-1β, signalling in an autocrine or paracrine manner, can induce long-lasting alterations to neuronal excitability through transcriptional, translational and post-translational mechanisms mediated by the activation of TNFR1 and IL1R1 expressed by nociceptors. Another TNF receptor, TNFR2, is silent under normal conditions however it is suggested that it contributes to TNF signalling following the induction of neuronal sensitisation (Schafers et al. 2008). Activation of NF-κB, p38 MAPK, ERK, JNK and PI3K pathways downstream from receptor activation (Pollock et al. 2002; Takahashi et al. 2006; Wei et al.
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2007) can induce the up-regulation of PGE$_2$ (Cunha et al. 1992), the bradykinin receptor B1 (Marceau 1995), BDNF (Lin et al. 2011), TrkB (Lin et al. 2011), TRPV1 (Khan et al. 2008), TRPA1 (Wu et al. 2015) and TRPV4 (Wu et al. 2015). Protein kinases such as PKA and PKC are also activated resulting in the post-translational sensitisation of TRPV1 (Nicol et al. 1997; Obreja et al. 2002), TRPA1 (see Lapointe & Altier 2011), P2X3 (Zhang et al. 2007) and Na$_V$ channels (Jin & Gereau 2006; Binshtok et al. 2008). In the superficial dorsal horn, both can promote central sensitisation by increasing the activity of NMDA and AMPA receptor activation as well as reducing inhibitory current induced by GABA and glycine (Kawasaki et al. 2008).

Further to their actions on neurons, cytokines can activate non-neuronal cells to indirectly promote pain hypersensitivity (see Hanisch 2002). The activation of peripheral neurons by cytokines can also indirectly promote the activation of non-neuronal cells via the release of glutamate and neurotransmitters. Activated glial cells display an increased production of many pro-nociceptive factors including NGF, NO, ATP and other pro-inflammatory cytokines following activation of receptors and signalling cascades that have been previously mentioned (see Milligan & Watkins 2009). TNF$\alpha$ can also inhibit the activity of glutamate transporters (Sitcheran et al. 2005) and potassium channels (Koller et al. 1998) in spinal cord glial cells to promote activation of AMPA and NMDA receptors on spinal neurons.

Both TNF$\alpha$ and IL-1$\beta$ are key orchestrators of the innate immune response and can therefore indirectly promote pain hypersensitivity by potentiating the local inflammatory response. Neuronal production of these cytokines would promote the local accumulation and activation of immune cells including macrophages, neutrophils, mast cells and T lymphocytes. For example, TNF$\alpha$ and IL-1$\beta$ promote the production of NGF, prostaglandins, bradykinin, histamine, matrix met-
allopeptidase (MMP)-9, NO and further promote their own synthesis by immune cells (see Moalem & Tracey 2006; Watkins et al. 2007). These, and other, pro-inflammatory mediators have a range of direct and indirect effects on neuronal and glial function, as detailed in chapter 1.2 of this thesis.

Despite the absence of an acute response to *P. gingivalis* LPS, the activation of neuronal TLRs represent an important mechanism in the development and maintenance of multiple orofacial pain states. Results presented in this chapter demonstrate the functional activation of TLR4, 2 and 7 expressed by TG sensory neurons and further support the hypothesis that primary nociceptors can directly detect and respond to bacterial and viral infection as well as a range of endogenous ligands. The most prevalent orofacial pain disorders, including TMJ disorders and carious tooth decay, are associated with the presence of multiple PAMPs and DAMPs. The dense innervation and unique location of many orofacial tissues mean that the activation of neuronal TLRs has the potential to occur rapidly and frequently during the progression of multiple orofacial diseases/disorders. The acute activation of neuronal TLRs and the subsequent induction of pro-inflammatory and pro-nociceptive cytokines may therefore contribute towards the initiation of an altered state of neuroimmune signalling to promote neuronal hypersensitivity.
Chapter 5

LPCAT1 & LPCAT2 expression
in trigeminal ganglion sensory
neurons

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5. LPCAT expression in TG neurons.

5.1 Introduction

The plasma membrane is a dynamic, fluid structure that undergoes constant remodelling in response to extracellular and intracellular signals. The general consensus on membrane organisation has moved on from that of a random homogeneous assortment of lipids towards that of an ordered and controlled assortment of multiple microdomains each with a specific structure and order of phospholipids and membrane proteins. Specific cholesterol- and sphingolipid-rich membrane microdomains, known as lipid rafts, possess a particularly ordered lipid structure that allows for controlled lateral trafficking of various membrane components (see Simons & Ikonen 1997). The high percentage of cholesterol, for example, within raft domains provides an increased thickness and stiffness of the phospholipid bilayer to optimise protein sorting. Lipid rafts are associated with a range of cell functions including cell signalling (see Simons & Toomre 2000). Lipid rafts are not only rich in receptor proteins but also facilitatory proteins such as coreceptors, enzymes and intracellular signal transduction molecules (see Anderson 1998). Therefore raft microdomains provide a highly ordered compartment to allow for the clustering and physical interaction of the necessary molecular components to generate a signalling response to a particular activatory stimulus.

Lipid rafts have been shown to play a vital role in numerous neuronal cell functions such as cell adhesion (Kasahara et al. 2000), synaptic transmission (Pato et al. 2008) and cellular signalling. Multiple membrane-bound receptors in sensory neurons cluster in lipid raft domains in order to initiate a signalling response. For example, P2X receptors (Garcia-Marcos et al. 2009), multiple TRP channels (Liu et al. 2006; Morenilla-Palao et al. 2009), TrkA (Limpert et al. 2007) and NaV1.8 (Pristera et al. 2012) have all been either identified in lipid rafts or their function has shown to be dysregulated following the removal of lipid raft components from
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TLRs, and other immune receptors (see Triantafilou et al. 2011), localise to lipid raft domains in a number of cell types in response to a range of stimuli (Triantafilou et al. 2004; citealpWong2009). All identified TLRs are type I transmembrane proteins with an intracellular TIR domain that associate with intracellular adaptor molecules such as TIRAP, MyD88, TRAM and TRIF, that are also clustered in raft domains, to initiate multiple signalling cascades (see Kawai & Akira 2007b). Bacterial recognition, via TLR2 and TLR4, is a complex process that involves interactions between multiple membrane proteins and also various serum proteins (see Park & Lee 2013). Upon ligand recognition by LBP-mediated LPS delivery to CD14 in raft domains, TLR4 is recruited into lipid rafts (Triantafilou et al. 2002) where it interacts with MD-2 and forms a homodimer to transduce a signal. TLR4 has also been shown to interact with atypical receptor components under certain circumstances (Byrd et al. 1999; Triantafilou et al. 2001; Heine et al. 2003). The dynamics of TLR2 activation are markedly different to those of TLR4 as TLR2 pre-exists as a heterodimer, with either TLR1 or TLR6. Ligand recognition then causes TLR2 heterodimers to translocate to lipid rafts where they interact with the resident raft proteins CD14 and CD36 to initiate MyD88-dependent signalling (Gupta et al. 1996; Soong et al. 2004; Hoebe et al. 2005). The involvement of raft domains in intracellular TLR signalling has not been shown however cholesterol-enriched endosomal raft-like domains have been identified (Nada et al. 2009). It has also been demonstrated that raft associated TLR7 enhances macrophage phagocytosis by interacting with HSP70 (Wang et al. 2006).

Currently the mechanisms of receptor movement within membranes are largely unknown however it has been shown that many proteins are targeted to raft domains via lipid modifications such as acyl and glycosylphosphatidylinositol (GPI) anchoring (see Levental et al. 2010). Members of a family of phospholipid remod-
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elling enzymes, known as lysophosphatidylcholine acyltransferases (LPCATs), reg-
ulate membrane structure and fluidity by converting lysophosphatidylcholine to
phosphatidylcholine using a diverse range of acyl-coenzyme A (CoA) donors (Ya-
mashita et al. 1997). Changes to the lysophospholipid/phospholipid ratio within
the membrane are known to affect lipid raft microdynamics (Stulnig et al. 2001) and
therefore LPCATs are suggested to modulate lateral movement of proteins within
the membrane. Indeed it has been shown that LPCATs are essential for the LPS
response in inflammatory cells and inhibition of LPCATs prevents the translo-
cation of TLR4 into lipid rafts and subsequent signalling (Schmid et al. 2003;
Jackson & Parton 2004; Jackson et al. 2008). LPCAT1 mediates the calcium-
dependent palmitoylation of histone proteins (Zou et al. 2011) and LPCAT2 has
recently been shown to directly modify TLR4 and TLR2 proteins (Abate & Jack-
son 2015). It is thought that these modifications facilitate targeting of these
receptors to lipid raft domains prior to the initiation of cell signalling (Abate & Jack-
son 2015).

To date four LPCAT isoforms have been identified, LPCAT1-4. LPCAT1 and LP-
CAT2 are from the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) fam-
ily of enzymes and contain LPAAT motifs whereas LPCAT3 and LPCAT4 lack
these motifs and are both from the membrane-bound O-acyltransferase (MBOAT)
family of acyltransferases. LPCAT1 and LPCAT2 are located on the endoplas-
mic reticulum and possess both acyltransferase and acetyltransferase activity as
demonstrated by their roles in membrane homeostasis and platelet activating fac-
tor (PAF) synthesis, respectively (Nakanishi et al. 2006; Shiindou et al. 2007).
LPCAT1 has been identified in a wide range of tissues and cell types although its
function remains largely unclear (Cheng et al. 2009). It displays acyltransferase
activity for lysophosphatidylcholine and lysophosphatidylethanolamine and has
a preference for saturated acyl-CoA donors. It has been shown to play a ma-
jor role in pulmonary surfactant production in lung alveolar cells by generating dipalmitoyl phosphatidylcholine (Bridges et al. 2010). LPCAT1 displays both acyltransferase and acetyltransferase activity in the non-immune biosynthesis and inactivation of PAF, respectively (Harayama et al. 2008; Cheng et al. 2009). LPCAT1 has also been shown to be overexpressed in multiple cancers (Mansilla et al. 2009; Zhou et al. 2011), play a role in retinal photoreceptor homeostasis (Cheng et al. 2009) and may also regulate mRNA synthesis (Zou et al. 2011). LPCAT2 is perhaps the best characterised LPCAT isoform. It is an inducible enzyme linked to inflammatory mechanisms due to its high expression levels in macrophages and neutrophils (Morimoto et al. 2010). Under normal conditions LPCAT2 participates in membrane remodelling by the acylation of lysophosphatidylcholine, lysophosphatidylserine and lysophosphatidylethanolamine with a preference for polyunsaturated fatty CoA donors. Following inflammatory stimuli such as LPS, PAF and ATP the acetyltransferase activity of LPCAT2 is induced which mediates the production of PAF from its pre-cursor lyso-PAF (Shindou et al. 2007; Morimoto et al. 2010; 2014). The enzyme is activated by phosphorylation following TLR4 (Morimoto et al. 2010) and PAF receptor (PAFR) (Morimoto et al. 2014) activation and the TLR4-dependent cytokine response in macrophages has been demonstrated to be dependent on LPCAT2 (Jackson et al. 2008; Abate & Jackson 2015).

LPCAT3 and LPCAT4 are poorly characterised in comparison to the previously mentioned isoforms. LPCAT3 expression has been ubiquitously identified in the mouse with particularly high expression in the testis, liver, adipose and pancreas (Hishikawa et al. 2008; Zhao et al. 2008). LPCAT4 expression is also widely distributed within the mouse however it shows particularly high levels of expression in brain, testis, ovary and epididymis (Hishikawa et al. 2008). The biological functions of LPCAT3 and LPCAT4 are unknown however LPCAT3 knock-
down results in liver inflammation (Rong et al. 2013). LPCAT3 displays acyltransferase activity for lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylserine with broad unsaturated-CoA specificity whereas LPCAT4 displays acyltransferase activity for lysophosphatidylcholine and lysophosphatidylethanolamine with a preference for oleoyl-CoA (Hishikawa et al. 2008; Gijon et al. 2008; Matsuda et al. 2008). When combined, LPCAT isoforms exhibit a broad substrate and donor specificity to provide phospholipid diversity within the cellular membrane (Harayama et al. 2014).

Previous chapters have demonstrated that neuronal TLR activation leads to inflammatory cytokine production however the mechanisms surrounding neuronal TLR activation are largely unknown. LPCAT2 has been demonstrated to regulate TLR4-dependent cytokine production in immune cells and it is therefore hypothesised that a similar mechanism may exist in sensory neurons. The expression of LPCAT isoforms within neuronal tissue and sensory neurons in particular has not been sufficiently described. LPCAT1 has been identified in the brain and spinal cord sensory neurons whereas LPCAT2 has been identified in spinal cord sensory neurons and microglia (Kihara et al. 2008; Cheng et al. 2009; Okubo et al. 2012). LPCAT2 is the only isoform to be identified in peripheral sensory neurons to date however this was only demonstrated following nerve injury (Hasegawa et al. 2010). The specific aim of this chapter was to describe the expression patterns of LPCAT1 and LPCAT2 in naïve sensory ganglia and in primary sensory neurons in vitro.
5.2 Results

5.2.1 Antibody characterisation

Antibodies specific for LPCAT1 and LPCAT2 were characterised using similar techniques to those in chapter 3. As the expression of LPCAT is identified in a wide range of tissues and cell types, western blotting was used to demonstrate the specificity of LPCAT antibodies. This was performed on tissues known to express LPCAT1 (cortex) and LPCAT2 (RAW 264.7 cells) as well as the specific tissues of interest, the TG and DRG. When LPCAT antibodies were used for western blotting a single, specific band was observed at the expected molecular weight (see Fig. 5.2). Antibody specificity was also determined by use in positive control tissues known to express LPCAT1 (lung) and LPCAT2 (spleen). Multiple positively stained cells were seen in the lung (LPCAT1, Fig. 5.1A-B) and spleen (LPCAT2, Fig. 5.1C-D). All other control experiments for primary and secondary antibodies have been detailed in Chapter 3.2.1.

5.2.2 Expression of LPCAT isoforms in naïve trigeminal ganglia

In order to investigate the expression of LPCAT1 and LPCAT2 within TG and DRG sensory neurons western blotting was performed followed by indirect single-labelling IHC. Co-expression of LPCAT1 and LPCAT2 with neurochemical markers of functional sensory neuron sub-populations was then demonstrated using dual-labelling IHC. LPCAT1 and LPCAT2 were identified in whole tissue lysates of the TG, DRG, cortex and RAW 267.4 cells (control). Cortex tissue and RAW cells were included as controls given that LPCAT1 and LPCAT2 are highly
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Figure 5.1. Fluorescent micrograph showing the distribution of LPCAT1 (green, A-B) and LPCAT2 (green, C-D) expression in lung and spleen tissue, respectively. Combined images include a DAPI counterstain to identify nuclear profiles (blue, B, D) Multiple positively stained profiles can be identified for both LPCAT1 and LPCAT2. Scale bar 50 µm and 15 µm, respectively.

Figure 5.2. Western Blot analysis for LPCAT isoform expression across multiple tissue types. LPCAT1 (A; 1:1000, ProteinTech) and LPCAT2 (B; 1:500, Novus Biologicals) expression in TG, DRG and cortex. 20µg protein was run in each lane. RAW264.7 cells were used as a positive control. β-III tubulin was used as a loading control. Images representative of three separate experiments.
expressed in brain and immune cells, respectively. A band just above 50 kDa was observed for LPCAT1 and LPCAT2 (Fig. 5.2). Whilst western blotting demonstrated the expression of LPCAT within the ganglia as a whole, immunofluorescence staining allowed for the description of the specific cellular localisation of expression for each isoform. LPCAT1-IR was identified in 50.4 ± 4.5% of total TG neurons (Fig. 5.3A-B). 14.1 ± 2.8% of total neurons displayed a more intense level of LPCAT1-IR; these were classed as high expressors (HE) of LPCAT1 as defined by fluorescent intensity. A proportion of non-neuronal cells also displayed weak LPCAT1-IR (Fig. 5.3A-B).

Further, the co-expression of LPCAT1 with common neurochemical markers was examined using dual-labelled IHC. Within the TG, LPCAT1 was expressed by 86.3 ± 4.7%, 67 ± 7.9% and 47.7 ± 5.3% of TRPV1-, P2X3- and NF200-IR neurons, respectively (Fig. 5.5). HE LPCAT1-IR was identified in 29.9 ± 3.3%, 19.4 ± 3% and 14.7 ± 2.7% of TRPV1-, P2X3- and NF200-expressing neurons, respectively.

LPCAT2 expression patterns were markedly different than that of LPCAT1. All neuronal profiles were negative for LPCAT2 however the majority of non-neuronal cells within the ganglia displayed strong positive LPCAT2-IR (Fig. 5.3C-D).

These data show that LPCAT1 is expressed by a sub-population of TG sensory neurons and co-localises with populations likely to be both nociceptors and non-nociceptors. LPCAT2 however is expressed exclusively by non-neuronal cells within the naïve TG.
Figure 5.3. Images show fluorescent micrographs of rat TG and DRG labelled with antibodies specific for LPCAT1 and LPCAT2. Multiple neuronal profiles within the TG (green, A) and DRG (green, B) displayed LPCAT1-IR, arrows show selected examples. A small proportion of neuronal profiles displayed high intensity of LPCAT1-IR, asterisks show selected examples. A proportion of non-neuronal cells also displayed weak LPCAT1-IR, arrowheads show selected examples. All neurons were negative for LPCAT2 but the majority of non-neuronal cells displayed strong LPCAT2-IR within the TG (green, C) and DRG (green, D), arrowheads show selected examples. Areas contained within the indicated borders (dashed lines) have been magnified in Fig. 5.4. Scale bar 50 µm. Images representative of three biological replicates.
Figure 5.4. Images show selected areas of Fig. 5.3 that have been magnified to give a more detailed representation of LPCAT isoform staining within the TG (A, C) and DRG (B, D). Similarly to the previous figure, arrows show selected examples of neuronal profiles that display positive LPCAT1-IR whilst asterisks show neuronal profiles that display high intensity of LPCAT1-IR, in the TG (A) and DRG (B). Arrowheads show selected examples of non-neuronal cells that display positive IR for LPCAT1 (A-B) and LPCAT2 (C-D). Scale bar 50 µm. Images representative of three biological replicates.
Figure 5.5. Images show fluorescence micrographs of rat TG double labelled for LPCAT1-IR (green, A, D, G) plus either TRPV1- (red, B), P2X3- (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localisation of LPCAT1-IR with neurochemical markers. LPCAT1-IR shows co-expression with a substantial proportion of TRPV1-IR neurons, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all neurons displaying TRPV1-IR neurons express LPCAT1-IR, examples indicated by open arrows (A, B, C). The majority of LPCAT1-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). A proportion of LPCAT1-IR neurons also co-express NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). There were also a population of NF200-IR neurons that did not co-express LPCAT1-IR, examples indicated by open arrows (G, H, I). Scale bar 50 µm. Images representative of three biological replicates.
5.2.3 LPCAT isoform gene expression in trigeminal sensory neurons in vitro

To assess whether LPCAT1 and LPCAT2 expression is altered following tissue dissociation qPCR was performed primary neuronal cultures at 12 h and 48 h in vitro, using acutely dissociated tissue as a reference sample (Fig. 5.6). LPCAT1 is detectable in the acutely dissociated sample (C\textsubscript{T} value = 25.7 ± 0.6) and shows no significant change in gene expression at both 12 h (-2.2 ± 0.4 fold change) and 48 h (-1 ± 1.2 fold change) in culture (one-way ANOVA with Dunnett’s post-hoc test). LPCAT2 is undetectable in the acutely dissociated sample (C\textsubscript{T} >40) however LPCAT2 gene expression is induced by approximately 35 ± 2.7 fold at 12 h post-plating (p < 0.001, one-way ANOVA with Dunnett’s post-hoc test. LPCAT2 reference C\textsubscript{T} > value was set at 40 for the purposes of statistical analysis.). At 48 h post-plating the level of LPCAT2 gene expression falls to 7.1 ± 0.6 fold but remains significantly increased compared to the acutely dissociated sample (p < 0.05, one-way ANOVA with Dunnett’s post-hoc test).

These data show that an increase in LPCAT2 gene expression is induced following nerve injury whereas LPCAT1 gene expression is not significantly different to control levels.

5.2.4 LPCAT isoform protein expression in TG sensory neurons in vitro

To assess changes in LPCAT isoform protein expression in vitro dual-labelling ICC was performed on primary neuron cultures 48 h post-plating, using β-III tubulin as a neuron-specific stain. Neurons from three individual culture preparations were manually counted and those displaying positive LPCAT1-/LPCAT2-IR
were expressed as a percentage of total neuronal profiles. There was no significant change in LPCAT1 expression in neurons after 48 h in culture compared to naïve tissue (Student’s t-test). LPCAT1 is expressed by 49.3 ± 5.4% of neurons 48 h post-plating (Fig. 5.7). In contrast to LPCAT1, LPCAT2 expression was significantly up-regulated in neurons after 48 h in culture compared to naïve tissue. LPCAT2-IR was entirely non-neuronal in naïve TG but following dissociation and 48 h in culture LPCAT2-IR was detected in 15.6 ± 3.8% of neurons (p < 0.05 compared to naïve tissue, Student’s t-test; Fig. 5.8).

These data show that LPCAT2 protein expression is induced in a proportion of neurons following nerve injury whereas LPCAT1 protein expression is not significantly different from control tissue. This is in accordance to the gene expression data and, collectively, these results suggest that the expression of LPCAT2 in TG sensory neurons is inducible whereas the expression of LPCAT1 is constitutive.
Figure 5.7. Images show fluorescence micrographs of cultured primary TG neurons at 48 h in vitro dual-labelled for LPCAT1-IR (green, A, D, G) plus β-III-tubulin-IR (red, B, E, H). The combined images (C, F, I) show co-localisation of LPCAT1-IR with the neuronal marker and includes a DAPI stain to identify nuclear profiles. Similarly to naïve neurons, positive LPCAT1-IR was detected in approximately half of all neurons after 48 h in vitro; arrows show positive neurons whilst open arrows show neurons that were considered to be negative. Images are representative of 3 independent cell cultures. Scale bar 15 µm.
Figure 5.8. Images show fluorescent micrographs of cultured primary TG neurons at 48 h in vitro dual-labelled for LPCAT2-IR (green, A, D, G) plus β-III-tubulin-IR (red, B, E, H). The combined images (C, F, I) show co-localisation of LPCAT2-IR with the neuronal marker and includes a DAPI stain to identify nuclear profiles. In contrast to naïve neurons, a small number of neurons displayed LPCAT2-IR after 48 h in vitro, arrows show LPCAT2-positive neurons whilst open arrows show LPCAT2-negative neurons. The majority of non-neuronal cells also display positive LPCAT2-IR, arrowheads show selected examples. Images are representative of 3 independent cell cultures. Scale bar 15 µm.
5.3 Discussion

The group of phospholipid modifying enzymes known as lysophosphatidylcholine acyltransferases are essential for a number of biochemical events at the membrane level. LPCAT2 in particular has been shown to mediate the TLR2 and TLR4 signalling capabilities of monocytes (Schmid et al. 2003; Jackson et al. 2008; Abate & Jackson 2015). Inhibiting this enzyme prevents TLR4 translocation to lipid raft domains within the cell membrane and causes a down-regulation of TLR4- and TLR2-mediated inflammatory cytokine production (Jackson et al. 2008; Abate & Jackson 2015). Here we have investigated whether the LPCAT isoforms, LPCAT1 and LPCAT2, are present in mammalian sensory ganglia in vivo and TG sensory neurons in vitro. Both isoforms are indeed present within naïve sensory ganglia however each have a distinct pattern of expression within the tissue. Approximately half of all sensory neurons were positive for LPCAT1; a small proportion of non-neuronal cells also displayed LPCAT1-IR. LPCAT1 expression was not restricted to a specific sub-population of cells given that it co-localised with all three of the neurochemical markers used suggesting that it is expressed by both nociceptor and non-nociceptor sub-populations. LPCAT2 was not expressed by neurons in either the TG or DRG. LPCAT2 was however expressed by non-neuronal cells that surrounded neurons, the morphology of which closely resemble satellite cells. The expression patterns of LPCAT1 and LPCAT2 in naïve tissue are intriguing as it would be expected that all neurons express one LPCAT isoform given their phosphatidylcholine remodelling properties and that phosphatidylcholine is a major phospholipid component of sensory neuron membranes (Calderon et al. 1995). Other, recently identified isoforms of LPCAT (LPCAT3 and LPCAT4) exist that possess phosphatidylcholine remodelling properties have not been included in this study due to antibody unavailability. LPCAT3 displays broad substrate and acyl-CoA donor specificity and has been suggested to be a key regulator of membrane
remodelling in certain tissues, particularly in the liver (Hishikawa et al. 2008; Gijon et al. 2008; Matsuda et al. 2008). LPCAT3 and LPCAT4 expression have not been characterised in sensory neurons and it is entirely feasible that they may be expressed. It is also possible that, given the variable staining intensity observed with LPCAT1, some neurons that were classed as negative may express a very low level of LPCAT1 that was not distinguishable above background staining.

The expression patterns of LPCAT1 and LPCAT2 in vivo are supported by the protein and gene expression of each respective isoform in TG sensory neurons in vitro. LPCAT1 gene expression was abundant in acutely dissociated neurons (C_T value) whereas LPCAT2 gene expression was undetectable (C_T > 40). This was expected given that the majority of non-neuronal cells are removed during the Percoll gradient spin of the dissociation process. As previously mentioned, the dissociation process inflicts an acute axotomy on sensory neurons as the peripheral and central processes are removed, first by dissection from the animal and second by enzymatic and mechanical digestion. Such an insult initiates a range of well documented cellular changes that mediate changes in membrane protein and neuropeptide expression (Bradbury et al. 1998; Shi et al. 2001; Kim et al. 2008). LPCAT1 gene expression remains comparable to control (pre-plating) at 12 and 48 h post-plating whereas LPCAT2 gene expression is significantly induced at both time points, with the greatest induction seen at 12 h. This is also demonstrated by a significant increase in LPCAT2 protein expression in sensory neurons after 48 h although the specific sub-populations of sensory neuron that this induction occurs in were not determined in this study. These data reflect previous studies performed on immune cells that show LPCAT1 to be constitutively expressed whereas LPCAT2 is inducible in response to LPS (Shindou et al. 2007). Okubo et al. (2012) have previously demonstrated these findings in spinal neurons using the SNI model of peripheral nerve injury. LPCAT1 and LPCAT2
mRNA were expressed by a proportion of spinal cord neurons in naïve animals and LPCAT2 mRNA expression increased following nerve injury whilst LPCAT1 mRNA remained unchanged (Okubo et al. 2012). It has also been shown that LPCAT2 is expressed by murine DRG neurons and non-neuronal cells 7 days post spinal nerve transection (Hasegawa et al. 2010). Therefore it appears that neuronal LPCAT1 and LPCAT2 share certain properties to those characterised in immune cells. Although nerve injury is sufficient to induce LPCAT2 expression, it is currently unknown whether an inflammatory stimulus, such as LPS, is also sufficient.

The results presented in this chapter do not provide any clear indications that either isoform may be associated with neuronal TLR signalling. In immune cells LPCAT2 mediates the translocation of TLR2 and TLR4 monomers into lipid raft domains of the phospholipid membrane that allow for intracellular signalling (Abate & Jackson 2015). The expression pattern of LPCAT1 overlaps somewhat with that of TLR4 (see Chapter 2) although, unlike TLR4, the expression of LPCAT is not specific to nociceptors. It is therefore likely that LPCAT1 has a more general function in these cells rather than one specifically related to nociception.

The observed pattern of LPCAT2 expression in naïve tissue initially suggests that the enzyme is not associated with TLR4. It was however shown that LPCAT2 expression is induced in neurons in vitro following nerve injury. This suggests that LPCAT2 may indeed be associated with a range of nerve-injury related signalling pathways. Due to antibody incompatibility it was not possible to demonstrate whether LPCAT2-expressing neurons co-localised with TLR4. It is not known whether the expression of LPCAT2 in neurons is up-regulated following PAMP-exposure or TLR activation and therefore the possibility of LPCAT2 playing a role in neuronal TLR4 activation can not be dismissed.
Current studies on neuronal LPCAT isoforms, including the present study, have focussed on enzyme expression rather than enzyme activity. Although determining enzyme expression is a necessary pre-cursor for further characterisation, investigating enzyme activity within neurons is key to further understanding their role in specific physiological and pathological processes. Indeed, in immune cells, an inflammatory stimulus such as LPS induces LPCAT2 activity as well as increased expression. LPS exposure also causes TLR4 to translocate to lipid rafts and it is now thought that LPCAT2 could mediate this movement (Jackson et al. 2008; Abate & Jackson 2015). The specific functions of both LPCAT1 and LPCAT2 in neurons are poorly understood. One function of LPCAT1 and LPCAT2 that is particularly relevant when discussing pain and inflammation is their ability to synthesis PAF, a lipid mediator which has been shown to contribute towards inflammatory and neuropathic pain (see Tsuda et al. 2011). LPCAT1 synthesises PAF in a constitutive, non-inducible manner whereas LPCAT2-dependent PAF synthesis is inducible (Shindou et al. 2007). Although the PAF-synthesising activity of neuronal LPCAT2 has not been directly demonstrated, LPCAT2 mRNA expression as well as PAFR mRNA is increased following nerve injury (Okubo et al. 2012). The contribution of PAF signalling to neuronal sensitisation and the generation of pain has been discussed (see Tsuda et al. 2011) and the application of a PAFR antagonist in this latter study suppressed mechanical allodynia following nerve injury. This study, and past studies, show that LPCAT2 is expressed by neurons and non-neuronal cells following nerve injury. Neuronal LPCAT2 may therefore increase the production of PAF by both neurons and tissue macrophages following a priming event such nerve injury, and possibly inflammation, leading to neuronal sensitisation via indirect mechanisms involving inflammatory cytokine production by tissue macrophages. The specific mechanism that mediates the induction of LPCAT2 following nerve injury is currently unknown however it is known that LPCAT2 function is Ca\textsuperscript{2+} dependent (Shindou et al. 2007). Calcium
ion influx is essential for normal neuronal function as well as the induction of neuronal plasticity and Tsuda et al. (2011) suggest a mechanism whereby LPCAT2 activity and PAF production is mediated by purinergic receptor and calcium channel activation. Following neuronal TLR activation, TRPV1 sensitisation has been demonstrated (Diogenes et al. 2011; Ferraz et al. 2011) and it is possible that TRPV1 activation-dependent calcium influx may also be sufficient to induce LPCAT2 activity and up-regulation.

This study, to the best of our knowledge, is the first to examine LPCAT1 and LPCAT2 expression in naïve TG sensory neurons and cultured sensory neurons in vitro. LPCAT1, but not LPCAT2, is shown to be expressed by trigeminal sensory neurons under normal conditions. Following nerve injury LPCAT1 expression levels remain unchanged whilst a small percentage of neurons begin to express LPCAT2. The calcium-dependent inducibility of LPCAT2 activity combined with its PAF-synthesising activity suggests that neuronal LPCAT2 is potentially involved in neuropathic and inflammatory pain. The involvement of LPCAT2 in neuronal TLR signalling should be explored further and a recent study has identified a range of potential LPCAT2-specific inhibitors which would greatly aid the field (Tarui et al. 2014). LPCAT2 specific inhibitors may also represent a potential future clinical target for pain management due to their antagonism of LPCAT2-dependent PAF synthesis and subsequent PAF-dependent pain hypersensitivity.
Chapter 6

General discussion and future perspectives
6. General discussion

6.1 Discussion

Traditionally it was thought that pathogens interact with sensory neurons through indirect mechanisms involving the activation of an innate immune response and production of pro-inflammatory mediators. It is now understood that sensory neurons can directly detect and respond to pathogenic challenge independently of the innate immune system through the activation of neuronal TLRs. The present study set out to investigate how the activation of neuronal TLRs within the trigeminal system alters neuronal signalling with the following core aims:

- To describe the degree of expression of TLR4, TLR2 and TLR7 within sensory ganglia and their distribution within functionally-identified neuronal sub-populations.

- To investigate pro-inflammatory cytokine production by trigeminal sensory neurons following exposure to agonists of the aforementioned TLRs.

- To investigate the potential involvement of LPCAT isoforms in the activation of neuronal TLRs.

Prior research has identified the expression of multiple TLRs in sensory neurons and the co-expression of certain TLRs with neurochemical markers such as TRPV1, NF200, CGRP and IB4 (Wadachi & Hargreaves 2006; Diogenes et al. 2011; Due et al. 2012; Lin et al. 2015; Xu et al. 2015). The majority of these past studies however do not attempt to quantify this expression of TLRs in neurons or the degree of co-expression of TLRs with neurochemical markers. A detailed, quantitative description of receptor expression is a pre-requisite for subsequent functional studies in order to fully understand the different functional cell types that may respond to TLR activation. Results presented in chapter 3 and in Helley et al. (2015) demonstrate that the expression of TLR4, TLR2 and TLR7 are
specific to C fibre nociceptor populations within the adult rat TG and DRG. The activation of these TLRs is therefore likely to primarily impact nociceptor signalling rather than that of non-nociceptor populations. The activation of these TLRs may however be able to indirectly alter the properties of larger non-nociceptors through paracrine signalling mechanisms downstream from receptor activation in nociceptors. TLR4, 2 and 7 shared similar patterns of expression and co-expression suggesting that C fibre nociceptors express a range of different TLRs and are therefore able to detect and respond to a range of exogenous and endogenous agonists. Indeed, results presented in chapter 4 demonstrate that the activation of the above TLRs in TG sensory neurons induces a transcriptional up-regulation of pro-inflammatory cytokines that are known to contribute towards peripheral and central sensitisation (see Fig. 6.1 for proposed mechanisms). An alteration in cytokine gene expression was observed in response to *E. coli* LPS but not to LPS isolated from the oral pathogen *P. gingivalis*. This suggests that neuronal TLRs may generate a differential response to agonists of varying potency as is the case for TLRs expressed by innate immune cells.

The lack of a neuronal cytokine response induced by exposure to *P. gingivalis* LPS provides an interesting insight into potential mechanisms of pathology associated with chronic periodontitis. Although the disease is associated with a chronic inflammatory response a majority of patients do not report a change in pain experienced (Abd El-Aleem et al. 2004). This is somewhat surprising given that the presence of an inflammatory response is often associated with peripheral sensitisation. This is indeed the case for some prevalent orofacial pain including acute conditions such as pulpitis and mucositis as well as chronic conditions affecting the TMJ (see Sessle 2011). The peripheral mechanisms of inflammatory pain are well established and the contribution of specific immune factors have been discussed in chapter 1 of this thesis. Many of these factors, including IL-1β
and TNFα, are found in increased concentrations within the GCF of periodontitis patients and actively contribute to tissue damage (see Gupta 2013). Despite this, the majority of pain reported with chronic periodontitis is associated with more advanced stages of the disease possibly due to more pronounced tissue damage to tooth supporting structures and abscess formation (see LeResche & Dworkin 2002).

Previous studies have demonstrated that the activation of neuronal TLR4 by *P. gingivalis* LPS sensitises TRPV1 and facilitates the associated release of CGRP (Diogenes et al. 2011; Ferraz et al. 2011). This would suggest that *P. gingivalis* promotes neuronal sensitisation however this does not appear to be replicated in the clinical setting. One potential explanation for this difference may involve the relatively high concentration of *P. gingivalis* LPS used in previous reports. This is in contrast to many immunological studies that routinely expose cells to concentrations in the nanogram range. Although a high concentration of agonist ensures a strong degree of receptor activation and is therefore useful for describing downstream mechanisms, it may not accurately represent the situation *in vivo*. This is also the case for the use of other agonists of neuronal TLRs. For example, excluding some preliminary data presented by Tse et al. (2014a) which shows that DRG neurons up-regulate COX-2 mRNA following 4 h exposure to 0.1 µg/mL of *E. coli* LPS, the lowest concentration of LPS used to activate neuronal TLR4 is 1 µg/mL. This study, and past studies, have also primarily used synthetic agonists to activate certain neuronal TLRs. Synthetic agonists are often used when investigating specific receptor signalling pathways as their receptor specificity and potency are well characterised. They cannot however be directly related to a pathological condition. Future research must therefore expand on these initial studies and utilise specific, pathologically relevant agonists at relevant concentrations to investigate their effects on nociceptor physiology.
A - Periphery

Activation by exogenous or endogenous ligands

Peripheral nerve terminal

Retrograde trafficking to neuronal soma

B - Sensory ganglion

Satellite cell

Peripheral axon

C - Central synapse

Central terminal of primary afferent

Second order neuron in trigeminal nucleus

Gliaal cell

Figure 6.1
Figure 6.1 – Summary of proposed mechanisms. The results presented in this thesis suggest that TNFα and IL-1β are produced by nociceptors, downstream from TLR activation. These cytokines exert a range of actions at multiple locations within the sensory nervous system to promote neuronal sensitisation. (A) TNFα and IL-1β may be released from the peripheral terminals of nociceptors and can act in an autocrine and paracrine fashion. The activation of TNFR1 and IL1R1 expressed by nociceptors is sufficient to induce spontaneous firing and also activate intracellular signalling pathways associated with mechanisms of peripheral sensitisation (see Fig. 1.1). The peripheral release of these cytokines can also stimulate innate immune cells such as macrophages to promote the inflammatory response. Inflammation is associated with the increased production of multiple factors that are known to directly increase neuronal excitability. (B) The release of TNFα and IL-1β by neuronal soma and satellite cells within the trigeminal ganglion can also promote neuronal sensitisation. Activation of TNFR1 and IL1R1 on neurons produces similar alterations to neuronal function as described above. These alterations are not limited to the neuron that initially releases the cytokines. Paracrine signalling may also promote neuronal sensitisation in neighbouring neurons which results in a more diffuse hyperalgesia. Satellite cells may also be activated resulting in the production of pro-inflammatory and pronociceptive factors which can further promote neuronal sensitisation. This can also promote a mild inflammatory response within the ganglion resulting in the sensitisation of neighbouring neurons. (C) TNFα and IL-1β may also be released from the central terminals of nociceptors to activate receptors expressed by second order neurons and non-neuronal cells. The activation of these receptors on neurons is sufficient to induce spontaneous firing but may also induce transcriptional and post-translational changes associated with central sensitisation (see Fig. 1.2). Activation of glial cells results in the production of factors that can further promote central sensitisation and an altered state of intercellular communication.
Specific cellular mechanisms that attempt to explain the apparent absence of pain hypersensitivity associated with periodontitis are lacking. One possible explanation for this relates to the microbiological aetiology of the disease. The keystone pathogen hypothesis states that no individual species of pathogen is responsible for the majority of tissue damage/immune activation (Hajishengallis et al. 2012). It is in fact the overall shift in plaque behaviour and breakdown of the symbiotic relationship with the host to a state of dysbiosis that promotes tissue destruction. The individual species of bacteria contained within the plaque are considered less potent when compared to the dysbiotic plaque as a whole. This is demonstrated by the relative potency of *P. gingivalis* LPS isoforms compared to that of enterobacterial pathogens such as *E. coli*. Generally *P. gingivalis* LPS is considered less immunologically potent than *E. coli* LPS (Bainbridge & Darveau 2001). Results presented in chapter 4 of this thesis suggest that this may also be the case for the activation of neuronal TLRs. Whilst *E. coli* LPS induced an increased expression of pro-inflammatory cytokine mRNA, two individual isoforms of *P. gingivalis* LPS did not. Therefore *P. gingivalis* alone may not possess sufficient TLR agonist activity in order to induce intracellular signalling pathways in neurons.

The relatively low potency of *P. gingivalis* LPS is suggested to be a host evasion mechanism employed by the pathogen to promote survival and plaque maturation (see Hajishengallis & Lamont 2014). The bacterium is able to actively alter its LPS isoforms in response to changes in the local environment that are associated with the diseased state (Al-Qutub et al. 2006; Curtis et al. 2011). Less potent isoforms of LPS, containing mono-phosphorylated, tetra-acylated lipid A for example, are more prevalent in the disease setting (Al-Qutub et al. 2006). This isoform has relatively weak TLR agonist activity compared to other isoforms (Darveau et al. 2004) and this is mirrored by the strength of the host immune response initiated in periodontitis. A strong cytokine response would promote
immune activation and subsequent clearance of the bacteria whereas a low level of immune activation is beneficial to bacterial survival. Tissue damage caused by the low level immune response releases essential molecules that can be utilised as nutrients by opportunistic species within the plaque, termed inflammophillic bacteria, to promote plaque maturation and disease progression (see Hajishengallis 2014). Therefore an absence of cytokine induction in neurons could promote bacterial survival by dampening the host response. The absence of neuronal cytokine production in response to *P. gingivalis* LPS may also contribute to the lack of clinical pain reported by chronic periodontitis patients.

The heterogeneity of bacterial species within sub-gingival plaques however means that may be possible for one or more of these other species to alter nociceptor physiology through the activation of TLRs. The contribution of many different microbial species to the pathogenesis of periodontitis have not been described due to the large number of implicated species in recent microbiome studies (De-whirst et al. 2010; Abusleme et al. 2013). The importance of previously under-appreciated microbial species in periodontal diseases are only just beginning to be explained (see Hajishengallis 2014). For example, *Filifactor alocis*, has recently been shown to induce a pro-inflammatory response (Moffatt et al. 2011) and it is therefore possible that it may also directly interact with sensory neurons. The focus of this study was on TLR activation however *P. gingivalis* and other periodontal pathogens also possess a range of other virulence factors. α-haemolysin and *N*-formylated peptides, for example, are able to directly induce calcium influx and action potential generation in nociceptors (Chiu et al. 2013). Periodontal pathogens, including *P. gingivalis*, secrete proteases such as gingipains (Guo et al. 2010) and express a range of different peptides on fimbriae (Hajishengallis 2007) that contribute towards tissue damage and these may also exert effects on nociceptors, similar to those demonstrated by Chiu et al. (2013).
Although *P. gingivalis* LPS was not sufficient to induce a transcriptional response in terms of the specific inflammatory cytokines within the time-frame studied, it is possible that alterations to other pro-nociceptive and/or pro-inflammatory factors may occur. As mentioned, *P. gingivalis* LPS is able to induce post-transcriptional alterations that are known to contribute towards peripheral and central sensitisation (Diogenes et al. 2011; Ferraz et al. 2011). A broad range of factors and mechanisms have been implicated in the development and maintenance of peripheral and central sensitisation (summarised in Chapter 1.2) and the effect of neuronal TLR activation on the expression of the majority of these factors has yet to be investigated. For example, in chapter 3, it is shown that TLR4, 2 and 7 all display a substantial amount of co-localisation with P2X3 in the TG and DRG. It is therefore possible that activation of these receptors, including the activation of TLR4 by *P. gingivalis* LPS, may alter P2X3 receptor function. P2X3 activation and sensitisation is central to the development and maintenance of both inflammatory and neuropathic pain states, including those induced by LPS (Franceschini et al. 2013). Activation of neuronal TLRs has been shown to activate signalling pathways such as p38 MAPK and ERK1/2 (Tse et al. 2014a) and it is well accepted that the activation of such pathways induces the sensitisation of P2X3 as well as multiple other receptors and ion channels on the neuronal membrane (see Fig. 1.1). Therefore the activation of TLRs may have implications for a wider range of receptors and channels than has been previously described.

A mechanistic role for neuronal TLRs in the initiation of pain associated with chronic periodontitis may still be proven by their ability to recognise DAMPs. Blocking TLR signalling in pain models associated with sterile inflammation, such as cancer pain and nerve injury, has been shown to reduce pain-like behaviour (Raghavendra et al. 2004; Tanga et al. 2005; Kim et al. 2007; Cao et al. 2009). This suggests that DAMPs may trigger a cycle of signalling events that
contributes to the potentiation of persistent pain. As previously mentioned, pain experienced in periodontitis patients is often associated with advanced disease and this, in turn, is associated with increasing amounts of DAMPs in the local micro-environment. In support of this, the ability of the specific DAMPs, S100 calcium-binding protein A8 (S100A8) and α2-macroglobulin, to directly activate sensory neurons has been demonstrated in DRG neurons innervating the knee joint (Miller et al. 2014). A role for HSP70, signalling via TLR4, has also been demonstrated in TG neurons in a model of tongue-referred pain associated with tooth pulp inflammation (Ohara et al. 2013). S100A8, α2-macroglobulin and HSP70, are often identified at increased levels within the GCF of periodontitis patients compared to healthy controls (Chen et al. 1998; Kojima et al. 2000; Kido et al. 2012), as have a wide range of other endogenous molecules (Kido et al. 2012) that are known to contribute towards pain (see Kato & Svensson 2015). Therefore neuronal TLRs may be activated by their respective endogenous agonists (see Table 1.1) and contribute to pain experienced in advanced periodontitis.

The activation of neuronal TLRs also remains relevant for the development of inflammatory and neuropathic pain in other orofacial pain conditions. This has been demonstrated for nociception associated with acute and chronic pulpitis (Ohara et al. 2013; Lin et al. 2015) and could also potentially be extended to include infectious conditions such as burning mouth syndrome, stomatitis and pericoronitis (see Drangsholt & LeResche 2009). Additionally, TMJ disorders associated with chronic tissue damage result in the release of endogenous TLR agonists including fibrinogen, HSP60, HSP70, hyaluronic acid and HMGB1 (see Haseeb & Haqqi 2013) some of which are associated with pain (see Kato & Svensson 2015). Further, the dense sensory neuron innervation of oral tissues means that a neuropathic component of an increasing number of orofacial pain conditions, such as idiopathic facial pain and burning mouth pain, is being identified. In these
situations, nerve injury, like other tissue damage, is associated with the release of endogenous TLR agonists. Sensory neurons express the necessary TLRs to detect the exogenous and endogenous TLR agonists associated with these conditions and the activation of these TLRs may directly contribute to the pain response.

In conclusion, the results presented in this thesis support the hypothesis that the activation of TLRs, expressed by trigeminal nociceptors, may contribute to the initiation and maintenance of pain hypersensitivity in a range of orofacial disorders. Superficial nerve endings within peripheral tissues are ideally placed to detect infection or tissue damage through the activation of TLRs. This is particularly relevant for many unique orofacial tissues given their relatively high density of innervation. The activation of these neuronal TLRs represents an innate sensing ability of nociceptors that not only has implications for neuronal excitability but also the orchestration of an inflammatory immune response. Neuronal TLR signalling has the potential to be exploited by pathogens to modify the host response, as is the case for TLRs expressed by immune cells (Hajishengallis & Lamont 2014). In addition, better understanding of the expression patterns of TLRs in sensory neurons has the potential for novel therapeutic approaches to pain control. For example, a recent study has utilised the specific expression of TLR5 on A-fibres to reduce the mechanical allodynia associated with nerve injury through the application of TLR5-targeted Na⁺ channel blockers (Xu et al. 2015).

The key findings and contributions of the present study to the greater research area report that:

- TG sensory neurons express TLR4, TLR2 and TLR7.
- This expression is exclusive to nociceptor populations as demonstrated by a significant degree of co-localisation with the neurochemical markers TRPV1
and P2X3, but not NF200.

- TLR4 and TLR2 are expressed by a proportion of non-neuronal cells within sensory ganglia, whereas TLR7 is only expressed by neurons.

- TG sensory neurons constitutively express a range of TLR-associated co-receptor and signalling adapter genes.

- TG sensory neurons in vitro up-regulate the TLR4-dependent expression of TNFα and IL-1β mRNA, but not IL-6 or IFNβ mRNA, in response to E. coli LPS.

- TG sensory neurons in vitro up-regulate the expression of TNFα and IL-1β mRNA, but not IL-6 or IFNβ mRNA, in response to synthetic TLR2 and TLR7 agonists.

- No cytokine induction was observed in response to two structurally different isoforms of P. gingivalis LPS.

- TG sensory neurons constitutively express LPCAT1 but not LPCAT2 under naïve conditions.

- LPCAT1 expression may not be specific to one sub-population of sensory neuron as it co-localises with TRPV1, P2X3 and NF200.

- LPCAT2 is expressed by the majority of satellite cells within sensory ganglia whereas LPCAT1 is expressed by a smaller proportion of non-neuronal cells.

- LPCAT2 gene and protein expression is induced in TG sensory neurons following nerve injury.
6. General discussion

6.2 Future perspectives

The expression of TLR4 in sensory neurons has been previously shown to be inducible by tissue damage. As mentioned previously, it has been demonstrated that 19% of neurons in the maxillary region of the TG and 29% of neurons innervating the gingivomucosa express TLR4 under normal conditions (Vindis et al. 2014). Following ligature induced periodontitis this expression increased to 32% and 41% respectively. Similarly, TLR4 is up-regulated in peptidergic neurons within the TG in the setting of acute pulpitis following dental injury (Lin et al. 2015). The inducibility of other neuronal TLRs has not been demonstrated following tissue damage. The alteration in neuronal expression following the onset of infection, such as periodontitis, has not been investigated. The expression of TLR1-10, excluding TLR7 and 8, are all increased in epithelial cells in patients with periodontitis (Beklen et al. 2008). Therefore investigating the periodontitis-induced alteration in neuronal TLR expression represents an interesting avenue of future research.

Despite recent advances in the field, the specific mechanisms of TLR activation in neurons remain poorly understood. For example, the specific mechanisms that govern receptor activation in neurons have not been described. In immune cells, TLR4 undergoes dimerisation and translocation to lipid raft domains within the cell membrane in order to activate intracellular signalling pathways following ligand binding. It is not known whether neuronal TLRs localise to lipid raft domains however a functional association between TLR7 and TRPA1, which is thought to be clustered in raft domains (Saghy et al. 2015), has recently been demonstrated (Park et al. 2014). Therefore it is likely that neuronal TLRs may also cluster in lipid raft domains to activate intracellular signalling cascades. Describing the specific mechanisms of TLR activation at the neuronal membrane may provide
6. General discussion

an interesting insight into the interaction of TLRs with other receptors and ion channels such as TRPV1 and P2X3. Previous studies have identified a central role for LPCAT2 in the activation and translocation of TLR4 and TLR2 within the phospholipid membrane (Abate & Jackson 2015). Chapter 5 of this thesis demonstrates that the expression of LPCAT2 in TG sensory neurons is not detectable under normal circumstances however the up-regulation of LPCAT2 mRNA and protein is induced by nerve damage. A potential role for LPCAT2 upstream of neuronal TLR activation therefore remains a possibility following nerve injury, however this requires further research. For example, the specific neuronal sub-populations that express LPCAT2 under these conditions and whether these cells also co-express TLRs needs to be demonstrated. Investigation into the expression of LPCAT2 in neurons under the setting of chronic inflammation is also needed in order to determine a potential role for LPCAT2 in inflammatory pain states.

Significant voids in current knowledge remain that are also particularly relevant for the transition to and maintenance of chronic pain. The current study focusses on the acute activation of neuronal TLRs however, in the setting of many chronic pain states, sensory neurons are exposed to multiple TLR agonists over time. This is indeed the case for common orofacial pain disorders associated with chronic inflammation and tissue damage such as TMJ disorders and carious tooth decay. Neuronal TLRs may therefore face repeated activation by the same or different agonists during the course of these disorders. The repeated activation of TLRs expressed by immune cells can either lead to a heightened sensitivity or a dampened response, through processes known as priming and tolerance, respectively. This is dependent on a range of factors including cell type, the agonist used and the agonist concentration. Repeated activation of nociceptors often results in a decreased activatory threshold and therefore increased excitation and neuronal sensitisation. Whether neuronal TLRs share this capacity for priming and toler-
ance with immune cells and how this may integrate with the multiple mechanisms responsible for neuronal plasticity is not currently known. Understanding this would significantly advance the knowledge of neuronal TLRs and may provide a valuable insight into potential mechanisms of the maintenance of chronic pain. This may also be particularly relevant in the case of chronic periodontitis. The acute application of \textit{P. gingivalis} LPS isoforms does not induce a transcriptional up-regulation of inflammatory cytokines in sensory neurons however the priming effect of this initial exposure is unknown. It is therefore possible that repeated exposure to LPS or subsequent exposure to a different ligand, such as an endogenous ligand associated with tissue damage, may indeed induce a different response.

Finally, it is also important to consider the recent findings that LPS may activate neurons through TLR-independent mechanisms. As previously mentioned, LPS has been shown to activate certain non-canonical receptors including TRPA1 (Meseguer et al. 2014) and various caspases (Shi et al. 2014). TRPA1 is considered to be a key transducer of a wide range of chemical stimuli associated with inflammation so it is perhaps not surprising that it may also directly detect foreign molecules such as LPS. Interestingly, TRPA1 has also been demonstrated to regulate the activation of TLR7 in sensory neurons (Park et al. 2014). Therefore, whilst TRPA1 may directly detect certain PAMPs or DAMPs, it may also play a key role in regulating the activity of neuronal TLRs. Further research is required to provide a more detailed description of the potential innate surveillance function of TRPA1. A broader study investigating the co-expression of TRPA1 and multiple neuronal TLRs as well as the ability of TRPA1 to directly detect other TLR agonists would provide an insight into this. Future work should also focus on the interaction between TRPA1 and neuronal TLRs in order to better understand the molecular mechanisms of neuronal TLR activation and regulation.
Appendix A

Publications
THE EXPRESSION OF TOLL-LIKE RECEPTOR 4, 7 AND CO-RECEPTORS IN NEUROCHEMICAL SUB-POPULATIONS OF RAT TRIGEMINAL GANGLION SENSORY NEURONS

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Abstract—The recent discovery that mammalian nociceptors express Toll-like receptors (TLRs) has raised the possibility that these cells directly detect and respond to pathogens with implications for either direct nociceptor activation or sensitization. A range of neuronal TLRs have been identified, however a detailed description regarding the distribution of expression of these receptors within sub-populations of sensory neurons is lacking. There is also some debate as to the composition of the TLR4 receptor complex on sensory neurons. Here we use a range of techniques to quantify the expression of TLR4, TLR7 and some associated molecules within neurochemically-identified sub-populations of trigeminal (TG) and dorsal root (DRG) ganglionic sensory neurons. We also detail the pattern of expression and co-expression of two isoforms of lysophosphatidylcholine acyltransferase (LPCAT), a phospholipid remodeling enzyme previously shown to be involved in the lipopolysaccharide-dependent TLR4 response in monocytes, within sensory ganglia. Immunohistochemistry shows that both TLR4 and TLR7 preferentially co-localize with transient receptor potential vanilloid 1 (TRPV1) in sensory neurons, a range of functional TLRs have been shown to be expressed by neurons in both the peripheral and central nervous system (Lafon et al., 2006; Mishra et al., 2006; Wadachi and Hargreaves, 2006; Cameron et al., 2007; Acosta and Davies, 2008; Barajon et al., 2009; Ochoa-Cortes et al., 2010; Diogenes et al., 2011; Ferraz et al., 2011; Qi et al., 2011; Due et al., 2012).

Toll-like receptors (TLRs) are a family of innate pattern recognition receptors that detect a wide range of exogenous pathogenic and endogenous damage-released ligands. Since the discovery by Wadachi and Hargreaves (2006) of TLR4 expression on trigeminal sensory neurons, a range of functional TLRs have been shown to be expressed by neurons in both the peripheral and central nervous system (Lafon et al., 2006; Mishra et al., 2006; Wadachi and Hargreaves, 2006; Cameron et al., 2007; Acosta and Davies, 2008; Barajon et al., 2009; Ochoa-Cortes et al., 2010; Diogenes et al., 2011; Ferraz et al., 2011; Qi et al., 2011; Due et al., 2012).

INTRODUCTION

Traditionally it is thought that pathogens interact with sensory neurons through indirect mechanisms involving the activation of an innate immune response and production of pro-inflammatory mediators (see Ren and Dubner, 2010 for review). These indirect mechanisms involve the release of inflammatory mediators and the peripheral sensitization of high-threshold sensory neurons, nociceptors, increasing excitability to sub-threshold stimuli and contributing toward the transition from acute to chronic pain states (for reviews see Marchand et al., 2005; Ren and Dubner, 2010). A complex web of interactions between neurons, non-neuronal cells and immune cells develops to maintain a state of pain hypersensitivity (Milligan and Watkins, 2009; Austin and Moalem-Taylor, 2010; Grace et al., 2011; Nicotra et al., 2012). Previous work on the impact of inflammatory mediators on nociceptor sensitization and pain generation has suggested that the degree of pain associated with infection is heavily influenced by the degree of immune activation (Marchand et al., 2005; Ren and Dubner, 2010). It is now understood that sensory neurons can directly detect and respond to pathogenic challenge independent of the innate immune system.

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The expression of TLRs in neurons, particularly primary sensory neurons, has uncovered a potential innate surveillance function with implications for both the acute nociceptive response and the maintenance of chronic pain states. TLR4 and the co-receptor CD14 are expressed on rat and human transient receptor potential vanilloid 1 (TRPV1)-positive and TRPV1-negative nociceptors within the trigeminal ganglion (TG) (Wadachi and Hargreaves, 2006; Diogenes et al., 2011). Activation of TLR4, by Porphyromonas gingivalis lipopolysaccharide (LPS), on TG sensory neurons both sensitizes TRPV1 and potentiates capsaicin-induced calcitonin gene-related peptide (CGRP) release (Diogenes et al., 2011; Ferraz et al., 2011). A role for TLR4, expressed by TG neurons, in mediating pain induced by tissue damage has also been demonstrated (Ohara et al., 2013; Miller et al., 2014). The activation of TLR4, by Escherichia coli LPS, on murine dorsal root ganglion (DRG) neurons has been shown to increase neuronal excitability (Ochoa-Cortes et al., 2010; Due et al., 2012), nociceptin expression (Acosta and Davies, 2008) and the myeloid differentiation primary response protein 88 (MyD88)-dependent production of pro-inflammatory mediators (Ochoa-Cortes et al., 2010; Tse et al., 2014a). The ability of TLR4 to influence the nociceptive response is not limited to TLR4. Activation of TLR3, 7 and 9 expressed by DRG neurons induces the production of pro-inflammatory mediators, up-regulates TRPV1 expression and sensitizes TRPV1 activation (Qi et al., 2011). Activation of TLR3 and TLR7 induces an itch response (pruritus) and directly activates DRG nociceptors (Liu et al., 2010, 2012). More recently the activation of TLR7, by endogenous murine macrophages, has been shown to rapidly activate DRG neurons through mechanisms that involve TRPA1 (Park et al., 2014).

In the DRG, the reported percentage of total TLR4-positive neurons varies widely (28–60%; Acosta and Davies, 2008; Due et al., 2012; Tse et al., 2014a,b). While it has been shown that 19% of neurons in the maxillary region of the TG and 29% of neurons innervating the gingivomucosa express TLR4, mainly in small- to medium-sized neurons (Vindis et al., 2014), there are currently no quantitative data for TLR4 expression within the TG as a whole. Previous studies have suggested that the expression of TLR7 is limited to small/medium sized neurons that express TRPV1 and TRPA1 although no quantitative analysis has been performed (Liu et al., 2010; Qi et al., 2011; Park et al., 2014).

There is also some debate regarding the composition of the TLR4 receptor complex expressed by sensory neurons. In innate immune cells a functional TLR4-signaling complex consists of TLR4, CD14 and myeloid differentiation protein (MD)-2 (Akira and Takeda, 2004). DRG neurons reportedly express CD14 and MD-1 messenger ribonucleic acid (mRNA) and protein but little MD-2 and no radioprotective 105 (RP105) mRNA or protein (Acosta and Davies, 2008). Upon activation, neuronal TLR4 is reported to form an atypical co-receptor complex with CD-14 and MD-1 (Acosta and Davies, 2008). MD-1 classically interacts with the TLR4 homolog, RP105 to regulate TLR4 signaling (Otto et al., 2011). More recent studies however have shown that DRG neurons do express MD-2 and RP105 mRNA and protein in addition to MD-1 and CD14 mRNA and protein (Ochoa-Cortes et al., 2010; Tse et al., 2014a). Additionally it was shown that the major part of TLR4-positive neurons co-localize with CD14 and MD-2, rather than MD-1 (Tse et al., 2014a).

Lysophosphatidylcholine acyltransferase 1 and 2 (LPCAT1 and LPCAT2) are two isoforms of a phospholipid-modifying enzyme that participates in membrane remodeling by mediating the acylation of lysophosphatidylcholine (see Shindou and Shimizu, 2009). The phospholipid/lysophospholipid composition of cellular membranes affects membrane function, including lipid raft functions (Stulnig et al., 2001), and therefore may impact upon multiple cell signaling pathways including TLR4 signaling (see Triantafillou et al., 2011). In macrophages, LPCAT2 is activated by phosphorylation following TLR4-dependent LPS recognition (Morimoto et al., 2010) and LPCAT activity is essential for the translocation of TLR4 to lipid rafts and subsequent generation of a TLR4 signaling response (Jackson et al., 2008). LPCAT1 and LPCAT2 have been identified in a range of tissues although a high level of expression has been demonstrated in lung alveolar cells and immune cells for LPCAT1 and LPCAT2, respectively (Nakanishi et al., 2006; Shindou et al., 2007; Morimoto et al., 2010). Both LPCAT1 and LPCAT2 expression have been demonstrated in a sub-set of spinal neurons (Okubo et al., 2012). Whereas LPCAT1 expression is constitutive, LPCAT2 is an inducible form of the enzyme (Shindou et al., 2005). Indeed, LPCAT2 is up-regulated in microglia following nerve injury while LPCAT1 expression remains unchanged (Okubo et al., 2012). LPCAT2 expression has also been identified in peripheral sensory neurons as well as non-neuronal cells within the DRG following nerve injury (Hasegawa et al., 2010). While previous studies have identified the expression of LPCAT isoforms in injured DRG neurons, the expression of LPCATs naive peripheral sensory neurons and the subsequent role they might play in the neuronal TLR response is unknown.

While it has been shown that nociceptors express a range of TLRs, a detailed analysis of TLR expression within multiple sensory neuron sub-populations has not been performed, particularly within the TG. A detailed analysis of TLR4 and TLR7 expression patterns within primary sensory neurons is a pre-requisite for further functional analysis of receptor activation. In the current study we explore the hypothesis that nociceptors possess the required molecular components to directly detect and respond to ligands of bacterial, viral and endogenous origin. Using well-defined neurochemical markers, we provide a semi-quantitative analysis of the expression of TLR4 and TLR7 within sensory neuron sub-populations. We also detail a gene expression profile of TLR signaling-associated components within the TG. Lastly, we describe the distribution of expression of two isoforms of a lysophosphatidylcholine acyltransferase (LPCAT) enzyme, LPCAT1 and LPCAT2 within sensory ganglia.
EXPERIMENTAL PROCEDURES

Materials
All materials, unless otherwise stated, were purchased from Sigma–Aldrich (Gillingham, UK). All real-time polymerase chain reaction (qPCR) reagents were purchased from Life Technologies (Carlsbad, CA, USA).

Animal care
Age-matched, adult male Sprague–Dawley rats (>3 months of age, 250–350 g, Charles River, UK) were group-housed in a temperature and humidity-controlled environment with a 12-h light/dark cycle (lights on at 8:00 A.M.) with food and water available ad libitum. All experiments adhered to guidelines described by Schedule 1 of the UK 1986 Animals (Scientific Procedures) Act. A total number of 6 animals were used for this study. 1 for TLR4 and LPCAT immunohistochemistry and 3 for qPCR analysis of TLR-associated gene expression.

Immunohistochemistry
Indirect single- and dual-labeled immunohistochemistry (IHC) was performed to determine the distribution of expression of TLR4, TLR7, LPCAT1 and LPCAT2 and their co-expression with the functional neurochemical markers neurofilament 200 (NF200), thermo-transducer TRPV1 and purinergic receptor P2X3 in the rat TG and DRG. Adult male Sprague–Dawley rats were euthanized in this study. For TLR and LPCAT immunohistochemistry and 3 for qPCR analysis of TLR-associated gene expression.

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was confirmed in merged images. Co-expression was expressed as a percentage of target markers expressing phenotypic markers and vice-versa.

TG dissociation
To investigate TLR-associated gene expression in TG sensory neurons, dissociated TG, rather than whole tissue samples were used in order to remove the majority of non-neuronal cells and non-cellular components. The protocol used for TG dissociation in this study was adapted from Malin et al. (2007). Briefly, male Sprague–Dawley rats were euthanized by exposure to rising concentrations of CO₂. Animals were transcardially perfused with ice-cold HBSS (Calcium and Magnesium-free), TG were rapidly removed and cut into 10–12 small pieces. Tissue was incubated with papain solution (60 units papain, 1 mg L-cysteine in HBSS) for 20 min at 37 °C with gentle mixing halfway. Tissue was pelleted by centrifugation at 800g for 3 min and the supernatant discarded. The tissue was resuspended in collagenase/disperse solution (12 mg collagenase type II and 14 mg dispase type II in HBSS) and incubated for 20 min at 37 °C with gentle mixing halfway. Pre-warmed trypsin inhibitor (1 mg/mL in HBSS) was added to stop enzymatic digestion and tissue was then pelleted by centrifugation at 800g for 3 min. Mechanical disruption by trituration with a sterile fire-polished, silicon-coated glass pipette in pre-warmed L15 medium created a single-cell suspension which was then passed through a 12.5/28% Percoll gradient at 1800 g for 10 min to yield a neuron-enriched cell pellet which was immediately processed for RNA isolation.

Real-time polymerase chain reaction (qPCR)
Total messenger RNA (mRNA) from dissociated TG neurons and whole spleen tissue was isolated using RNAqueous-Micro kit according to the manufacturer’s instructions. Isolated RNA was treated with DNase I for 20 min at 37 °C to remove any genomic DNA contamination. Total RNA concentration was determined using a Qubit 2.0 Flurometer with the Qubit RNA Assay Kit. Sample purity was assessed using a nanodrop 2000 (Thermo-Fisher Scientific, Waltham, MA, USA). Only samples with a 260/280 ratio of > 1.8 were used for reverse transcription. First-strand cDNA was synthesized from 100 ng RNA using the SuperScript III reverse transcriptase (Reaction conditions: 25 °C for 10 min, 42 °C for 2 h, 85 °C for 5 min). cDNA was stored at −20 °C until used for qPCR. Relative quantification was achieved with qPCR using TaqMan Gene Expression Assays for TLR4 (Rn00569848_m1), MD-1 (Rn01434815_m1), MD-2 (Rn01448830_m1), CD14 (Rn00572656_m1), MyD88 (Rn01640049_m1) and TRIF-related adapter molecule (TRAM) (Rn02082474_s1) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn01775763_g1) as the endogenous control. All qPCR experiments used TaqMan Fast Advanced Master Mix and were performed in triplicate on a QuantStudio 12 k Flex system with a Fast 96-well Block using 2.5 ng of template cDNA in a total reaction volume of 10 μL per well. The reaction parameters were as follows – 50 °C for 2 min, 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s. An initial no RT control for each sample type was performed to assess the effectiveness of the DNase treatment used which returned an undetectable result. No template controls were also included in every run and returned undetectable results. A gene expression profile from each tissue type was created using ΔΔCt values, with GAPDH set at 100. Analysis of gene expression data between tissue types was performed using the 2−ΔΔCt method (Livak and Schmittgen, 2001) with target gene expression in each sample normalized against the endogenous control gene GAPDH.

Statistical analysis
Data are displayed as mean ± SEM, n = 3. Comparisons between groups were made using a Student’s t-test, as appropriate, performed on IBM SPSS statistics software version 21.0 (1 animal = 1 unit for statistical analysis). Differences were considered statistically significant when p < 0.05.

RESULTS
Expression of TLR4 & TLR7 within the adult mammalian TG & DRG
Indirect single-labeling IHC was used to investigate the expression of TLR4 and TLR7 within the naïve adult rat TG and DRG. Multiple neuronal profiles displayed TLR4- and TLR7-IR in the TG and DRG (Fig. 1).
Whereas the expression of TLR7 was strictly neuronal, a proportion of non-neuronal cells that were morphologically identified as satellite cells (elongated fusiform cells that envelope neuronal soma) also expressed TLR4 (Fig. 1A, B). Non-neuronal staining was differentiated from neuronal membrane staining by carefully examining the co-expression patterns with neuronal markers and also by identifying the non-neuronal cell nucleus. No overlap of staining with neuronal markers was observed and clear, distinguishable nuclei were identified hence we characterized this staining as non-neuronal.

TLR4 was expressed by 29.3 ± 3.4% and 32 ± 2.9% of total sensory neurons in the TG and DRG, respectively. TLR7 was expressed by 32.4 ± 1.8% and 35 ± 3.9% of sensory neurons in the TG and DRG, respectively. There was no significant difference in expression levels of TLR4 and TLR7 between the TG and DRG (Student’s t-test). TLR4-TLR7-IR neurons are expressed as a percentage of total neuronal profiles as defined using a DAPI counterstain. Rat spleen tissue was used as a positive control and displayed multiple TLR4- and TLR7-IR cells, respectively (Fig. 2). Red blood cells were used as a negative control tissue as they do not express TLR4 or TLR7. No staining was observed in the negative control tissue (Fig. 3). Spleen tissue was stained in the absence of primary antibodies to assess the specificity of the secondary antibodies used. No staining was observed under these conditions (Fig. 4).

Co-expression of TLR4 & TLR7 with neurochemical markers of functional sensory neuron populations

Indirect dual-labeling IHC was used to characterize the degree of co-expression of TLR4 and TLR7 with neurochemical markers that are indicative of functional sensory neuron populations within the naïve adult rat TG and DRG. TLR4-IR was commonly observed in TRPV1- and P2X3-expressing neurons, respectively (Fig. 5A–F). However, in contrast to TRPV1 and P2X3, TLR4 was virtually absent from all NF200-expressing neurons (Fig. 5G–I). Similar to TLR4, TLR7-IR was identified in a large proportion of TRPV1- and P2X3-expressing neurons, respectively (Fig. 6A–F). TLR7 and NF200 expression was also mutually exclusively (Fig. 6G–I). Similar patterns of co-expression were observed for TLR4 and TLR7 within the TG and DRG. A full characterization of TLR4 and TLR7 co-localization within the TG and DRG is shown in Table 1.

There was a significant difference between TLR4 and TLR7 in their relative levels of co-expression with the nociceptor markers used. TLR4 co-localized more frequently with P2X3-positive neurons compared to TRPV1-positive neurons (TLR4/TRPV1 co-expression vs. TLR4/P2X3 co-expression, p < 0.0001 for TG and DRG, Student’s t-test). In the TG, TLR7 co-localized more frequently with TRPV1-positive neurons compared to P2X3-positive neurons (TLR7/TRPV1 co-expression vs. TLR7/P2X3 co-expression, p < 0.05 for TG, Student’s t-test).

Expression of TLR4 signaling-associated molecules in TG sensory neurons

We performed qPCR on dissociated TG sensory neurons in order to create a gene expression profile for TLR4 signaling-associated components. We evaluated the gene expression of TLR4 and three TLR4 co-receptor molecules, MD-1, MD-2 and CD14, as well as two intracellular signaling molecules that represent two pathways of the TLR4 intracellular signaling cascade, MyD88 and TRAM. The MyD88 pathway is also downstream from all other TLRs, with the exception of TLR3. For reference purposes, we have also included the pain-associated genes TRPV1, P2X3 and TrkA. The expression of these target genes relative to the housekeeping gene GAPDH (set at 100) is shown for TG sensory neurons (Fig. 7A) and control (spleen tissue, Fig. 7B), respectively. All target genes are expressed in TG sensory neurons (Ct < 35) albeit at lower levels than TRPV1, P2X3 and TrkA expression, respectively (Fig. 7A). Gene expression levels of the
Fig. 3. TLR expression in red blood cells as a negative control. Phase-contrast microscopy was used to identify red blood cells (A). No positive staining was observed in red blood cells for TLR4 (B) or TLR7 (C) antibodies. Scale bar 50 μm.

Fig. 4. Secondary antibody control staining in spleen tissue. Tissue staining was performed in the absence of primary antibody in order to observe non-specificity of secondary antibodies. Minimal levels of non-specific background staining were observed for Donkey anti-Rabbit AF488 (A), Donkey anti-Mouse AF555 (B) and Donkey anti-Guinea Pig AF594 (C). Scale bar 50 μm.

Fig. 5. Fluorescence micrograph of rat TG dual-labeled for TLR4-IR (green, A, D, G) plus either TRPV1- (red, B), P2X3- (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localization of TLR4-IR with neurochemical markers. A substantial proportion of TLR4-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Not all TRPV1-IR neurons express TLR4, examples of co-expressing neurons are indicated by filled arrows (A, B, C). A substantial proportion of TLR4-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E, F) and by asterisks in the combined image (F). Also however, not all P2X3-IR neurons express TLR4, examples indicated by open arrows (D, E, F). There was minimal co-localization between TLR4-IR and NF200-IR; examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The majority of NF200-IR neurons did not express TLR4-IR, examples indicated by open arrows (G, H, I). A similar pattern of expression and co-expression was observed in the DRG (images not shown). Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
TLR-associated components within TG sensory neurons were also compared to those of the primary spleen tissue which is known to contain a broad range of TLR-expressing immune cells. All genes are expressed at lower levels in TG sensory neurons than in the spleen tissue (Fig. 7C). MD-1 was expressed 290.1 ± 36.3-fold lower in TG sensory neurons compared to primary spleen tissue although this is not surprising given the high B lymphocyte content of the spleen tissue (Cesta, 2006). TLR4 gene expression was the most comparable between the different tissue types with a 9.1 ± 1.1-fold lower expression in TG sensory neurons. The remaining targets were expressed 9.8 ± 2.1 (TRAM), 13 ± 2.1 (MD-2), 24.5 ± 3.1 (MyD88) and 40 ± 8.2 (CD14) fold lower in TG sensory neurons than the spleen.

Expression of LPCAT isoforms in the TG

We performed indirect single- and dual-labeling IHC to investigate the expression of LPCAT1 and LPCAT2 within the TG and DRG and their co-expression with neurochemical markers. LPCAT1-IR was identified in

![Fig. 6. Fluorescence micrograph of rat TG double labeled for TLR7-IR (green, A, D, G) plus either TRPV1- (red, B), P2X3- (red, E) or NF200-IR (red, H). The combined images (C, F, I) show co-localization of TLR7-IR with neurochemical markers. A substantial proportion of TLR7-IR neurons co-express TRPV1-IR, examples of co-expressing neurons are indicated by filled arrows (A, B) and by asterisks in the combined image (C). Some neurons expressing low levels of TRPV1-IR neurons do not display TLR7-IR, examples indicated by open arrows (A, B, C). A substantial proportion of TLR7-IR neurons also co-express P2X3-IR, examples of co-expressing neurons are indicated by filled arrows (D, E) and by asterisks in the combined image (F). Also however, not all P2X3-IR neurons display TLR7-IR, examples indicated by open arrows (D, E, F). There was minimal co-localization between TLR7-IR and NF200-IR, examples of co-expressing neurons are indicated by filled arrows (G, H) and by asterisks in the combined image (I). The vast majority of NF200-IR neurons did not express TLR7-IR, examples indicated by open arrows (G, H, I). A similar pattern of expression and co-expression was observed in the DRG (images not shown). Scale bar 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1. Summary of TLR4 and TLR7 co-localization with the neurochemical markers TRPV1, P2X3 and NF200 within the naïve adult rat TG and DRG. Co-expression was expressed as a percentage of target markers expressing phenotypic markers and vice-versa

<table>
<thead>
<tr>
<th>Neurochemical label</th>
<th>% of labeled cells that express TLR4</th>
<th>% of TLR4-positive cells that express label</th>
<th>% of labeled cells that express TLR7</th>
<th>% of TLR7-positive cells that express label</th>
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<tbody>
<tr>
<td><strong>TG</strong></td>
<td></td>
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<tr>
<td>TRPV1</td>
<td>53.7 ± 0.9</td>
<td>57.8 ± 0.9</td>
<td>75.7 ± 2.5</td>
<td>42.6 ± 8.1</td>
</tr>
<tr>
<td>P2X3</td>
<td>76.6 ± 0.4</td>
<td>58.2 ± 2</td>
<td>61.6 ± 2.1</td>
<td>33.4 ± 3.1</td>
</tr>
<tr>
<td>NF200</td>
<td>3.5 ± 0.7</td>
<td>9.2 ± 1.5</td>
<td>–</td>
<td>–</td>
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<tr>
<td><strong>DRG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV1</td>
<td>56.1 ± 1.4</td>
<td>68.2 ± 4.7</td>
<td>78.6 ± 2.6</td>
<td>45.5 ± 10.5</td>
</tr>
<tr>
<td>P2X3</td>
<td>81.9 ± 0.6</td>
<td>76.5 ± 2.5</td>
<td>54.8 ± 9.4</td>
<td>32.5 ± 4.8</td>
</tr>
<tr>
<td>NF200</td>
<td>4 ± 1.3</td>
<td>6.9 ± 2.1</td>
<td>–</td>
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</table>
50.4 ± 4.5% of total neurons (Fig. 8A, B). A proportion of non-neuronal cells that were morphologically identified as satellite cells also displayed weak IR for LPCAT1 (Fig. 8A, B).

LPCAT1 was expressed by 86.3 ± 4.7%, 67 ± 7.9% and 47.7 ± 5.3% of TRPV1-, P2X3- and NF200-expressing neurons, respectively (Fig. 9).

All neuronal profiles were negative for LPCAT2 but the majority of non-neuronal cells displayed strong LPCAT2-IR (Fig. 8C, D).

Rat lung and spleen tissue were used as positive control tissues for LPCAT1 and LPCAT2, respectively. Multiple positively stained cells can be identified (Fig. 2E–H). As LPCAT isoforms are expressed across a range of tissue types we used western blotting to demonstrate the specificity of the primary antibodies. A single band at the expected molecular weight was identified for both LPCAT1 and LPCAT2 in the TG, DRG, cortex and RAW264.7 cells (Fig. 10).

**DISCUSSION**

Previous studies have shown both TLR4 and TLR7 to be expressed by nociceptors within mammalian sensory ganglia (Wadachi and Hargreaves, 2006; Liu et al., 2010; Qi et al., 2011; Due et al., 2012; Park et al., 2014) however this is the first quantitative description of TLR4 and TLR7 expression within adult mammalian...
Here we show that TLR4 and TLR7 are expressed by both capsaicin- and ATP-responsive nociceptors due to a high degree of co-expression with TRPV1 and P2X3, respectively. We show that dissociated sensory neurons from the TG express a range of TLR4-associated genes that are required for a full TLR4 signaling response. We also show that LPCAT1 and LPCAT2 are expressed within sensory ganglia although only LPCAT1 displays neuronal expression in native tissue.

Our TLR4 expression data fall within the range of expression observed in previous studies on DRG tissue sections (28–34% of total neurons; Due et al., 2012; Tse et al., 2014b). Previous studies have reported that non-neuronal cells do not express TLR4 (Due et al., 2012; Tse et al., 2014a) however here we show that a proportion of satellite cells do indeed display TLR4-IR. There was no difference in expression levels of TLR4 and TLR7 between the TG and DRG. Interestingly however there was a significant difference between TLR4 and TLR7 in their relative levels of co-expression with the nociceptor markers used. TLR4 co-localized more frequently with P2X3-positive neurons whilst TLR7 co-localized more frequently with TRPV1-positive neurons.
The functional significance of this observation is currently unknown although Park et al. (2014) have recently shown that TLR7 interacts with TRPA1, expressed by a proportion of TRPV1-positive neurons, to directly activate sensory neurons. We found minimal co-localization between TLR4/TLR7 and NF200 suggesting that TLR4 and TLR7 selectively localize to C-fiber nociceptors. Preferential and direct activation and/or sensitization of C-fiber nociceptors by pathogens and damage-released endogenous molecules (DAMPs) via TLR4 and TLR7 is therefore potentially possible.

There is now extensive evidence for the involvement of neuroimmune signaling in the development and maintenance of chronic pain states (reviewed by Nicotra et al., 2012). TLRs are an important receptor family involved in the complex intercellular signaling network that develops in the setting of chronic inflammatory pain. TLR4 is the most widely studied member of the receptor family in this regard and it is rapidly up-regulated following the onset of both inflammatory and neuropathic pain (DeLeo et al., 2004; Raghavendra et al., 2004; Zhao and Zhang, 2015). TLR4 deletion (Tanga et al., 2005) and pharmacological inhibition have both been shown to prevent (Bettoni et al., 2005) and reverse behavioral hypersensitivity (Hutchinson et al., 2007, 2008, 2010; Lan et al., 2010) and decrease the production of pro-inflammatory mediators in models of neuropathic pain (Tanga et al., 2005; Lan et al., 2010). Importantly, the involvement of TLR4 in the development and maintenance of chronic pain has also been demonstrated in a human model. A low intravenous dose of LPS sensitized nociceptors to a subsequent capsaicin-dependent flare, allodynia and hyperalgesia however the effect of LPS on neurons in this study is likely to be mainly indirect (Hutchinson et al., 2013). These findings have been extended to include the contribution of TLR2 and TLR3 to central nervous system preclinical pain models (Kim et al., 2007; Obata et al., 2008; Mei et al., 2011). TLR3 (Liu et al., 2012) and TLR7 (Park et al., 2014) activation has also been shown to directly activate peripheral sensory neurons in the murine DRG. Early research in this field has focussed mainly on TLR4 however more TLRs are emerging as important mediators of pain hypersensitivity. Whilst the role, for example, of TLR7 in clinical pain models has yet to be studied, peripheral blood mononuclear cells from chronic pain patients display increased responsiveness to TLR7 ligand stimulation, as well as TLR2 and TLR4 (Kwok et al., 2012).

Based on the findings of the current study, plus others, it is likely that TLR activation may occur directly and preferentially on nociceptors without the necessary intervention of the innate immune system. The ability of neurons to directly detect and respond to pathogenic ligands represents a rapid response mechanism that has significant consequences for acute nociceptor activation, sensitization and altered neuronal/non-neuronal cell communication. There is also potential for sensory neurons to detect DAMPs (Goh and Midwood, 2011) and this has been demonstrated in TG (Ohara et al., 2013) and DRG (Miller et al., 2014; Park et al., 2014) sensory neurons. Following TLR4 activation, for example, peripheral sensory neurons display increased excitability (Ochoa-Cortes et al., 2010; Due et al., 2012), sensitization of TRPV1 and potentiation of TRPV1-dependent neuropeptide production (Diogenes et al., 2011; Ferraz et al., 2011). Various pro-inflammatory mediators are also induced downstream from TLR4 activation (Ochoa-Cortes et al., 2010; Tse et al., 2014a). Neuronal TLR7 activation induces rapid inward currents and action potentials to increase neuronal excitability through mechanisms involving TRPA1 (Liu et al., 2010, 2012; Park et al., 2014). An up-regulation and sensitization of TRPV1 and induction of pro-inflammatory mediators is also observed following neuronal TLR7 activation (Qi et al., 2011). It is likely that these mediators can further act through autocrine and/or paracrine mechanisms to further alter nociceptive responses and contribute to the local inflammatory response (Opree and Kress, 2000; Sommer and Kress, 2004; Binshok et al., 2008; Milligan and Watkins, 2009; Uceyler et al., 2009; Zhang et al., 2011; Ji et al., 2013).

This study is the first to demonstrate a co-localization between TLR4 and TLR7 with P2X3-expressing neurons. P2X3 activation, by ATP, induces rapid nociceptor depolarization which plays a prominent role in the sensitization of nociceptors and alteration of neuronal/non-neuronal cell communication following the onset of neuroinflammation (Fabbretti, 2013). It is known that LPS evokes an up-regulation and sensitization of P2X3 receptors in primary TG neuron cultures although it is not known whether this is through a direct action on neurons or secondary to the activation of non-neuronal cells and subsequent release of ATP and inflammatory mediators (Franceschini et al., 2011). Here we show that a large proportion of P2X3-positive neurons co-express TLR4 and TLR7 suggesting that bacterial and viral infection, respectively, could directly modulate P2X3 receptor function. We suggest that the activation of TLR4 and TLR7 could be sufficient to sensitize P2X3 in a similar manner to that of TLR4-dependent TRPV1 sensitization.

The exact composition of the TLR4 receptor complex that is required for neuronal TLR4 activation remains unclear. The co-localization of TLR4 with the co-receptor CD14 has been identified in capsacin-responsive nociceptors within the TG (Wadachi and Hargreaves, 2008) and the involvement of CD14 in neuropathic pain has been demonstrated (Cao et al., 2009). However there is conflicting evidence as to whether MD-1 or MD-2 is involved in neuronal TLR4 signaling. In innate immune cells the TLR4 receptor complex consists of TLR4, CD14 and MD-2 (Akashi-Takamura and Miyake, 2008). MD-1, a MD-2 homolog, normally forms a complex with RP105, a TLR4 homolog that lacks an intracellular Toll/Interleukin-1 receptor (TIR) homology domain (Medzhitov, 2001). One study has shown that neuronal TLR4 interacts with MD-1 rather than the conventional co-receptor MD-2 (Acosta and Davies, 2008). This latter study also showed lack of MD-2 expression in DRG sensory neurons. However more recent studies have identified both MD-1 and MD-2 mRNA and protein expression in DRG nociceptors and shown a preferential co-
localization of TLR4 with MD-2 (Ochoa-Cortes et al., 2010; Tse et al., 2014a). The present study shows that acutely dissociated TG sensory neuron preparations express TLR4, CD14, MD-1, MD-2, MyD88 and TRAM mRNA. This in agreement with Tse et al. (2014a) and Ochoa-Cortes et al. (2010) that sensory neurons express both MD-1 and MD-2. MyD88 and TRAM are also expressed by sensory neurons suggesting that both the MyD88-dependent and MyD88-independent branches of the TLR4-signaling pathway are available following neuronal TLR4 activation. MyD88-dependent signaling downstream of neuronal TLR4 and interleukin-1β (IL-1β) receptor activation has been shown (Davis et al., 2006; Qi et al., 2011; Tse et al., 2014a) however the role of MyD88-independent signaling in peripheral sensory neurons is yet to be demonstrated. Given that the acutely dissociated sensory ganglia cell preparations used in this study contain a proportion of non-neuronal cells and we have shown that a small number of satellite cells display TLR4-IR it is therefore not possible to directly assign this expression purely to neurons. However, given the TLR expression patterns observed in this study as well as the techniques used when dissociating ganglia we believe that it is reasonable to suggest that the gene expression is largely neuronal. It is worth noting that co-receptor components, such as MD-2 and CD14, exist in soluble forms largely neuronal. It is worth noting that co-receptor components do not necessarily need to be expressed by neurons to mediate neuronal TLR4 signaling, a phenomenon which has been demonstrated in lung epithelial cells (Kennedy et al., 2004).

In monocytes, the group of phospholipid modifying enzymes known as lysophosphatidylcholine acyltransferases (LPCAT) is essential for the TLR4 response (Schmid et al., 2003; Jackson et al., 2008). TLR4 associates with lipid raft membrane domains, in an LPCAT-dependent manner, in order to activate intracellular signaling cascades (Triantafilou et al., 2002; Jackson et al., 2008). We have investigated whether specific isoforms of this enzyme, LPCAT1 and LPCAT2, are present in mammalian sensory ganglia and may therefore be involved in neuronal TLR signaling. Although multiple studies show that neuronal TLRs are functional, it is yet to be demonstrated that neuronal TLRs associate with lipid raft microdomains, as is seen in innate immune cells, to initiate these responses. Both isoforms are expressed within sensory ganglia however each displays a distinct expression pattern within the tissue. Approximately half of all TG sensory neurons counted in this study expressed LPCAT1. The expression of LPCAT1 was not exclusive to any one particular sub-population of sensory neuron; LPCAT1 co-localized to a proportion of TRPV1-, P2X3- and NF200-positive neurons within the TG. This suggests that LPCAT1 does not specifically mediate TLR4 signaling in sensory neurons and we suggest that LPCAT1 may play a role in more general membrane physiology common to all sensory neuron populations. Interestingly, we demonstrate a complete absence of LPCAT2 expression in sensory neurons within the naïve TG, suggesting little/no LPCAT2 activity in neurons under normal conditions. Therefore, based on the results presented in this study, we cannot suggest a role for LPCATs in neuronal TLR signaling. It has however been shown that, unlike LPCAT1, LPCAT2 is an inducible enzyme and both the expression and PAF synthesizing activity of LPCAT2 is up-regulated following LPS priming in monocytes (Shindou et al., 2005). Indeed, the expression of LPCAT2 has been identified in sensory neurons and non-neuronal cells in the DRG and spinal cord following nerve injury (Hasegawa et al., 2010; Okubo et al., 2012). Therefore nerve injury appears sufficient to provide a priming signal that increases LPCAT2 expression within peripheral and spinal sensory neurons. It is not known whether LPS exposure is sufficient to induce LPCAT2 expression in neurons and understanding this may be key to uncovering an association of LPCAT2 with neuronal TLR4 signaling.

CONCLUSIONS

The full extent of direct pathogen-neuron interactions is unknown and recently a mechanism for direct activation of nociceptors by bacterial N-formylated peptides and the pore-forming toxin ⧫-hemolysin was identified (Chiu et al., 2013). Pain experienced in a murine model of Staphylococcus aureus skin infection correlated with bacterial load rather than immune involvement (Chiu et al., 2013). Therefore, direct pathogen-neuron interactions may play a more prominent role in the onset of pain hypersensitivity and may not be as reliant on immune cell involvement as was previously thought. A growing body of evidence suggests that TLRs expressed by nociceptors are able to directly influence nociceptor function and contribute to a neuroimmune signaling network involving neurons, non-neuronal and immune cells that act both centrally and peripherally to maintain chronic pain hypersensitivity. In this study we have described in detail the expression of TLR4, TLR7 and other TLR-associated molecules within the adult mammalian TG and DRG. Our results support a mechanism whereby nociceptors can directly detect and respond to pathogenic challenge independent of innate immune activation.

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Appendix B

Conferences attended
Conference presentations:

2015:

*September: Cardiff, UK.* British Society for Oral and Dental Research Biennial Meeting.
Poster presentation: *(Commendation - Unilever Poster Prize)*
Direct bacteria-neuron interaction via Toll-Like Receptors: consequences for orofacial pain.

*September: Plymouth, UK.* Biomedical and Healthcare Science Research Day
Poster presentation:
TLR2-dependent production of pro-inflammatory cytokines in trigeminal sensory neurons.

*May: Edinburgh, UK.* British Neuroscience Association Annual Meeting
Poster presentation:
Lipopolysaccharide-induced production of pro-inflammatory cytokines in trigeminal ganglion neurons via Toll-like receptor activation.

*February: Amsterdam, Netherlands.* Oral Microbiology and Immunology Group Postgraduate Prize Meeting
Oral presentation:
Lipopolysaccharide-induced production of pro-inflammatory cytokines in trigeminal ganglion neurons via Toll-like receptor activation.
Conferences attended

2014:

*September: Dubrovnik, Croatia.* Pan European Regional Congress of the International Association for Dental Research.

Poster presentation:
Toll-like Receptor & Co-receptor Expression in Trigeminal Sensory Neurons.


Poster presentation:
Toll-like Receptor & Co-receptor Expression in Trigeminal Sensory Neurons.

*July: Plymouth, UK.* PU PSMD Research Day.

Poster and Oral presentation: (Winner - Best Poster Prize)
Expression of Toll-like Receptors & Associated Molecules in Trigeminal Ganglion Sensory Neurons.

*February: Plymouth, UK.* Transfer Presentation

Oral presentation:
Expression of Toll-like Receptors & Associated Proteins in Dorsal Root and Trigeminal Ganglion Sensory Neurons.

2013:

*September: Bath, UK.* British Society for Oral and Dental Research Biennial Meeting

Oral presentation:
Expression Patterns of TLR Complex Components Within Trigeminal Sensory Neurons.
**July: Plymouth, UK.** Centre for Research in Translational Biomedicine Research Day

Poster presentation:

Expression Patterns of Toll-like Receptor 4 Complex Components within Trigeminal Sensory Neurons.

**March: Torquay, UK.** Peninsula Schools of Medicine and Dentistry Annual Research Event

Oral presentation:

Neuronal Toll-like Receptors: Mediators of Nociceptor Sensitisation?
Appendix C

Neuronal culture optimisation
C. Neuronal culture optimisation

Purpose

As stated in the main methods section of this thesis, the protocol used to isolate and culture sensory neurons was adapted from Malin et al. (2007). Multiple adaptations were made to this original protocol in order to meet the requirements of the experiments performed. These are detailed below.

Percoll spin

As stated in the original protocol, the procedure for isolating rat TG is slightly different to that regarding murine TG. The recommended Percoll gradient for murine tissue is 12.5%/28% whereas, for rat tissue, it is 30%/60% (Eckert et al. 1997). Therefore this latter composition was selected for use. Following the first Percoll spin, neurons are mainly located at the 30%/60% interface as well as within the 30% layer. A thick ‘axon matt’ is formed on top of the 30% layer (see Fig. 2.1. Eckert et al. (1997) also recommend harvesting this ‘axon matt’ and performing a second Percoll spin in order to maximise the final neuronal yield. Initially this was performed however the neuronal cultures performed using this method contained a large proportion of cellular debris. In order to remove this debris an optimisation experiment was performed whereby individual layers of harvested cells were isolated and cultured individually.

The 30%/60% interface from the first Percoll spin, the 30% layer from the first Percoll spin and the 30%/60% interface from the second Percoll spin were isolated, cultured under the same conditions as previously described and examined using phase contrast microscopy. Both the first interface (Fig. C.1) and 30% layer (Fig. C.2) contained a larger neuron:debris ratio compared to the second interface (Fig. C.3). The second interface also added an extra 30 min to the protocol. Given the extra time required for the second Percoll spin and the low yield of neurons obtained, the second Percoll spin was removed from the protocol.
C. Neuronal culture optimisation

Culture media

The original protocol recommends the use of F12 culture media, supplemented with foetal calf serum and Pen/Strep, for the maintenance of isolated neurons. This was used initially however a very limited amount of neurite outgrowth was observed (see Fig. C.4). Therefore this media was replaced with NBA media supplemented with 2% v/v B-27, 0.5 mM GlutaMAX, 1% v/v Pen/Strep. A further reason for this change in media was to eliminate the need to use serum. Serum is known to contain multiple substances, including NGF for example, that
can alter neuronal function. B-27 serum-free supplement was therefore used in combination with extra glutamine in order to support neuronal survival \textit{in vitro}. The observed amount of neurite outgrowth using NBA media was much higher than previously observed (see Fig. C.5).

![Figure C.4](image1)

**Figure C.4.** Phase micrograph of neurons maintained in complete F12 media.

![Figure C.5](image2)

**Figure C.5.** Phase micrograph of neurons maintained in complete NBA media.

**Conclusions**

From these optimisation experiments it was concluded that the second Percoll spin should be removed from the isolation protocol and that NBA, serum-free culture medium better supports the survival and regeneration of primary sensory neurons \textit{in vitro}. The final protocol for the isolation and culture of rat TG neurons is detailed below.
C. Neuronal culture optimisation

Protocol for the isolation and culture of rat primary sensory neurons.

Stocks required for culture:

- Laminin stock (200 µg/mL). Reconstitute 5 mg laminin powder in 5 mL HBSS and mix thoroughly. Aliquot at 100 µL and store at -20 °C.

- Poly-D-lysine stock (2 mg/mL). Reconstitute 5 mg poly-D-lysine powder in 2.5 mL sterile water and mix thoroughly. Aliquot at 20 µL and store at -20 °C.

- Papain stock (5 mg/mL). Reconstitute 50 mg papain powder in 10 mL sterile water and mix thoroughly. Calculate the volume equivalent of 60 U depending on the specifications of the batch. Aliquot and store at -20 °C.

- L-cysteine stock (6.67 mg/mL). Weigh out 20 mg of L-cysteine powder and add 1.5 mL HBSS. Mix thoroughly, aliquot at 150 µL and store at -20 °C.

- Collagenase stock (50 mg/mL). Reconstitute 50 mg collagenase type II powder in 1 mL HBSS and mix thoroughly. Aliquot at 200 µL and store at -20 °C.

Materials preparation:

- Soak glass coverslips in 1% v/v phosphate-free detergent for approximately 3 h. Wash 5x 10 min in sterile water and allow to air dry in a fume hood. Wrap 7 coverslips in a small amount of foil and sterilise in the hot air oven overnight.

- Fire polish the tips of glass pipettes using a bunsen burner until the bore is visibly rounded but a clear hole is still visible. Allow to cool to room temperature. Silicon coat the pipettes by pipetting RepelCote solution 3
times. Expel as much of the solution as possible and allow to dry with the fine tip facing upwards in a fume hood. Sterilise in a hot air oven overnight.

Day prior to culture:

- Thaw one aliquot of laminin and poly-D-lysine on ice. Add 100 µL of laminin stock and 10 µL of poly-D-lysine stock to 890 µL HBSS and mix thoroughly. This coating solution must be used within 10 min.
- Add one coverslip to each well of a 6 well plate and add 100 µL of coating solution to each coverslip, ensuring that the entire coverslip is covered.
- Incubate the coverslips overnight at 4 °C.

Day of primary culture:

**Solution preparation (For 1 animal, 2x TG)**

1. Add 500 µL Pen/Strep stock and 1 mL HEPES stock to a final volume of 50 mL L15 medium.
2. Add 400 µL B27 stock, 200 µL Pen/Strep stock and 50 µL GlutaMAX stock to a final volume of 20 mL NBA medium.
3. Prepare papain solution by adding 923 µL papain stock (amount equivalent to 60 U, changes with each new batch), 150 µL L-cysteine stock and 3 µL saturated sodium bicarbonate to 424 µL HBSS.
4. Prepare collagenase/dispase solution by adding 14 mg dispase and 200 µL collagenase stock to 2.8 mL HBSS. Mix thoroughly.
5. Prepare trypsin inhibitor solution by adding 7 mg trypsin inhibitor to 7 mL HBSS. Mix thoroughly.
6. Incubate L15, NBA, papain and collagenase/dispase at 37 °C. Incubate
C. Neuronal culture optimisation

trypsin inhibitor at 4 °C.

7. For each animal that is to be dissected, fill a 15 mL tube with 1.5 mL HBSS and keep on ice.

8. Fill small Petri dish with HBSS and keep on ice.

**Tissue dissection**

9. Perform schedule 1 on adult rat by rising concentration of CO₂ and confirm the permanent cessation of a heartbeat.

10. Transcardially perfuse with chilled HBSS for approximately 5 mins.

11. Dissect the two trigeminal ganglia and place in the Petri dish on ice.

12. Repeat the above process if more tissue is required.

13. Chop tissue into approximately 10 pieces and transfer to 15 mL tubes containing HBSS on ice.

**Tissue dissociation**

14. During the below enzyme incubation stages make the Percoll gradient. Make one complete gradient per animal. For the 30% layer, add 1.2 mL Percoll to 2.8 mL warm L15 media in 15 mL tube. For the 60% layer, add 2.4 mL Percoll to 1.6 mL warm L15 media in 15 mL tube.

15. Gently layer 30% layer on top of the 60% layer using a plastic Pasteur pipette. This should be done slowly and carefully to ensure the two layers do not mix. A clear interface between the two layers should be visible. Store at room temperature.

16. Add 1.5 mL of pre-warmed papain solution to each 15 mL tube and mix thoroughly.
17. Incubate at 37 °C for 20 min with vigorous mixing halfway.

18. Centrifuge at 800 x g for 5 min.

19. Remove supernatant and resuspend pellet in 3 mL collagenase/dispease so-

lution.

20. Incubate at 37 °C for 20 min with vigorous mixing halfway.

21. Remove trypsin inhibitor from the fridge and warm to 37 °C during this

incubation.

22. Add 3 mL warm trypsin inhibitor to the tissue to inactivate the enzymes.

23. Centrifuge at 800 x g for 5 min.

24. Remove supernatant and add 500 µL of warm L15 media.

25. Pre-wet a silicon-coated, flame-polished glass pipette with L15 media.

26. Triturate tissue 8-10 times being careful not to introduce air bubbles. The

solution should become cloudy but small tissue chunks may still be visible.

27. Gently layer this cloudy suspension onto the Percoll gradient and centrifuge

at 1800 x g for 10 min.

28. During this spin, remove the coverslips and cell culture-grade water from

the fridge and warm to room temperature.

29. Following the Percoll spin a cloudy layer should be discernible at the 30%/60%

layer interface. A thick, white layer will be located on top of the 30% layer

(see Fig. 2.1). Discard this first layer, trying to remove as much of the
debris as possible.

30. Collect the cloudy layer at the 30%/60% layer interface and dilute into 12

mL L15 media. Mix thoroughly.
31. Centrifuge at 1800 x g for 6 min to pellet the cells.

32. During this spin remove coating solution from the coverslips and wash 2x with cell culture-grade water. Remove final wash and air dry coverslips.

33. Remove supernatant and resuspend pellet in 75 µL NBA medium per coverslip.

34. If processing multiple animals, pool the final cell pellets together before plating.

**Cell culture**

35. Add 75 µL of cell suspension to each coverslip in a ring or doughnut shape. This will prevent cells from grouping together on the middle of the coverslip. Mix cells thoroughly before adding to coverslip.

36. Incubate at 37 °C, 5% CO₂ for 2 h to allow cells to adhere.

37. Add 3 mL of warm NBA media to each well.

38. Maintain cells in culture at 37 °C, 5% CO₂.
Appendix D

Real-time PCR optimisation
D. Real-time PCR optimisation

Quantity of template

Before performing the main qPCR experiments in it was necessary to optimise the amount of template (cDNA) that was loaded into each reaction so that the \( C_T \) values of each target were within a suitable range. Further, due to the limited amount of RNA obtained from one culture preparation (roughly 18 \( \mu \)L of RNA at a concentration of 10 \( \mu \)g/mL) optimisation was required to determine a suitable number of target genes and total reactions performed for each sample. The master mix and PCR run conditions were pre-optimised by the supplier as probe (either TaqMan or geNORM) sets were used. Therefore, using the reaction conditions detailed in chapter 2.11, the \( C_T \) values of CD14 and MD-1 were determined when loading 1.25 ng, 2.5 ng and 5 ng of template into each reaction. Reactions were run in duplicate.

For both target genes the \( C_T \) values increased as the amount of template was decreased (see Fig. D.1). CD14 had a higher \( C_T \) value when compared to MD-1 for each of the three conditions. From these optimisation experiments it was decided that 2.5 ng of template cDNA would be loaded into each reaction. This allowed each of the 7 target genes (4 genes of interest and 3 control genes) to be run in triplicate with enough sample remaining to repeat the run if necessary.

Probe efficiencies

For the cytokine gene expression experiments performed in chapter 4 the amplification efficiencies of the primer and probe sets were considered. Efficiencies were calculated using an algorithm based on the kinetics of each individual reaction without the need for a standard curve (Zhao & Fernald 2005). This was performed on PCR miner online software. Efficiencies were calculated for each experiment and the mean efficiency updated accordingly. Efficiencies can be found below in table D.1.
D. Real-time PCR optimisation

(a) CD14

(b) MD-1

Figure D.1. Amplification graph showing the C_T values of CD14 (a) and MD-1 (b) when loading 1.25 ng (green), 2.5 ng (yellow) and 5 ng (red) of template into the reaction.
### Table D.1. Calculated efficiencies for primer/probe sets used for qPCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Mean efficiency</th>
<th>n</th>
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<th>Standard error</th>
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</thead>
<tbody>
<tr>
<td>TNFα</td>
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<td>0.0114</td>
<td>0.0022</td>
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<tr>
<td>IL-1β</td>
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<td>27</td>
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<td>0.0016</td>
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<tr>
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<td>0.0348</td>
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</tbody>
</table>
Appendix E

*E. coli* LPS-dependent induction of TNFα gene expression in RAW 264.7 cells
Purpose

The bio-activity of the *E. coli* LPS, used in chapter 4.2.4, was demonstrated by exposing the murine macrophage cell line RAW 264.7 cells to LPS and measuring the change in TNFα gene expression.

Methods

RAW 264.7 cells were plated at a density of $1 \times 10^5$ cells per well in a 6 well plate in DMEM culture medium (10% foetal calf serum, 1mM L-glutamine) and allowed to adhere overnight. The following day, culture media was removed and cells were exposed to 1 µg/mL of LPS for 2 hours. Cells were then processed for qPCR as described previously in chapter 2.11.

qPCR was then performed to measure the gene expression of TNFα relative to the endogenous control gene GAPDH. 12.5 ng of template (in 3 µL) and 9 µL of master mix (containing 6 µL Fast SyBr Green master mix, 0.5 µL of primer mix (containing 10 µM of each the forward and reverse primers) and 2.5 µL water) were loaded into each reaction (TNFα forward: AGGACCCAGTGTGGGAAGCT, reverse: AAAGAGGAGGCAACAAGGTAGAGA. GAPDH forward: CCTCGTC-CCGTA-CAAAAATG, reverse: TCTCCACTTTGCCACTGCAA). Each reaction was run in duplicate. Run conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Melt curve: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s.

Analysis was performed as described in chapter 2.11.4. The calculated efficiencies for the TNFα and GAPDH primers were 1.792 and 1.669, respectively.

Results

TNFα was up-regulated by 33.92 fold following exposure to *E. coli* LPS (Fig. E.1). Melt curve analysis for each primer pair produced a single peak (Fig. E.2).
Figure E.1. Relative gene expression of TNFα in RAW 264.7 cells induced by E. coli LPS (1 µg/mL, 2 h). TNFα mRNA is up-regulated by exposure to E. coli LPS. Data is presented as mean of reactions run in duplicate, n = 1.

Figure E.2. Melt curve analysis of TNFα and GAPDH primers. A single peak is observed for each primer pair.
Conclusions

This preliminary experiment shows that the *E. coli* LPS used is able to induce an expected response in a well characterised cell line. This suggests that the results presented in chapter 4.2.4.1 are a true reflection on the physiological situation.
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