



The role of resolvins in chronic pain

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A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

The University of Sheffield
Faculty of Medicine, Dentistry and Health
School of Clinical Dentistry

March 2023

ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisors, Professor Fiona Boissonade, Professor Nikita Gamper, and Professor Dan Lambert, for their guidance, advice, and expertise throughout my PhD, as well as their insightful feedback for this thesis. I would also like to express my gratitude to Dr Matt Worsley for his laboratory assistance and support, particularly with the *in vivo* work. I would also like to thank Dr Emma Bird for her guidance, and Brenka McCabe for her help with molecular biology techniques.

I would like to express my appreciation to Dr Oscar Solis Castro for his advice and help, particularly during the initial stages of my PhD. Also a big thank you to Sara Memarpour Hobbi and Martina Morchio for their support and encouragement. I would also like to thank members of Gamper lab for their warm welcome and assistance during my visit, with a special thanks to Dr Shihab Sarwar Shah for his training and guidance in calcium imaging. I would like to acknowledge Mark Barber for the provision of tooth pulp tissues.

I would also like to extend my gratitude to the MRC DiMeN DTP for their financial support and provision of various training courses.

Last but not least, I would like to thank my family for their continuous support and belief throughout the years.

ABSTRACT

Derived from omega-3 fatty acids, resolvins are instrumental in the active process of terminating inflammation. Their actions are mediated by G-protein coupled receptors (GPCR): BLT1, GPR32, and ChemR23. Recent studies indicate resolvins alleviate pain symptoms in pre-clinical models, suggesting therapeutic potential in chronic pain. However, little is known about the resolvin receptors in human pain-relevant tissues, and the mechanisms by which resolvins may modify pain are poorly understood.

Dental caries can lead to pulpal inflammation and spontaneous pain, but not all patients with caries report symptoms of pain. By comparing receptor expression in normal and inflamed tooth pulp from patients with and without pain, this study aimed to identify receptors that may be important in the modulation of inflammation and pain in man. A series of experiments was first conducted to validate the specificity of the antibodies against BLT1, ChemR23, and GPR32. Using these antibodies, expression of BLT1, ChemR23, and GPR32 was identified in nerve axons and blood vessels in human tooth pulp tissues. Notably, BLT1 expression in the vasculature was found to be higher in patients with pain, indicating its potential role in inflammatory pain.

To explore the responses initiated by the resolvin-BLT1 axis, rodent trigeminal ganglion (TG) cells containing cell bodies of sensory neurons that supply the tooth pulp were used. BLT1 was identified in neurons and satellite glial cells in TG cells. Additionally, E-series resolvins (RvE1 and RvE2) could activate Gq-coupled signalling cascades and induce calcium mobilisation in TG neurons and non-neuronal cells. RvE2 could also activate Gi/o-coupled signalling cascades and reduce cytosolic cyclic AMP in TG cells.

In conclusion, the findings presented in this thesis demonstrate that BLT1 is upregulated in human pain-relevant tissues; furthermore, resolvins can modify activity in sensory neurons via BLT1, further supporting its potential as a novel therapeutic target for chronic pain.

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LIST OF ABBREVIATIONS

5-HT	Serotonin (5-hydroxytryptamine)
5-LOX	5-lipoxygenase
15-LOX	15-lipoxygenase
17-HDHA	17-hydroxy-docosahexaenoic acid
18-HEPE	18-hydroxyeicosatetraenoic acid
AC	Adenylyl Cyclase
AIA	Adjuvant-induced arthritis
ALX/FPR2	Lipoxin receptor (ALX)/N-formyl peptide receptor (FPR)-2
ANOVA	Analysis of variance
B2M	β -2 microglobulin
MPA	Amino-Hydroxy-Methyl-Isoxazolepropionic Acid
ASIC	Acid-sensing ion channel
ATP	Adenosine 5'-triphosphate
AT-Rv	Aspirin triggered-resolvin
BDNF	Brain-derived neurotrophic factor
BK	Bradykinin
BLT1	Leukotriene B4 receptor 1
BSA	Bovine serum albumin
Ct	Cycle threshold
CaMKII	Calcium/calmodulin-dependent protein kinase II
cADDIS	cyclic AMP Difference Detector <i>in situ</i>
cAMP	Cyclic adenosine 3',5'-monophosphate
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
ChemR23	Chemerin receptor 23
CHO	Chinese Hamster Ovary
CHRNA3	Cholinergic receptor nicotinic alpha 3 subunit
CMKLR1	Chemokine receptor-like 1
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CRG	Carrageenan
CTX	Cholera toxin
Cy3	Cyanine 3
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Growth Medium
DRG	Dorsal root ganglion
EC	Extracellular solution
ECL	Enhanced chemiluminescent
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
GDNF	Glial cell-derived neurotrophic factor

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GPCR	G-protein coupled receptor
GPR18	G protein-coupled receptor 18
GPR32	G protein-coupled receptor 32
HBSS	Hank's Balanced Salt Solution
HEK293	Human embryonic kidney 293
HUVEC	Human umbilical vein endothelial cell
IASP	International Association for the Study of Pain
IB4	Isolectin B4
ICC	Immunocytochemistry
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IMS	Industrial Methylated Spirit
IP₃	Inositol 1,4,5-triphosphate
LTB₄	Leukotriene B ₄
LXA₄	Lipoxin A4
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MCP	Monocyte chemoattractant protein
MIA	Monosodium iodoacetate
MIP	Macrophage Inflammatory Protein
miRNA	microRNA
MNX	Medial meniscal transection
NAGly	N-arachidonoyl glycine
NDS	Normal Donkey Serum
NF	Neurofilament
NGF	Nerve growth factor
NK	Natural killer
NK1	Neurokinin-1
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTRK1	Neurotrophic receptor tyrosine kinase 1
NPPB	Natriuretic peptide B
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PENK	Proenkephalin
PFA	Paraformaldehyde
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
PTX	Pertussis toxin
PMN	Polymorphonuclear neutrophils
PUFA	Polyunsaturated fatty acid

qPCR	Quantitative Polymerase Chain Reaction
RT	Reverse Transcription
Rv	Resolvin
RvD	D-series resolvins
RvE	E-series resolvins
SCN10A	Sodium voltage-gated channel alpha subunit 10
SEM	Standard error of the mean
siRNA	Small interfering RNAs
SMA	Smooth Muscle Actin
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SP	Substance P
TBS	Tris-buffered saline
TG	Trigeminal ganglion
TNF-α	Tumour Necrosis Factor alpha
trkA	Tyrosine kinase A
trkB	Tyrosine kinase B
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPM8	Transient receptor potential melastatin 8
UEA I	Ulex Europaeus Agglutinin I
VAS	Visual analogue scale
Vc	Trigeminal spinal subnucleus caudalis
VGCC	Voltage-gated calcium channel
VIP	Vasoactive intestinal polypeptide

CHAPTER 1: INTRODUCTION

Pain is defined by the International Association for the Study of Pain (IASP) as an 'unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage'. Pain sensation is subjective, and can be categorised based on the initiating conditions. For example, neuropathic pain is associated with nerve damage in the somatosensory nervous system, whereas inflammatory pain results from tissue damage and the associated inflammatory response (Xu and Yaksh, 2011). Chronic pain is commonly recognised as pain that persists longer than the normal healing time, which is usually 3 – 6 months. It represents a major global health problem that poses a significant economic burden to society, affecting patients across all clinical disciplines. It is estimated that one in ten adults are newly diagnosed with chronic pain globally every year (Goldberg and McGee, 2011), and approximately 28 million people live with a degree of chronic pain in the UK, and its prevalence increases with age (Fayaz et al., 2016). Furthermore, according to the 2019 Global Burden of Disease Study, chronic pain remains the single greatest cause of years lived with disability (Disease et al., 2020).

Chronic pain is a complex and multifactorial phenomenon. Despite progression in the understanding of the induction mechanisms that produce the perception of pain, the mechanism by which acute pain is resolved remains to be elucidated. Failure in the resolution of acute inflammation is thought to contribute to the transition from an acute to a chronic pain state (Ji et al., 2011). Furthermore, the presence of inflammatory infiltrates at the site of injury is common in both neuropathic and inflammatory pain, and contributes to the generation of behavioural hypersensitivity (Ellis and Bennett, 2013, Vora et al., 2007). Promotion of the resolution of inflammation may therefore aid the resolution of both pain states. Resolution of acute inflammation was once thought to be a passive process, whereby antigen is eliminated to terminate inflammation. However, in light of recent studies, resolution of inflammation is now considered to be an active process regulated by the biosynthesis of specialised pro-resolving mediators such as resolvins. These mediators can control inflammation and promote its resolution, thereby helping to return sites of inflammation to homeostasis (Serhan et al., 2008). Resolvins have also been found to have the ability to attenuate symptoms of inflammation and neuropathic pain (Xu et al., 2013) in animal models, which indicates the therapeutic potential of resolvins as an analgesic. However, the molecular basis by which resolvins regulate activities in sensory neurons is still not well understood.

Chronic pain is a clinical problem that is difficult to treat, particularly with neuropathic pain, wherein pharmacological treatments such as non-steroidal anti-inflammatory drugs (NSAIDs), antidepressants, anticonvulsants, and opioid analgesics do not provide adequate pain relief for all patients (Coles et al., 2022). Non- pharmacological treatments such as

electrical nerve stimulation, acupuncture, and cognitive behavioural therapy are also available, but further clinical studies are needed to demonstrate their effectiveness (Hange et al., 2022, Eller et al., 2022). Furthermore, current medications for chronic pain are limited by several adverse side effects such as the risks of addiction, respiratory depression, and constipation associated with opioids (Benyamin et al., 2008), and the cardiovascular or gastrointestinal adverse effects associated with non-steroidal anti-inflammatory drugs (Wongrakpanich et al., 2018). Further research is therefore needed to improve the understanding of pain mechanisms and identify novel targets as alternative pharmacological management options for treating chronic pain. Resolvins-related drugs represent one potential class of novel analgesics. Therefore, the overall aim of my project is to further investigate the potential role of resolvins in the modification of pain.

1.1. Pain

There are two types of chronic pain: neuropathic pain and inflammatory pain, both of which can occur throughout the body. The neuroscience group at the School of Clinical Dentistry has considerable expertise in investigating both types of pain in the context of orofacial pain. The work has utilised two archives of human tissues: lingual nerve neuromas relevant to neuropathic pain, and tooth pulp relevant to inflammatory pain. In my project, I will use the archive of human tooth pulp tissues, as well as a previously established animal model of lingual nerve injury. In the following section, I will attempt to elaborate on these two types of chronic pain in the context of two examples of orofacial pain: neuropathic pain arising due to lingual nerve injury, and inflammatory pain arising as a result of dental caries. Thereafter, in section 1.2, I will attempt to explore the potential role of resolvins in both neuropathic and inflammatory pain.

1.1.1. Pathways and generation of orofacial pain

Orofacial pain describes pain within the trigeminal system, which can be induced by trigeminal nerve injury or orofacial inflammation, as recently reviewed by Rotpenpian and Yakkaphan in 2021. The orofacial pain transmission pathway associated with the trigeminal nerve is illustrated in Figure 1.1. The trigeminal nerve (V) is the largest cranial nerve, and is formed by three divisions: ophthalmic (V_1), maxillary (V_2), and mandibular (V_3). These divisions join in the trigeminal ganglion (TG), which contains clusters of cell bodies of first-order sensory neurons from all three divisions. Axons from these cell bodies then enter the pons, and descend further into the brainstem to relay information to either the principal sensory nucleus or the trigeminal spinal nucleus (Sessle, 2000). The principal sensory nucleus is situated in the dorsal pons and receives innocuous input such as pressure and light touch from A β -fibres (Bathla and Hegde, 2013). The trigeminal spinal nucleus spans from the pontomedullary junction to the upper cervical cord, and is subdivided rostrocaudally

into three subnuclei: oralis (Vo), interpolaris (Vi), and caudalis (Vc). Neurons in the trigeminal spinal subnucleus caudalis (Vc) receive most of the noxious sensory input from the orofacial region including: deep touch, temperature, and pain from A δ - and C-fibres (Henssen et al., 2016). Historically, Vc was described to be further subdivided into three main subnuclei: subnucleus marginalis, subnucleus gelatinosa, and subnucleus magnocellularis, which correspond to the Rexed's lamina I, lamina II, and laminae III-IV of the spinal dorsal horn respectively (Hockfield and Gobel, 1982). Vc is considered to be analogous to the dorsal horn of the spinal cord and has the same laminated structure. The ophthalmic division terminates in the ventral portion of Vc, the maxillary division terminates in the middle portion; whereas the mandibular division terminates in the dorsal part of Vc (Iwata et al., 2017). Axons of the second-order neurons from the trigeminal nucleus then cross the midline and ascend in the trigeminothalamic tract, terminating in the ventral posteromedial nucleus in the thalamus. Finally, axons from these third-order neurons project to the primary somatosensory cortex where pain information is recognised and processed.

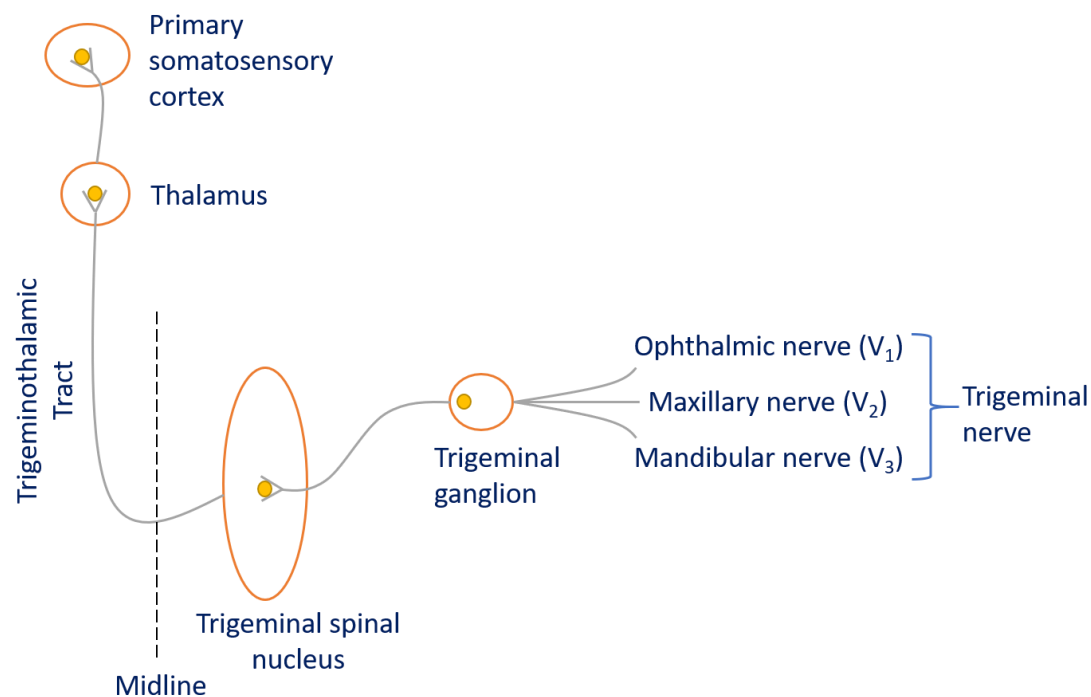


Figure 1.1. Orofacial pain transmission pathway.

Pain impulses in the orofacial region first transmit in the peripheral nerve fibres in the three divisions of the trigeminal nerve, which converge in the trigeminal ganglion. Impulses are then conducted to the spinal trigeminal nucleus and from there, reach the thalamus via the trigeminothalamic tract after crossing the midline. The pain pathway then reaches the primary somatosensory cortex.

The trigeminal nerve provides the majority of sensory innervation to the scalp, face, and mouth, and transmits nociceptive input from these regions to the brain. Sensory

information is carried by three different types of peripheral afferent fibres: A β -, A δ -, and C-fibres. Most A β -fibres respond to low-threshold, innocuous stimuli (Webster, 2002), but some A β -fibres also respond to noxious stimuli (Nagi et al., 2019, Djouhri and Lawson, 2004). A δ - and C-fibres mediate potentially noxious stimuli. A δ -fibres are lightly myelinated and mediate acute, well-localised pain. C-fibres are unmyelinated so they conduct impulses slower than A δ fibres and mediate slow pain which is poorly localised (Basbaum et al., 2009). The peripheral afferent fibres that respond to noxious stimuli are nociceptors, which are 'high-threshold sensory receptors of the peripheral somatosensory nervous system that are capable of transducing and encoding noxious stimuli', as defined by the IASP. A recent more definitive approach characterises human dorsal root ganglion (DRG) neurons using spatial transcriptomics to identify transcripts within individual cells (Tavares-Ferreira et al., 2022). Based on the expression profiles of receptors and ion channels, one A β -nociceptor [characterised by high expression of sodium voltage-gated channel alpha subunit 10 (SCN10A)], one A δ high-threshold mechanoreceptor [characterised by high expression of neurotrophic receptor tyrosine kinase 1 (NTRK1)], and five C-nociceptor subtypes [characterised by high expression of transient receptor potential melastatin 8 (TRPM8), proenkephalin (PENK), transient receptor potential ankyrin 1 (TRPA1), cholinergic receptor nicotinic alpha 3 subunit (CHRNA3), and natriuretic peptide B (NPPB)] have been identified. Furthermore, cell types within the human TG have also been transcriptionally profiled at single-cell resolution using single-nucleus RNA sequencing (Yang et al., 2022). Based on the expression of various neuronal subtype marker genes, a few clusters of nociceptors have been identified: peptidergic nociceptor (enriched with tachykinin precursor 1 that codes for substance P and neurokinin A), non-peptidergic nociceptor [enriched with sodium voltage-gated channel alpha subunit 11 (SCN11A)], and TRPM8+ nociceptor. While there is a high degree of functional similarity between DRG and TG neurons (Lopes et al., 2017), differences between DRG and TG nociceptors have been highlighted in the literature (Megat et al., 2019). However, in the study carried out by Yang et al. (2022), the subtypes of nociceptors within human TG neurons were not characterised in such a detailed manner as the study conducted by Tavares-Ferreira et al. (2022) in human DRG neurons. Therefore, a comparison of the nociceptor subtypes in human DRG and TG neurons could not be made.

The peripheral terminals of nociceptors express receptors and ion channels that can detect mechanical, thermal, and chemical stimuli. For example, transient receptor potential (TRP) channels are attuned to detect specific physical and chemical stimuli. TRPV1 channels are activated by heat, capsaicin, and H⁺ (Julius and Basbaum, 2001), whereas TRPA1 channels are activated by cold, heat, natural compounds in garlic, mustard oil, and noxious chemicals such as tear gas (Zhang et al., 2022). Acid-sensing ion channels (ASICs), ATP-gated P2X receptors, serotonin (5-HT) receptors, tyrosine kinase A (trkA) receptors,

and numerous G-protein coupled receptors (GPCRs) that indirectly activate ion channels are also expressed in peripheral terminals of nociceptors (Sessle, 2011). Activation of these receptors and ion channels thus contributes to pain sensation through the generation of action potentials that propagate to the central nervous system (CNS) (Raouf et al., 2010), as illustrated in Figure 1.2.

Upon detection of acute painful stimuli by nociceptors at the periphery, action potentials are generated, which are then relayed to the central terminals of the nociceptive neurons, as shown in Figure 1.2. This triggers the release of excitatory neurotransmitters such as glutamate, activating postsynaptic AMPA glutamate receptors, resulting in the generation of excitatory postsynaptic potentials (EPSPs) in the second-order neurons. Summation of sub-threshold EPSPs eventually results in the generation of postsynaptic action potentials, pain signals are thus transmitted to higher-order neurons (Iwata et al., 2017). In the conditions where there is prolonged activation of nociceptors, modifications occur in the peripheral and central nervous systems that promote increased excitability, and ultimately lead to central sensitisation.

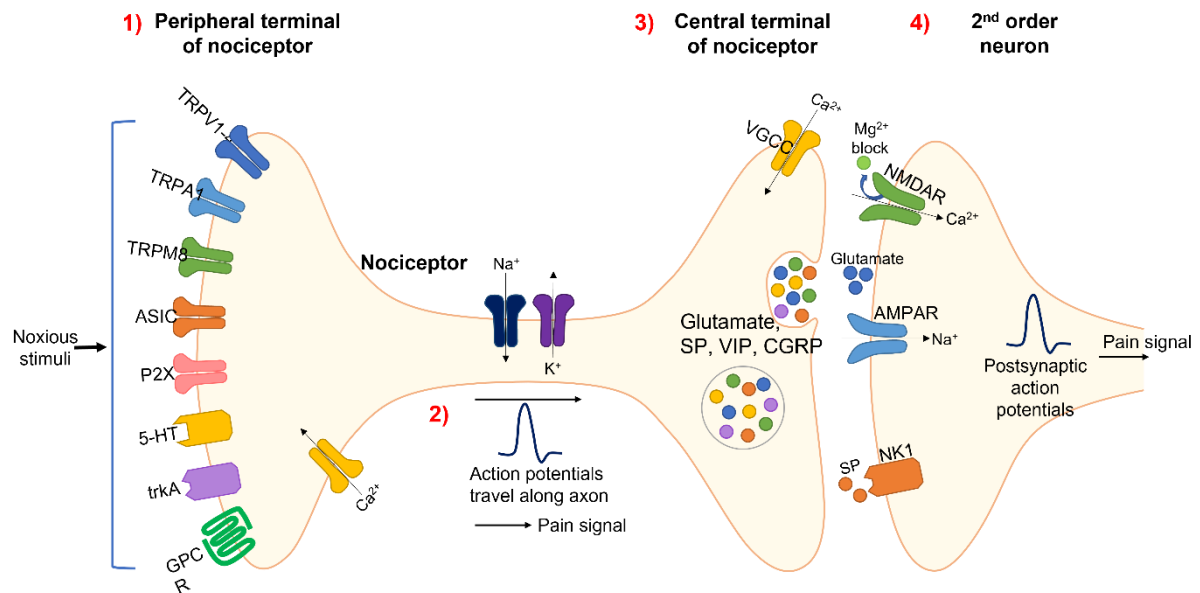


Figure 1.2. Transmission of pain signals from the periphery to the CNS.

1. Peripheral terminals of nociceptors detect noxious stimuli via various ion channels and receptors such as TRP channels, ASICs, ATP-gated P2X receptors, 5-HT receptors, trkA receptors, and numerous GPCRs that activate ion channels indirectly. Noxious stimuli are transduced to electrical signals, eliciting action potentials that propagate to the CNS.
2. Action potentials are propagated along the axon of nociceptors to the presynaptic terminals that synapse with second-order neurons.
3. The release of neurotransmitters (e.g., glutamate) and neuropeptides (e.g., SP, VIP, and CGRP) from the presynaptic terminals is triggered by Ca^{2+} influx via voltage-gated calcium channels (VGCCs).
4. Glutamate activates AMPA receptors (AMPARs), and SP binds to NK1 receptors, resulting in the generation of excitatory postsynaptic potentials (EPSPs) in the second-order neurons to transmit pain signals. Constant noxious afferent input depolarises the membrane, leading to postsynaptic depolarisation, upon which the voltage-dependent Mg^{2+} block in NMDARs is removed. Postsynaptic nociceptive neurons are thus hyperactivated, resulting in central sensitisation, which facilitates the transmission of pain signals to higher CNS regions.

The schematic diagram was adapted with permission based on Olesen et al., 2012 and Raouf et al., 2010.

1.1.2. Lingual nerve injury and neuropathic pain

Neuropathic pain is defined as 'pain caused by a lesion or disease in the somatosensory nervous system' by the IASP (Jensen et al., 2011), and can be initiated by peripheral nerve injury. The trigeminal nerve is susceptible to damage during surgical procedures in the orofacial region and as a consequence of facial fractures. The lingual nerve lies in close proximity to the lower wisdom teeth and may be damaged as a complication of wisdom tooth removal.

The lingual nerve is a branch of the mandibular division of the trigeminal nerve (Figure 1.3A). The lingual nerve provides somatosensory innervation to the lingual gingiva, mucous membranes of the floor of the mouth, and anterior two-thirds of the tongue (Figure 1.3C) through its wide range of nociceptive, mechanosensitive, and thermosensitive fibres (Martos-Fernandez et al., 2014). In addition, the lingual nerve innervates the sublingual and submandibular salivary glands (Figure 1.3C) via secretomotor parasympathetic fibres conveyed from the chorda tympani branch of the facial nerve (Zur et al., 2004). The lingual nerve also carries taste fibres from the chorda tympani to the mucous membrane of the tongue (Zur et al., 2004). The lingual nerve runs in the lingual soft tissue adjacent to the lower third molar (Figure 1.3B) (Mendes et al., 2013). Therefore, it is susceptible to trauma through routine oral surgery procedures (Atkins and Kyriakidou, 2021), most commonly the extraction of the mandibular third molar (Mason, 1988), and more rarely via inadvertent injection of local anaesthetics into the nerve (Pogrel et al., 2003).

Patients with lingual nerve injuries usually have various degrees of sensory disturbances on the ipsilateral side of the tongue, such as numbness in the tongue (hypoesthesia), abnormal sensations such as itching and tingling (paraesthesia), and unpleasant abnormal sensation such as pricking and burning (dysaesthesia) (Loescher et al., 2003). These symptoms profoundly affect the ability to speak and eat, and cause damage to the tongue via inadvertent tongue biting. This can cause difficulty with speech, chewing, and pronunciation. The perception of taste may also be disturbed as the lingual nerve also carries taste fibres from the chorda tympani to the tongue (Scrivani et al., 2000).

The majority of the patients regain normal sensation within three to six months (Kipp et al., 1980, Mason, 1988). However, a small group of patients (approximately 0.5 – 1%) are left with permanently impaired sensation or sensory loss and discomfort (Robinson et al., 2004a), causing significant distress in patients due to the vital role of the lingual nerve in normal sensory perception and functions such as speech and chewing. Within this group of patients, some develop chronic neuropathic pain induced by the nerve injury, with a reported incidence of 45% in a previous study (Jaaskelainen et al., 2005). The combination of sensory deficits and disturbances, their effect on speech and eating, and pain impact work, social life and well-being, leading to anxiety, depression, isolation and reduced quality of life. Nevertheless, not all patients with lingual nerve injury report symptoms of pain, so tissues from patients with and without pain can be compared to investigate neuropathic pain.

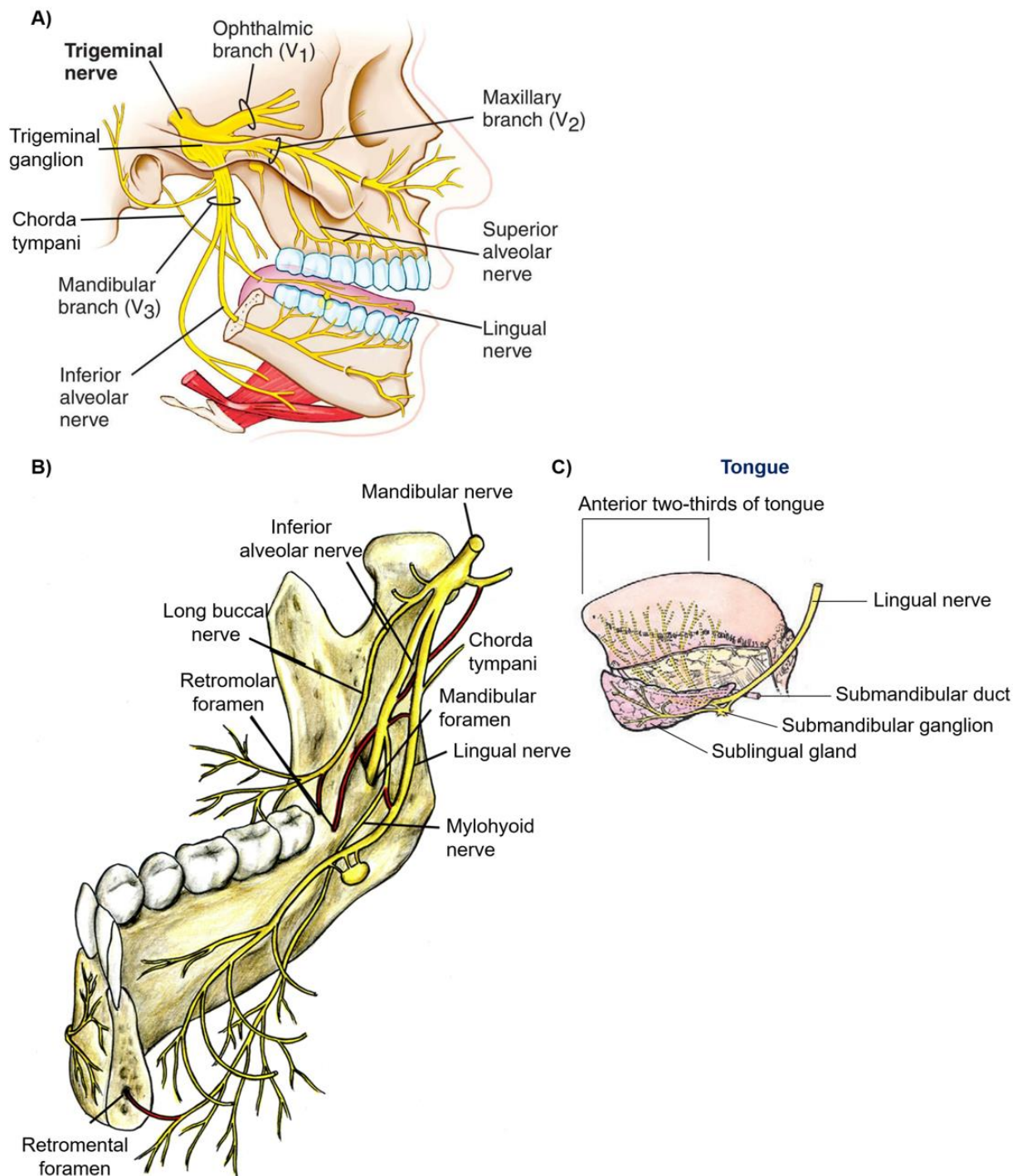


Figure 1.3. Anatomical position of the lingual nerve, and its innervation.

- A) The lingual nerve branches from the mandibular division of the trigeminal nerve (V₃). It is also joined by the chorda tympani, a branch of the facial nerve, which conveys secretomotor parasympathetic fibres and taste fibres.
- B) The lingual nerve runs anterior and medial to the inferior alveolar nerve. It passes anteriorly under the cover of the oral mucosa, situated just inferior to the lower third molar.
- C) The lingual nerve innervates the anterior two-thirds of the tongue. It also innervates the sublingual and submandibular glands, and receives taste information from the anterior two-thirds of the tongue via the chorda tympani.

(A) and (C) were taken with permission from Curtis, 2009, (B) was modified with permission from Rodella et al., 2012.

1.1.2.1. Nerve regeneration following lingual nerve injury

In response to lingual nerve injury, Schwann cells release pro-inflammatory cytokines and chemokines, which recruit and activate macrophages from circulation. As shown in Figure 1.4, the proximal end of the nerve degenerates within the zone of injury. The distal end of the nerve undergoes Wallerian degeneration, during which macrophages and Schwann cells contribute to removing axonal and myelin debris from the distal injury site by phagocytosis (Caillaud et al., 2019). This prepares the distal nerve stump for reinnervation and provides a supportive microenvironment allowing axonal regrowth.

A growth cone is then formed at the end tip of the proximal axonal stump, which is promoted by calcium (Menorca et al., 2013). The growth cone receives guidance from various neurotrophic factors such as ephrins, semaphorins, and netrins, which are secreted by Schwann cells. The secretion of regenerating factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) by Schwann cells is also upregulated, which in turn stimulates the production of new Schwann cells (Lee and Wolfe, 2000). Schwann cells then align longitudinally and form Bands of Büngner, which act as a guide for the proliferating proximal axon to reach the distal end, allowing axonal regeneration to take place (Gordon, 2016). After reinnervation, maturation occurs which includes remyelination and axon enlargement, after which functional recovery is completed (Burnett and Zager, 2004), and patients can regain some sensory functions.

1.1.2.2. Neuroma formation

Many factors can affect the process of lingual nerve regeneration. If the sprouting axon regenerates abnormally or in an uncontrolled manner, a lingual nerve neuroma may form in the junction between two ends of the nerve, as illustrated in Figure 1.5. It is thought that neuroma formation occurs when regenerating sprouts are prevented from reaching the distal stump, for example by scar formation (Foltan et al., 2008). The neuroma is an abnormal part of the nerve. It is a bulbous mass of regenerating axons, scar tissue, Schwann cells, blood vessels, fibroblasts, and immune cells (Vora et al., 2005). It represents frustrated attempts at sprouting regeneration to bridge the gap after nerve injury. Neuromas can be classified into two subtypes, neuroma-in-continuity and nerve-end-neuroma, based on the extent of the lingual nerve injury. Neuroma-in-continuity forms when the nerve remains partially intact and its continuity remains, whereas nerve-end-neuroma forms when neurotmesis occurs and the nerve is transected completely (Chhabra et al., 2010).

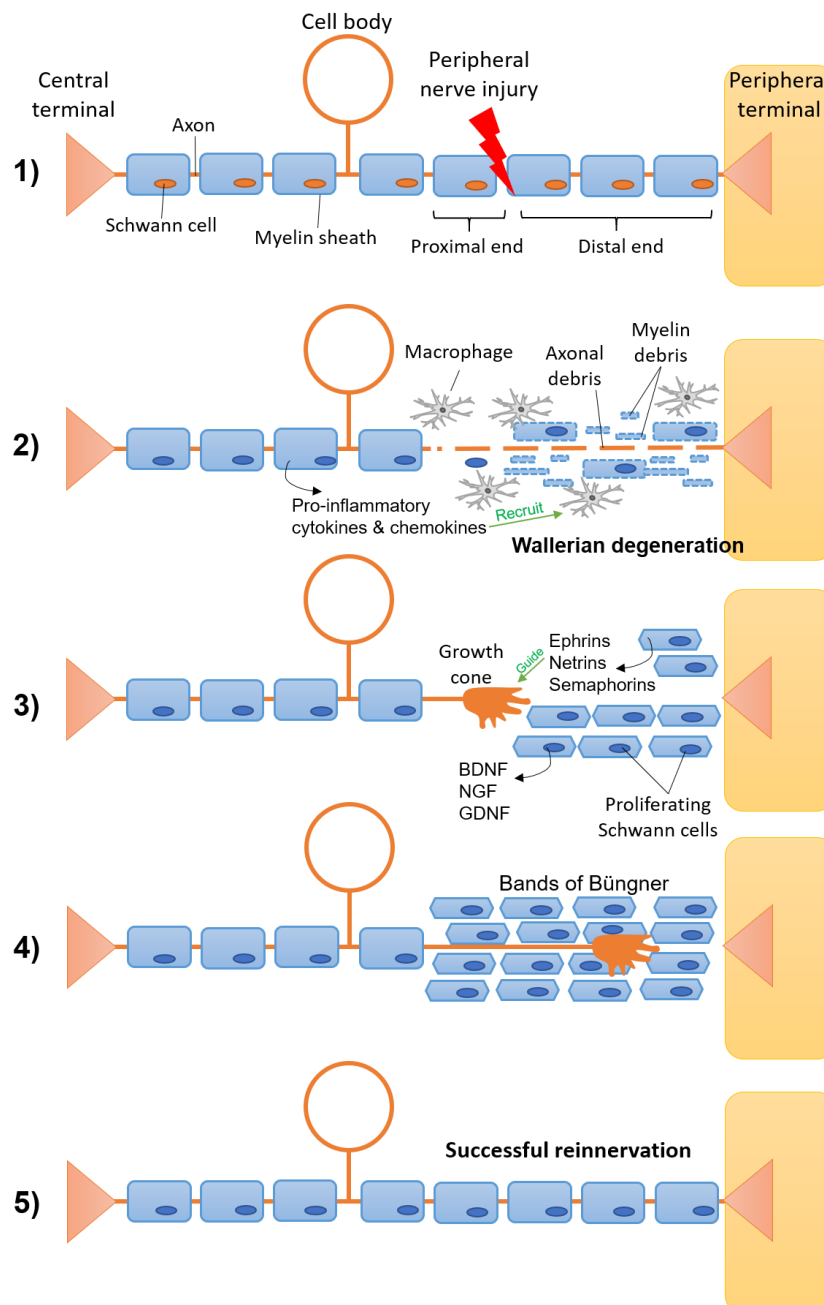


Figure 1.4. Simplified schematic of nerve regeneration following peripheral nerve injury.

1) Following peripheral nerve injury, pro-inflammatory cytokines and chemokines are released, which triggers the recruitment of macrophages from the circulation.

2) The proximal end of the nerve degenerates within the zone of injury, while the distal end undergoes Wallerian degeneration, during which macrophages and Schwann cells clear axonal and myelin debris from the distal injury site by phagocytosis.

3) A growth cone is formed at the distal tip of the proximal bud, and its growth is guided by neurotrophic factors (e.g., ephrins, netrins, and semaphorins). Regenerating factors (e.g., BDNF, NGF, and GDNF) are also secreted, which stimulates Schwann cell proliferation.

4) Schwann cells then align longitudinally to form Bands of Büngner, which act as a guide for the regenerating axon to reach the distal end, allowing **(5)** axonal regeneration to take place.

The schematic diagram was adapted from Caillaud et al., 2019 under the Creative Commons Attribution-NonCommercial-ShareAlike License.

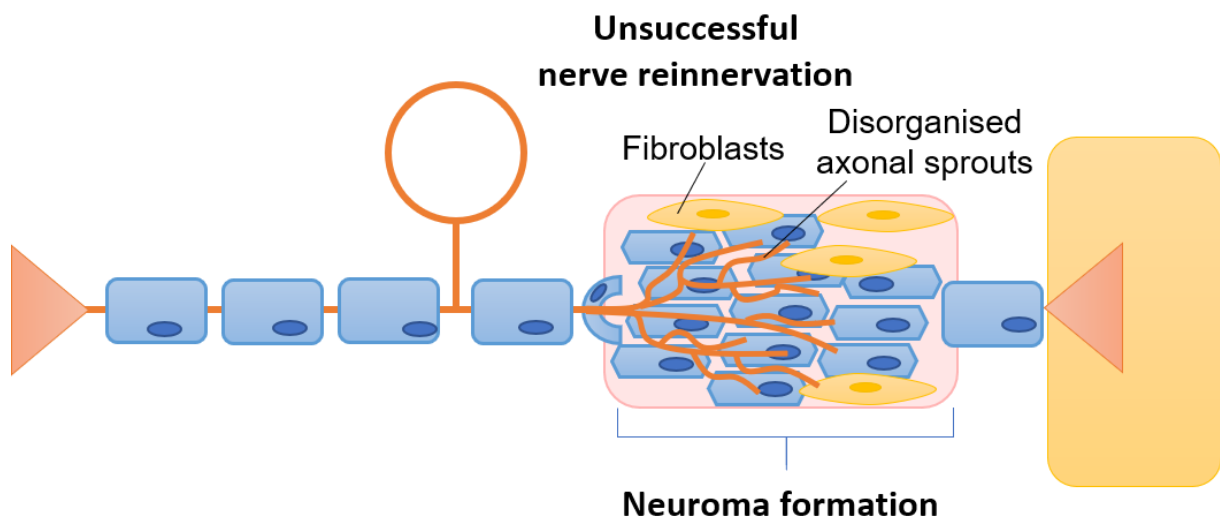


Figure 1.5. Simplified schematic diagram of neuroma formation.

A neuroma may form in the junction between two ends of the nerve if the sprouting axon regenerates abnormally in an uncontrolled manner. It is a swollen mass of regenerating axons, scar tissue, Schwann cells, fibroblasts, and blood vessels, which represent frustrated attempts at sprouting regeneration to bridge the gap after peripheral nerve injury.

The schematic diagram was adapted from Caillaud et al., 2019 under the Creative Commons Attribution-NonCommercial-ShareAlike License.

In some patients, the formation of lingual nerve neuroma is accompanied by positive symptoms (reflect an abnormal level of excitability) such as dysaesthesia, allodynia, and hyperalgesia; as well as negative symptoms (reflect loss of sensation) such as hypoesthesia, which is indicative of nerve damage in the somatosensory nervous system. Dysaesthesia is defined as unpleasant sensations of burning and pricking, which can be spontaneous or evoked by touching the tongue or tongue movement. Allodynia refers to pain elicited by a stimulus that does not provoke pain normally, such that even a slight contact or light compression causes pain; whereas hyperalgesia is defined as increased pain response to a stimulus that causes pain normally (Coulthard et al., 2014). These symptoms may arise due to the structural changes within the neuroma and pain-sensing neurons (Vora et al., 2005).

1.1.2.3. Mechanisms of chronic neuropathic pain following lingual nerve injury

The mechanisms underpinning the development of neuropathic pain are complex and poorly understood. They involve the development of increased excitability in both the peripheral and central nervous systems, as recently reviewed by Shinoda et al. in 2021. A wide range of changes have been documented at the site of nerve injury and many of these are thought to be linked to the initiation and maintenance of neuropathic pain. Following lingual nerve injury, spontaneous ectopic activity is observed in injured lingual nerve axons (Robinson et al., 2004a, Biggs et al., 2008, Yates et al., 2005), intact afferents that supply the affected region (Hossain et al., 2017), as well as in the cell bodies of the TG (Fried et al.,

2001), and thus contribute to spontaneous pain. TRPV1 expression is found in human lingual nerve neuromas (Biggs et al., 2007), and administration of a TRPV1 antagonist can attenuate the level of spontaneous activity that arises in the lingual nerve following injury (Biggs et al., 2008), which strengthens the concept that TRPV1 is involved in the generation of spontaneous activity. Structural changes within the region of neuromas include upregulation of voltage-gated Na^+ and Ca^{2+} channels (Dib-Hajj et al., 2010, Fried et al., 2001), and downregulation of K^+ channels (Chien et al., 2007). In particular, $\text{Na}_v1.8$ expression in lingual nerve neuroma has been shown to correlate positively with pain symptoms (Bird et al., 2013). It has been proposed that changes in these ion channels following lingual nerve injury could reduce the threshold for action potential generation and the subsequent increase in spontaneous ectopic firing (Fried et al., 2001). Expression of non-coding microRNA is also altered following lingual nerve injury, and the levels of miR-29a and miR-500a in neuromas have a negative correlation with pain symptoms, indicating that microRNA can also contribute to neuropathic pain (Tavares-Ferreira et al., 2019). Another structural change includes the presence of inflammatory infiltrates within the neuroma. This consists of immune cells such as neutrophils, macrophages, and lymphocytes (Vora et al., 2007). Various inflammatory mediators including pro-inflammatory cytokines [e.g., Interleukin (IL)-1 β , Tumour necrosis factor alpha (TNF- α), bradykinin (BK), prostaglandin (PG) E_2 , and NGF] (Cheng and Ji, 2008, Tracey and Walker, 1995), as well as neuropeptides (e.g., SP, VIP, and CGRP) (Robinson et al., 2004a) also accumulate within the TG and the site of lingual nerve injury. Accumulation of inflammatory mediators and neuropeptides activates and sensitises nociceptors (Cheng and Ji, 2008), leading to peripheral sensitisation. These structural and inflammatory changes can therefore result in neuronal hyperexcitability and hypersensitivity to various physical, chemical, and mechanical stimuli.

Sustained peripheral sensitisation can trigger central sensitisation in Vc in the brainstem, leading to the development and maintenance of chronic pain. Under these conditions, the central release of neurotransmitters (e.g., glutamate) and neuropeptides [e.g. substance P (SP), vasoactive intestinal polypeptide (VIP), and calcitonin gene-related peptide (CGRP)] are enhanced (Latremoliere and Woolf, 2009b). The voltage-dependent Mg^{2+} block in postsynaptic NMDA glutamate receptors is also removed upon postsynaptic depolarisation (Petrenko et al., 2003), which increases Ca^{2+} permeability (depicted in Figure 1.2), and thus postsynaptic nociceptive neurons are hyperactivated, resulting in central sensitisation (Latremoliere and Woolf, 2009b). This strengthens synaptic transmission and increases the excitability of central nociceptive neurons, facilitating the nociceptive transmission to higher CNS regions (Basbaum et al., 2009). Additionally, glial cells such as microglia, astrocytes, and oligodendrocytes in the trigeminal nucleus are activated following lingual nerve injury (Hossain et al., 2017). Activated microglia release BDNF, which activates

trkB receptors expressed in second-order neurons (Latremoliere and Woolf, 2009a), thereby promoting disinhibition (Ferrini and De Koninck, 2013). Activated glial cells also release various pro-inflammatory cytokines (e.g., TNF- α and IL-1 β) and thus contribute to central sensitisation (Shinoda et al., 2019). Together, these changes that occur following lingual nerve injury enhance neuronal excitability and reduce inhibition, generating symptoms such as hyperalgesia and allodynia.

Lingual nerve injury is a clinical condition with no universal satisfactory treatment. Surgical interventions to repair the nerve can improve the degree of neuropathic pain and the perception of various sensory stimuli, and patients also reported a reduction in the incidence of inadvertent tongue biting and speech impairment (Atkins and Kyriakidou, 2021). However, patients do not have a complete recovery. In some cases, symptoms of sensory discomfort such as paraesthesia, dysaesthesia, and allodynia persist after surgical repair despite general improvement in symptoms (Hillerup and Stoltze, 2007, Zuniga et al., 2014), so some patients are left with chronic pain that does not respond well to current pharmacological management strategies (Robinson et al., 2004b). Therefore, it is important to explore alternative pharmacological approaches for alleviating nerve injury-induced neuropathic pain.

1.1.3. Pulpal inflammation and inflammatory pain

Pain is one of the cardinal signs of inflammation, and inflammatory pain is a component of several types of pain such as postoperative pain, pain associated with trauma, arthritis, and pain associated with localised infection including toothache. Inflammatory pain is caused by tissue damage and the associated inflammatory response, wherein the release of various sensitising immune cells and inflammatory mediators activate and reduce the threshold of nociceptors that innervate the inflamed tissue. Pulpal inflammation is usually initiated by decay of the enamel surface on a tooth leading to the formation of dental caries, which allows oral microorganisms to penetrate the tooth structure and reach the tooth pulp, resulting in irritation and infection. This causes inflammation in the tooth pulp, or pulpitis, which can progress to irreversible chronic pulpal inflammation if it remains untreated (Park et al., 2015). The swollen, inflamed tooth pulp tissues can cause spontaneous pain or dental sensitivity. However, a tooth can have an inflammatory reaction without manifesting pain, and not all patients with carious teeth report symptoms of pain (Michaelson and Holland, 2002). This allows comparisons to be drawn between non-inflamed tooth pulp from normal teeth and inflamed pulp from carious painful and non-painful teeth to further the understanding of the mechanisms of inflammatory pain.

The tooth pulp is a highly innervated, unmineralized oral tissue which is housed inside the pulp chamber within the centre of each tooth, as illustrated in Figure 1.6A. It is composed of soft connective tissues, blood vessels, and nerve fibres. Sensory fibres and

blood vessels that supply the tooth pulp enter via the apical foramen. These pulpal nerve fibres progress into the periphery along the route of the blood vessels. They branch extensively to form the nerve plexus of Raschkow underneath the cell-rich zone (Yu and Abbott, 2007), as shown in Figure 1.6B. The plexus of Raschkow mostly consists of thinly myelinated A δ -fibres, a small amount of thickly myelinated A β - fibres and thinner unmyelinated C-fibres (Hossain et al., 2019). The myelinated pulpal nerve fibres lose their myelin sheath gradually as they progress to the cell-rich zone (Yu and Abbott, 2007). The cell-rich zone is rich in fibroblasts and undifferentiated mesenchymal cells, which support the odontoblast layer by differentiation and proliferation (Ghannam and Bordoni, 2019). In the cell-free zone, the pulpal nerve fibres form a rich network of free nerve fibres, some of which may extend further into the dentine layer. These nerve fibres act as specific receptors for pain, and can transduce pain signals from external thermal, mechanical, and chemical stimuli (Ahlquist and Franzen, 1994). An extensive capillary network is also found in the cell-free zone, enabling it to maintain and support the subjacent odontoblast layer, which in turn maintains the overlying dentine layer (Park et al., 2015).

Under normal conditions, intense thermal stimuli applied to the surface of intact teeth can induce pain. On the other hand, pain can arise in response to thermal, mechanical, and chemical stimuli applied to the surface of exposed dentine. Transduction of stimuli leading to activation of intradental sensory nerve terminals may be brought about directly (via fluid movement in dentinal tubules activating sensory pulpal nerve mechanoreceptors) (Hossain et al., 2019) or indirectly, with the odontoblast acting as a receptor that in turn activates pulpal sensory nerves via the release of glutamate or ATP (Shibukawa et al., 2015). Pulpal sensory nerves are primary afferent nerve fibres of the trigeminal nerve with cell bodies in the TG, as shown in Figure 1.6C. The upper teeth are innervated by superior alveolar nerves, which branch from the maxillary division (V₂) of the trigeminal nerve. Sensations to the lower teeth are supplied by inferior alveolar nerves, which branch from the mandibular division (V₃) of the trigeminal nerve. The majority of the sensory pulpal axons are A δ - and C-fibre, but the few A β -fibres in the pulp are also thought to act as nociceptors (Jain et al., 2013). Activation of these fibres is associated with two different types of dental pain. Stimulation of A δ -fibres is associated with acute, sharp, and well-localised pain (Basbaum et al., 2009) in response to external stimuli as they predominately terminate in the periphery of the tooth pulp close to the dentine layer (Hildebrand et al., 1995). In contrast, C-fibres mediate dull, prolonged pain which is poorly localised to the affected tooth (Basbaum et al., 2009). Since C-fibre endings are predominately found within the centre of the tooth pulp (Hildebrand et al., 1995), they are thought to be activated by noxious stimuli that affect the pulp, such as the inflammatory mediators released during pulpal inflammation (Yu and Abbott, 2007).

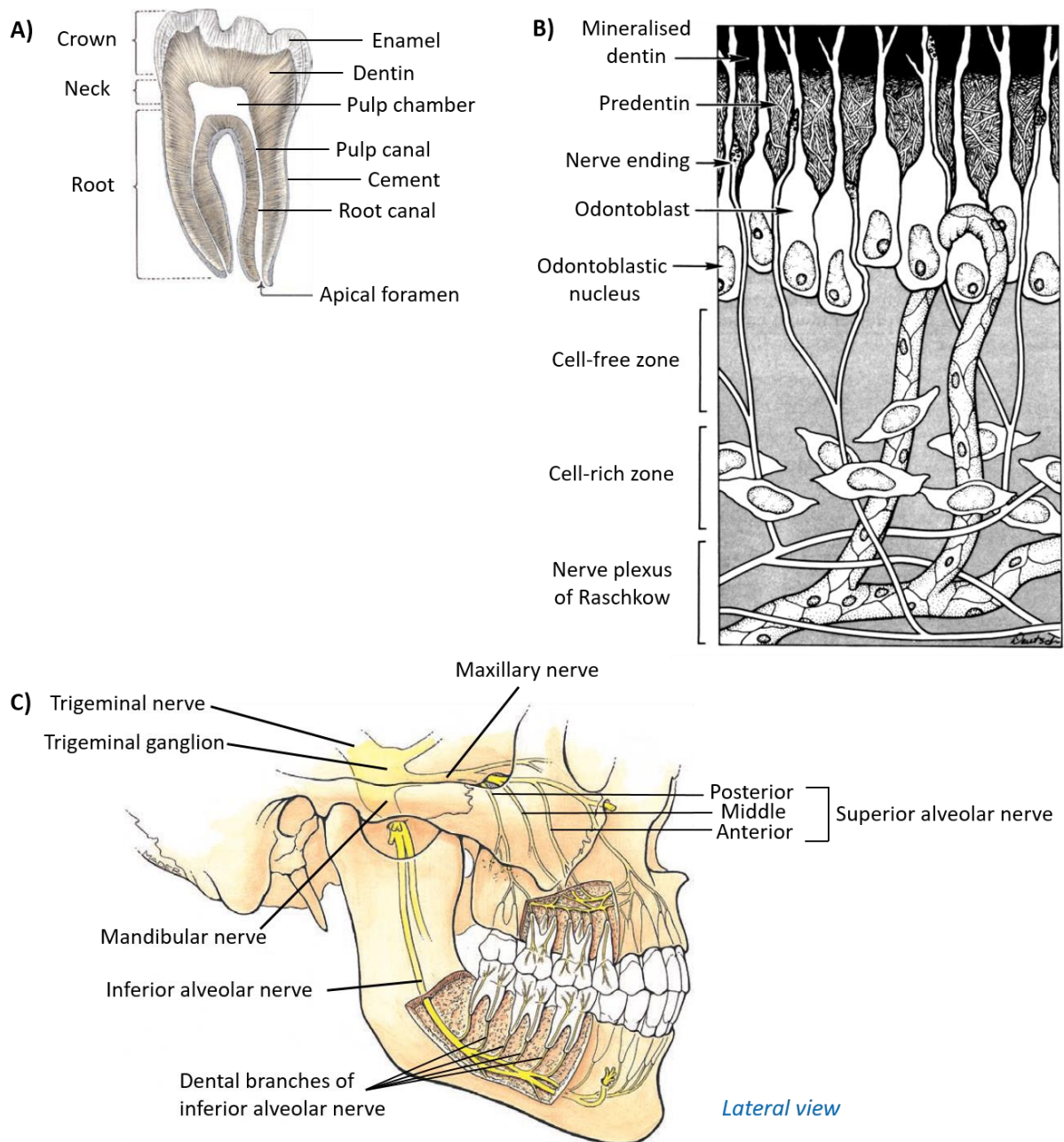


Figure 1.6. Tooth morphology and innervation of teeth

- A) Longitudinal view of a molar tooth, anatomical drawing taken with permission from Curtis, 2009. The tooth pulp is located inside the pulp chamber of the tooth. It is innervated by various sensory fibres, which reach the root canal via the apical foramen. Blood vessels that supply the tooth pulp also pass through this foramen.
- B) Schematic drawing of the morphology of the tooth pulp, taken from with permission Pashley et al., 2002. The tooth pulp is highly vascularised and innervated. It is organised into a layer of dentine-forming odontoblasts, the cell-free zone, the cell-rich zone, and the nerve plexus of Raschkow.
- C) Lateral view of innervation of teeth, anatomical drawing taken with permission from Curtis, 2009. The upper teeth are innervated by three superior alveolar nerves: posterior, middle, and anterior superior alveolar nerves, which supply sensations to different teeth in the upper jaw. They branch from the maxillary division (V_2) of the trigeminal nerve. The lower teeth are innervated by the inferior alveolar nerves, which is a branch of the mandibular division (V_3) of the trigeminal nerve.

1.1.3.1. Mechanism of painful pulpitis

Painful pulpitis is characterised by spontaneous or provoked pain, which is usually accompanied by mechanical and thermal hyperalgesia and allodynia, as well as increased resistance to local anaesthetics (Galicía et al., 2016). Expression of various types of thermosensitive (e.g., TRPV1, TRPV2, TRPV4, TRPA1, TRPC5, TRPM8) (Hossain et al., 2019, Bernal et al., 2021), mechanosensitive (e.g., PIEZO1, PIEZO2) (Sun et al., 2022, Han et al., 2022), acid-sensing (e.g., ASIC3) (Ichikawa and Sugimoto, 2002), purinergic (e.g., P2X2, P2X3) (Lee et al., 2019), and glutamate (e.g., mGLUR5) (Kim et al., 2009) receptors have been reported in pulpal nerve terminals and odontoblasts, and are implicated in dental pain. In the presence of caries, immune cells such as neutrophils, macrophages, T-cells, and dendritic cells are recruited to the tooth pulp (Rechenberg et al., 2016). Together with the resident cells, they release various inflammatory mediators such as NGFs, PGs, BK, ATP, H⁺, and pro-inflammatory cytokines (e.g., IL-1 β , TNF- α , and IL-6). These mediators act on their respective receptors expressed in pulpal nerve terminals and odontoblasts. Activation of these receptors sensitises and activates the nociceptors, leading to peripheral sensitisation, which represents a heightened response to subsequent noxious stimuli and increased excitability in response to sensitising inflammatory mediators (Takeda et al., 2011), resulting in allodynia and hyperalgesia. Vasoactive neuropeptides such as SP and CGRP are also released locally, resulting in vasodilation and an increase in vascular permeability (Hildebrand et al., 1995). This lead to an increase in intrapulpal pressure and can activate the pulpal nerves spontaneously (Zhan et al., 2021). Sustained input from the peripheral pulpal nerves to the trigeminal spinal nucleus in the brainstem results in central sensitisation (Latremoliere and Woolf, 2009b). This can manifest as secondary hyperalgesia, wherein the enhanced response to noxious stimuli extends to the surrounding undamaged tissue area. When pulpal inflammation persists for a period of time and becomes chronic, the inflammatory mediators activate the pulpal nerve endings continually and thus cause ongoing, increased pain.

Dental caries is a common chronic oral disease, which can typically lead to inflammation of the tooth pulp and elicit inflammatory pain. The treatment of dental caries depends on the depth of the caries. It is typically treated by removing the dental decay and restoring it with a filling. For more extensive dental caries, a crown, root canal treatment, or even tooth extraction may be needed (Renton and Wilson, 2016), allowing tooth pulp tissues to be studied. Investigating changes induced by pulpal inflammation in the tooth pulp will hopefully help to identify potential new targets for the modulation of painful pulpitis and potentially other inflammatory pain conditions caused by chronic inflammation.

1.1.4. Commonalities between neuropathic and inflammatory pain

Chronic pain is a complex condition and represents an unmet clinical need. Current medication fails to provide clinically meaningful pain relief for all patients, and a large proportion of patients are left with insufficient pain relief (Finnerup et al., 2010). The development of more efficacious and safer analgesics, as well as the identification of novel targets as alternative pharmacological management options are therefore required to improve treatment strategies. The causes of neuropathic and inflammatory pain are fundamentally different, but some mechanisms are common to the generation of both types of pain states. For example, the presence of inflammatory infiltrates at the site of injury is common in both chronic neuropathic and inflammatory pain, and contributes to the generation of pain in man and behavioural hypersensitivity in animal models. Furthermore, failure to resolve acute inflammation is thought to contribute to the transition from acute to chronic pain (Ji et al., 2011). Promotion of the resolution of inflammation may therefore aid the resolution of both pain states. This process was once presumed to be a passive process, however, in the light of recent studies, it is now considered to be an active process regulated by the biosynthesis of specialised pro-resolving mediators such as resolvins to stimulate the resolution of inflammation and the subsequent healing process (Serhan, 2017).

1.2. Resolvins

Coined by the Serhan lab, resolvins (short for resolution-phase interaction products) are a family of structurally related bioactive products that are generated endogenously during the resolution phase of acute inflammation by cells of the innate immune system (Serhan et al., 2002). They were originally identified in inflammatory exudates from murine air pouch and isolated human polymorphonuclear neutrophils (PMN) (Serhan et al., 2000), and were later identified in human plasma (Arita et al., 2005, Mas et al., 2012, Colas et al., 2014). Resolvins are enzymatically derived from omega-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Their biosynthetic pathways are summarised in Figure 1.7.

Two molecular series of resolvins with chemically unique structures have been identified: D-series (RvD) and E-series (RvE) resolvins (Serhan et al., 2008). E-series members such as resolvin E1 and E2 are derived from EPA with the aid of a series of synthetic enzymes such as aspirin-acetylated COX-2 or cytochrome P450, and 5-LOX. Two different groups of D-series members are derived from DHA via different enzymatic pathways, as illustrated in Figure 1.7. Resolvin D1, D2, D3, and D5 are derived via the lipoxygenase-initiated pathways, producing intermediates in the S configuration at carbon 17. In the presence of aspirin, intermediates in the R configuration at carbon 17 are produced via the aspirin-acetylated COX-2 or cytochrome P450 pathway, generating aspirin-triggered D-series resolvins (e.g., AT-RvD1).

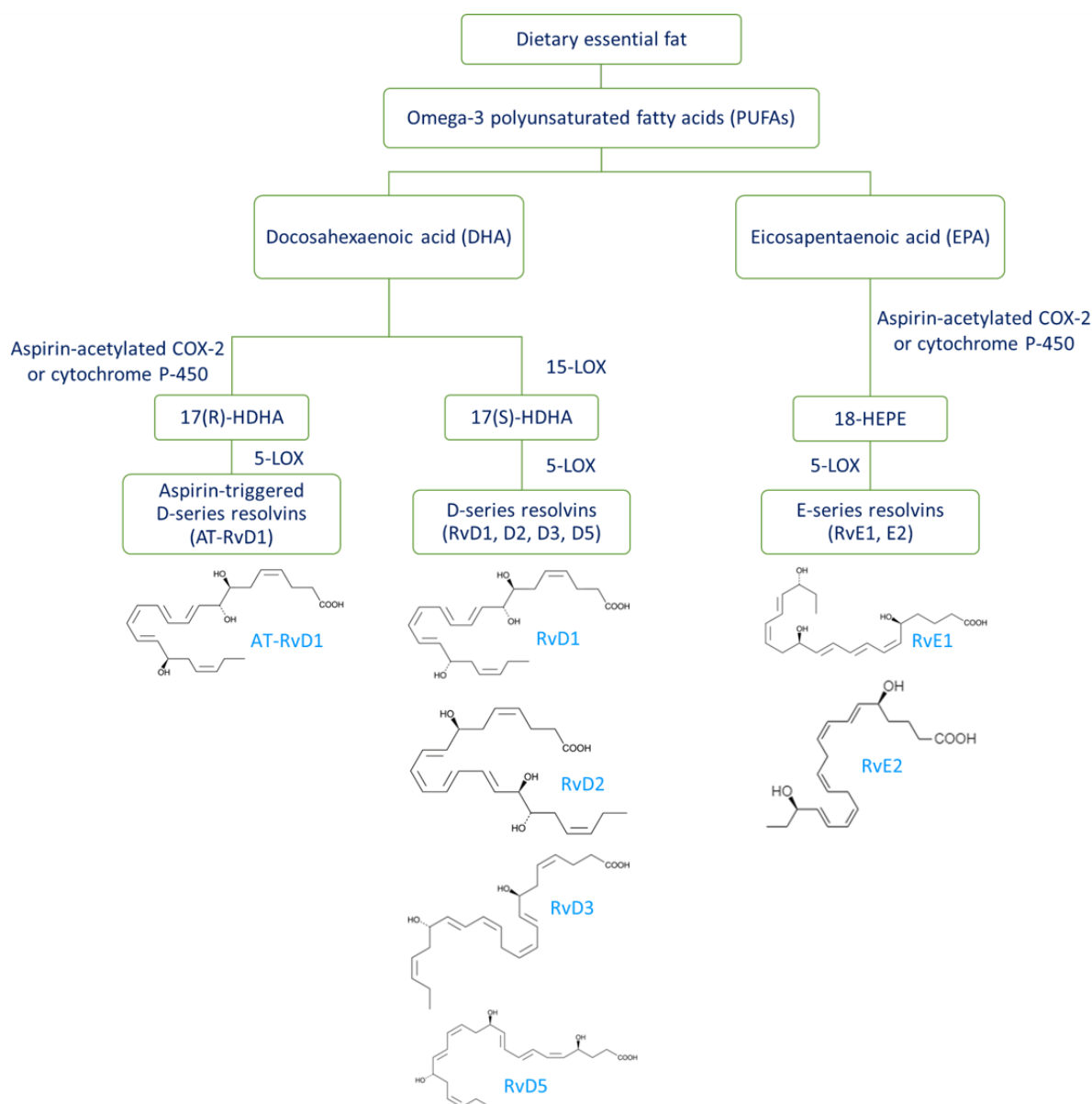


Figure 1.7. Biosynthesis cascade of resolvins originating from omega-3 PUFAs.

Resolvins are derived from omega-3 PUFAs, DHA and EHA, which are dietary essential fat mainly found in fish. DHA and EHA serve as substrates for the synthesis of two series of resolvins with structures chemically unique to each other: D-series (RvD) and E-series (RvE) resolvins. Biosynthesis of resolvins requires a series of enzymatic conversions, with the aid of synthetic enzymes such as aspirin-acetylated COX-2, cytochrome P450, 5-LOX, and 15-LOX. Structural drawings of resolvins were taken with permission from Kohli and Levy, 2009.

Abbreviations:

17-HDHA: 17-hydroxy-docosahexaenoic acid; 18-HEPE: 18-hydroxyeicosatetraenoic acid; COX-2: Cyclooxygenase-2; 15-LOX: 15-lipoxygenase; 5-LOX: 5-lipoxygenase

D- and E-series resolvins and their pathway precursors are identified in human blood serum and lymphoid tissues from healthy subjects without diet supplementation (Colas et al., 2014). Interestingly, the levels of resolvins and their precursors in serum from these subjects are similar to the levels found in serum from healthy subjects following three weeks of

omega-3 PUFA supplementation (Mas et al., 2012). This suggests that DHA and EPA are not automatically metabolised into resolvins under normal conditions, and increased production of resolvins may only occur in the presence of inflammation. One of the contributing factors could be the low expression levels of the synthetic enzymes (e.g., COX-2 and lipoxygenases) that are crucial for the biosynthesis of resolvins in normal, non-inflamed tissues (Dubois et al., 1998, Levy et al., 2001). After an injury, circulating DHA and EPA in the blood appear rapidly at sites of inflammation within the local inflammatory exudates (Kasuga et al., 2008). The expression levels of those biosynthetic enzymes are also up-regulated during acute inflammation (Dubois et al., 1998, Levy et al., 2001), allowing them to catalyse the rapid production of resolvins from DHA and EPA, and contribute to the resolution of inflammation. This is shown in a study comparing the levels of PUFAs-derived metabolites between patients with aggressive periodontitis (inflammatory disease) and healthy subjects, where the levels of EPA, DHA, D-series resolvins precursor 17-HDHA, and E-series resolvins 18-HEPE in gingival crevicular fluid and serum from aggressive periodontitis patients are significantly higher than that from healthy subjects, indicating that the pathway precursors of resolvins are upregulated near the inflamed site and in circulation in the presence of inflammation (Elabdeen et al., 2013). Interestingly, this study reported that the levels of RvD1 and RvD2 do not differ significantly between aggressive periodontitis patients and healthy individuals (Elabdeen et al., 2013). While in another study, it was reported that the levels of RvD1 and RvE1 in serum from patients with rheumatoid arthritis (chronic inflammatory disorder) are significantly lower than that from healthy individuals and patients in remission (Ozgul Ozdemir et al., 2020). This discrepancy may arise as resolvins have short biological half-lives (Valdes et al., 2017), making it difficult to measure their levels accurately. However, it is also possible that there may be a defect in resolvin synthesis in those patients with chronic inflammation (rheumatoid arthritis), but further studies are needed to evaluate this.

1.2.1. Anti-inflammatory and pro-resolving actions of resolvins

Resolvins are specialised pro-resolving mediators that actively regulate the resolution of inflammation. Resolution of inflammation is a highly coordinated and active process that is considered to begin at the later stages of inflammation, controlled by endogenously produced specialised pro-resolving mediators including resolvins. The anti-inflammatory (reduction of cell recruitment) and pro-resolving (enhancement of cell clearance) actions of resolvins have been demonstrated in various animal models of inflammation, as summarised in Table 1.1. They act locally to limit further recruitment of leukocytes to the injury site (e.g., neutrophils, macrophages, eosinophils, and lymphocytes) and reverse vascular changes, which reflect their anti-inflammatory actions. They also have properties that are thought to be pro-resolving, such as enhancing the phagocytosis of apoptotic cells and tissue debris by macrophages (Oh et al., 2012) and promoting the clearance of leukocytes by natural killer (NK) cells (Lawrence et al., 2002, Basil and Levy, 2016). Therefore, resolvins can restrain inflammation and accelerate the return to homeostasis at the injury site. Disruption of this active process to resolve acute inflammation may lead to uncontrolled, chronic inflammation, which is implicated in a variety of chronic inflammatory diseases.

Table 1.1. Anti-inflammatory and pro-resolving actions of resolvins in animal models of inflammation.

Resolvins	Inflammatory conditions	Anti-inflammatory/Pro-resolving effects	References
RvD1	Peritonitis [mice]	- Reduces leukocyte infiltration	(Recchiuti et al., 2011, Spite et al., 2009b)
	Allergic airway inflammation [mice]	- Reduces infiltration of eosinophils and the levels of pro-inflammatory mediators (IL-17, IL-23, and LTB ₄) - Promotes phagocytic clearance of allergen by macrophages	(Rogerio et al., 2012)
	Lung injury [mice]	- Reduces neutrophil infiltration - Reduces pro-inflammatory cytokine production (IL-6, IL-1 β , and TNF- α)	(Xia et al., 2019)
	Dorsal air pouch [mice]	- Reduces neutrophil recruitment	(Serhan et al., 2002)
	Arthritis [mice]	- Reduces the levels of pro-inflammatory cytokines (IL-6, IL-17, IL-1 β , TNF- α , IFN- γ , and PGE ₂) in the serum - Reduces paw swelling and redness - Reduces cartilage destruction in joints	(Benabdoun et al., 2019, Sun et al., 2020)
RvD2	Peritonitis [mice]	- Reduces PMN infiltration and the production of pro-inflammatory mediators (IL-6, IL-23, IL-1 β , TNF- α , PGE ₂ and LTB ₄) - Enhances microbe clearance	(Spite et al., 2009a)
	Periodontitis [rats & mice]	- Reduces infiltration of neutrophils, regulates levels of anti- and pro-inflammatory cytokines, and promotes M2 macrophage accumulation - Prevents alveolar bone loss	(Siddiqui et al., 2019, Mizraji et al., 2018)
RvD3	Arthritis [mice]	- Reduces leukocyte infiltration, eicosanoid levels, and paw oedema	(Arnardottir et al., 2016)
	Psoriasis [mice]	- Reduces infiltration of immune cells and mRNA expression of pro-inflammatory cytokines (IL-17C, IL-17F, IL-22, and IL-23a) in the skin	(Lee et al., 2020)
RvD5	Peritonitis [mice]	- Enhances phagocytosis of microbes and reduces TNF- α	(Chiang et al., 2012)
RvE1	Pulpitis [rats]	- Reduces neutrophil recruitment and pro-inflammatory cytokines (IL-6, IL-1 β , and TNF- α), and restricts the extent of inflammation	(Xu et al., 2019, Dondoni et al., 2014)
	Femoral arterial injury [mice]	- Reduces neointima formation and vascular neutrophils infiltration - Restrains vascular smooth muscle cell migration	(Liu et al., 2018)
	Peritonitis [mice]	- Promotes engulfment of apoptotic PMN by macrophages	(Schwab et al., 2007)
	Periodontitis [rats & rabbits]	- Reduces inflammatory cell infiltration and alveolar bone loss - Promotes regeneration of bone and connective tissues	(Hasturk et al., 2006, Hasturk et al., 2007, Lee et al., 2016)

	Corneal inflammation [mice]	- Reduces chemokine CXCL1 production and neutrophil recruitment	(Lee et al., 2015)
	Allergic airway inflammation [mice]	- Reduces leukocyte infiltration and the production of pro-inflammatory cytokines (IL-6, IL-17, and IL-23) - Reduces inflammation-induced lung morphological changes - Enhance trafficking of NK cells	(Haworth et al., 2011, Haworth et al., 2008)
RvE2	Peritonitis [mice]	- Reduces neutrophil infiltration	(Tjonahen et al., 2006)

Abbreviations: IL: Interleukin; IFN- γ : Interferon- γ ; LTB₄: Leukotriene B₄; TNF- α : tumour necrosis factor- α ; PGE₂: Prostaglandin E₂; PMN: Polymorphonuclear neutrophils

1.2.2. Analgesic actions of resolvins

Inflammation is associated with both chronic neuropathic and inflammatory pain. Therefore, uncontrolled inflammation that arises from failed resolution of acute inflammation may also contribute to the transition from acute to chronic pain, as well as the maintenance of neural plasticity and chronic pain (Ji et al., 2011). Resolvins have been shown to alleviate symptoms of neuropathic and inflammatory pain or pain-like behaviour in several established animal models of pain, as summarised in Table 1.2.

Systemic and peripheral administration of resolvins has anti-nociceptive effects in various animal models of inflammatory pain, as indicated by their ability to attenuate mechanical and thermal hypersensitivity (Oehler et al., 2017, Xu et al., 2010, Bang et al., 2010, Lima-Garcia et al., 2011, Abdelmoaty et al., 2013, Luo et al., 2019, Ikeda et al., 2020) or reduce pain-related behavioural changes (Xu et al., 2010, Park et al., 2011). The analgesic effects of resolvins are also accompanied by their inflammation-resolving effects. Pre-treating the paw with RvE1 prior to induction of inflammatory pain with carrageenan (CRG) attenuates heat hyperalgesia, as well as reduces paw oedema, infiltration of neutrophils, and the expression of pro-inflammatory chemokines [e.g., Monocyte chemoattractant protein (MCP)-1, Macrophage Inflammatory Protein (MIP)-1 α] and cytokines (e.g., IL-6, IL-1 β , and TNF- α) in the inflamed paw (Xu et al., 2010). Administration of AT-RvD1 (RvD1 epimer) after the induction of inflammatory pain with CRG also reduces mechanical hyperalgesia and levels of pro-inflammatory cytokines (IL-1 β , and TNF- α) (Lima-Garcia et al., 2011). Additionally, in a rat model of radicular pain, intrathecal administration of RvD1 (Liu et al., 2016) and RvD2 (Zhang et al., 2018) alleviate mechanical allodynia and reduces the levels of pro-inflammatory cytokines (IL-6 and TNF- α) in the spinal dorsal horn as well. These studies further demonstrate that resolvins have both analgesic and inflammation-resolving properties.

Studies on the analgesic actions of resolvins in neuropathic pain are less extensive compared to studies on inflammatory pain. Nevertheless, systemic administration of resolvins can alleviate neuropathic pain induced by nerve trauma (Xu et al., 2013, Wang et al., 2022b, Doyle et al., 2014, Pang et al., 2023, Tao et al., 2021) and chemotherapy (injections of paclitaxel) (Luo et al., 2019), demonstrated by a reduction in mechanical hypersensitivity and allodynia. Furthermore, in a chronic constriction injury (CCI) model of neuropathic pain, pre-treatment with RvD2 reduces astrocyte activation and the levels of pro-inflammatory mediators (IL-17 and CXCL1) in the spinal dorsal horn (Pang et al., 2023), indicating that resolvins can also dampen neuroinflammation induced by nerve injury.

Interestingly, the dose required to alleviate neuropathic pain appears to be higher than the dose required for inflammatory pain. 100 ng of RvD5 (Luo et al., 2019) and RvE1

(Luo et al., 2019, Xu et al., 2013) given intrathecally are required to reduce mechanical hyperalgesia and allodynia in neuropathic pain models, but only 10 ng of intrathecal RvD5 (Luo et al., 2019) or RvE1 (Xu et al., 2010) are needed to alleviate inflammatory pain. The differential potency of RvD5 and RvE1 in neuropathic and inflammatory pain suggests that even though both types of pain respond to resolvins, inflammatory pain appears more sensitive to the administration of resolvins.

Resolvins have short biological half-lives as they are further metabolised and inactivated via enzymatic pathways (Sun et al., 2007, Arita et al., 2006, Hong et al., 2008). Their metabolic instability may thus limit their therapeutic potential. This is evidenced in a previous study investigating the effects of RvE1 treatment in a neuropathic pain model, wherein RvE1 treatment reduces mechanical hypersensitivity and cold allodynia induced by CCI of the sciatic nerve, but its effects are transient and not sustained for more than one hour post-administration; while the anti-nociceptive effects of a synthetic ChemR23 ligand are sustained for at least 24 hours post-administration (Doyle et al., 2014). In order to overcome this limitation, other more stable and longer-lasting alternatives should be explored. One of the alternatives is the epimer of RvD1, AT-RvD1, which has the same bioaction and mechanism of action as RvD1, differing only in the stereochemical configuration at carbon 17 (Rogerio et al., 2012). This makes it more resistant to enzymatic degradation, subsequently, AT-RvD1 has a longer half-life than RvD1 (Sun et al., 2007). Alternatively, 17(R)-HDHA, a precursor of AT-RvD1, also has a longer half-life than RvD1 (Huang et al., 2017), and thus could have more long-lasting beneficial effects. Intraperitoneal administration of both AT-RvD1 and 17(R)-HDHA can attenuate symptoms of inflammatory pain in animal models (Huang et al., 2017, Lima-Garcia et al., 2011), as summarised in Table 1.2. Interestingly, only the level 17-HDHA, but not the other D-series resolvins (RvD1, RvD2, RvD3, and RvD5) and RvE1, is associated with sensitivity to acute heat pain in the general population (Valdes et al., 2017). In the same study, a negative correlation between the level of 17-HDHA and pain scores in patients with osteoarthritis (chronic inflammatory pain condition) has also been found, but this association is not evident for the level of RvD1 or RvD2 (Valdes et al., 2017). Further studies are therefore needed to investigate the mechanism of action of 17-HDHA, and determine whether exogenous administration of resolvins in human can produce the analgesic and inflammation resolution actions observed in animal models of pain and inflammation.

Table 1.2. Analgesic actions of resolvins in animal models of neuropathic and inflammatory pain.

Resolvins	Pain models	Animals	Pain state	Pain-like symptoms improved by resolvin treatment	References
RvD1	Chemotherapy	Mice	Neuropathic	- Reduces mechanical allodynia	(Luo et al., 2019)
	SNL	Rat		- Reduces mechanical hyperalgesia	(Wang et al., 2022b)
	SNI	Rat		- Reduce mechanical allodynia	(Tao et al., 2021)
	CFA	Rats/Mice	Inflammatory	- Reduces mechanical allodynia and hyperalgesia - Reduces heat hyperalgesia	(Oehler et al., 2017, Xu et al., 2010, Bang et al., 2010, Ikeda et al., 2020)
	CRG	Mice		- Reduces heat hyperalgesia	(Xu et al., 2010)
	Formalin*	Mice		- Reduces phase II formalin-induced spontaneous pain behaviour	(Xu et al., 2010)
				- Reduces formalin-induced spontaneous pain (Both phase I & II)	(Bang et al., 2010)
	Mustard oil	Mice	- Reduces spontaneous pain behaviour	(Park et al., 2011)	
	Lumbar disc herniation	Rats	Radicular	- Reduces mechanical allodynia - Reduces the levels of pro-inflammatory cytokine (IL-1β & TNF-α) in the spinal dorsal horn and dorsal root ganglion	(Liu et al., 2016)
AT-RvD1	Chemotherapy	Rats	Neuropathic	- Inhibits responses of spinal wide dynamic range neurons to non-noxious mechanical stimulation of the hind paw	(Meesawatsom et al., 2020)
	CFA	Mice	Inflammatory	- Reduces heat hyperalgesia	(Bang et al., 2012)
		Rats		- Reduces mechanical hyperalgesia - Reduces the levels of pro-inflammatory cytokine (IL-1β & TNF-α) in inflamed paw (not seen in the spinal cord)	(Lima-Garcia et al., 2011)
				- Reduces nociceptive responses of dorsal horn spinal neurons	(Meesawatsom et al., 2016)
	CRG	Rats		- Reduces mechanical hyperalgesia	(Abdelmoaty et al., 2013)
RvD2	Chemotherapy	Mice	Neuropathic	- Reduces mechanical allodynia	(Luo et al., 2019)
	CCI			- Reduces mechanical allodynia and heat hyperalgesia	(Pang et al., 2023)

				- Reduces astrocyte activation and the levels of pro-inflammatory mediators (IL-17 and CXCL1) in the spinal dorsal horn	
	CFA	Mice	Inflammatory	- Reduces mechanical allodynia and heat hyperalgesia	(Park et al., 2011)
	CRG			- Reduces mechanical allodynia and heat hyperalgesia	
	Capsaicin			- Reduces spontaneous pain behaviour (flinching/licking)	
	Mustard oil			- Reduces spontaneous pain behaviour (flinching/licking)	
	Formalin			- Reduces phase II formalin-induced spontaneous pain behaviour	
	Lumbar disc herniation	Rats	Radicular	- Reduces mechanical allodynia - Reduces the levels of pro-inflammatory cytokines (IL-6 & TNF- α) in the spinal dorsal horn	(Zhang et al., 2018)
RvD5	Chemotherapy	Mice	Neuropathic	- Reduces mechanical allodynia (male mice only)	(Luo et al., 2019)
	Formalin		Inflammatory	- Reduces phase II formalin-induced spontaneous pain behaviour (male mice only)	
RvE1	CCI	Mice	Neuropathic	- Reduces mechanical allodynia	(Xu et al., 2013)
	SNL			- Reduces mechanical allodynia and heat hyperalgesia	
	CCI			- Reduces mechanical hypersensitivity and cold allodynia	(Doyle et al., 2014)
	CFA		Inflammatory	- Reduces mechanical allodynia and heat hyperalgesia	(Xu et al., 2010)
	CRG			- Reduces heat hyperalgesia - Reduces paw oedema, neutrophil infiltration, and the expression of pro-inflammatory chemokines (MCP-1, MIP-1 α) & cytokines (IL-6, IL-1 β , and TNF- α) in the inflamed paw	
	Formalin			- Reduces phase II formalin-induced spontaneous pain behaviour	
	Capsaicin			- Reduces spontaneous pain behaviour	(Park et al., 2011)

17(R)-HDHA	CFA/AIA	Mice	Inflammatory	- Reduces mechanical hyperalgesia - Reduces the levels of pro-inflammatory cytokine (IL-1β & TNF-α) in the inflamed paw (not seen in the spinal cord)	(Lima-Garcia et al., 2011)
	MIA	Rats		- Reduces established weight-bearing asymmetry between affected ipsilateral and contralateral paw - Reduces mechanical allodynia	(Huang et al., 2017)
	MNX			- Reduces mechanical allodynia	

Abbreviations: CFA: Complete Freund's adjuvant; CRG: Carrageenan; CCI: Chronic constriction injury; SNL: Spinal nerve ligation; SNI: Spared nerve injury; MCP-1: Monocyte chemotactic protein1; MIP-1 α : Macrophage inflammatory protein-1 α ; AIA: Adjuvant-induced arthritis; MIA: Monosodium iodoacetate; MNX: Medial meniscal transection

*Two phases of spontaneous pain behaviour (intensive licking of the injected paw) are identified after formalin injection. Phase I (first 5 minutes post-injection) is thought to be associated with direct activation of peripheral nociceptors, whereas phase II (20 – 30 minutes post-injection) is thought to be driven by both peripheral and central sensitisation processes (Hunskar and Hole, 1987).

1.2.3. Resolvin receptors

The biological actions of resolvins are mediated by their specific membrane receptors via downstream signal transduction pathways. All known receptors for resolvins are seven-transmembrane spanning G-protein coupled receptors (GPCRs), as shown in Figure 1.8. These GPCRs include: Lipoxin receptor (ALX)/N-formyl peptide receptor (FPR)-2, G protein-coupled receptor 32 (GPR32), chemerin receptor 23 (ChemR23), leukotriene B4 receptor 1 (BLT1), and G protein-coupled receptor 18 (GPR18).

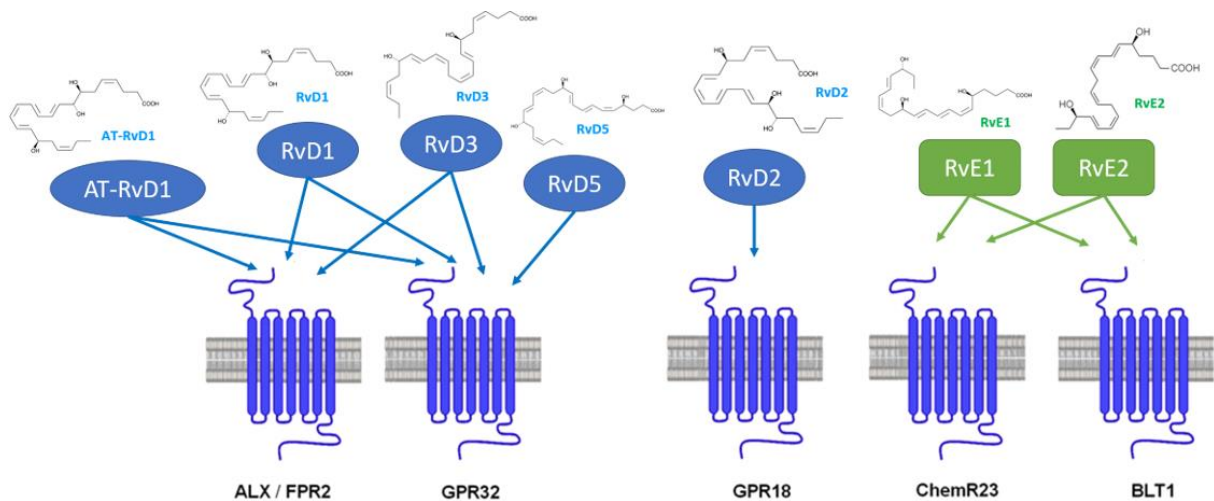


Figure 1.8. Resolvins and their receptors

Resolvins bind and signal through their specific seven-transmembrane GPCRs. AT-RvD1, RvD1, and RvD3 bind to ALX/FPR2 and GPR32, RvD5 binds to GPR32, and RvD2 binds to GPR18. Actions of RvE1 and RvE2 are mediated by ChemR23 and BLT1. GPCR schematic drawings were taken with permission from Chen et al., 2018.

GPCRs transduce extracellular signals into the cells by coupling with the heterotrimeric G protein, which is composed of guanosine diphosphate (GDP)-bound $G\alpha$ subunit that is associated with the $G\beta\gamma$ heterodimer. Upon activation of GPCRs by their ligand, a conformational change occurs, which triggers the exchange of GDP for guanosine triphosphate (GTP). This results in the dissociation of the heterotrimeric G protein, releasing α -GTP and $\beta\gamma$ subunits in their active forms. They then diffuse in the membrane and interact with various enzymes or ion channels that are involved in signal transduction (Calebiro et al., 2021). G proteins are classified into four families according to their constituent $G\alpha$ subunit: G_s , $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Kim et al., 2020). Each of the four families of G proteins activates different signalling pathways. G_s proteins activate adenylyl cyclase, leading to an increased intracellular level of cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA). On the contrary, $G_{i/o}$ proteins inhibit adenylyl cyclase activity and thus reduce the level of intracellular cAMP. $G_{q/11}$ activates phospholipase C (PLC), which

hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Sanchez-Fernandez et al., 2014). IP₃ triggers the release of Ca²⁺ from the intracellular stores such as endoplasmic reticulum into the cytosol, whereas DAG is necessary for the activation of protein kinase C (PKC). On the other hand, G12/13 activates the small GTPase Rho and modulates the reorganisation of actin cytoskeleton (Kamoto et al., 2015). Originally, it was thought that GPCRs can only couple to one type of Gα protein, but it has now been established that GPCRs can couple to more than one subtype of Gα protein and activate multiple signalling pathways (Fonin et al., 2019).

As shown in Figure 1.9, resolvins receptors are widely expressed in various cell types such as immune cells, endothelial cells, neurons, and glial cells, which may explain the diverse actions of resolvins.

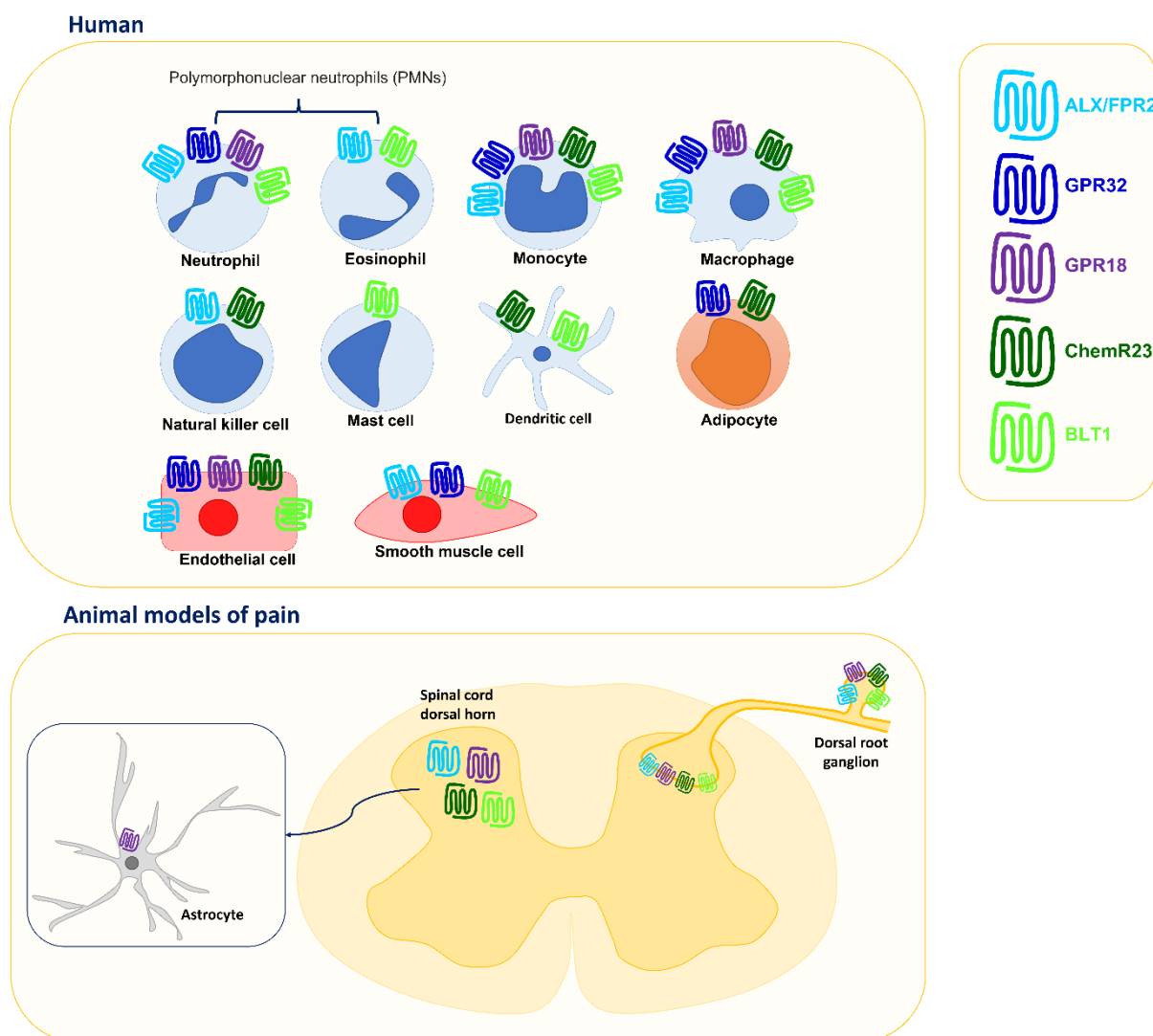


Figure 1.9. Examples of various cell types that express resolvin receptors in human and animal models of pain.

Resolvin receptors (ALX/FPR2, GPR32, GPR18, ChemR23, and BLT1) are widely expressed in various cell types such as immune cells (PMNs, monocytes, macrophages, natural killer cells, mast cells, and dendritic cells), adipocytes, endothelial cells, and vascular smooth muscle cells. In various animal models of pain, resolvin receptor expression has also been identified in dorsal root ganglion (DRG), as well as neurons and astrocytes in the spinal cord dorsal horn.

Actions of RvD1 and AT-RvD1 are mediated by both ALX/FPR2 and GPR32 (Krishnamoorthy et al., 2012), but in a context-dependent manner (Norling et al., 2012). This has been demonstrated in isolated human PMN, where high concentrations (higher than 10 nM) of RvD1 differentially activate ALX/FPR2, but low concentrations of RvD1 (nanomolar concentrations lower than 10 nM) act via GPR32 (Norling et al., 2012), as illustrated in Figure 1.10. ALX/FPR2 is also activated by RvD3 (Arnardottir et al., 2016). Both RvD3 (Dalli et al., 2013) and RvD5 (Chiang et al., 2012) also activate GPR32, whereas RvD2 binds to and signals through GPR18 (Chiang et al., 2015).

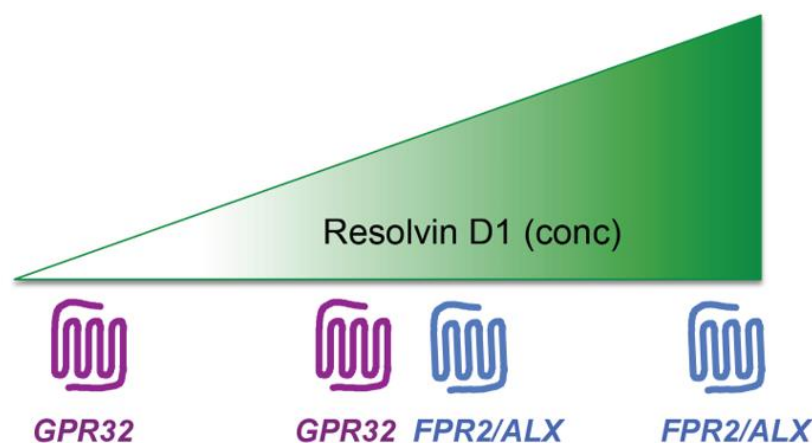


Figure 1.10. Concentration-dependent binding of RvD1 on GPR32 and FPR2/ALX.

Schematic diagram representing the concentration-dependent binding of RvD1 on GPR32 and FPR2/ALX, taken with permission from Norling et al., 2012. Low concentrations of RvD1 elicit GPR32-dependent responses, whereas higher concentrations of RvD1 elicit ALX/FPR2-dependent responses. This is established based on a previous study conducted using isolated human polymorphonuclear neutrophils (PMN) (Norling et al., 2012).

The biological actions of RvE1 and RvE2 are mediated by both ChemR23 and BLT1. RvE1 and RvE2 act as agonists for ChemR23, but they act as antagonists for BLT1 by competing for the binding of BLT1 with LTB₄, a full agonist for BLT1 (Sawada et al., 2018, Oh et al., 2012). RvE1 binds with high affinity to ChemR23 but binds to BLT1 with lower affinity (Arita et al., 2007). RvE2 is thought to be a partial agonist of ChemR23 as it produces weaker activation of ChemR23 than RvE1 (Oh et al., 2012). However, RvE2 interacts with BLT1 in similar potency as RvE1 and is thus equipotent with RvE1 (Oh et al., 2012).

1.2.3.1. ALX/FPR2

ALX/FPR2 is expressed in: monocytes, macrophages (Waechter et al., 2012), neutrophils, eosinophils, NK cells (Duvall and Levy, 2016), vascular smooth muscle cells, and endothelial cells (Baker et al., 2009) in human. Apart from RvD1 and RvD3, ALX/FPR2 can also interact with and transmit intracellular signals from other ligands including lipoxin A₄ (LXA₄) and annexin A1 to transduce their anti-inflammatory and pro-resolving effects (Pirault and Back, 2018). Interestingly, the anti-inflammatory action of RvD1 in mice with induced peritonitis is enhanced in transgenic mice that overexpress human ALX-FPR2 (Krishnamoorthy et al., 2012), but its anti-inflammatory activity is abolished in mice that lack the receptor (Norling et al., 2012). This indicates the crucial role of ALX/FPR2 in mediating the actions of RvD1, and suggests that upregulation of ALX/FPR2 may be beneficial in resolving inflammation.

Both RvD1 and LXA₄ act as full agonists for ALX/FPR2, activating the receptor with high affinity and equipotency (Krishnamoorthy et al., 2010a). Activation of ALX/FPR2 by RvD1 and LXA₄ is blocked by an ALX/FPR2 antagonist (Krishnamoorthy et al., 2010a),

indicating that RvD1 is indeed an agonist for ALX/FPR2. The action of RvD3 is less extensively studied, but its anti-inflammatory actions in arthritic mice are lost in ALX/FPR2-deficient mice (Arnardottir et al., 2016), which suggests that ALX/FPR2 also mediates RvD3 actions.

ALX/FPR2 is coupled to Gi protein, which is demonstrated by a recent study that resolved the cryo-electron microscopy structure of ALX/FPR2 in complex with a Gi protein in an active conformation, using WKYMVm (synthetic selective agonist for ALX/FPR2) for binding (Zhuang et al., 2022). A previous study showed that stimulation of ALX/FPR2 with WKYMVm in CaLu-6 cells induces phosphorylation of intracellular signalling molecules including phosphoinositide 3-kinase (PI3K), Akt, PLC, and PKC (Cattaneo et al., 2019), which suggests that the signal transduction pathways of ALX/FPR2 involve G $\beta\gamma$ -mediated activation of PLC-Ca²⁺ pathway and PI3K-Akt pathway (Ye et al., 2009). These signalling events are largely inhibited after pre-treatment with Gi protein inhibitor pertussis toxin (PTX) (Cattaneo et al., 2019), providing further evidence that ALX/FPR2 is coupled to Gi protein. Furthermore, application of a selective ALX/FPR2 agonist ACT-389949 inhibits forskolin-induced cAMP production in a dose-dependent manner in CHO cells transfected with human ALX/FPR2 (Lupisella et al., 2022), indicating both G α_i and G $\beta\gamma$ subunits are involved in the signalling pathways of ALX/FPR2, as illustrated in Figure 1.11.

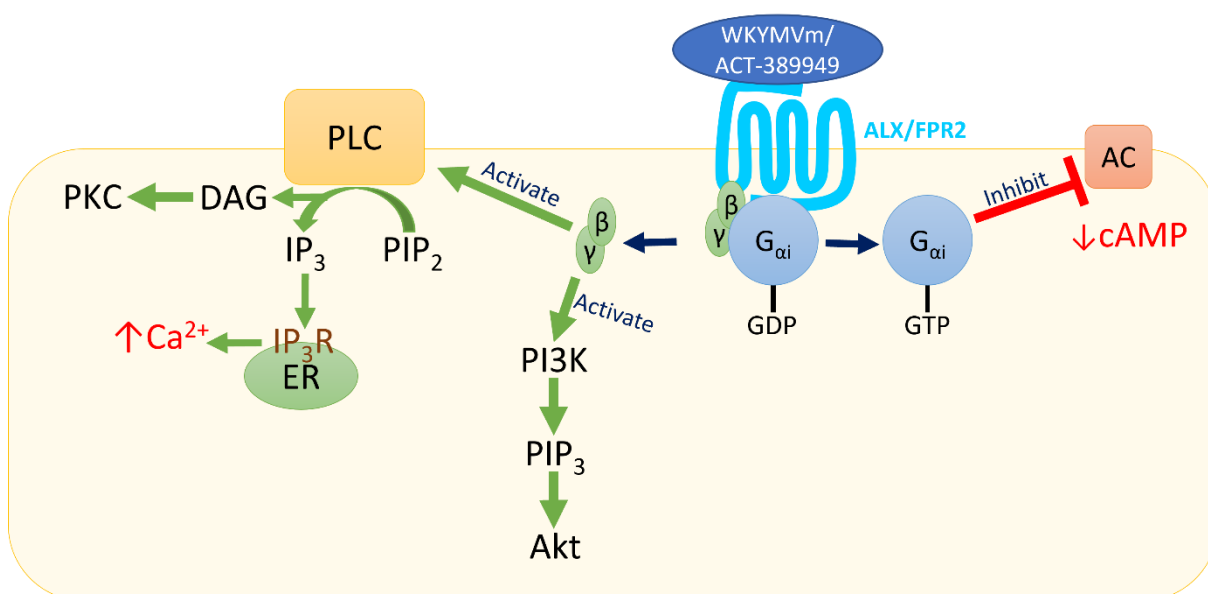


Figure 1.11. ALX/FPR2 signal transduction pathway.

Upon activation of ALX/FPR2 by its selective agonists, WKYMVm and ACT-389949, GDP-GTP exchange occurs, releasing α -GTP from G $\beta\gamma$. Activated G α_i then inhibits adenylyl cyclase (AC), reducing the level of cyclic AMP (cAMP), which in turn reduces the activity of protein kinase A (PKA). G $\beta\gamma$ activates the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway. G $\beta\gamma$ also interacts with phospholipase (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). DAG is necessary for the activation of protein kinase C (PKC), whereas IP₃ is critical to the mobilisation of Ca²⁺ from the endoplasmic reticulum.

One of the downstream signalling pathways activated by binding of RvD1 is the inhibition of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and the subsequent suppression of phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) (Fredman et al., 2014). However, the mechanisms by which RvD1 and RvD3 transduce signals via ALX/FPR2 are still unclear as there are limited functional studies. In human vascular smooth muscle cells, application of AT-RvD1 (RvD1 epimer) leads to an increase in the level of cAMP and PKA activity, and the effects of AT-RvD1 are abolished by a PKA inhibitor Rp-8-Br-cAMP and the blockade of ALX/FPR2 with its specific antibody (Mottola et al., 2017). This indicates that AT-RvD1 activates cAMP-PKA signalling via ALX/FPR2, but it is not known if adenylyl cyclase is involved in this pathway, and thus it cannot be concluded if ALX/FPR2 is also coupled to Gs protein. Conversely, RvD3 activity in dissociated mouse DRG neurons is abolished by pre-treatment with PTX and ALX/FPR2 inhibitor PBP10 (Lee et al., 2020), indicating that the RvD3 action is mediated by Gai-coupled ALX/FPR2. These give some indication that RvD1, AT-RvD1, and RvD3 may act on ALX/FPR2 via different signalling pathways, and suggest that ALX/FPR2 signalling may differ depending on the type of ligand used. Further studies are therefore needed to investigate the signalling mechanisms of resolvins via ALX/FPR2.

In the rat MIA model of osteoarthritis pain, ALX/FPR2 mRNA expression in the ipsilateral spinal cord dorsal horn 14 days following model induction is higher than that in saline-treated rats (Huang et al., 2017). Furthermore, positive correlations are found between synovial ALX/FPR2 mRNA expression and inflammatory markers such as COX2, IL-1 β , and TNF- α (Huang et al., 2017). Consistent with the findings from the animal model, ALX/FPR2 is present in osteoarthritis joint tissues from patients (Hashimoto et al., 2007), where a positive correlation between ALX/FPR2 level and IL-6 (biomarker associated with knee pain) is found in the synovium and medial tibial plateau bone from patients with end-stage osteoarthritis (Huang et al., 2017), which is indicative of its involvement in chronic inflammatory pain.

FPR2 gene expression in blood samples from neuropathic pain patients is found to be significantly higher than that from control participants (Young et al., 2023). Additionally, the *FPR2* gene located on chromosome 19 has been identified as a predicted target for the microRNA (miRNA) hsa-miR-29a using target prediction and gene enrichment analysis (Tavares-Ferreira et al., 2019). The expression of hsa-miR-29a is found to be reduced in human lingual nerve neuromas from patients with pain compared to neuromas from patients without pain. Furthermore, miR-29a expression is negatively correlated with the intensity of neuropathic pain reported by the patients (Tavares-Ferreira et al., 2019), which suggests that downregulation of miR-29a expression could be related to neuropathic pain. Since the regulation of ALX/FPR2 expression is mediated by another miRNA (miR-181b) (Pierdomenico et al., 2015), it is possible that miR-29a can also regulate ALX/FPR2

expression. Furthermore, ALX/FPR2 expression is upregulated in the L5 spinal cord and DRG 7 days after L5-spinal nerve ligation in rats in comparison to sham-operated rats (Wang et al., 2022b). These suggest that ALX/FPR2 is involved in neuropathic pain, and may potentially be a therapeutic target for both inflammatory and neuropathic pain.

1.2.3.2. GPR32

Expression of GPR32 is identified in neutrophils, macrophages, monocytes, lymphocytes, adipocytes, vascular smooth muscle cells, and endothelial cells in human (Krishnamoorthy et al., 2012). Similar to ALX/FPR2, it also mediates the anti-inflammatory and pro-resolving actions of LXA₄, RvD1 (Krishnamoorthy et al., 2010a), and RvD3 (Dalli et al., 2013). Additionally, activation of GPR32 by RvD5 can also lead to the enhancement of the phagocytic activity of human macrophages (Chiang et al., 2012), promoting efferocytosis, and thus potentiating the resolution of the inflammatory process. This demonstrates that GPR32 also mediates the pro-resolving actions of RvD5.

A previous study using an electrical cell-substrate impedance sensing assay indicates that GPR32 is coupled to $G_{\alpha s}$, as incubation with RvD1 in CHO cells transfected with human GPR32 leads to a reduction in impedance (Chiang et al., 2019). However, additional studies are needed to determine the downstream intracellular signals evoked by RvD1, RvD3, and RvD5 via GPR32 as they have not been well characterised in previous studies.

RvD1 can activate both ALX/FPR2 and GPR32 in a concentration-dependent manner in isolated human PMN, wherein lower RvD1 concentrations preferentially activate GPR32 (illustrated in Figure 1.10) (Norling et al., 2012). Interestingly, RvD1 fails to reduce PMN infiltration (Krishnamoorthy et al., 2012) and promote macrophage phagocytosis (Norling et al., 2012) in mice that lack ALX/FPR2, which raises doubt about the role of GPR32. However, an orthologue for human GPR32 has only been identified in chimpanzees (Krishnamoorthy et al., 2010a). In mice and rats, GPR32 is present as a pseudogene (non-functional gene with high sequence similarity) (Haitina et al., 2009), and the counterparts of human GPR32 in rats and mice remain unknown. On the contrary, human ALX/FPR2 orthologues have been identified in mice (Takano et al., 1997) and rats (Chiang et al., 2003). Therefore, it is possible that the anti-inflammatory and analgesic actions of RvD1 (Rogerio et al., 2012, Xu et al., 2010) and RvD3 (Arnardottir et al., 2016) found in murine models of inflammation and pain may depend on ALX/FPR2 activation. Effects of RvD5 administration seen in mouse models of inflammation (Chiang et al., 2012) and pain (Luo et al., 2019) would need to be interpreted with caution due to the absence of human GPR32 counterparts in mice. Further studies to investigate RvD5 actions in GPR32 knockout mice are therefore needed to explore whether those anti-inflammatory and analgesic effects of RvD5 may be mediated by another RvD5 receptor that has yet to be identified. Nevertheless, it may be

challenging to investigate if GPR32 mediates the anti-inflammatory and analgesic actions of RvD1, RvD3, and RvD5 in human, as it is not possible to study GPR32-mediated effects in murine models.

1.2.3.3. GPR18

GPR18 expression has been identified in neutrophils, macrophages, monocytes (Chiang et al., 2015), and endothelial cells (Wilhelmsen et al., 2014, Ulu et al., 2019) in human.

Using a GPCR- β -arrestin-based screening, RvD2 was found to activate GPR18 selectively, with similar potency as the full agonist of GPR18, N-arachidonoyl glycine (NAGly) (Chiang et al., 2015). Furthermore, the effect of RvD2 in enhancing PMN apoptosis in mice is abolished in mice that lack GPR18, indicating that GPR18 mediates the pro-resolving actions of RvD2 (Chiang et al., 2015, Chiang et al., 2017). Pre-treatment of cholera toxin (CTX), a $G_{\alpha s}$ protein inhibitor, inhibits changes induced by GPR18 while pre-treatment with PTX has no effects (Chiang et al., 2015). This suggests that GPR18 is coupled to $G_{\alpha s}$ protein. Administration of RvD2 also increases the level of cellular cAMP in cultured conjunctival goblet cells (Botten et al., 2019). This suggests that binding of RvD2 activates the cAMP-dependent pathway by activating adenylyl cyclase, activating downstream signalling pathways mediated by PKA (Chiang et al., 2017), as shown in Figure 1.12.

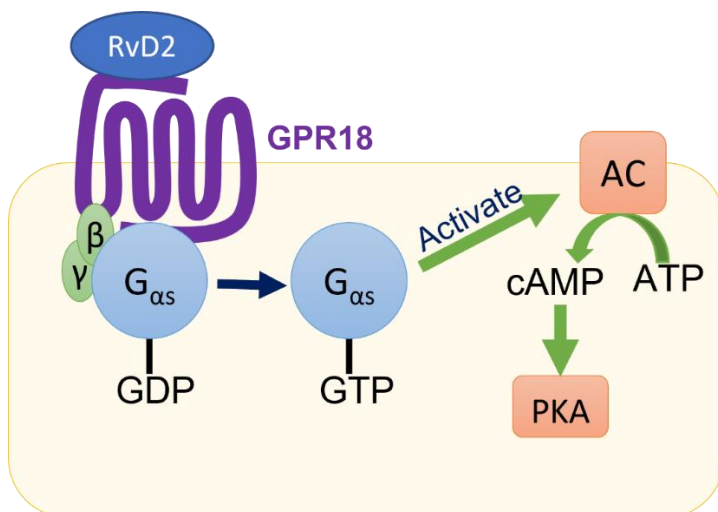


Figure 1.12. GPR18 signal transduction pathway.

Upon activation of GPR18 by its ligand RvD2, GDP-GTP exchange occurs, releasing α -GTP from $G\beta\gamma$. Activated $G_{\alpha s}$ then activate adenylyl cyclase (AC), leading to an increase in second messenger cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA), which regulates a wide variety of physiological processes.

In a rat model of radicular pain, GPR18 is found to be expressed in neurons and astrocytes in the spinal dorsal horn (Zhang et al., 2018), which could be one of the sites of action of RvD2. Additionally, the level of GPR18 mRNA is elevated in the ipsilateral spinal cord following sciatic nerve injury in rats (Malek et al., 2016), providing further evidence that GPR18 may be a potential target for pain treatment.

1.2.3.4. ChemR23

ChemR23, also known as chemokine receptor-like 1 (CMKLR1), is found to be expressed in macrophages, dendritic cells, monocytes, NK cells, adipocytes, and endothelial cells in human (Herova et al., 2015).

ChemR23 was initially discovered as a receptor for the chemoattractant protein chemerin (Wittamer et al., 2003), but later studies identify RvE1 and RvE2 as other ligands for the receptor, where RvE1 is a full agonist like chemerin, and RvE2 acts a partial agonist for ChemR23 (Oh et al., 2012). Like RvE1, chemerin also reduces the second phase of pain behaviour induced by formalin (Xu et al., 2010). Additionally, reducing ChemR23 expression by small interfering RNAs (siRNAs) treatment reverses the analgesic effect of RvE1 in the same model (Xu et al., 2010). These suggest that ChemR23 mediates the analgesic action of RvE1 in this inflammatory pain model.

In CHO cells transfected with ChemR23, chemerin application inhibits cAMP accumulation and calcium mobilisation (Wittamer et al., 2003). Additionally, the effects of RvE1 are abolished by pre-treatment with PTX in HEK293 cells transfected with ChemR23 (Arita et al., 2005). These provide evidence that ChemR23 is coupled to Gi protein. Furthermore, application of RvE1 leads to an increase in intracellular calcium in rat conjunctival goblet cells, and this increase is abolished in cells transfected with ChemR23 siRNA (Yang et al., 2020). This suggests that binding of RvE1 to Gi-coupled ChemR23 activates the $G\beta\gamma$ -PLC β -Ca²⁺ signalling pathway, as illustrated in Figure 1.13. Additionally, application of RvE1 stimulates Akt phosphorylation in CHO cells transfected with ChemR23 (Ohira et al., 2010). Phosphorylation of Akt is inhibited when the cells are pre-treated with the PI3K inhibitor wortmannin (Ohira et al., 2010), indicating that RvE1 also activates the PI3K/Akt signalling pathway via ChemR23, as depicted in Figure 1.13. In contrast to chemerin and RvE1, the interaction between RvE2 and ChemR23 has not been studied extensively, and thus requires further investigation.

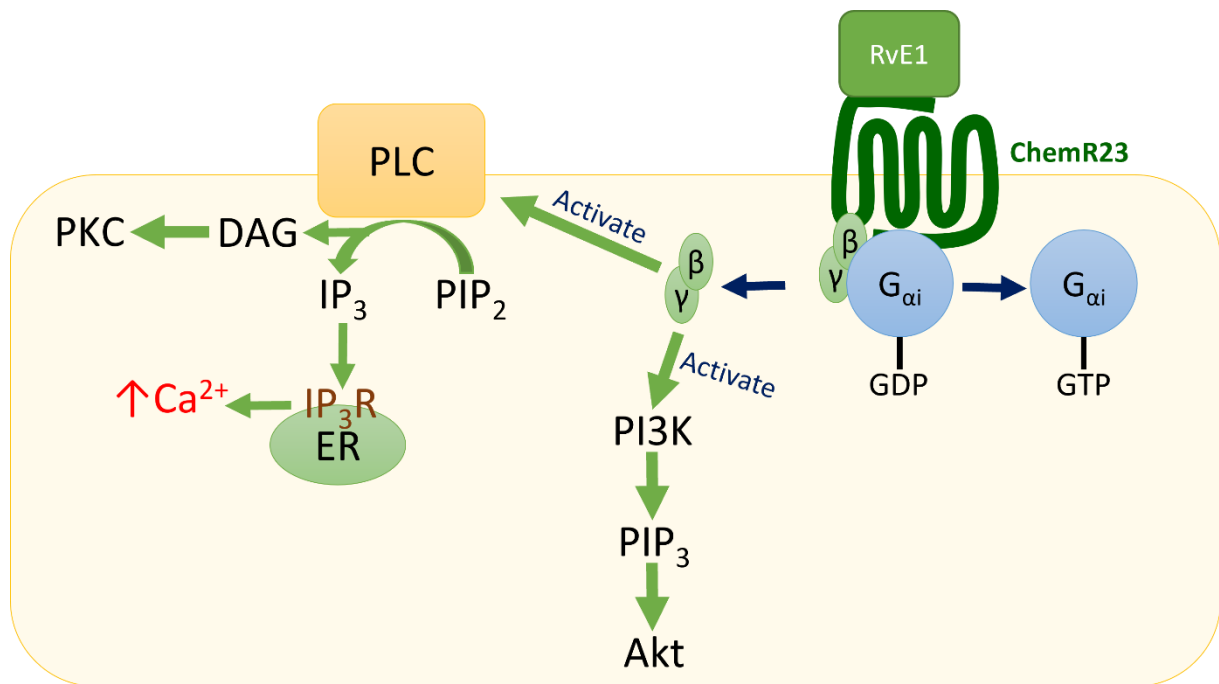


Figure 1.13. ChemR23 signal transduction pathway.

Upon activation of ChemR23 by RvE1 (full agonist), GDP-GTP exchange occurs, releasing α -GTP from $G\beta\gamma$. $G\beta\gamma$ then interacts with phospholipase (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG is necessary for the activation of protein kinase C (PKC), whereas IP₃ is critical to the mobilisation of Ca^{2+} from the endoplasmic reticulum. $G\beta\gamma$ also activates the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway.

Differential expression of ChemR23 has been observed in inflammatory conditions, ChemR23 is found to have higher expression levels in gingival tissues from patients with periodontitis compared to healthy tissues (Ozcan et al., 2018). There is also a positive correlation between the extent of ChemR23 staining and the periodontal clinical parameters of tissue samples, where patients with more severe periodontal disease have greater levels of ChemR23 in their gingival tissues (Ozcan et al., 2018). Furthermore, ChemR23 expression is upregulated in the early stages of pulpal inflammation (after 24 hours) in rats. Interestingly, this upregulation can be prevented by early treatment of RvE1 (Xu et al., 2019), which demonstrates the anti-inflammatory action of RvE1 when it is administered at the early stages of inflammation. However, ChemR23 expression in an osteoarthritis inflammatory pain rat model is more variable (Huang et al., 2017). ChemR23 expression is lower in the synovium of MIA-treated rats compared to rats treated with saline at later time points (Day 14 and 35 after induction of the model), but its expression in the ipsilateral spinal cord dorsal horn increases 35 days after induction of the model (Huang et al., 2017). This suggests that ChemR23 expression may change at different stages of inflammation. While ChemR23 is likely to be involved in the early stages of inflammation to mediate the effects of its ligand, its role in later stages of inflammation remains to be investigated.

ChemR23 gene expression is elevated in the ipsilateral lumbar (L4-L6) dorsal part of the spinal cord 3 and 14 days after sciatic nerve ligation in rats in comparison to naïve rats (Parkitna et al., 2006). Furthermore, ChemR23 is found to be expressed in mice DRG and spinal cord neurons (Xu et al., 2010, Ji et al., 2011), and its gene expression in paw skin, DRG, and ipsilateral dorsal horn of the spinal cord is upregulated in the carrageenan model of inflammatory pain after injection of carrageenan (Meesawatson et al., 2016, Xie et al., 2021). Similarly, from a recent unpublished study in our laboratory, ChemR23 is found in neurons within the spinal cord dorsal horn and trigeminal nucleus in rats, and its expression in the ipsilateral trigeminal nucleus is higher following lingual nerve injury in rats 3, 7, and 28 days post-injury than in naïve rats (Tavares Ferreira, 2017). Accumulation of ChemR23 is also observed in axons near the sciatic nerve ligation site in mice (Ji et al., 2011), which suggests that ChemR23 may be transported axonally to peripheral terminals after nerve injury. Moreover, ChemR23 is expressed in joint tissues in osteoarthritis patients, where there is a positive correlation between ChemR23 expression and IL-6 (biomarker associated with knee pain) in medial tibial plateau bone from patients with end-stage osteoarthritis (Huang et al., 2017). Collectively, data from these studies suggest that ChemR23 may potentially be a target for treating pain.

1.2.3.5. BLT1

BLT1 is found to be expressed in neutrophils (Freire et al., 2017), eosinophils, macrophages, monocytes, dendritic cells, mast cells (Qiu et al., 2006), vascular smooth muscle cells, and endothelial cells (Back et al., 2005) in human.

Both RvE1 and RvE2 exhibit similar potency for BLT1 (Oh et al., 2012). They have been suggested to act as partial agonists for BLT1 and can counteract the effects of LTB₄, a BLT1 full agonist (Arita et al., 2007). However, other studies in the literature suggested that they are antagonists at BLT1 (Sawada et al., 2018, Archambault et al., 2019), as RvE1 can abolish the effects of LTB₄ to a similar extent as a BLT1 antagonist (Sawada et al., 2018). Despite these conflicting claims, it can be established that RvE1 and RvE2 can compete with LTB₄ and dampen LTB₄-BLT1 signalling. LTB₄ is a pro-inflammatory mediator that acts by promoting the chemotaxis of PMN and macrophages to the sites of inflammation (Yokomizo et al., 1997). Therefore, RvE1 and RvE2 may exert their anti-inflammatory and pro-resolving actions by inhibiting LTB₄-BLT1 signalling, as illustrated in Figure 1.14. This is demonstrated by a previous study, where it was shown that the increase in IL-23 production by dendritic cells induced by LTB₄ is reversed by RvE1 (Sawada et al., 2018).

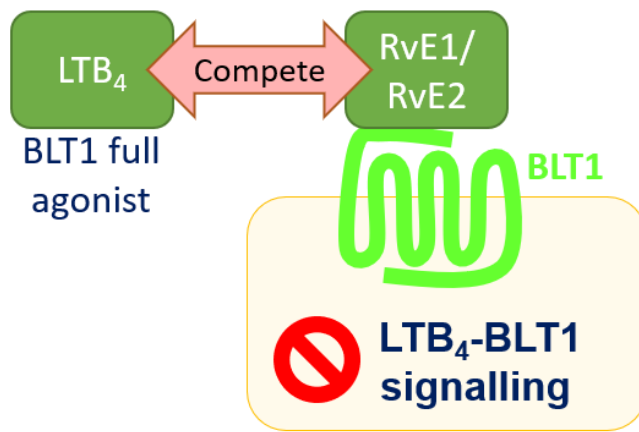


Figure 1.14. Inhibition of LTB₄-BLT1 signalling by RvE1 and RvE2.

RvE1 and RvE2 compete with LTB₄, a BLT1 full agonist, for binding to BLT1, and thus inhibit LTB₄-BLT1 signalling.

In CHO cells transfected with BLT1, application of LTB₄ inhibits forskolin-induced cAMP accumulation in a dose-dependent manner, and this inhibitory action is abolished by pre-treatment with PTX, indicating that BLT1 is coupled to Gi protein. This is further evidenced in a recent study that resolved the cryo-electron microscopy structure of LTB₄-bound BLT1 in complex with a Gi protein in an active conformation (Wang et al., 2022a). LTB₄ has also been shown to increase intracellular calcium concentration and IP₃ accumulation in CHO cells transfected with BLT1, but these increases are not affected by pre-treatment with PTX (Yokomizo et al., 1997, Yokomizo et al., 2001), indicating that the Gi-Gβγ-PLCβ-Ca²⁺ signalling pathway is not involved. These also indicate that BLT1 couples to both PTX-sensitive and PTX-insensitive G-proteins, and thus BLT1 may be coupled to both Gi and Gq proteins (illustrated in Figure 1.15). In HEK293 cells transfected with BLT1, application of RvE1 can inhibit forskolin-induced cAMP accumulation (Arita et al., 2007), and can also lead to an elevation in intracellular calcium level (Merlin et al., 2022). Application of RvE1 in rat and human conjunctival goblet cells also leads to an increase in intracellular calcium, and this increase is abolished by pre-treatment with BLT1 antagonist (Yang et al., 2020), indicating that the RvE1-induced calcium signalling is mediated by BLT1 (illustrated in Figure 1.15). In contrast to LTB₄ and RvE1, the interaction between RvE2 and BLT1 has not been studied extensively.

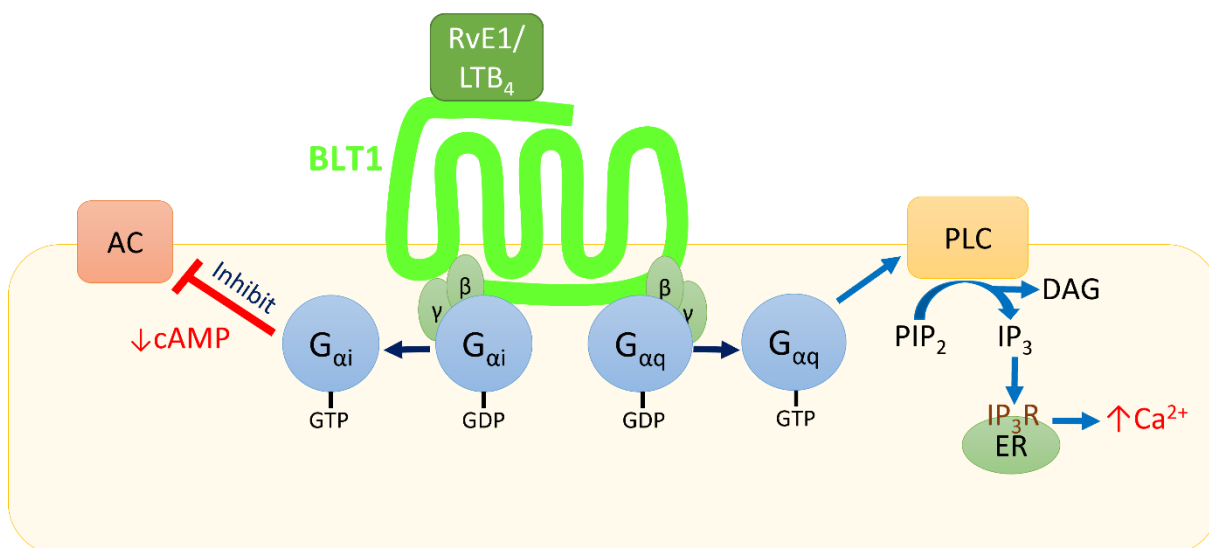


Figure 1.15 BLT1 signal transduction pathway.

BLT1 is coupled to both Gi and Gq proteins. Upon activation of BLT1 by LTB₄ (full agonist) and RvE1 (possible partial agonist), GDP-GTP exchange occurs, releasing α-GTP from Gβγ. Activated G_{αi} then inhibits adenylyl cyclase (AC), reducing the level of cyclic AMP (cAMP), which in turn reduces the activity of protein kinase A (PKA). On the other hand, activated G_{αq} activates phospholipase (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). DAG is necessary for the activation of protein kinase C (PKC), whereas IP₃ is critical to the mobilisation of Ca²⁺ from the endoplasmic reticulum.

BLT1 is expressed in TRPV1-positive neurons in mouse DRG (Andoh and Kuraishi, 2005) and non-neuronal cells in rat DRG (Okubo et al., 2010a), and its expression in ipsilateral spinal cord neurons increases after spared nerve injury in rats (Okubo et al., 2010b). Furthermore, administration of a BLT1 antagonist reduces allodynia after spare nerve injury in rats (Okubo et al., 2010b) and phase II pain behaviour induced by formalin in mice (Asahara et al., 2015). This suggests that BLT1 may be involved in both neuropathic and inflammatory pain, and that LTB₄-BLT1 signalling may potentially be a therapeutic target.

1.2.4. Mechanisms of action of resolvins

The analgesic actions of resolvins are mediated by their specific GPCRs, which have a wide expression pattern, including immune cells, neurons, and glial cells (Ji et al., 2011). Therefore, resolvins may be able to dampen pain via multiple mechanisms such as modulating spinal synaptic plasticity and glial activation (Xu et al., 2013), in addition to dampening inflammation. However, the mechanisms and signalling pathways by which resolvins mediate their pro-resolving and analgesic actions remain to be elucidated.

One of the proposed mechanisms is via inhibition of TRP channel signalling, as summarised in Table 1.3. TRP channels are expressed in sensory neurons (C- and Aδ-fibres) throughout the nociceptive pathways within the peripheral nervous system. Since different TRP channels detect different physical and chemical stimuli, they mediate a range

of different pain phenotypes. For example, TRPV1 is a polymodal nociceptive channel activated by heat, low pH, and chemical compounds such as capsaicin. Similarly, TRPA1 channels are polymodal sensors activated by thermal stimuli, irritant chemicals, and mechanical force. TRPV4 is also a polymodal channel, and contributes to heat, mechanical, and hypotonicity-induced pain. Additionally, TRPV3 channels are also responsible for heat pain (Mickle et al., 2016). In the literature, the interaction between resolvins and TRP channels was mainly studied using mouse DRG neurons. RvD1 inhibits the activities of TRPA1, TRPV3, and TRPV4 in mouse DRG neurons (Bang et al., 2010, Payrits et al., 2020, Pyo et al., 2022); while AT-RvD1 only attenuates TRPV3 activity in HEK293 cells transfected with various TRP channels (Bang et al., 2012). RvD2 suppresses TRPV1 channel activity, in addition to attenuating TRPA1 activity, with a higher potency than RvD1 in mouse DRG neurons (Park et al., 2011, Perna et al., 2021, Payrits et al., 2020). RvD3 suppresses TRPV1 activity in mouse DRG neurons and cultured human DRG neurons (Lee et al., 2020). On the other hand, RvE1 inhibits TRPV1 channel activity, with a higher potency than RvD2 in mouse DRG neurons (Xu et al., 2010, Park et al., 2011). RvE1 can inhibit the potentiation of TRPV1 currents induced by capsaicin (TRPV1 agonist) and SP in mouse DRG neurons (Jo et al., 2016), and can also reduce spontaneous pain induced by capsaicin (Xu et al., 2010, Park et al., 2011). RvE1 can also induce TRPA1 inhibition in DRG neurons, but a higher concentration is required in comparison to RvE1-induced TRPV1 inhibition (Pyo et al., 2022). Interestingly, RvE1 fails to inhibit SP-induced TRPV1 currents in DRG neurons when they are pre-treated with PTX (Jo et al., 2016). Furthermore, the inhibitory effects of RvE1 on TRPV1, RvD2 on TRPV1 and TRPA1, and RvD3 on TRPV1 are abolished by the administration of GDP β S, a G-protein inhibitor (Park et al., 2011, Jo et al., 2016, Lee et al., 2020). These findings suggest that resolvins do not act directly on TRP channels, but instead act via a GPCR-mediated signalling pathway to exert their inhibitory effects on TRP channels (Park et al., 2011). This is supported by the fact that ChemR23 and TRPV1 are co-expressed in mice DRG neurons (Xu et al., 2010, Jo et al., 2016). On the other hand, Bang et al. proposed that the inhibitory effects of AT-RvD1 on TRPV3 may not be mediated by GPCR-mediated signalling as the effects are not affected by gallein, a G $\beta\gamma$ inhibitor (Bang et al., 2012). However, the evidence is not very conclusive. This is because gallein can only block G $\beta\gamma$ subunit signalling, and does not affect G α signalling (Campbell and Smrcka, 2018). Therefore, it can only be interpreted that AT-RvD1-mediated TRPV3 inhibition does not depend on G $\beta\gamma$ -mediated signalling. Since TRP channels are involved in mediating different pain phenotypes, the inhibitory actions of resolvins on different TRP channels may explain how resolvins alleviate pain. However, the detailed mechanisms by which resolvins mediate the inhibitory actions on TRP channels in the peripheral terminals require further investigation.

Table 1.3. Inhibitory effects of different resolvins on different TRP ion channels.

TRP ion channels	Resolvins
TRPA1	RvD1 (Bang et al., 2010, Payrits et al., 2020, Pyo et al., 2022) RvD2 (Park et al., 2011, Payrits et al., 2020) RvE1 (Pyo et al., 2022)
TRPV1	RvD2 (Park et al., 2011, Perna et al., 2021, Payrits et al., 2020) RvD3 (Lee et al., 2020) RvE1 (Xu et al., 2010, Park et al., 2011, Pyo et al., 2022, Jo et al., 2016)
TRPV3	AT-RvD1 (Bang et al., 2012) RvD1 (Bang et al., 2010)
TRPV4	RvD1 (Bang et al., 2010)

Another suggested mechanism by which resolvins exert their analgesic actions is via downregulation of postsynaptic NMDA receptor (NMDAR) activities in the central terminals. NMDAR activation in the central nociceptor terminals is essential in central sensitisation and thereby the development of chronic pain (Iwata et al., 2017). A member of the MAPK family, extracellular signal-regulated kinase (ERK), is phosphorylated in central nociceptive neurons when they are hyperactivated. Phosphorylated ERK is thus widely considered to be a marker of neuronal plasticity in central sensitisation (Ji et al., 1999). RvE1 can suppress TNF- α -induced potentiation of NMDAR-mediated currents in spinal dorsal horn neurons (Xu et al., 2010). This inhibitory action appears to be mediated by the ERK signalling pathway, as it is blocked by inhibiting the ERK pathway with mitogen-activated protein kinase kinase (MAP2K) inhibitors (Xu et al., 2010). Furthermore, RvE1 treatment reduces ERK phosphorylation in dorsal horn neurons after TNF- α treatment (Xu et al., 2010), which is indicative of a reduction in NMDAR-mediated activity. This was similarly observed in a later experiment with RvD1, where RvD1 treatment reduces phosphorylation of two NMDAR subunits, NR1 and NR2B, in spinal dorsal horn neurons in a chronic pancreatitis rat model (Quan-Xin et al., 2012). Therefore, resolvins may suppress hyperactivation of NMDAR in central postsynaptic neurons by inhibiting the ERK signalling pathway, but the detailed signalling mechanisms await further investigation.

The anti-inflammatory and pro-resolving actions of RvE1 and RvE2 can be mediated by both ChemR23 and BLT1. These two receptors have different expression patterns, whereby ChemR23 is predominantly expressed in antigen-presenting cells such as macrophages and dendritic cells, and not as highly expressed in PMN as BLT1 (Arita et al., 2005). Therefore, ChemR23 and BLT1 may have different functional roles. Activation of ChemR23 by RvE1 and RvE2 promotes macrophage phagocytosis of apoptotic cells such as neutrophils (Herova et al., 2015), whereas BLT1 activation reduces infiltration of leukocytes by displacing the binding of LTB₄ (Arita et al., 2007). Interestingly, binding of RvD1 to

ALX/FPR2 can also reduce the level of LTB₄ (BLT1 agonist) (Fredman et al., 2014), indicating that different resolvins may act in tandem to help to resolve inflammation and transduce their analgesic actions via their respective receptors.

1.3. Summary

Despite progress in improving our understanding of the induction mechanisms that lead to pain perception, our understanding of how pain is resolved is still limited. The active process to resolve acute inflammation is mediated by the family of endogenously produced lipid mediators such as resolvins (Serhan, 2014). When this process is disrupted, inflammation can be sustained, leading to uncontrolled, chronic inflammation, and is thought to contribute to the transition from acute to chronic pain state (Ji et al., 2011). This may occur because of defects in resolvin receptor expression, intracellular signalling, or production of synthetic enzymes required to generate resolvins (Basil and Levy, 2016, Ozgul Ozdemir et al., 2020). Therefore, the endogenous anti-inflammatory and pro-resolution pathways mediated by activation of resolvin receptors can potentially be leveraged to provide new therapeutic targets for pain treatment. This project will further investigate the mechanisms via which resolvins can modulate pain, using human tissues and an animal model to address the four aims described below.

Aim 1: Validation of antibody specificity

Expression of resolvin receptors in human pain-related tissues, such as osteoarthritis joint tissues, has mostly been described using qPCR (Hashimoto et al., 2007, Huang et al., 2017). In the literature, there are only a few studies that used immunohistochemistry to label resolvin receptors in human tissues. These include studies that evaluate ALX/FPR2 expression in human skin biopsies (Motwani et al., 2018) and minor salivary glands (Nelson et al., 2014). Unfortunately, details about the antibody used to label ChemR23 in human gingival tissues via immunohistochemistry are not described in the article (Ozcan et al., 2018). In our laboratory, BLT1 and GPR32 expression in human lingual nerve neuromas were only evaluated in a small pilot study (Tavares Ferreira, 2017). Further studies are therefore needed to demonstrate that the antibodies selected to be used in this project are specific and can selectively bind to their respective resolvin receptors (ALX/FPR2, GPR32, ChemR23, and BLT1). Therefore, the first aim of my project was to validate the specificity of the antibodies using a human cell line previously shown to express these receptors.

Aim 2: Characterisation of resolvin receptor expression in human tissues from patients with inflammatory pain

Studies on the action of resolvins in animal models of inflammation and pain suggest that they can alleviate inflammatory pain (Xu et al., 2010, Park et al., 2011, Luo et al., 2019). Expression of their receptors including ALX/FPR2 (Huang et al., 2017), BLT1 (Okubo et al., 2010a), and ChemR23 (Meesawatsom et al., 2016, Parkitna et al., 2006) has also been identified in the spinal cord and DRG in those animals. However, the expression of resolvin receptors at sites of inflammation and pain, versus healthy tissues in patients is less

extensively studied. One study found that ChemR23 is differentially expressed in tissues with and without inflammation, wherein ChemR23 expression is higher in tissues with periodontitis compared to that in healthy tissues, and that the extent of ChemR23 staining correlates positively with the severity of periodontal disease (Ozcan et al., 2018). Even though a positive correlation between ALX/FPR2 and ChemR23 expression with IL-6 (a biomarker thought to associate with knee pain) has been identified in joint tissues from osteoarthritis patients (Huang et al., 2017), this study did not correlate the expression of ALX/FPR2 and ChemR23 with pain symptoms in those patients.

In order to address this, the second aim of my project was to characterise the expression of resolvins receptors in normal tooth pulp tissues from non-carious teeth and inflamed tooth pulp tissues from carious painful and non-painful teeth. The expression of resolvins receptors can then be quantified and correlated with the clinical pain history of the patients, allowing for the identification of resolvins receptors that are differentially expressed in inflammatory pain. I hypothesised that resolvins receptors are expressed in human tooth pulp tissues, and that there would be a difference in resolvins receptor expression in tooth pulp tissues from carious painful and non-painful teeth.

Aim 3: Investigation on the intracellular signalling cascades by which resolvins modify activity in sensory neurons

Previous studies mainly used cell lines transfected with specific resolvins receptors (Chiang et al., 2019, Arita et al., 2007, Merlin et al., 2022, Arita et al., 2005) or immune cells (Fredman et al., 2014) to investigate the downstream signalling pathways by which resolvins exert their actions via their receptors. However, there is evidence that the types of G-proteins that resolvins receptors couple to may vary depending on the type of cell (Yokomizo et al., 1997); thus, findings from other cell types may not be relevant to neuronal cells. Therefore, the third aim of my project was to investigate the signalling cascades through which resolvins modify activity in TG neurons. Rodent TG cells were used as they contain cell bodies of neurons that supply the tooth pulp, and are involved in the orofacial pain transmission pathway. I hypothesised that, application of resolvins could modify activity in TG cells via their specific receptors.

Aim 4: Development of a pre-clinical model of lingual nerve injury

As inflammatory infiltrates are present at the site of affected nerve or tissue in neuropathic and inflammatory pain conditions, resolvins and their specific receptors may be a novel approach for pain treatment, with potentially broad clinical applications in both neuropathic and inflammatory pain. Current pharmacological treatments for chronic neuropathic pain that arises as a result of lingual nerve injury are not universally efficacious and are limited by their adverse effects, so there is an unmet clinical need for effective and

safe pain medications. Previous studies in the literature investigating the effects of resolvins in animal models of neuropathic pain usually induce nerve injury by constricting the sciatic nerve (Pang et al., 2023, Doyle et al., 2014) or ligating the spinal nerve (Xu et al., 2013). Since the lingual nerve innervates the tongue, its receptive field is extremely difficult to access using conventional behavioural assessments (e.g., testing for withdrawal thresholds to thermal and mechanical stimuli using the plantar test and von Frey test respectively). Therefore, a pre-clinical model was previously established in our laboratory (Tavares-Ferreira et al., 2019) using the Orofacial Stimulation Test to monitor changes in drinking behaviour following lingual nerve CCI, linked with allodynia-like symptoms found in neuropathic pain.

Our previous work (using chromic catgut for the constriction) revealed a significant change in drinking behaviour (Tavares-Ferreira et al., 2019); however, the behavioural change was less marked than those reported following sciatic nerve CCI (Medeiros et al., 2021). Studies of CCI have used a range of different sutures for constriction (Kukkar et al., 2013, De Vry et al., 2004), and in some cases, more extensive and longer-term changes are reported when using non-resorbable sutures (Kupers et al., 1992) (as detailed in chapter 6). In addition, at the time of these experiments, chromic catgut sutures were not readily available to purchase. Therefore, the aim of this study was to compare behavioural changes following lingual nerve CCI using chromic catgut (resorbable) and black silk (non-resorbable) sutures and determine their relative effectiveness in inducing pain-related behavioural changes; with a view to establishing a model to investigate the effects of resolvins on pain-like behaviour change following lingual nerve injury.

CHAPTER 2: MATERIALS AND METHODS

This chapter describes the materials and methods used to carry out the studies reported in this thesis.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-human BLT1	Alomone	Cat# ALR-001 LOT# ALR001AN0150
Rabbit anti-human GPR32	Genetex	Cat# GTX71225 LOT# 821705764 LOT# 821905162
Rabbit anti-human FPR2/ALX	Alomone	Cat# AFR-002 LOT# AFR002AN0225
Rabbit anti-human ChemR23	Invitrogen	Cat# PA5-33438 LOT# UH2819701
Fluorescein labelled Ulex Europaeus Agglutinin I (UEA I)	Vector Laboratories	Cat# FL-1061 Lot# ZF0829
Monoclonal Anti-Actin, α -Smooth Muscle - FITC antibody produced in mouse	Sigma-Aldrich	Cat# F3777 Lot# 086M4820V
Mouse anti-Tubulin β 3	Biolegend	Cat# 801202 Lot# B249869
Rabbit anti-LTB4-R1/BLTR (BLT1)	Bioss	Cat# bs-2654R
Mouse anti- β -Tubulin III	Sigma-Aldrich	Cat# T8578 Lot# 029M4843V
Mouse anti-Neurofilament 160/200 (NF160/200)	Sigma-Aldrich	Cat# N2912 Lot# 054M4865V
Mouse anti-Glutamine Synthetase	Abcam	Cat# ab64613 Lot# MAB-03452
Donkey anti-rabbit Cy3	Jackson Immunoresearch	Cat# 711-165-152
Donkey anti-mouse FITC	Jackson Immunoresearch	Cat# 711-095-150
Donkey anti-rabbit IgG, Alexa Fluor 488	Invitrogen	Cat# A-21206 Lot# 1796375
Donkey anti-mouse IgG, Alexa Fluor 555	Invitrogen	Cat# A-31570 Lot# 1905844
BLT1 (ext) ₁₆₈₋₁₈₁ peptide (Control antigen for ALR-001)	Alomone labs	LOT# ALR001AG0140
L-fucose	Vector Laboratories	Cat# S-9007
Mouse anti-GAPDH	Protein tech	Cat# 60004-1-Ig
Anti-rabbit IgG, HRP linked antibody	Cell signalling	Cat# 7074
Anti-mouse IgG, HRP linked antibody	Cell signalling	Cat# 7076
Biological samples		
Human tooth pulp	Charles Clifford Dental Hospital, Sheffield, UK	N/A
Experimental Models: Primary cell cultures		
Human umbilical vein endothelial cells (HUVECs)	Kind gift from Dr Simon Whawell	N/A

Primary rat trigeminal ganglion (TG) cell culture	Generated in house, see Section 2.3	N/A
Experimental Models: Organisms/Strains		
Sprague-Dawley rats	Charles River	N/A
Wistar rats	Charles River	N/A
Chemicals		
Medium 199 (M199)	Sigma-Aldrich	Cat# M4530
L-Glutamine solution (0.2M)	Sigma-Aldrich	Cat# G7512
Penicillin-Streptomycin (10,000 units penicillin and 10 mg streptomycin/ml)	Sigma-Aldrich	Cat# P0781
HEPES solution (1M, pH 7.0 – 7.6)	Sigma-Aldrich	Cat# H0887
Foetal Bovine Serum (FBS)	Sigma-Aldrich	Cat# F7524
Trypsin EDTA solution	Sigma-Aldrich	Cat# T4049
Opti-MEM I Reduced Serum Medium	Gibco	Cat# 31985062
Oligofectamine Transfection Reagent	Invitrogen	Cat# 12252011
Precision Plus Protein™ Dual Color Standards	Bio-Rad	Cat#1610374
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat# 34579
Restore Western Blot stripping buffer	Thermo Fisher Scientific	Cat# 21059
OCT compound	Sakura Finetek, Europe BV	Cat# S-9007
Vectashield mounting medium with DAPI	Vector Laboratories	Cat# H-1200 Lot# ZF0515
Modified Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	Cat# H9394
Dulbecco's Modified Eagle's Growth Medium (DMEM)	Gibco	Cat# 10566016
Dispase II	Gibco	Cat# 17105041
Collagenase Type 1A	Sigma-Aldrich	Cat# C9891
Poly-D-lysine	Sigma-Aldrich	Cat# A-003-M
Laminin	Sigma-Aldrich	Cat# L2020
Tissue culture-grade water	Cytiva	Cat# SH30529.02
Paraformaldehyde Solution, 4% in PBS	Thermo Fisher Scientific	Cat# J61899.AK
Mounting medium with DAPI - Aqueous, Fluoroshield	Abcam	Cat# ab104139
Fluo-4, AM	Invitrogen	Cat# F14201 Lot# 1361001
Pluronic F-127	Sigma	Cat# P2443
Resolvin E1	Cayman Chemical	Cat# CAY10007848
Resolvin E2	Cayman Chemical	Cat# CAY13827
20-OH-LTB4	Santa Cruz	Cat# sc-200959

U-75302	Cayman Chemical	Cat# CAY70705
Green Downward cADDIS cAMP sensor BacMam	Montana molecular	Cat# 96-001-01
Sodium butyrate	Sigma-Aldrich	Cat# B5887
hD2 receptor BacMam	Montana molecular	Cat# 96-001-01
Quinpirole hydrochloride	Sigma-Aldrich	Cat# Q102
Forskolin	Sigma-Aldrich	Cat# F6886 Lot# SLBP3308CV
Critical commercial assays		
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23225
Monarch Total RNA Miniprep Kit	New England Biolabs	Cat# T2010
High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat# 4368814
Rotor-Gene Probe PCR Kit	QIAGEN	Cat# 204574
cADDIS cAMP Assay for Gi kit	Montana molecular	Cat# X0200G
Oligonucleotides		
<i>LTB4R</i> : Hs00609525_m1	Thermo Fisher Scientific	Cat# 4331182
<i>FPR2</i> : Hs00971276_mH	Thermo Fisher Scientific	Cat# 4351372
<i>GPR32</i> : Hs00265986_s1	Thermo Fisher Scientific	Cat# 4331182
<i>CMKLR1</i> : Hs01386063_m1	Thermo Fisher Scientific	Cat# 4351372
<i>B2M</i> : Hs99999907_m1	Thermo Fisher Scientific	Cat# 4326319
LTB4R siRNA: Hs.567248	Thermo Fisher Scientific	Cat# 146855
GPR32 siRNA: Hs.515555	Thermo Fisher Scientific	Cat# 144721
CMKLR1 siRNA: Hs.197143	Thermo Fisher Scientific	Cat# 146307
Silencer™ Negative Control No. 1 siRNA	Invitrogen	Cat# AM4611
Silencer™ siRNA Labeling Kit with FAM dye	Invitrogen	Cat# 1634
Software		
Image-Pro Plus	Media Cybernetics	N/A
ZEN software	ZEISS Microscopy	N/A
ZEN lite	ZEISS Microscopy	N/A
Leica Application Suite X (LAS X)	Leica Microsystem	N/A
NIS-Elements AR	Nikon	N/A
Magellan V7.2	Tecan	N/A
NanoDrop 2000 V1.6	Thermo Fisher Scientific	N/A
Rotor-Gene Q Series Software 2.1.0	QIAGEN	N/A
ORO software 1.3	Ugo Basile	Cat# 31300
GraphPad Prism 9.5.1	GraphPad Software	N/A

2.1. Validation of antibody specificity using human umbilical vein endothelial cell (HUVEC) culture

In the literature, expression of resolvins receptors (ALX/FPR2, GPR32, ChemR23, and BLT1) in human tissues was predominantly identified using qPCR (Hashimoto et al., 2007, Huang et al., 2017), and there were only a few studies that used immunohistochemistry to evaluate their expression in human tissues. ALX/FPR2 and ChemR23 are the only two receptors that have been identified in human tissues (human minor salivary glands and gingival tissues respectively) using immunohistochemistry (Nelson et al., 2014, Ozcan et al., 2018), but the uses of antibodies or controls were not well described in these studies. Therefore, antibodies that are suitable for use in pain-related tissues from human need to be selected. The specificity of these antibodies would also need to be validated to demonstrate that they can selectively bind to their respective resolvins receptors. The antibody against GPR32 was selected as it was used in a small pilot study in our laboratory to evaluate its expression in human lingual nerve neuromas, in which clear positive staining was observed (Tavares Ferreira, 2017). The antibody against ALX/FPR2 was selected as the quality of ALX/FPR2 labelling using this antibody in human minor salivary glands is good (Nelson et al., 2014). Since there are limited studies for the use of BLT1 and ChemR23 antibodies in immunohistochemistry, the antibodies against BLT1 and ChemR23 were selected based on the good quality of labelling in images provided by their manufacturers.

In order to validate the specificity of these antibodies raised against the resolvins receptors used in the immunohistochemistry assays, they were tested using human umbilical vein endothelial cells (HUVECs), which have been previously shown to express the resolvins receptors, GPR32 (Krishnamoorthy et al., 2010b), ALX/FPR2 (Baker et al., 2009), BLT1 (Qiu et al., 2006), and ChemR23 (Hao et al., 2015) in the literature using qPCR and western blot.

2.1.1. Cell culture

A vial containing approximately 1 million HUVECs at passage 4 was kindly provided by Dr Simon Whawell. The cells were thawed and cultured in a T-75 flask, and grown in Medium 199 (M199) supplemented with 20% FBS, 1% 0.2 M L-Glutamine, 1.5% 1 M HEPES, and 1% Penicillin-Streptomycin. The flask was placed in a 37°C, 5% CO₂ humidified incubator, and the medium was replaced with fresh medium every other day. Upon reaching approximately 80% confluency, the cells were dissociated with Trypsin EDTA, and subcultured in new T-75 flasks at a passage split ratio of 1:2.

2.1.2. qPCR

Total RNA was extracted from confluent HUVECs grown in T-75 flasks. At approximately 80% confluency, cells were collected by scraping the surface of a flask with a scraper after adding RNA lysis buffer.

RNA was extracted using the Monarch Total RNA Miniprep Kit following the manufacturer's protocol for cultured mammalian cells. Briefly, the sample mixture was passed through the gDNA removal column and then the RNA purification column. Residual gDNA was then removed enzymatically through DNase I treatment. Subsequently, the column matrix was purified using RNA priming buffer and RNA wash buffer. Finally, RNA was eluted into 50 µl nuclease-free water. The amount and purity of the eluted RNA were then assessed by spectrometry at 260 and 280 nm using a NanoDrop Spectrophotometer.

RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription (RT) Kit. 300 ng of total RNA was used per 20 µl reaction, and the RT reaction mix was prepared as described in Table 2.1. The reactions were incubated in a thermocycler for 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and then held at 4°C. No template control (RNA template being replaced with RNAase-free water) and reaction mixture substituting MultiScribe reverse transcriptase with RNAase-free water were also run as negative controls. cDNA samples synthesised were then stored at -20°C until ready for use in qPCR assays.

Table 2.1. Reaction mix for RNA reverse transcription (RT)

RT reaction mix components	Volume per 20 µl reaction (µL)
10X RT buffer	2
25X dNTP mix (100 mM)	0.8
10X RT random primers	2
MultiScribe reverse transcriptase	1
RNA	10
Nuclease-free water	4.2
<i>Total</i>	<i>20</i>

qPCR assays were carried out in triplicates for each gene-specific TaqMan probe, following the Rotor-Gene Probe two-step RT-PCR protocol. Reactions were carried out in a Rotor-Gene Q under standard cycling conditions. The following TaqMan probes were used: FAM-conjugated probes for *LTB4R* (BLT1; Hs00609525_m1), *FPR2* (Hs00971276_mH), *GPR32* (Hs00265986_s1), and *CMKLR1* (ChemR23; Hs01386063_m1). These probes were selected as they span the exon-intron boundary so all splice variants could be detected. Gene expression levels of resolvin receptors were normalised to the VIC-conjugated β -2 microglobulin (*B2M*; Hs99999907_m1) as an endogenous control. In the subsequent analysis using the Rotor-Gene Q Series Software, the threshold was set to 0.04, which was set at the position where the exponential amplification across all amplification plots is in parallel.

2.1.3. Immunocytochemistry

HUVECs were seeded on 16 mm round glass coverslips placed in 12-well plates. A preliminary experiment was first conducted to compare cell adhesion in coated glass coverslips and plain uncoated coverslips. The coated coverslips were prepared by incubating them with 0.01% poly-L-Ornithine for 30 minutes at room temperature in the well plate. They were subsequently rinsed with phosphate-buffered saline (PBS) prior to being seeded with HUVECs. Since no difference was found between coated and uncoated coverslips, HUVECs were seeded on plain uncoated glass coverslips in the experiments thereafter. The cells were cultured in supplemented M199 until they reached approximately 50% confluency. After removing the medium, the cells were first washed with PBS twice, and then fixed with 100% ice-cold methanol for 15 minutes at -20°C. After aspirating the methanol, the cells were washed thrice with PBS for 5 minutes at room temperature, and were stored at 4°C until ready for use within two weeks.

The cells were first incubated with 20% normal donkey serum (NDS) in PBS containing 0.5% Triton X-100 (PBST) for 1 hour at room temperature. They were next incubated with primary antibodies (diluted in 5% NDS in PBST) (Table 2.2) overnight at 4°C. Based on recommendations on the data sheets provided by the manufacturers, various dilutions of primary antibodies were first evaluated to determine the optimal dilutions of these antibodies (detailed in Table 2.2). On the next day, the cells were first washed thrice in PBS for 5 minutes, and then incubated with the corresponding secondary antibodies (diluted in 1.5% NDS in PBST) (Table 2.2) in the absence of light for 90 minutes at room temperature. The cells were washed thrice in PBS for 5 minutes in the absence of light. Finally, the coverslips were mounted on slides using a Vectashield medium with DAPI to preserve fluorescence and stain cell nuclei. In order to keep the coverslips in place on the microscope slides, they were sealed using nail varnish after being mounted. The slides were stored covered in a slide box at 4°C until ready to be examined under the microscope.

Table 2.2. Details of antibodies used in immunocytochemistry (ICC) of HUVECs and immunohistochemistry (IHC) of human tooth pulp tissues

Primary antibody	Antibody type	Dilutions tested	Optimal dilution	Immunogen	Secondary antibody	Dilution
Rabbit anti-human BLT1	Polyclonal	<u>ICC/ IHC:</u> 1:200, 1:400	<u>ICC/ IHC:</u> 1:200	Synthetic peptide mapping to 168-181 amino acids of human BLT1 receptor (2 nd extracellular loop)	Donkey anti-rabbit Cy3	1:500
Rabbit anti-human GPR32	Polyclonal	<u>ICC:</u> 1:50, 1:100	<u>ICC:</u> 1:50	Synthetic peptide mapping to 281-300 amino acids of human GPR32 receptor	Donkey anti-rabbit Cy3	1:500
		<u>IHC:</u> 1:100	<u>IHC:</u> 1:100			
Rabbit anti-human FPR2/ALX	Polyclonal	<u>ICC:</u> 1:50, 1:100, 1:200	N/A	Synthetic peptide mapping to 184-196 amino acids of human FPR2/ALX receptor (2 nd extracellular loop)	Donkey anti-rabbit Cy3	1:500
Rabbit anti-human ChemR23	Polyclonal	<u>ICC/ IHC:</u> 1:200, 1:400	<u>ICC/ IHC:</u> 1:200	Synthetic peptide to C-terminal cytoplasmic domain of human ChemR23 receptor	Donkey anti-rabbit Cy3	1:500
Mouse anti-tubulin β 3	Monoclonal	1:100	1:100	Raised against microtubules derived from rat brain	Donkey anti-mouse FITC	1:300
Monoclonal Anti-Actin, α -Smooth Muscle - FITC antibody produced in mouse (α -SMA)	Monoclonal	1:500	1:500	N-terminal synthetic decapeptide of α -smooth muscle actin	- (Direct immunofluorescence)	
Fluorescein labelled UEA I	- (Lectin)	1:50, 1:100	1:50	Lectin specific for glycocompounds containing α -linked fucose residues	- (Direct immunofluorescence)	

2.1.4. Western blot

Total protein was extracted from confluent HUVECs grown in T-75 flasks. At approximately 80% confluency, HUVECs were trypsinised and resuspended in PBS. Proteins were then isolated from HUVECs by lysing in ice-cold RIPA buffer containing protease inhibitors for 30 minutes on ice, and then centrifuged for 10 minutes at 4°C. The supernatant was then collected and stored at -20°C, with its protein concentration being determined with BCA protein assay using the Magellan software. Protein samples were then denatured with SDS lysis buffer and incubated at 95°C for 5 minutes. The protein samples (30 µg per well) were loaded on 12% SDS-polyacrylamide gel along with the protein ladder. Samples were then separated based on their molecular weight by electrophoresis at 120V for the first 15 minutes, and 150V for 1 hour. The proteins were then electroblotted onto a PVDF membrane (Trans-Blot Turbo Midi PVDF Transfer Pack, Bio-Rad). Protein transfer was confirmed with Ponceau staining.

After rinsing with distilled water, membranes were blocked with 5% milk in tris-buffered saline (TBS) for 1 hour at room temperature with agitation. Membranes were then rinsed with TBS and incubated overnight at 4°C with the primary antibodies (Table 2.3, further details about the antibodies are provided in Table 2.2) with agitation. A range of dilutions was first tested to determine the optimal dilution for the primary antibodies (detailed in Table 2.3). Negative control was conducted by completing the full process of the assay but omitting the primary antibody. After three washes with TBS-Tween (TBST), membranes were incubated with the HRP-linked secondary antibody (Table 2.3) for 30 minutes at room temperature with agitation. After another three TBST washes for 5 minutes each, the membranes were covered with enhanced chemiluminescent (ECL) reagent for 5 minutes so immunoreactive bands could be visualised. This was followed by overnight x-ray film exposure and film development in the dark room.

Table 2.3. Details of antibodies used in western blot assays

Primary antibody	Dilutions tested	Optimal dilution	Secondary antibody	Dilution
Rabbit anti-human BLT1	1:100 1:200 1:500	1:100	Anti-rabbit IgG, HRP-linked antibody	1:3000
Rabbit anti-human GPR32	1:200 1:500	N/A		
Rabbit anti-human ChemR23	1:200 1:500	N/A		

*Further details about the primary antibodies are provided in Table 2.2. These are the same antibodies used in immunocytochemistry.

2.1.5. siRNA transfection

In order to further establish the specificity of the antibodies, the expression of BLT1, GRP32, and ChemR23 in HUVECs was knocked down respectively in HUVECs via small interfering siRNA (siRNA) transfection. Immunocytochemistry and western blot assays were then repeated to test if there is a difference in the signal produced.

For further immunocytochemistry assays, HUVECs were seeded on 25 mm round glass coverslips placed in 6-well plates, whereas HUVECs were seeded in 6-well plates for further western blot assays. The cells were grown in M199 supplemented with 20% FBS, 1% 0.2 M L-Glutamine, and 1.5% 1 M HEPES without antibiotics, and were at ~60% confluence at the time of transfection. 50 μ M stock siRNA (BLT1 siRNA, GPR32 siRNA, ChemR23 siRNA, and negative control siRNA) was prepared by adding 100 μ l nuclease-free water to the 5 nmol siRNA. 50 nM diluted siRNA mixture was then prepared by adding 0.5 μ l stock siRNA to 500 μ l Opti-MEM. As mock transfection control, nuclease-free water was used in place of siRNA during the preparation of the diluted siRNA mixture. The oligofectamine transfection mixture was prepared by combining the diluted oligofectamine mixture (10 μ l oligofectamine per 40 μ l Opti-MEM) with the diluted siRNA mixture. After 30 minutes of incubation, 300 μ l Opti-MEM was added to the transfection mixture. HUVECs were then washed twice with Opti-MEM prior to the addition of 300 μ l Opti-MEM per well. 200 μ l transfection mixture was subsequently added dropwise to the side of the well. After the initial 4 hours of incubation at 37°C in the 5% CO₂ humidified incubator, M199 supplemented with 40% FBS, 1% 0.2 M L-Glutamine, and 1.5% 1 M HEPES was added. The cells were then ready to be used experimentally after a further 48 hours in the 37°C, 5% CO₂ humidified incubator, following the protocols described above to prepare for further immunocytochemistry and western blot experiments.

2.2. Human tissues

Human tooth pulp tissues were obtained from the archive of human tissues from Charles Clifford Dental Hospital. Permanent molar teeth were collected from children who required dental extractions under general anaesthesia at Sheffield Children's Hospital (n = 35; 12 boys and 23 girls; mean age = 9.86 ± 0.85 years). The study received ethical approval by East of England – Cambridge Central Research Ethics Committee (17/EE/0238) STH19847 (The use of lingual nerve and tooth pulp tissue to identify molecules linked with chronic oro-facial pain). Informed consent was obtained from accompanying guardians for the use of extracted teeth for research purposes. Tooth extraction was required due to gross caries, and compensating extractions were needed in some cases, providing non-carious teeth from some patients. Prior to tooth extraction, pain visual analogue scale (VAS) score assessment was conducted by the clinician to obtain the pain history. Therefore, teeth were divided into three groups according to caries and pain status: non-carious (n = 11), carious painful (n = 9), and carious non-painful (n = 15).

After tooth extraction, teeth were sectioned longitudinally and the two halves were immersed in Zamboni's fixative (4% paraformaldehyde, 0.2% picric acid in 0.1M phosphate buffer, pH 7.4) for 24 hours at 4°C. Each tooth was given a sample number so subsequent analysis could be conducted blind. Following fixation, the teeth were rinsed with numerous washes of 0.2M PBS to remove the residual yellow colour from the fixative. The tooth pulp tissue was then carefully extracted from the pulp chamber using a dental excavator and placed in PBS for another 24 hours at 4°C.

2.2.1. Human tooth pulp tissue processing

The tooth pulp samples collected were cryoprotected in 30% sucrose for 5 hours at 4°C. They were then embedded in OCT compound on a cryostat in fast-freezing mode. Serial 10 µm longitudinal tooth pulp sections were cut on a cryostat and collected onto poly-D-lysine (PDL) coated microscope slides in 20 slides with 3 sections per slide, as shown in Figure 2.1. 60 sections were collected from each tooth pulp sample and each section was approximately 200 µm apart from each other. Slides were left to air dry at room temperature for 1 hour and were then stored at -80°C until ready for use.

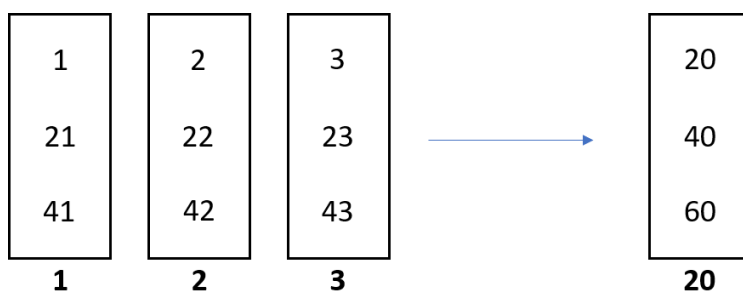


Figure 2.1. Representation of the serial collection of human tooth pulp sections. 60 sections were collected from each tooth pulp sample in 20 slides, with three sections per slide.

2.2.2. Immunohistochemistry of human tooth pulp tissues

Indirect immunofluorescence assays were used to determine and compare the expression of resolvin receptors, BLT1, ChemR23, and GPR32 in human tooth pulp tissues. Based on recommendations on the data sheets provided by the manufacturers, different dilutions of antibodies against resolvin receptors were first evaluated to determine the optimal dilution for each antibody (detailed in Table 2.2).

Sections were removed from -80°C storage and left to air dry at room temperature for 1 hour before staining. They were then washed twice in PBST for 10 minutes on a rocker. The sections were subsequently incubated with 20% NDS in PBST for 1 hour at room temperature to reduce non-specific binding of the antibodies. They were next incubated with primary antibodies (diluted in 5% NDS in PBST) (Table 2.2) overnight at 4°C. On the next day, the sections were first washed twice in PBS for 10 minutes, and then incubated with the corresponding secondary antibodies (diluted in 1.5% NDS in PBST) (Table 2.2) in the absence of light for 90 minutes at room temperature. The sections were washed twice in PBS for 10 minutes in the absence of light. In dual-labelling studies, the incubation steps were repeated with markers of specific cell types and their respective secondary antibodies (Table 2.2). Finally, they were mounted in Vectashield medium with DAPI to preserve fluorescence and stain cell nuclei and coverslipped, and stored covered in a slide box at 4°C until ready to be examined under the microscope.

2.2.2.1. Specificity control

To confirm that the immunostaining was specific, where possible, primary antibodies were pre-absorbed with their corresponding blocking peptides. The antibodies were first prepared in the desired dilution (Table 2.2) in 5% NDS in PBST, and were then mixed with their corresponding blocking peptides (1 µg peptide per 1 µg antibody). The solution was then left at 4°C for approximately 18 hours prior to performing the immunofluorescence staining assay. However, only the BLT1 antibody has a corresponding blocking peptide, so

the negative controls for other primary antibodies were conducted by completing the full process of the assay but omitting the primary antibodies.

UEA-I is a type of lectin that binds to α -fucose and thus its specificity was tested by pre-absorption with L-fucose. UEA-I was first prepared in its desired dilution (Table 2.2) and was then mixed with 0.2 M L-fucose dissolved in PBST. The solution was incubated at room temperature for an hour prior to performing the immunofluorescence staining assay.

2.3. Primary trigeminal ganglia culture and transduction

Wistar rats (6 – 10 days old) of both sexes were anaesthetised by isoflurane inhalation. After the loss of the pedal withdrawal reflex, the animals were sacrificed via Schedule 1 procedures (cervical dislocation followed by decapitation) in accordance with the UK Animals (Scientific Procedures) Act 1986. The left and right trigeminal ganglia (TG) embedded at the base of the skull were exposed after removing the brain. After cutting the maxillary branch, mandibular branch, and trigeminal root, both TGs were excised and placed into ice-cold modified Hank's Balanced Salt Solution (HBSS).

Following tissues extraction, TGs were cut into small pieces and transferred to a dissociation solution comprised of pre-warmed (37°C) modified HBSS, dispase II (10 mg/ml), and collagenase Type 1A (1 mg/ml), and had been sterile-filtered through a 0.22 µm filter. The TG-containing dissociation solution was then placed in a 37°C, 5% CO₂ humidified incubator for 30 minutes. To facilitate the cell dissociation process, the solution was gently triturated approximately 10 times with a P1000 pipette until the solution turned cloudy. The solution was placed in the incubator for a further 5 minutes. The TG-containing solution was transferred to ice-cold Dulbecco's Modified Eagle's Growth Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 50 µg/ml penicillin-streptomycin to terminate the dissociation process. The solution was then centrifuged at 800 rpm for 5 minutes at 4°C. The pellet was resuspended in ice-cold supplemented DMEM and centrifuged at 900 rpm for 5 minutes at 4°C. The pellet was then resuspended in 500 µl pre-warmed supplemented DMEM.

The TG cell mixtures were then plated onto poly-D-lysine and laminin-coated 10mm round coverslips in a 12-well cell culture plate, at a volume of 100 µl per coverslip. The cell culture plate was placed in the incubator for 4 hours. After 4 hours, the wells were flooded with pre-warmed supplemented DMEM (1 ml per well), and cells were cultured in the incubator for a further 2-3 days. All experiments involving primary TG cultures were carried out 2-3 days after preparation.

For cAMP imaging experiments, TG cells were transduced with a cyclic AMP Difference Detector *in situ* (cADDiS) cAMP sensor in a BacMam vector (green downward sensor), which is a biosensor that changes in fluorescence intensity in response to changes in cAMP level. The same cell dissociation process described above was followed. Following the second centrifugation, the pellet was resuspended in 400 µl pre-warmed supplemented DMEM, and mixed with a viral transduction mixture containing cADDiS cAMP sensor, sodium butyrate, hD2 receptor BacMam, and supplemented with DMEM (according to the instructions of the manufacturer). The TG cell mixtures were then plated onto pre-coated coverslips in a 12-well cell culture plate, which was covered with foil and placed into the

incubator for 4 hours. After 4 hours of incubation, the wells were flooded with pre-warmed supplemented DMEM. The cells were then cultured in the incubator, protected from light, for a further 2 days.

2.3.1. Immunocytochemistry of rat TG cells

The media was removed 48 hours after the preparation of primary TG cultures. The cells were washed twice with PBS and fixed by adding 4% PFA into the wells. The well plate was then placed on ice for 20 minutes. After aspirating the PFA, the cells were washed thrice for 5 minutes on the rocking table at room temperature.

The cells were subsequently incubated with a blocking buffer containing 0.05% Tween20, 0.25% Triton X, and 5% donkey serum in PBS for 45 minutes on the rocking table for 1 hour at room temperature. They were next incubated with primary antibodies (diluted in 50 mg/ml bovine serum albumin (BSA) in PBS) (Table 2.4) overnight at 4°C on the rocking table in a cold room. On the following day, the cells were first washed thrice with PBS for 5 minutes on the rocking table at room temperature. The cells were then incubated with their respective fluorochrome-conjugated secondary antibodies (diluted in 10 mg/ml BSA in PBS) (Table 2.4) for 2 hours in the absence of light on the rocking table at room temperature. Subsequently, the cells were washed thrice with PBS for 5 minutes in the absence of light on the rocking table at room temperature. Finally, the coverslips were mounted on slides using fluoroshield mounting medium with DAPI, and sealed with nail varnish. The slides were stored covered in a slide box at 4°C until ready to be visualised under the microscope.

Table 2.4. Details of antibodies used in immunocytochemistry of primary TG culture

Primary antibody	Antibody type	Dilution	Immunogen	Secondary antibody	Dilution
Rabbit anti-LTB4-R1 (BLT1)	Polyclonal	1:100	KLH conjugated synthetic peptide derived from human LTB4-R1	Donkey anti-rabbit, Alexa Fluor 488	1:1000
Mouse anti- β -Tubulin III	Monoclonal	1:1000	Synthetic peptide corresponding to a fragment of neuronal specific β III tubulin	Donkey anti-mouse, Alexa Fluor 555	1:1000
Mouse anti-Neurofilament 160/200 (NF160/200)	Monoclonal	1:500	Purified mid-size rat neurofilament (NF-M) subunit	Donkey anti-mouse, Alexa Fluor 555	1:1000
Mouse anti-Glutamine Synthetase	Monoclonal	1:500	Recombinant fragment corresponding to Human Glutamine Synthetase aa 274-373	Donkey anti-mouse, Alexa Fluor 555	1:1000

2.3.2. Calcium imaging

Prior to calcium imaging, each coverslip was transferred to a small dish containing 2 μ M Fluo-4 AM (green fluorescent Ca^{2+} indicator dye) and 0.01% pluronic F-127 in standard extracellular solution containing (in mM): 160 NaCl, 2.5 KCl, 1 MgCl_2 , 10 HEPES, 2 CaCl_2 , and 10 Glucose (adjusted to pH 7.4 with HCl or NaOH, 310-320 mOsm). It was then placed in the incubator for 45 minutes, protected from light. The coverslip with Fluo-4 loaded TG cells was then mounted in the camera chamber of a Nikon TE-2000E inverted microscope fitted with a LiveScan Swept-field confocal system for imaging. During imaging, the cells were perfused continuously with extracellular solution or drugs at room temperature through a gravity-driven perfusion system. The cells were excited at 488 nm using an argon laser, and images were acquired at 2-second intervals using an electron multiplying CCD camera (DQC-FS) through a 40x objective. Time-lapse sequences were recorded using the NIS-Elements AR software.

The timeline of calcium imaging experiments is shown in Figure 2.2. In order to establish the baseline, the cells were perfused with the standard extracellular solution for 1 minute in the beginning. Neuronal cells were identified by switching (for 3 minutes) to perfusion of 'high K^+ ' extracellular solution, in which 47.5 mM NaCl was removed from the standard extracellular solution and replaced with KCl. After a recovery period of 5 minutes in which the cells were perfused with extracellular solution, 10 nM of RvE1, RvE2, or selective BLT1 agonist 20-OH-LTB₄ (diluted in extracellular solution) was applied to assess their effects on calcium signalling, as depicted in Figure 2.2A. To determine if their effects were mediated by BLT1, the cells were first pre-incubated with BLT1 antagonist U75302 during the Fluo-4 loading process. The cells were then perfused with 1 μ M U75302 for 1 minute prior to the application of RvE1, RvE2, or 20-OH-LTB₄ in the presence of U75302, followed by a 5-minute wash with extracellular solution, and finally perfusion with high K^+ extracellular solution for 3 minutes, as described in Figure 2.2B. In order to eliminate the effects of extracellular Ca^{2+} , the cells were perfused with Ca^{2+} -free extracellular solution for 2 minutes after the initial 1-minute perfusion with extracellular solution. The Ca^{2+} -free extracellular solution was made by removing CaCl_2 from the extracellular solution and adding 1 mM EGTA and increased NaCl (165 mM). RvE1, RvE2, or 20-OH-LTB₄ diluted with Ca^{2+} -free extracellular solution were then applied. After the 5-minute recovery period when the cells were perfused with extracellular solution, the cells were incubated with high K^+ extracellular solution for 3 minutes to identify neuronal cells, as depicted in Figure 2.2C.

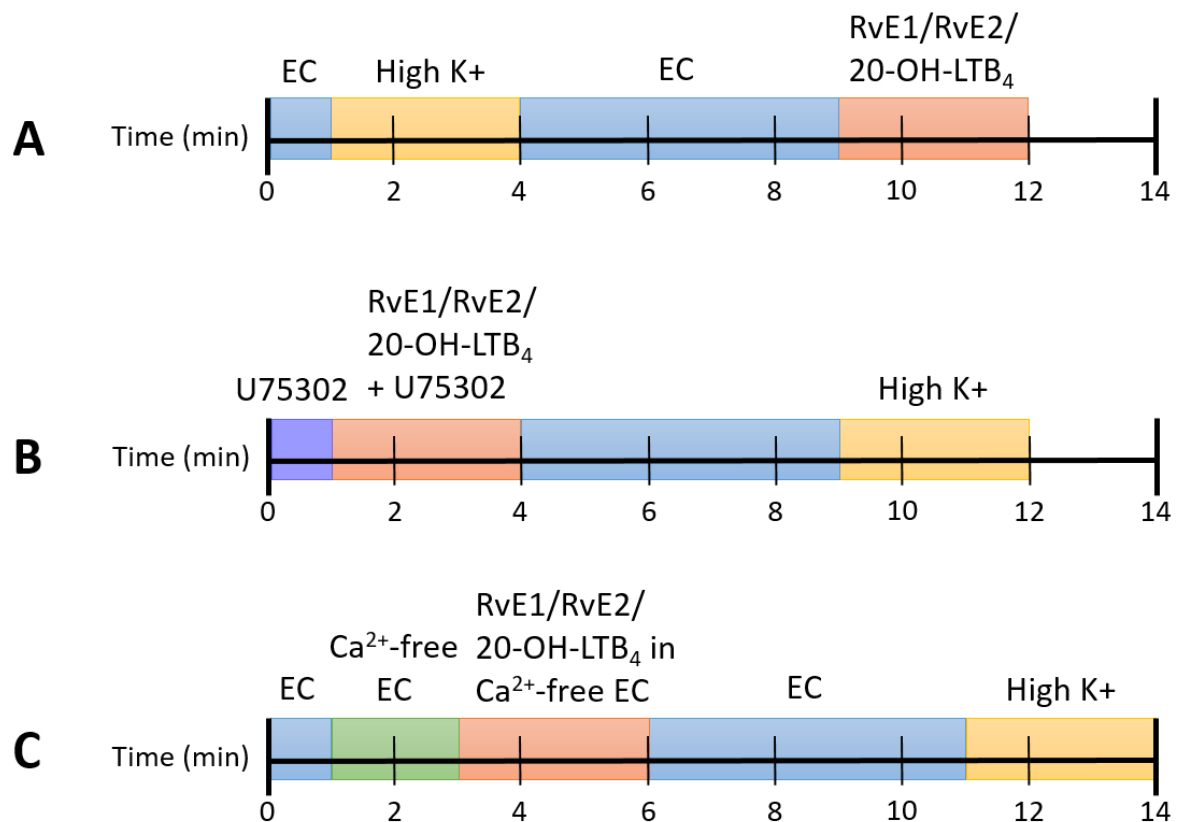


Figure 2.2. Timeline of calcium imaging experiments

The cultured TG cells were perfused with extracellular solution (EC) for a minute to establish a baseline, before switching to various conditions to:

- (A)** Establish the effects of RvE1 (10 nM), RvE2 (10 nM), or 20-OH-LTB₄ (10 nM) on calcium signalling.
- (B)** Establish if the responses are mediated by BLT1. The cells were perfused with BLT1 antagonist U75302 (1 μ M) prior to application of RvE1, RvE2, or 20-OH-LTB₄ in the presence of U75302.
- (C)** Eliminate the influence of extracellular Ca²⁺ by testing the drugs in calcium-free condition following incubation in Ca²⁺-free EC.

2.3.3. cAMP imaging (cADDIs)

As described above in section 2.3, the TG cells were transduced with a cADDIs cAMP sensor during preparation. The cADDIs is a genetically encoded fluorescent protein that changes in fluorescence intensity in response to changes in cAMP level. A downward fluorescent sensor was used, and thus a decrease in cADDIs fluorescence corresponds to an increase in cytosolic cAMP level, while an increase in fluorescence corresponds to a reduction in the level of cytosolic cAMP. This allows changes in cAMP level in the TG cells to be observed in real-time. Prior to cAMP imaging, each coverslip was transferred to a small dish containing extracellular solution. The dish was covered with foil and allowed to rest at room temperature for ~25 minutes so the cells could adjust to the new environment. The

coverslip was then mounted in the camera chamber, and imaging was conducted in the same manner as mentioned above in section 2.3.2.

The timeline of cAMP imaging experiments is shown in Figure 2.3. The transduced cells were perfused with extracellular solution for 1 minute to establish a baseline. The adenylyl cyclase activator forskolin (50 μ M diluted in extracellular solution) was then applied, which raised the basal level of cAMP in cells. This allows the potential activation of Gi/o-coupled signalling cascade following application of RvE1, RvE2, or 20-OH-LTB₄ to be observed. The reliability of cAMP level reporting by cADDis could also be tested. RvE1, RvE2, or 20-OH-LTB₄ (10 nM diluted in extracellular solution) were then applied to investigate their effects on the level of cAMP by measuring changes in fluorescence intensity. Extracellular solution was also applied following forskolin application to check if the cAMP level reduced during the recovery period. As a positive control, quinpirole was used to stimulate Gi signalling via the hD2 receptor that the TG cells were also transduced with (described above in section 2.3).

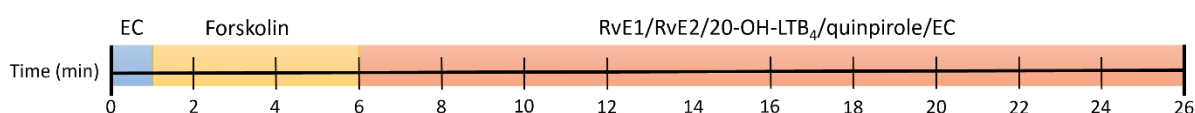


Figure 2.3. Timeline of cAMP imaging experiments.

The cultured TG cells transduced with a cADDis cAMP sensor were perfused in extracellular solution (EC) for 1 minute, followed by application of 50 μ M forskolin for 5 minutes, and then application of RvE1 (10 nM), RvE2 (10 nM), 20-OH-LTB₄ (10 nM), quinpirole (10 μ M) or EC for 20 minutes.

2.4. Animal experiments

A total of 22 adult male Sprague-Dawley rats (250-275 g) were used in the behavioural studies reported in this thesis. They were acquired from Charles River UK and were allowed to acclimatise for at least a week after they arrived at the Biological Services Unit of the University of Sheffield. The rats were housed in groups of 2 or 3 per cage with environmental enrichment in the same room, with the temperature (19-23°C) and relative humidity (40-70%) controlled. They were kept on a 12-hour light/dark cycle with *ad libitum* access to food and water. All efforts were made to minimise animal suffering and to reduce the number of animals used in the study. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. The animal model of lingual nerve injury used was adapted from the previously established model in our laboratory (Tavares-Ferreira et al., 2019).

2.4.1. Surgical procedure

Prior to the surgery, all surgical instruments were sterilised in an autoclave (Prestige Medical Classic Autoclave) to prevent the occurrence of infection and contamination. The surgical bench and the surrounding area were also disinfected with 70% industrial methylated spirit (IMS). Anaesthesia was first induced with 4% isoflurane (IsoFlo 100% w/w) and 2% O₂ via inhalation in the chamber of an anaesthetic machine. The animals were then transferred to a heating pad covered with a surgical drape to maintain their body temperature at 37±2°C during the surgery. The area of incision (beneath the left side of the mandible) was then shaved. Animals were maintained under general anaesthesia for the duration of the surgical procedure with 2-2.5% isoflurane and 0.5% O₂ via inhalation. After a surgical plane of anaesthesia was reached (i.e. absence of pedal withdrawal reflex), an incision was made below the left side of the mandible. The heartbeat of the animal was also monitored throughout the surgery. Under an operating microscope, the left lingual nerve was exposed by blunt dissecting through the muscle layer and connective tissues. The animals were assigned to CCI silk (n=8), CCI catgut (n=8), or sham group (n=6) randomly. For the rats in the CCI groups, two 6/0 black silk or chromic catgut sutures were tied loosely around the lingual nerve 2 mm apart (Figure 2.4). For the rats in the sham group, no sutures were tied around the lingual nerve after it was exposed. The subcutaneous tissue and overlying skin were then closed with 4/0 vicryl sutures. The animals were then left to recover for 28 days after the surgery.

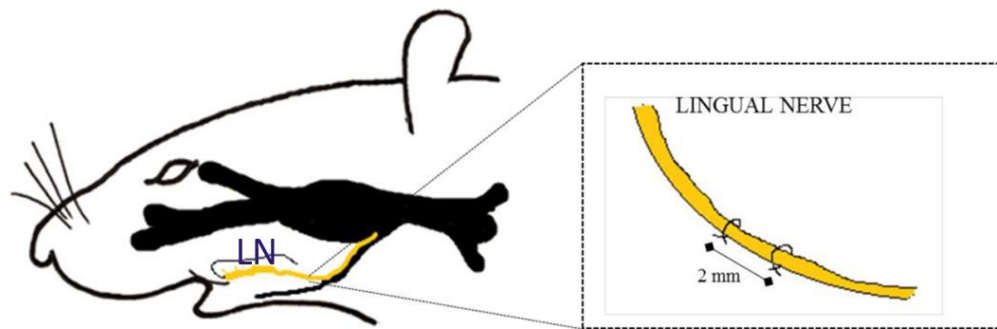


Figure 2.4. Chronic constriction injury of the rat lingual nerve.

In the group of animals that received chronic constriction injury, two sutures were tied loosely around the left lingual nerve (labelled with LN on the diagram) 2 mm apart. The schematic drawing of the rat was modified from Csati et al., 2015 under the Creative Commons CC BY License, not to scale.

2.4.2. Behavioural testing

Animals with a CCI to the sciatic nerve show symptoms that closely mimic those observed in human neuropathic pain, including a reduction in withdrawal thresholds to non-noxious mechanical stimuli that can be interpreted as allodynia (Attal et al., 1990). Based on clinical observations in human, lingual nerve CCI may also induce pain hypersensitivity. Since the lingual nerve innervates the tongue, lingual nerve CCI may affect drinking behaviour.

Using the Orofacial Stimulation Test (Ugo Basile), the drinking behaviour of animals were assessed before and after the surgery to investigate the effect of lingual nerve injury. Briefly, the rats (n=22) inserted their snout voluntarily through an opening to access the positive reward (Sainsbury's chocolate milkshake) with their tongue from a drinking bottle, as shown in Figure 2.5. Drinking behaviour was recorded by a computer and quantified automatically by the orofacial (ORO) software. This software automatically recorded the number and duration of drinking attempts by measuring the interruption of an infrared barrier traversing the opening to the reward bottle, and thus investigator bias was avoided. Animals were first trained to use the equipment and develop an interest in the reward for five days prior to surgery. Behavioural testing then took place immediately before the surgery (day 0), and subsequently on days 1, 3, 5, 7, 10, 14, 16, 18, 21, 25, and 28 after the surgery, as described in Figure 2.6. Sessions consisted of 10 minutes of acclimatisation period in the testing cage, and then 10 minutes of recording period when animals have free access to the reward bottle, and had a choice between receiving the reward and avoiding tongue contact with the drinking bottle. Avoidance of drinking the reward, therefore, provides an indication of altered pain threshold to mechanical stimuli, linked with allodynia-like symptoms found in neuropathic pain. The training and testing sessions were conducted at approximately the same time every day between 8:00 – 11:00 am.

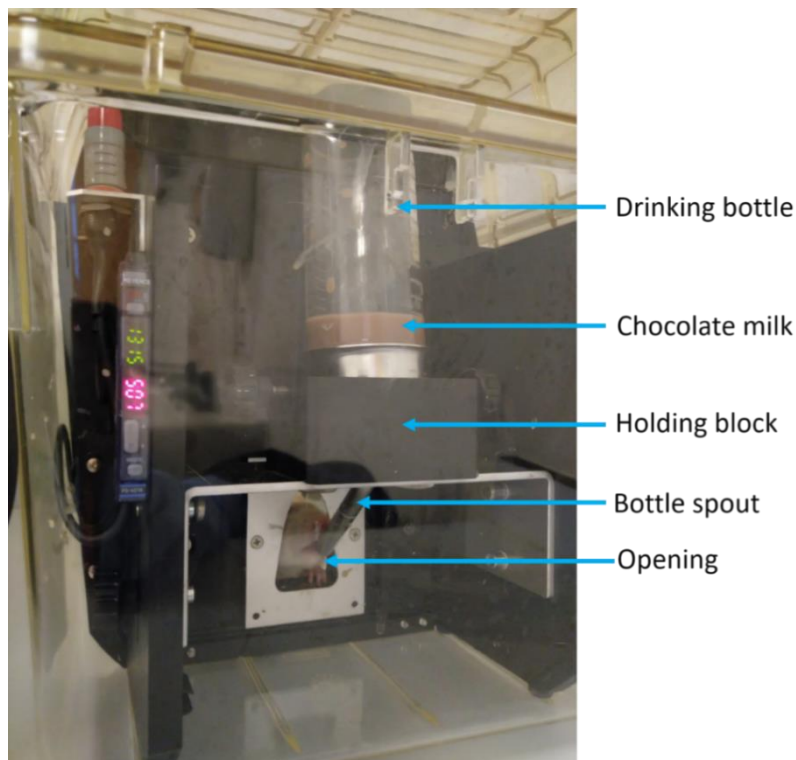


Figure 2.5. Ugo Basile Orofacial Stimulation Test equipment setup.

The drinking bottle with chocolate milk was positioned on the holding block so the bottle snout was centred with respect to the opening. The rat inserted its snout through the opening voluntarily to access the positive reward (chocolate milk).

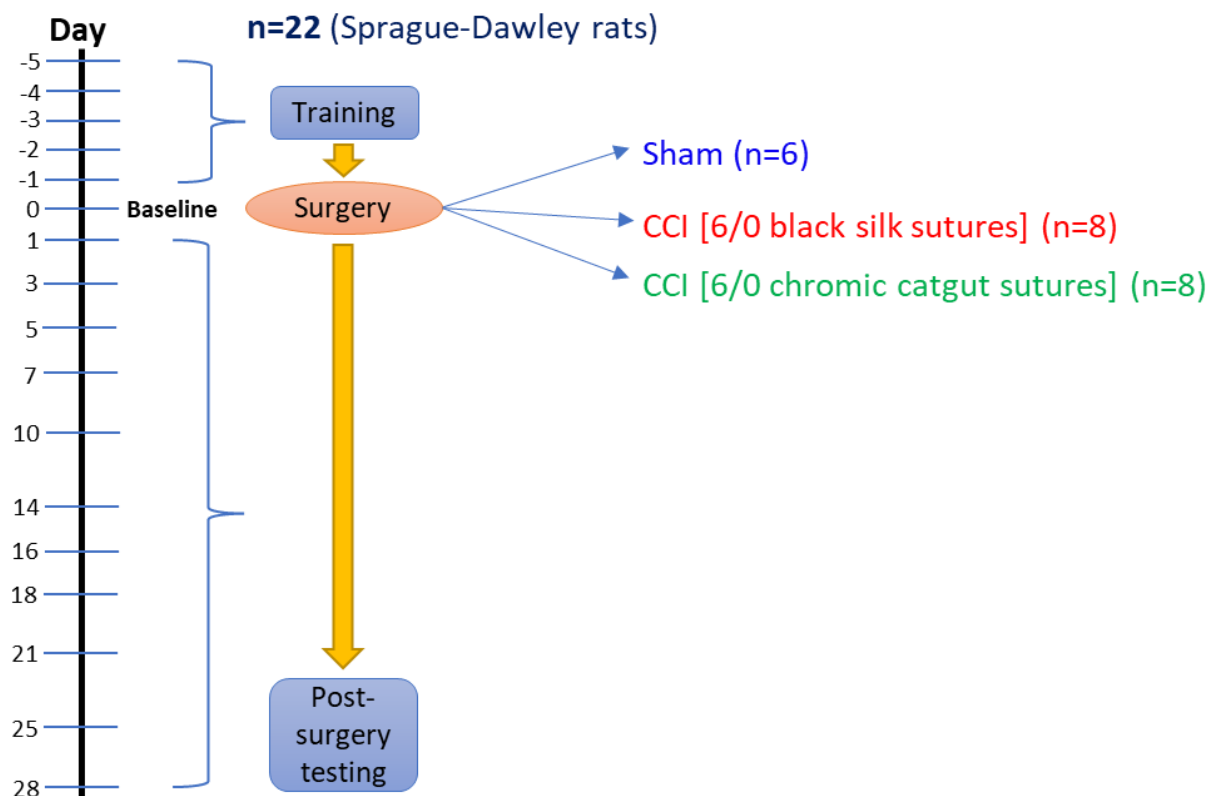


Figure 2.6. Time points of training and post-surgery behaviour testing.

Animals were trained to use the equipment for five days consecutively prior to surgery. Behavioural testing was then performed on the day of the surgery (day 0), and on days 1, 3, 5, 7, 10, 14, 16, 18, 21, 25, and 28 after the surgery.

2.4.3. Terminal procedures

At the end of the experiment, the animals were anaesthetised by isoflurane inhalation (4%), followed by an overdose of pentobarbitone (pentobarbitone sodium). After the loss of the pedal withdrawal reflex, the animal was transcardially perfused with 100 ml PBS followed by 150 – 200 ml 4% paraformaldehyde (PFA). After perfusion, the left and right lingual nerves, trigeminal ganglia, and brainstem were collected and placed in 4% PFA immediately.

2.4.4. Rat tissues processing

The tissues collected were placed in 4% PFA for 4 hours at room temperature, and then placed in 30% sucrose solution overnight at 4°C to cryoprotect the tissues. Before freezing the tissues, the meninges of trigeminal ganglion and brainstem were removed, and the right side of the brainstem was marked lightly to allow for identification of ipsilateral and contralateral sides. Tissues were then embedded in OCT compound on a cryostat in fast-freezing mode, and were stored at -80°C until ready for sectioning.

2.5. Image acquisition and analysis

2.5.1. Widefield fluorescence microscopy

Images of HUVECs and human tooth pulp sections were taken on a Zeiss Axioplan 2 imaging microscope (Carl Zeiss International) fitted with a QI Click colour CCD camera (Q-imaging). Lumen 200 fluorescent illumination system (Prior Scientific Inc) was used to illuminate the samples and filter the appropriate wavelength emission for DAPI (4',6-diamidino-2-phenylindole), FITC (Fluorescein isothiocyanate), and Cy3 (Cyanine 3). Digital images obtained were processed using Image-Pro Plus and quantified using Image J.

Images of human tooth pulp sections were also acquired using a Leica DMI8 inverted microscope, fitted with a Leica K5 sCMOS microscope camera system. The microscope was equipped with a LED 3 fluorescence light source which offers white light in the 390-680 range, and three filter cubes for epifluorescence illumination: FITC, Y3, and LED 405. Digital images obtained were processed using Leica Application Suite X (LAS X) software, where tile scan and z-stack images taken were merged and processed. The labelling was quantified using Image J subsequently to determine the area of positive staining. A suitable automated thresholding algorithm that applied to all images was selected during image analysis, so the data obtained would be consistent and replicable. Quantification was also repeated once to check that the measurements were consistent and reproducible.

2.5.2. Confocal microscopy

Images of rat TG cells were acquired using a Zeiss LSM880 upright confocal microscope with the ZEN software in the Bioimaging and Flow Cytometry Facility at the University of Leeds. The system has a blue diode 405 nm laser, an argon 488 nm laser, and a HeNe 543nm laser. The images were then processed using the ZEN lite software.

Confocal images of human tooth pulp tissues were acquired using a Zeiss LSM880 microscope with Airyscan in the Light Microscope Facility at the University of Sheffield. Images acquired were processed using Airyscan with the ZEN software. The system has a blue diode 405 nm laser, an argon 488 nm laser, and a HeNe 543nm laser. The images were then processed using the ZEN lite software.

2.5.3. Calcium imaging and cAMP imaging analysis

Regions of interest were placed around individual cells that responded and in the background to analyse time-lapse sequences of images taken during calcium imaging and fluorescence imaging. Background fluorescence intensity was subtracted from that measured at regions of interest at each time point to measure changes in fluorescence intensity (ΔF) over time. They were then normalised to the baseline fluorescence intensity recorded at $t=0$ ($\Delta F/F_0$) to account for differences in the starting fluorescence intensity of each individual cell.

2.6. Statistical analyses

2.6.1. Statistical analysis of quantified images

Sample size (n) refers to the number of samples in each group of human tooth pulp tissues. Group data were presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism. One-way analysis of variance (ANOVA) was used to determine if the average area of positive staining was significantly different between tissues from different groups (under the assumptions of normally distributed data and equal variances). Tukey's multiple comparisons test was then used to define the source of significance when there was a statistically significant difference between groups. If the data were not normally distributed (tested using Shapiro-Wilk test for normality), comparisons between tissues from different groups were made using Kruskal-Wallis ANOVA. If the variances across the groups were not equal (tested using Bartlett's test), Brown-Forsythe and Welch ANOVA was performed instead of ordinary one-way ANOVA. Both Kruskal-Wallis ANOVA and Brown-Forsythe and Welch ANOVA were followed by Dunn's multiple comparisons test to define the source of significance when there was a statistically significant difference between groups. Results with $p < 0.05$ were considered statistically significant.

2.6.2. Statistical analysis of calcium imaging data

Sample size (n) refers to the number of TG preparations, and one rat was used per preparation of primary TG cultures. Data were presented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism. Unpaired t-test was used to determine if there was a significant difference in the amplitudes of Ca^{2+} transients (under the assumptions of normally distributed data and equal variances). If the variance between the groups was not equal (tested using the F test), Welch's t-test was performed instead of unpaired t-test. One-way ANOVA was used to determine if there was a statistically significant difference in the amplitudes of Ca^{2+} transients induced by various test compounds (under the assumptions of normally distributed data and equal variances). Tukey's multiple comparisons test was then used to define the source of significance when there was a statistically significant difference between groups. Results with $p < 0.05$ were considered statistically significant.

2.6.3. Statistical analysis of behavioural testing

Sample size (n) refers to the number of animals in each experimental group (sham, CCI silk, CCI catgut). Group data were presented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism. Two-way ANOVA repeated measures was used to determine if there was a significant difference in total time spent drinking between experimental groups. Paired two-tailed t-test was used to determine if there was a significant difference in total time spent drinking within each group, comparing results obtained at each

timepoint of post-surgery behavioural testing with the baseline. Results with $p < 0.05$ were considered statistically significant.

CHAPTER 3: VALIDATION OF ANTIBODY SPECIFICITY

3.1. Introduction

As described in chapter 1 the resolution of inflammation is now considered to be an active process regulated by the biosynthesis of specialised pro-resolving mediators such as resolvins. Resolvins are derived from two omega-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) – giving rise to the D-series (RvD) and E-series (RvE) resolvins respectively (Serhan et al., 2008). RvE resolvins act on the receptors BLT1 and ChemR23, whereas RvD resolvins act on the receptors ALX/FPR2 and GPR32 (see Figure 1.8). The overarching aim of the work described in this thesis is to further investigate the mechanisms via which resolvins may influence pain, and this includes the use of immunohistochemistry to characterise resolvin receptor expression in human tooth pulp tissues linked with inflammatory pain.

Previous studies have used qPCR to identify the expression of resolvin receptors (ALX/FPR2, GPR32, ChemR23, and BLT1) in tissues related to pain from human (Hashimoto et al., 2007, Huang et al., 2017). In the literature, there are only a few studies that have used immunohistochemistry to label resolvin receptors in human tissues, with ALX/FPR2 (in human skin biopsies and minor salivary glands) and ChemR23 (in human gingival tissues) being the only two receptors identified with immunohistochemistry (Motwani et al., 2018, Nelson et al., 2014, Ozcan et al., 2018). However, these studies did not include details of the antibodies or describe the use of controls to demonstrate binding specificity of the antibodies used. Additional studies are therefore needed to validate the specificity of antibodies against resolvin receptors, prior to their use in immunohistochemistry of human tooth pulp tissues. The rationale behind antibody selection is detailed in the methods in Section 2.1. These antibodies have been shown to produce good quality of labelling based in previous pilot studies in the laboratory (Tavares Ferreira, 2017), data from the literature (Nelson et al., 2014), or images provided by the manufacturer of the antibodies.

In order to validate the specificity of the antibodies selected to be used in immunohistochemistry assays, the antibodies were tested using human umbilical vein endothelial cells, (HUVECs). These cells have been previously shown to express the resolvin receptors, GPR32 (Krishnamoorthy et al., 2010b), ALX/FPR2 (Baker et al., 2009), BLT1 (Qiu et al., 2006), and ChemR23 (Hao et al., 2015), using qPCR and western blot. Therefore, HUVECs were used to establish the specificity of the antibodies raised against human resolvin receptors, which would be used in subsequent immunohistochemistry assays on human tooth pulp tissues.

This chapter describes:

1. Assessment of resolvin receptor transcript expression in HUVEC cultures using qPCR
2. Assessment of resolvin receptor labelling using immunocytochemistry in cultured HUVECs
3. Determination of antibody specificity using western blot
4. Further validation of antibody specificity by selective knockdown of resolvin receptors

3.2. Results

3.2.1. Analysis of resolvin receptor expression in cultured HUVECs by qPCR

As described in the methods chapter qPCR was used to establish if cultured HUVECs express the resolvin receptors, BLT1, GPR32, ChemR23, and ALX/FPR2. As shown in Table 3.1, BLT1, GPR32, and ChemR23 transcripts were detected in the cultured HUVECs, with cycle thresholds (Cts). The Ct value is defined as the number of cycles required for the fluorescent signal to rise above the fluorescence threshold (above the background level), and thus indicates that a detectable amount of BLT1, GPR32, and ChemR23 transcripts has been amplified. Although ALX/FPR2 has been found to be expressed in HUVECs in previous studies (Baker et al., 2009), ALX/FPR2 transcript was not found in the cultured HUVECs, as no amplification was observed (Figure 3.1A).

Amplification was seen for the endogenous control, β -2 microglobulin (B2M), across all samples (Figure 3B). Therefore, Ct values for resolvin receptors can be normalised to the Ct values for B2M in the same sample (Table 3.1), allowing for a comparison of the mRNA transcription levels of resolvin receptors in the cultured HUVECs.

Table 3.1. Quantification of transcript levels of resolvin receptors in cultured HUVECs. Each sample was run in triplicates, and the individual Ct values are represented as mean $\pm$ standard deviation (SD). The Ct values for resolvin receptors (target) were normalised to the Ct values for the endogenous control β -2 microglobulin (B2M) in the same sample, obtaining Δ Ct values for each resolvin receptor.

Resolvins receptors (Target)	Target Mean Ct $\pm$ SD	B2M Mean Ct $\pm$ SD	Δ Ct $\pm$ SD Target – B2M
BLT1	30.1 $\pm$ 0.07	21.4 $\pm$ 0.29	11.9 $\pm$ 0.04
GPR32	33.3 $\pm$ 0.07	21.3 $\pm$ 0.15	8.68 $\pm$ 0.32
ChemR23	31.4 $\pm$ 1.25	21.8 $\pm$ 0.30	9.62 $\pm$ 0.99
ALX/FPR2	-	21.8 $\pm$ 0.19	-

It is important to note that reactions were prepared using two negative controls, wherein the RNA or reverse transcriptase were substituted with RNAase-free water. The no RNA template controls failed to amplify any detectable product (Figure 3.1A), indicating that there was no contamination of extraneous nucleic acid. However, there was some background amplification from one of the no reverse transcriptase control for ALX/FPR2 (Ct = 35), which suggests that there may be some contamination of genomic DNA in this sample.

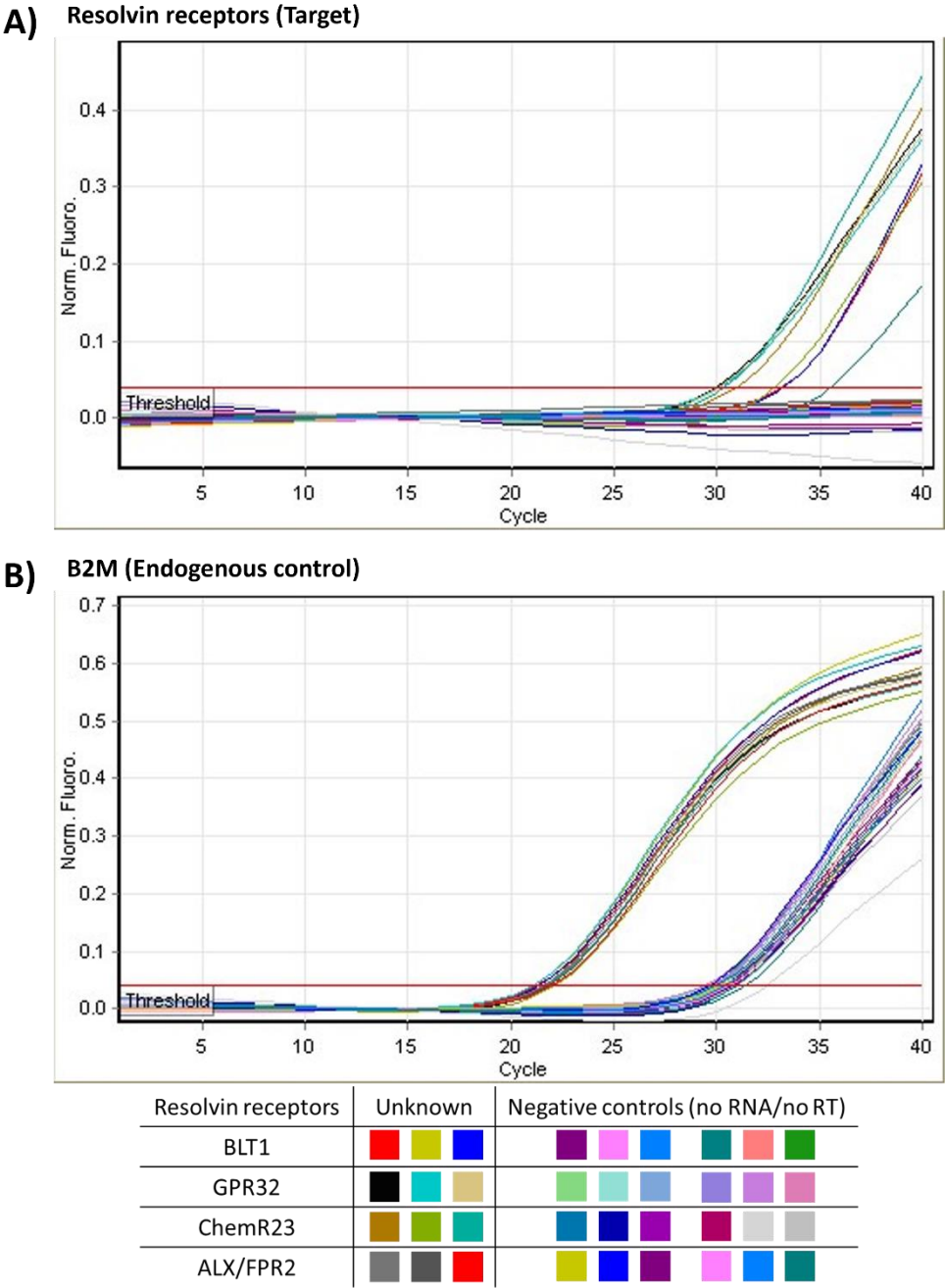


Figure 3.1. qPCR amplification plots of resolvin receptors and B2M

- A) Amplification was seen for BLT1, GPR32, and ChemR23, but no amplification was observed for ALX/FPR2. The two negative controls, RNA template control and no reverse transcriptase (RT) control failed to amplify any detectable product in most samples, apart from one of the no RT control for ALX/FPR2 (Ct = 35).
- B) Amplification was also seen for the endogenous control, B2M, across all samples.

3.2.2. Analysis of resolvin receptor expression in cultured HUVECs by immunocytochemistry

The expression of resolvin receptors in cultured HUVECs was then examined with immunocytochemistry using the resolvin receptor antibodies and methods described in the methods chapter (Section 2.1.3). In summary, cultured HUVECs were incubated overnight with primary antibodies raised in rabbit against synthetic peptides mapping to amino acid sequences from specific domains of human BLT1, GPR32, ChemR23, and ALX/FPR2 receptors. Antibody binding was visualised via incubation with anti-rabbit conjugated to cyanine 3 (Cy3).

Positive BLT1, GPR32, and ChemR23 labelling were found in HUVECs (Figures 3.2, 3.3, and 3.4 respectively). In all cases (BLT1, ChemR23, and GPR32) staining was seen in the cytosol. Additionally, there was some indication that BLT1 was also present in the cellular membrane. These images show staining with primary antibody dilutions of 1:200 (BLT1 and ChemR23) and 1:50 (GPR32). As detailed in the methods chapter (Section 2.1.3), different dilutions of primary antibodies were first tested in preliminary studies to determine the optimal dilution that produces the best quality of staining. The labelling of resolvin receptors in HUVECs also acts as optimisation for the use of antibodies in human tooth pulp tissues, which will be described in chapter 4.

No clear positive staining was identified following pre-absorption of the BLT1 antibody (Figure 3.2D), and in negative controls where the primary antibody was omitted (Figures 3.3D and 3.4D).

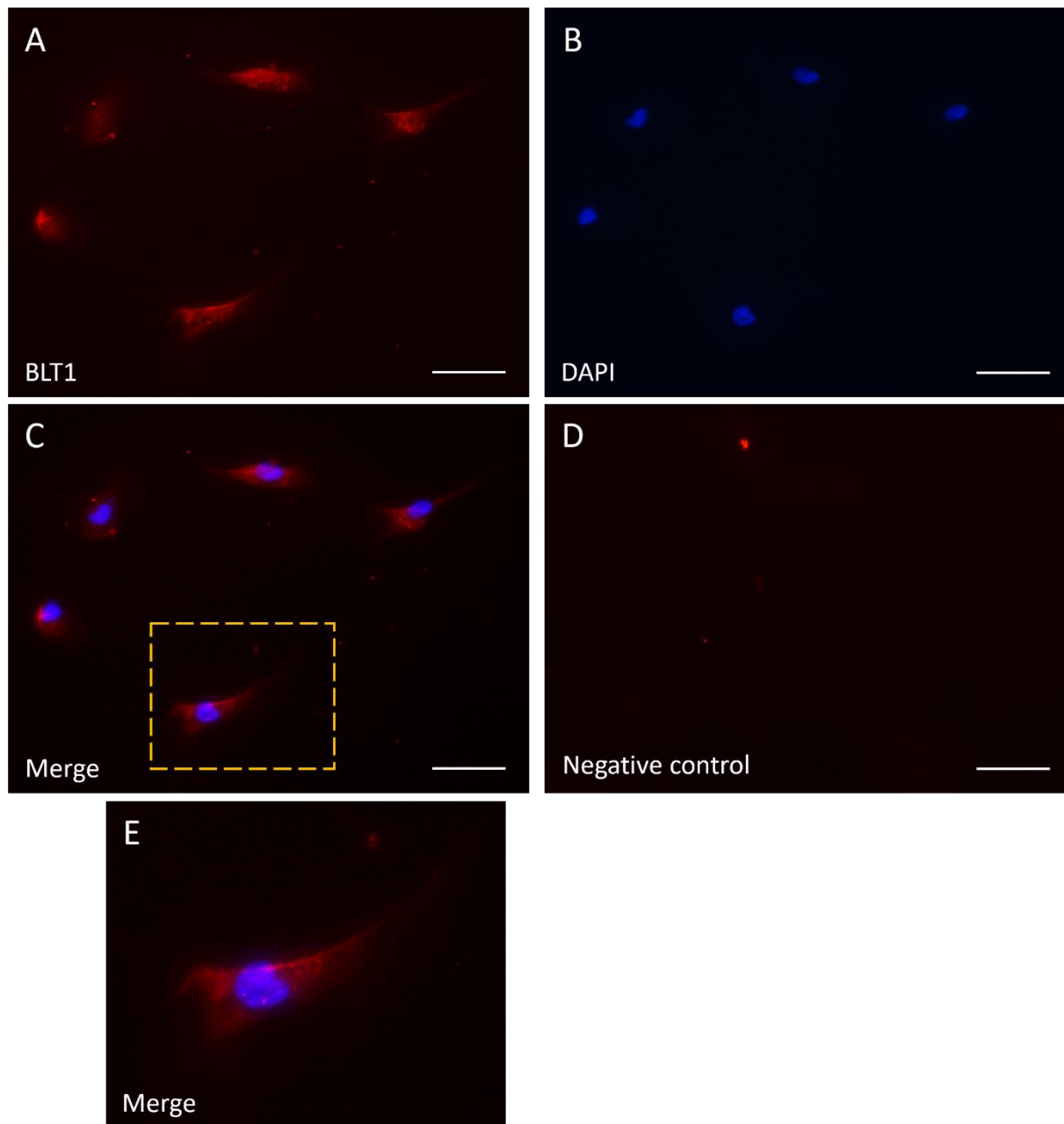


Figure 3.2. BLT1 expression in HUVECs.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with BLT1 (1:200) (visualised with Cy3 – red) and DAPI (blue), showing co-localisation between BLT1 and DAPI-stained nuclei. (D) Pre-absorption with the blocking peptide for the antibody against BLT1. (E) Enlargement of the area marked by the box in (C), showing BLT1 expression in the cytosol, with some indication of labelling in the cellular membrane. Scale bar = 50 µm.

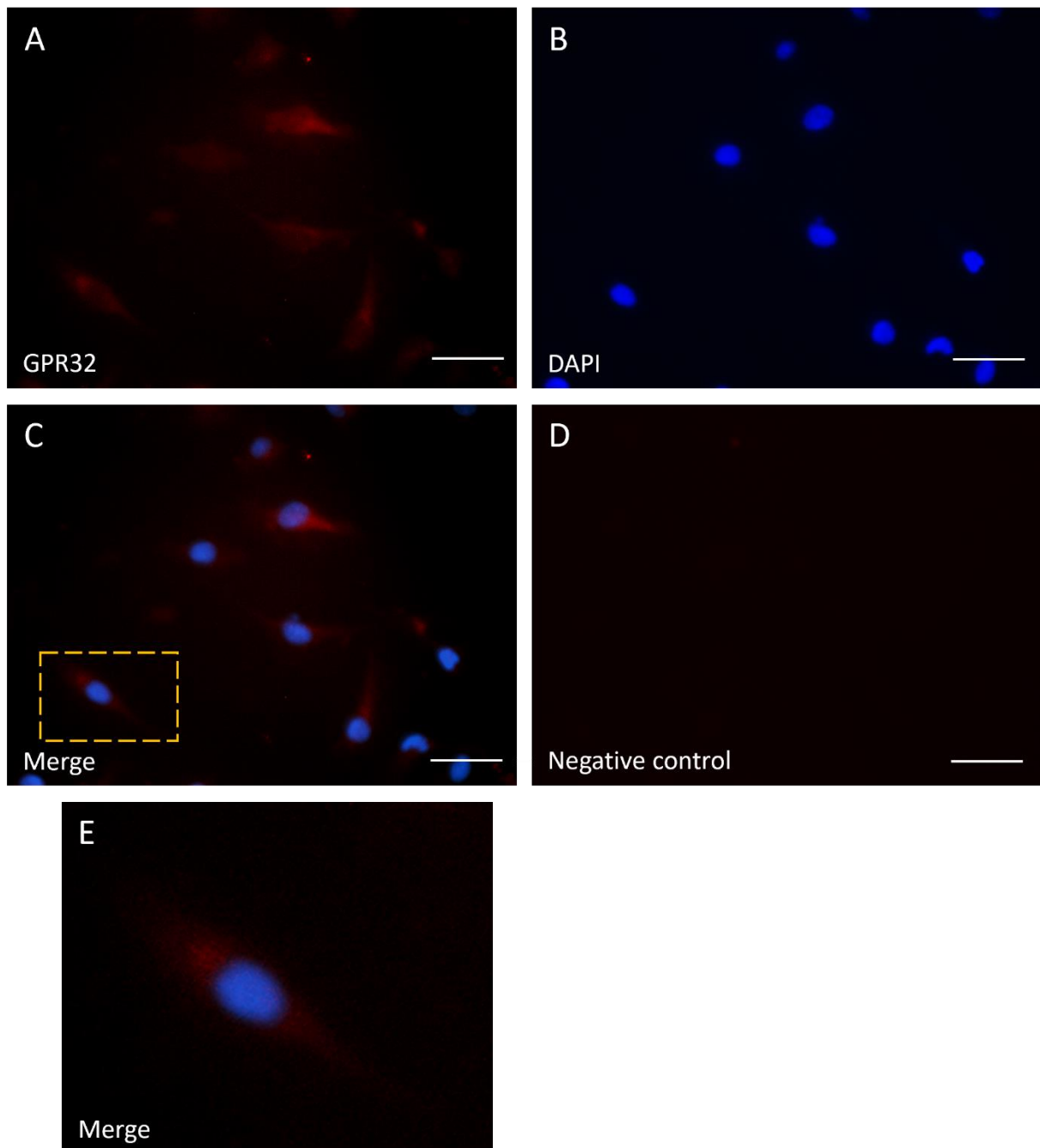


Figure 3.3. GPR32 expression in HUVECs.

(A) Positive staining with the antibody against GPR32. (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with GPR32 (1:50) (visualised with Cy3 – red) and DAPI (blue). (D) Negative control by omitting the antibody against GPR32. (E) Enlargement of the area marked by the box in (C), showing GPR32 expression in the cytosol. Scale bar = 50 μ m.

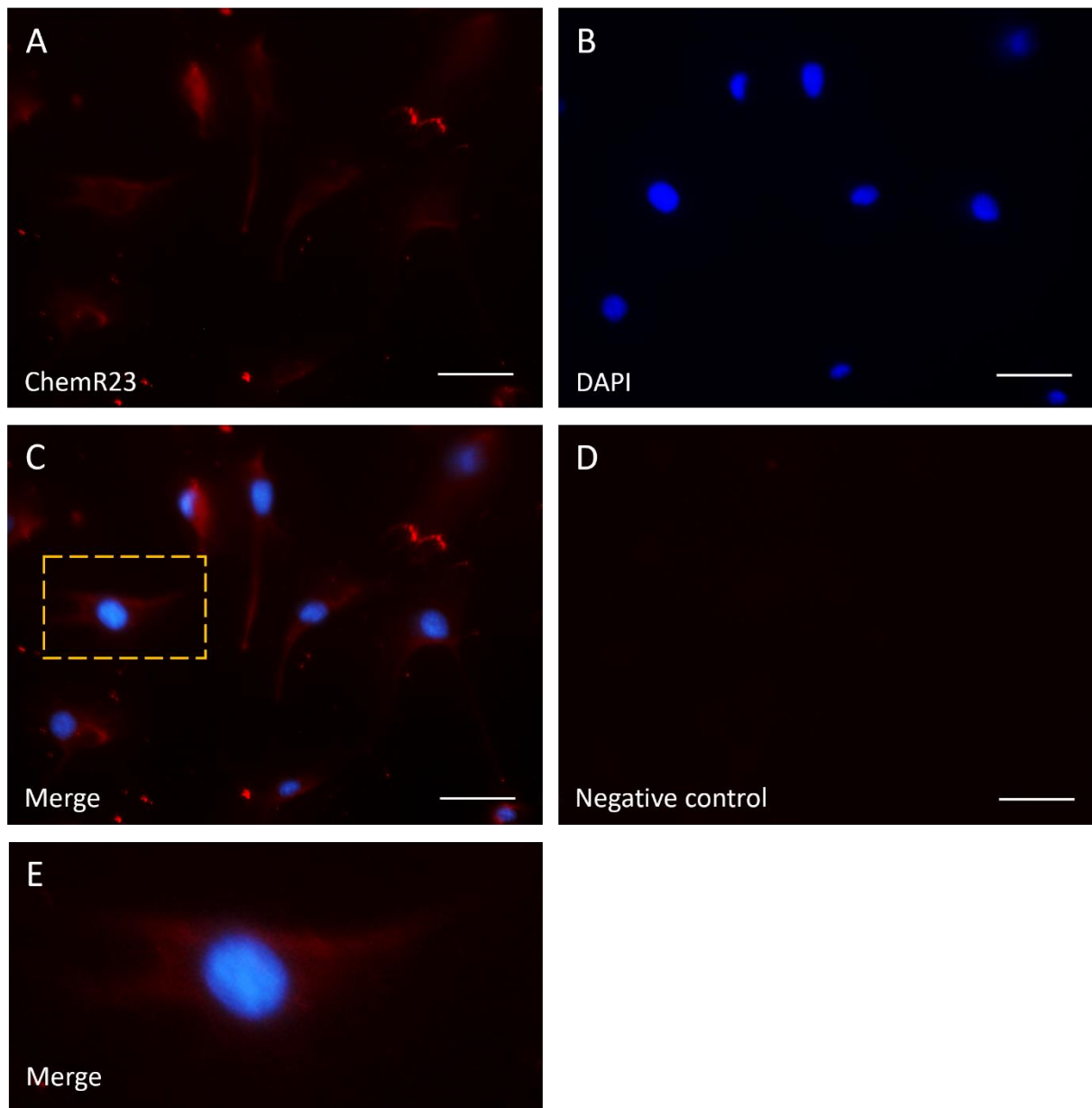


Figure 3.4. ChemR23 expression in HUVECs.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with ChemR23 (1:200) (visualised with Cy3 – red) and DAPI (blue). (D) Negative control by omitting the antibody against ChemR23. (E) Enlargement of the area marked by the box in (C), showing ChemR23 expression in the cytosol. Scale bar = 50 μ m.

ALX/FPR2 expression was not seen in the cultured HUVECs, since the level of fluorescence intensity from varying dilution of ALX/FPR2 antibody (Figure 3.5A, B) and pre-absorbed ALX/FPR2 antibody (Figure 3.5C) were similar.

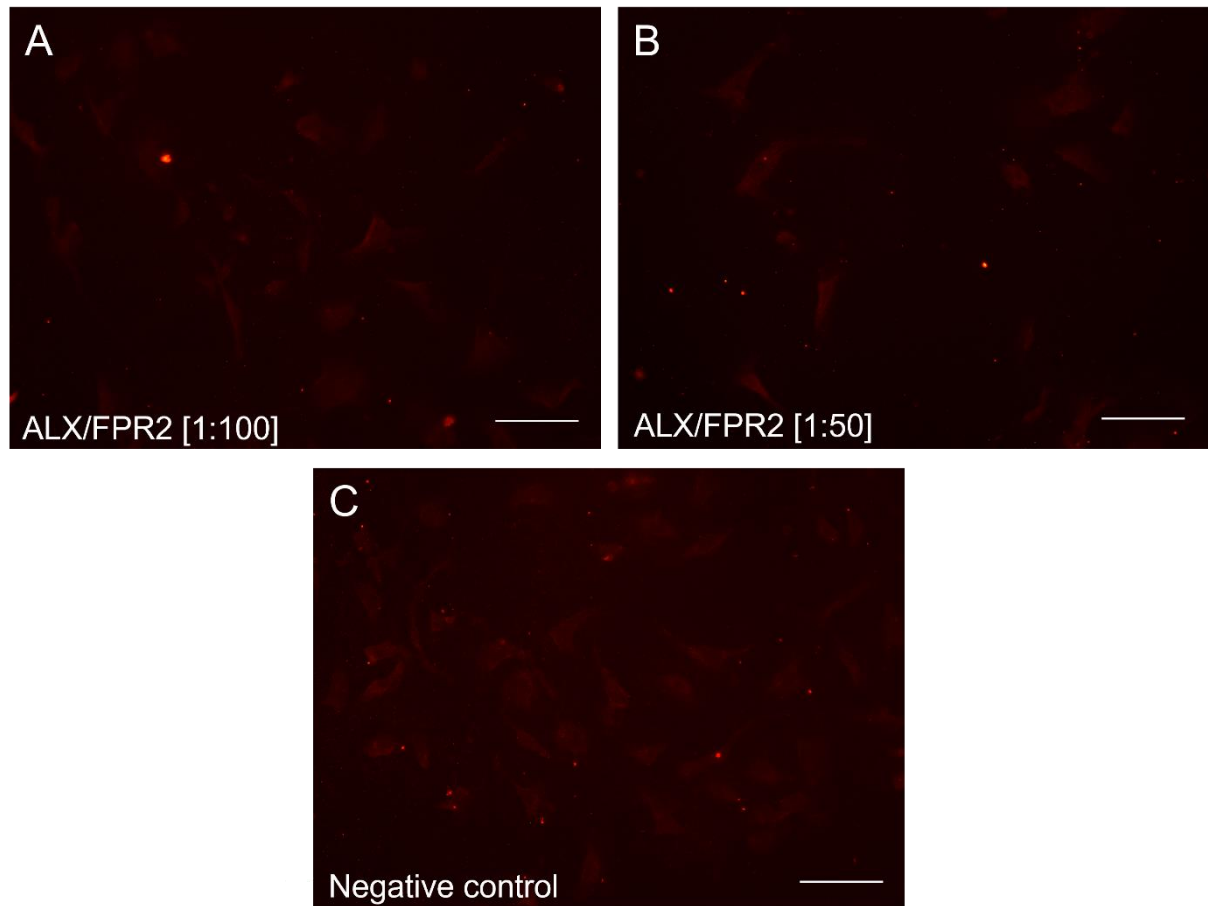


Figure 3.5. ALX/FPR2 did not appear to be expressed in the cultured HUVECs.

(A) Weak positive staining with the antibody against ALX/FPR2 (1:100). (B) Weak positive staining with the antibody against ALX/FPR2 (1:50), with a similar fluorescence intensity as the 1:100 ALX/FPR2 antibody. (C) Pre-absorption with the blocking peptide for the antibody against ALX/FPR2, with a similar intensity as ALX/FPR2 antibody. Scale bar = 100 μ m.

3.2.3. BLT1 protein expression was identified by western blot in HUVECs

The antibodies for resolvin receptors, BLT1, GPR32, and ChemR23 were assessed for their ability to selectively bind to the relevant resolvin receptor proteins in HUVECs using western blot, as detailed in the methods chapter. In preliminary studies, various concentrations of primary antibodies were tested to establish the optimal concentration that could produce a clear band.

The BLT1 antibody labelled a clear band at approximately 37 kDa, the expected molecular weight of BLT1 protein (Figure 3.6), indicating binding to BLT1. Additionally, it also labelled a faint band at approximately 45 kDa. However, the antibodies for GPR32 and ChemR23 failed to label any bands even after several additional repeats with increasing concentrations of antibodies (1:200 and 1:500 dilutions). In the negative control where the primary antibody was omitted, no band was observed.

Since results from both qPCR and immunocytochemistry experiments indicate that ALX/FPR2 is not present in cultured HUVECs, western blot experiments were not conducted using the ALX/FPR2 antibody.

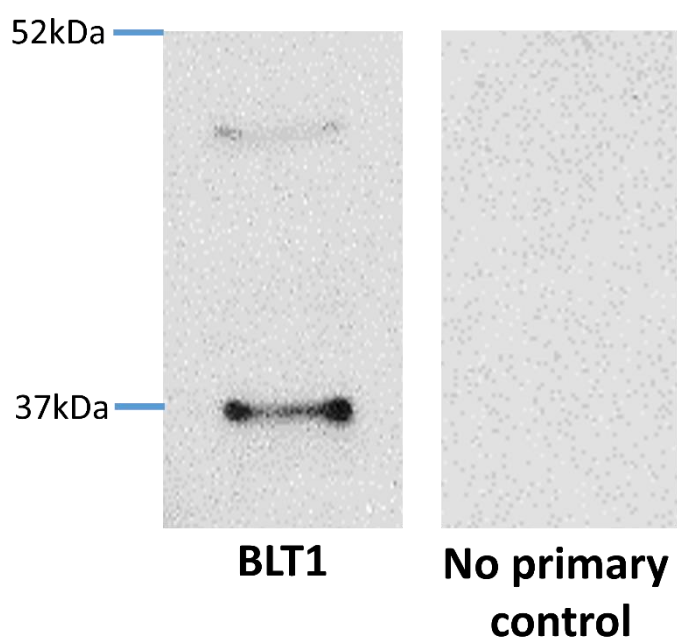


Figure 3.6. Western blot analysis of resolvin receptor protein expression in HUVECs. BLT1 protein expression was identified in the cultured HUVECs, wherein a clear band near 37 kDa and a faint band at approximately 45 kDa were labelled by BLT1 antibody. No band was observed in the negative control where the primary antibody was omitted.

3.2.4. Evaluation of immunocytochemical staining following siRNA-mediated knockdown of resolvins receptors

To further establish the specificity of antibodies raised against BLT1, GPR32, and ChemR23, immunocytochemical assays were performed after knocking down resolvins receptor expression in cultured HUVECs via siRNA transfection (see methods for details).

Following siRNA-mediated knockdown of BLT1 (Figure 3.7), GPR32 (Figure 3.8), and ChemR23 (Figure 3.9), a reduction in positive signal for each of those receptors respectively was observed in comparison to cells transfected with negative control non-targeting siRNA and mock transfection control. Expression of BLT1, GPR32, and ChemR23 in these cells was analysed by quantifying the area of positive staining per coverslip. Several images were taken per coverslip so approximately 50 cells were included in total.

The area of positive BLT1 staining was reduced by 92% and 96% after siRNA-mediated knockdown of BLT1 (Figure 3.7G), compared to cells transfected with negative control non-targeting siRNA and mock transfection control, respectively. Additionally, negative control non-targeting siRNA appeared to affect BLT1 expression in the cells, as the area of BLT1 labelling was reduced by 43% in comparison to the mock transfection control.

Interestingly, BLT1 labelling was mainly seen in the nuclei in the cells transfected with negative control non-targeting siRNA (Figure 3.7B, 3.7H) and mock transfection control (Figure 3.7C, 3.7I), whereas in naïve cells BLT1 labelling was mainly seen in the cytoplasm (Figure 3.2).

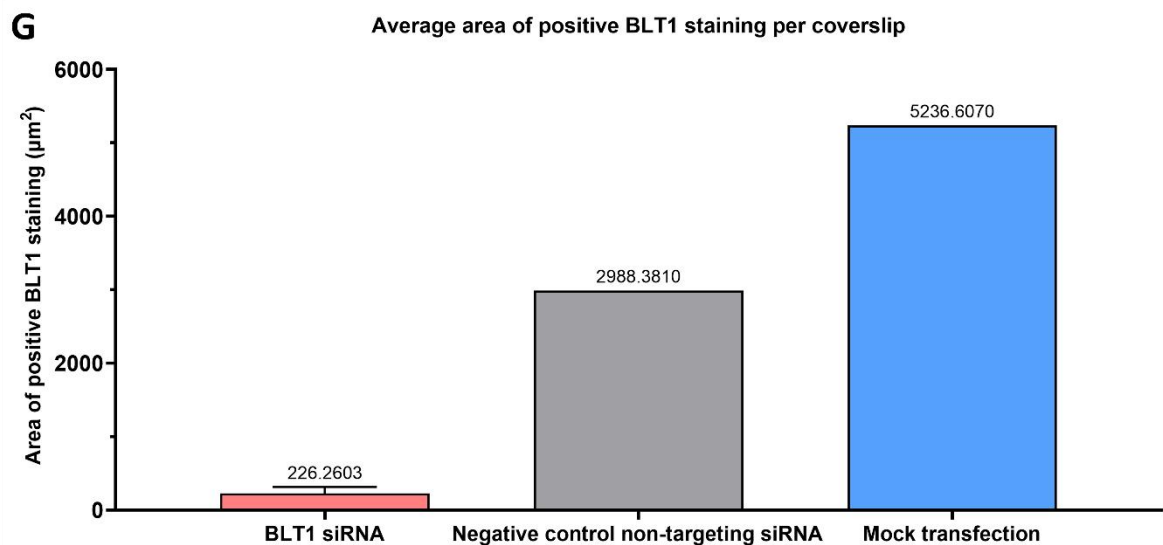
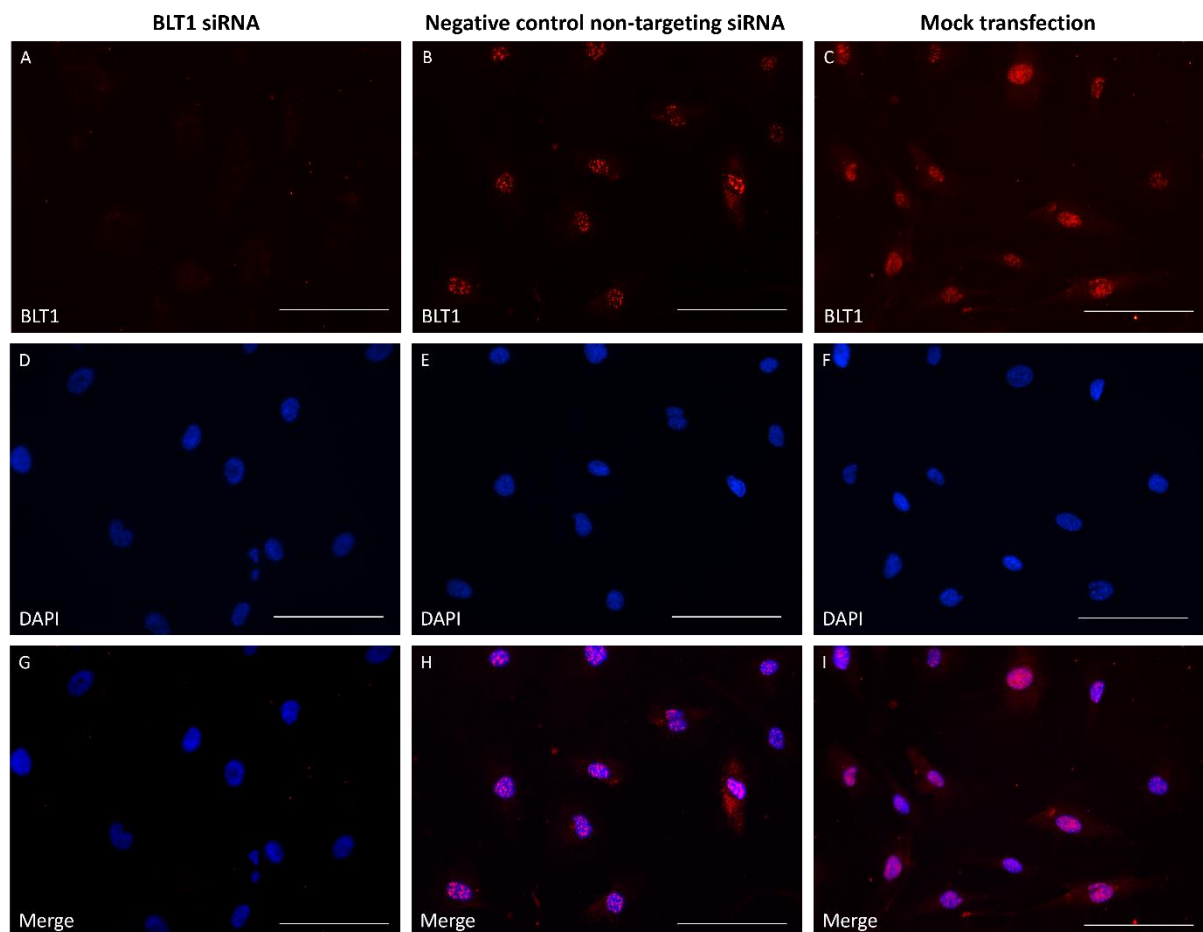


Figure 3.7. Reduction in BLT1 signal following siRNA-mediated knockdown of BLT1 expression in HUVECs.

(A – I) Cultured HUVECs were transfected with BLT1 siRNA (A, D, G), negative control non-targeting siRNA (B, E, H), and nuclease-free water as mock transfection control (C, F, I). Red fluorescence represents BLT1 staining, while blue fluorescence indicates DAPI-stained nuclei. Scale bar = 100 μm .

(G) Quantification analysis of positive BLT1 staining in cultured HUVECs transfected with BLT1 siRNA, negative control non-targeting siRNA, and mock transfection control. Error bar represents mean $\pm$ SEM (n=number of coverslips).

The area of positive GPR32 staining was reduced by 81% and 85% after siRNA-mediated knockdown of GPR32 (Figure 3.8G), compared to cells transfected with negative control non-targeting siRNA and mock transfection control, respectively.

In cells transfected with negative control non-targeting siRNA (Figure 3.8B, 3.8H) and mock transfection control (Figure 3.8C, 3.8I), GPR32 labelling was mainly seen in the cytoplasm and around the nuclei, which is similar to that seen in naïve cells (Figure 3.3).

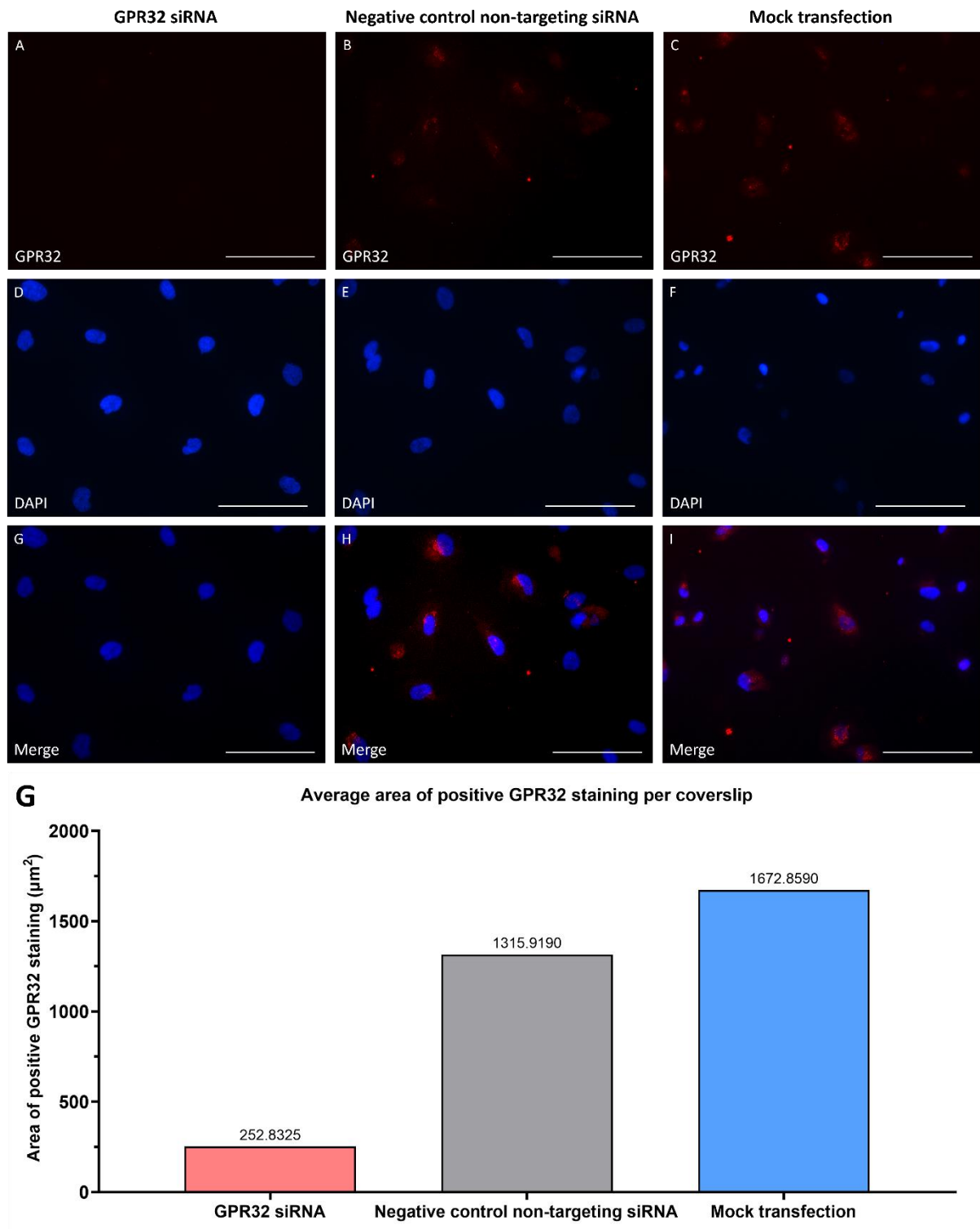


Figure 3.8. Reduction in GPR32 signal following siRNA-mediated knockdown of GPR32 expression in HUVECs.

(A – I) Cultured HUVECs were transfected with GPR32 siRNA (A, D, G), negative control non-targeting siRNA (B, E, H), and nuclease-free water as mock transfection control (C, F, I). Red fluorescence represents GPR32 staining, while blue fluorescence indicates DAPI-stained nuclei. Scale bar = 100 μm .

(G) Quantification analysis of positive GPR32 staining in cultured HUVECs transfected with GPR32 siRNA, negative control non-targeting siRNA, and mock transfection control.

The area of positive ChemR23 staining was reduced by 82% and 83% after siRNA-mediated knockdown of ChemR23 (Figure 3.9G), compared to cells transfected with negative control non-targeting siRNA and mock transfection control, respectively.

In cells transfected with negative control non-targeting siRNA (Figure 3.9B, 3.9H) and mock transfection control (Figure 3.9C, 3.9I), ChemR23 labelling was mainly seen in the cytoplasm and around the nuclei, which is similar to that seen in naïve cells (Figure 3.4).

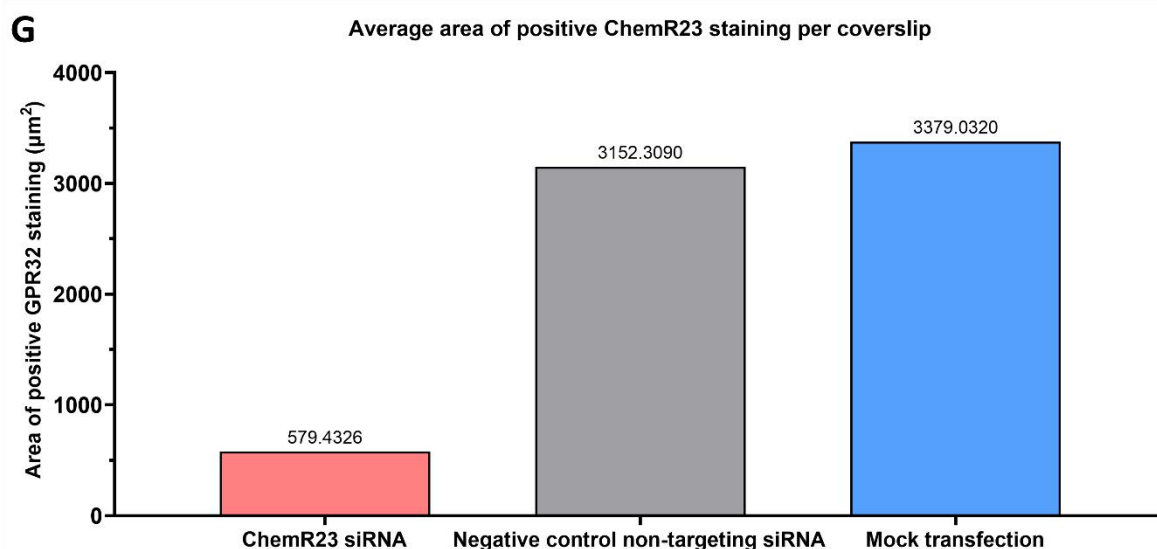
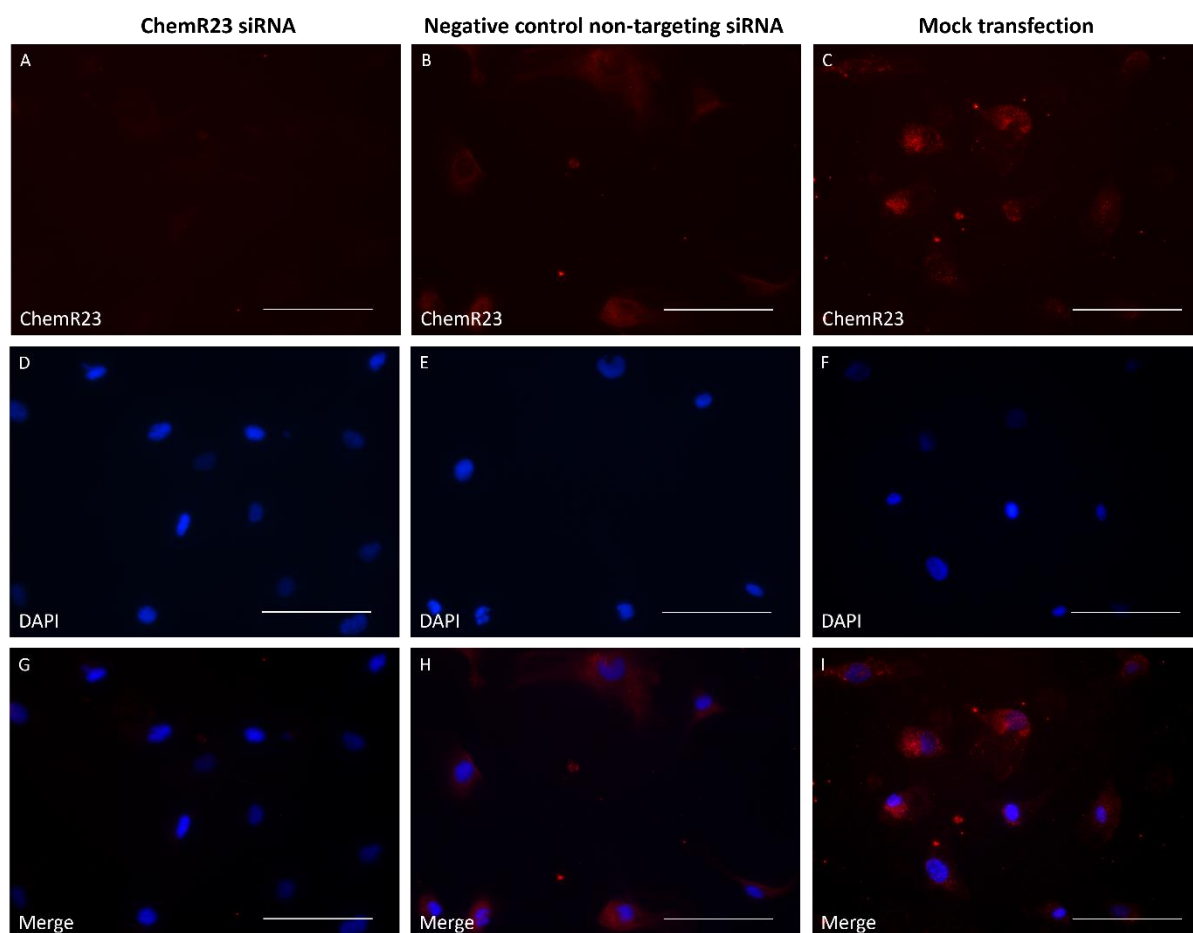


Figure 3.9. Reduction in ChemR23 signal following siRNA-mediated knockdown of ChemR23 expression in HUVECs.

(A – I) Cultured HUVECs were transfected with ChemR23 siRNA (A, D, G), negative control non-targeting siRNA (B, E, H), and nuclease-free water as mock transfection control (C, F, I). Red fluorescence represents ChemR23 staining, while blue fluorescence indicates DAPI-stained nuclei. Scale bar = 100 μm .

(G) Quantification analysis of positive ChemR23 staining in cultured HUVECs transfected with ChemR23 siRNA, negative control non-targeting siRNA, and mock transfection control.

3.2.5. Western blot analysis following siRNA-mediated knockdown of BLT1

BLT1 antibody detected a band at approximately 45 kDa in the cultured HUVECs transfected with negative control non-targeting siRNA and mock transfection control (Figure 3.10). However, the band was not observed or was very faint after knocking down BLT1 expression in the cells via siRNA transfection.

Western blot experiments were not carried out for knockdown of GPR32 and ChemR23 in HUVECs. This is because GPR32 and ChemR23 antibodies were not able to detect the relevant proteins in western blot experiments described above in 3.2.3

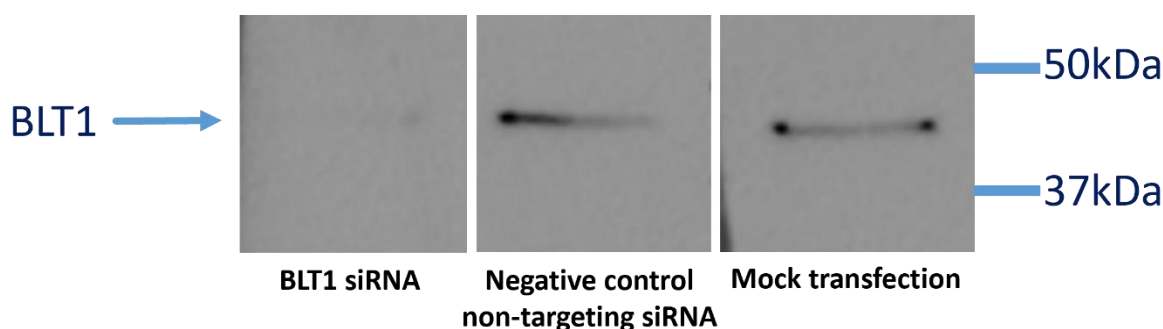


Figure 3.10. Western blot showing knockdown of BLT1 expression in HUVECs after transfection with BLT1-targeting siRNA.

BLT1 antibody detected a band at approximately 45 kDa following transfection with negative control non-targeting siRNA and nuclease-free water as mock transfection control. However, no band was detected or was very faint after transfection with BLT1-targeting siRNA.

Since the membrane was cut into three strips prior to incubation with BLT1 antibody (Figure 3.10), the comparison between the lanes might be less reliable. Therefore, three repeats of the experiment were conducted, where the samples were loaded directly next to each other for better comparison. However, each of those repeats failed to replicate what was shown in Figure 3.10, for reasons unknown. In one of the repeats, the BLT1 antibody detected a band at around 37 kDa in cells transfected with mock transfection control, but no bands were detected in cells transfected with negative control non-targeting siRNA or BLT1 siRNA (Figure 3.11A) In this repeat, equal loading of protein samples was demonstrated by re-probing the membrane with the GAPDH antibody, which labelled three bands at approximately 36 kDa, the expected molecular weight of GAPDH protein (Figure 3.11A). In other repeats of the experiments, the BLT1 antibody failed to detect any bands in all the samples despite confirmation of protein transfer with Ponceau S staining (Figure 3.11B). Due to time constraints, I was not able to repeat the experiment again.

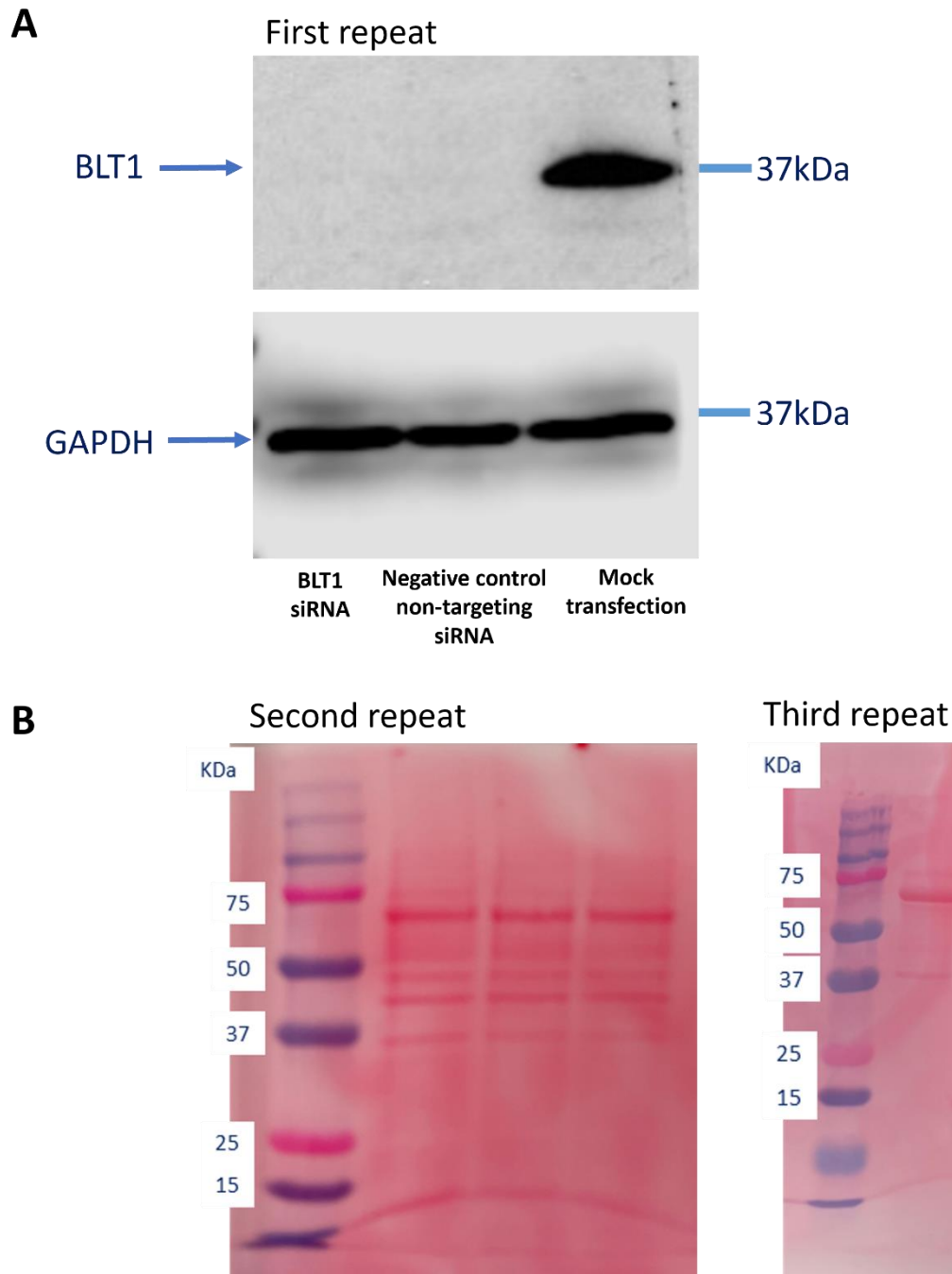


Figure 3.11. Unsuccessful repeats of Western blot experiments following siRNA-mediated knockdown of BLT1

(A) In one of the repeats, BLT1 detected a band at around 37 kDa in cells transfected with mock transfection control, but no bands were detected in cells transfected with negative control non-targeting siRNA or BLT1 siRNA. Equal loading of protein samples was demonstrated by re-probing the membrane with the GAPDH antibody, which detected three bands at around 36 kDa, the expected molecular weight of GAPDH protein.

(B) Confirmation of protein transfer with Ponceau S staining in the other two repeats, where no bands were detected by the BLT1 antibody.

3.3. Discussion

Data from this study indicated that the antibodies raised against BLT1, GPR32, and ChemR23 were specific to their respective receptors. The antibodies were tested using HUVECs. Previous studies using qPCR and western blot have demonstrated that this cell line expresses the resolvins receptors, GPR32 (Krishnamoorthy et al., 2010b), ALX/FPR2 (Baker et al., 2009), BLT1 (Qiu et al., 2006), and ChemR23 (Hao et al., 2015). Therefore, HUVECs were thought to be a good candidate to validate the specificity of antibodies.

Gene expression for BLT1, GPR32, and ChemR23 was identified in the cultured HUVECs by qPCR (Table 3.1), which is consistent with findings from previous studies in the literature (Qiu et al., 2006, Hao et al., 2015, Krishnamoorthy et al., 2010b). However, the TaqMan probe for ALX/FPR2 failed to amplify any detectable product, indicating that ALX/FPR2 was either not expressed or expressed at a very low level in the cultured HUVECs. This was unexpected as ALX/FPR2 expression was detected by qPCR in a previous study, albeit with different primers (Baker et al., 2009). However, the details of the primers used were not provided by Baker et al. (2009), so the TaqMan probe for ALX/FPR2 that spans the exon-intron boundary was used instead.

The specificity of the antibodies against BLT1, GPR32, and ChemR23 was then established using immunocytochemical staining, wherein these antibodies produce clear positive staining in HUVECs (Figures 3.2 – 3.4). However, the ALX/FPR2 antibody failed to produce immunostaining that was different to what was seen with the pre-absorption control (Figure 3.5), which is consistent with the findings from qPCR that ALX/FPR2 was not expressed in the cultured HUVECs. Therefore, the specificity of ALX/FPR2 antibody could not be assessed using the cultured HUVECs and the antibody could not be used in immunohistochemistry of human tooth pulp tissues.

Western blot analysis was also conducted to validate the specificity of the antibodies raised against resolvins receptors. Western blot using the BLT1 antibody labelled a protein band at approximately 37 kDa, the expected molecular weight of BLT1 protein (Figure 3.6). An additional faint band at approximately 45 kDa was also observed, which was not seen in the previous study that analysed BLT1 protein level in HUVECs by western blot using a different BLT1 antibody (Qiu et al., 2006). However, since the whole blot was not shown, the possibility that a weak band might have been observed at a higher molecular weight cannot be eliminated. The data sheet provided by Alomone labs for the BLT1 antibody describes a clear band at approximately 55 kDa in western blot analysis of three other cell lines, which could be related to the weak band observed at approximately 45 kDa in the present study. In subsequent western blot analysis conducted after siRNA-mediated transfection, the BLT1 antibody could only detect the band at around 45 kDa in cells transfected with negative

control non-targeting siRNA and mock transfection control (Figure 3.10), indicating that the band is likely to be specific. The higher molecular weight band observed suggests that the BLT1 protein may be post-translationally modified. Nevertheless, the experiment could be repeated to further confirm if the ~45 kDa band was specific by pre-incubating the BLT1 antibody with its blocking peptide. Unexpectedly, the antibodies for GPR32 and ChemR23 failed to label any bands even after several additional repeats using increasing concentrations of antibodies and protocol modification. This includes an increase in the incubation time of primary antibodies and the amount of protein sample loaded. Since results from both qPCR and immunocytochemistry experiments indicate that GPR32 and ChemR23 are present in HUVECs, it is likely that these antibodies might not be suitable for western blot.

To further demonstrate the specificity of antibodies raised against BLT1, GPR32, and ChemR23, further immunocytochemistry assays were conducted after selectively knocking down the expression of BLT1, GPR32, and ChemR23 in the cultured HUVECs via siRNA transfection (Figures 3.7 – 3.9). The cells were also transfected with negative control non-targeting siRNA and nuclease-free water as mock transfection control each time to provide a baseline for comparison with siRNA-treated cells. A reduction in positive signal for BLT1, GPR32, and ChemR23 was observed in comparison to cells transfected with negative control non-targeting siRNA and mock transfection control, which further establishes that the antibodies used were specific to their respective receptors. It is important to note that even though the negative control non-targeting siRNA has no significant sequence similarity to mammalian gene, it still appeared to have some effects in BLT1 expression, as the area of BLT1 labelling was reduced by 43% in comparison to the mock transfection control (Figure 3.7G), which might be due to non-specific effects related to the delivery of siRNA to the cells.

Western blot analysis was also conducted after selectively knocking down BLT1 expression via siRNA transfection. The BLT1 antibody detected a band at around 45 kDa in cells transfected with negative control non-targeting siRNA and mock transfection control (Figure 3.10), but it failed to detect this band in cells transfected with BLT1 siRNA. However, it should be noted that the data represented in Figure 3.10 are from an experiment where each sample was loaded next to a protein ladder and the membrane was cut into three strips prior to incubation with BLT1 antibody, making a comparison between the lanes less reliable. Attempts were made to repeat the experiment, but they all failed to replicate (Figure 3.11) what was seen in Figure 3.10. Due to time constraints, I was not able to repeat the western blot experiment again.

In summary, the data in this chapter demonstrate that the antibodies raised against BLT1, GR32, and ChemR23 specifically label the relevant proteins. Therefore, these

antibodies were used to investigate the expression of resolvins receptors in human tooth pulp tissues via immunohistochemistry, as described in the next chapter.

CHAPTER 4: CHARACTERISATION OF RESOLVIN RECEPTOR EXPRESSION IN HUMAN TOOTH PULP TISSUES

4.1. Introduction

As described in section 1.2 resolvins are specialised pro-resolving mediators that actively regulate the resolution of inflammation (Serhan, 2014). They are enzymatically derived from two omega-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) – giving rise to the D-series (RvD) and E-series (RvE) resolvins respectively (Serhan et al., 2008). The biological actions of resolvins are mediated by their specific receptors, which are all G-protein coupled receptors. RvE1 and E2 act on BLT1 and ChemR23, whereas RvD1, D3, and D5 bind and signal through to GPR32 (see Figure 1.8). Recent studies have also shown that resolvins can alleviate pain symptoms in pre-clinical models (Luo et al., 2019, Oehler et al., 2017, Xu et al., 2013, Park et al., 2011), demonstrating their therapeutic potential in chronic pain. However, the expression of resolvins receptors in sites of inflammation and pain, versus healthy tissues in patients is much less studied. Previous studies have shown that resolvins receptors are differentially expressed in tissues with and without inflammation (Ozcan et al., 2018), and a correlation has been shown between resolvins receptor expression and expression of biomarkers linked with knee pain, in joint tissues from osteoarthritis patients (Huang et al., 2017). However, this joint tissue study did not demonstrate upregulation of receptors in this chronic pain condition as it did not investigate expression in healthy tissue. Therefore, the aim of the experiments presented in this chapter was to characterise resolvins receptor expression in three groups of human tooth pulp tissue: non-inflamed human tooth pulp tissues from non-carious healthy teeth, inflamed tooth pulp tissues from carious painful teeth, and inflamed tooth pulp tissues from carious non-painful teeth (depicted in Figure 4.1). This will allow identification of any correlation between receptor expression and the presence of pain and inflammation.

Human tooth pulp samples were labelled using immunohistochemistry with antibodies raised against the resolvins receptors BLT1, ChemR23 and GPR32. The specificity and selectivity of the resolvins receptor antibodies were determined as described in chapter 3. Association of receptors with nerve bundles and blood vessels was investigated using dual labelling with antibodies raised against the neuronal marker β -tubulin III, smooth muscle marker cell marker α -smooth muscle actin (α -SMA), and the endothelial cell marker ulex europaeus agglutinin I (UEA I).

4.2. Experimental protocol

As described in detail in section 2.2 in the methods, teeth were divided into three groups according to caries and pain status: non-caries, carious painful, and carious non-painful. The grouping of human tooth pulp tissues is depicted in Figure 4.1.

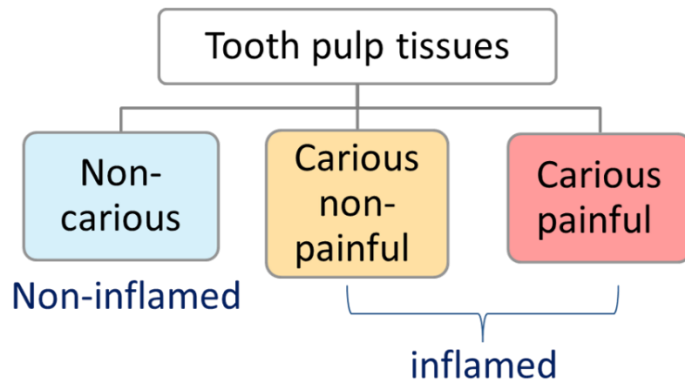


Figure 4.1. Grouping of human tooth pulp tissues

Resolvin receptor expression was characterised in three groups of human tooth pulp tissue:

1. Non-inflamed human tooth pulp tissues from non-caries healthy teeth
2. Inflamed tooth pulp tissues from carious painful teeth
3. Inflamed tooth pulp tissues from carious non-painful teeth

Further details of experimental protocol and analyses were described in the methods in sections 2.2, 2.5.1, and 2.6.1. Tile-scan images of the longitudinally sectioned tooth pulp tissues were acquired to obtain images of the whole tissue section. Images were also taken from the pulp horn and central region of the human tooth pulp sections (described in Figure 4.2).

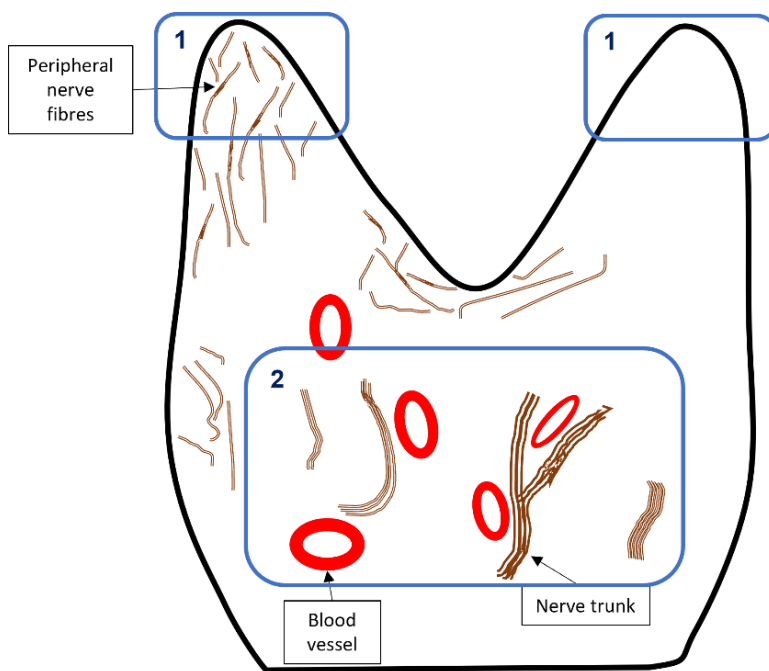


Figure 4.2. Schematic diagram of a longitudinally sectioned human tooth pulp.

Boxed areas: 1. Pulp horn; 2. Central region. The drawing was adapted from Rodd and Boissonade, 2003.

BLT1 expression in human tooth pulp tissues was characterised following the steps detailed below:

1. Preliminary staining to identify the structures that express BLT1.
2. Dual labelling with an antibody raised against the neuronal marker β -tubulin III was carried out to investigate BLT1 expression associated with nerve fibres.
3. Quantification of BLT1 labelling associated with nerve fibres.
 - a. Images of whole tissue sections were taken from the three groups of human tissues, and areas of BLT1 and β -tubulin III labelling were quantified. 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.
 - b. In the pulp horn, two areas of interest around two-thirds of the pulp horns were drawn based on the processed image that indicates positive β -tubulin III staining. These two areas of interest were copied onto the image of BLT1 labelling so the same regions were analysed for the area of positive BLT1 and β -tubulin III labelling in the pulp horns (see Figure 4.3A – B). 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.
 - c. In the central region, areas of interest were drawn around nerve bundles based on the processed image that indicates positive β -tubulin III staining. These areas of interest were copied onto the image of BLT1 labelling so the same regions were analysed for the area of positive BLT1 and β -tubulin III labelling in the central region of the tooth pulp (see Figure 4.3C – D). On average, 15 – 20 nerve bundles were sampled per section. 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.
4. Dual labelling with an antibody raised against the smooth muscle cell marker α -smooth muscle actin (α -SMA) and the endothelial cell marker ulex europaeus agglutinin I (UEA I) was carried out to investigate BLT1 expression in blood vessels.
5. Quantification of BLT1 labelling in blood vessels. Images were taken in areas rich in blood vessels (Figures 4.16 – 4.18) from the pulp horn and central region to quantify areas of BLT1 and FITC (α -SMA and UEA I) labelling in these two regions of the tooth pulp. 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.

ChemR23 expression in human tooth pulp tissues was characterised following the steps detailed below:

1. Preliminary staining to identify the structures that express ChemR23.
2. Dual labelling with neuronal marker β -tubulin III to investigate ChemR23 expression associated with nerve fibres.
3. Quantification of ChemR23 labelling associated with nerve fibres.
 - a. Images of whole tissue sections were taken from the three groups of human tissues, and areas of ChemR23 and β -tubulin III labelling were quantified. 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.
 - b. In the pulp horn, two areas of interest around two-thirds of the pulp horns were drawn based on the processed image that indicates positive β -tubulin III staining. These two areas of interest were copied onto the image of ChemR23 labelling so the same regions were analysed for the area of positive ChemR23 and β -tubulin III labelling in the pulp horns (depicted in Figure 4.3A – B). 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.
 - c. In the central region, areas of interest were drawn around nerve bundles based on the processed image that indicates positive β -tubulin III staining. These areas of interest were copied onto the image of ChemR23 labelling so the same regions were analysed for the area of positive ChemR23 and β -tubulin III labelling in the central region of the tooth pulp (depicted in Figure 4.3C – D). On average, 15 – 20 nerve bundles were sampled per section. 3 – 4 tooth pulp sections were analysed for each tooth pulp sample, and an average was taken.

GPR32 expression in human tooth pulp tissues was characterised as follows:

1. Preliminary staining to identify the structures that express GPR32.
2. Dual labelling with neuronal marker β -tubulin III to investigate GPR32 expression associated with nerve fibres.

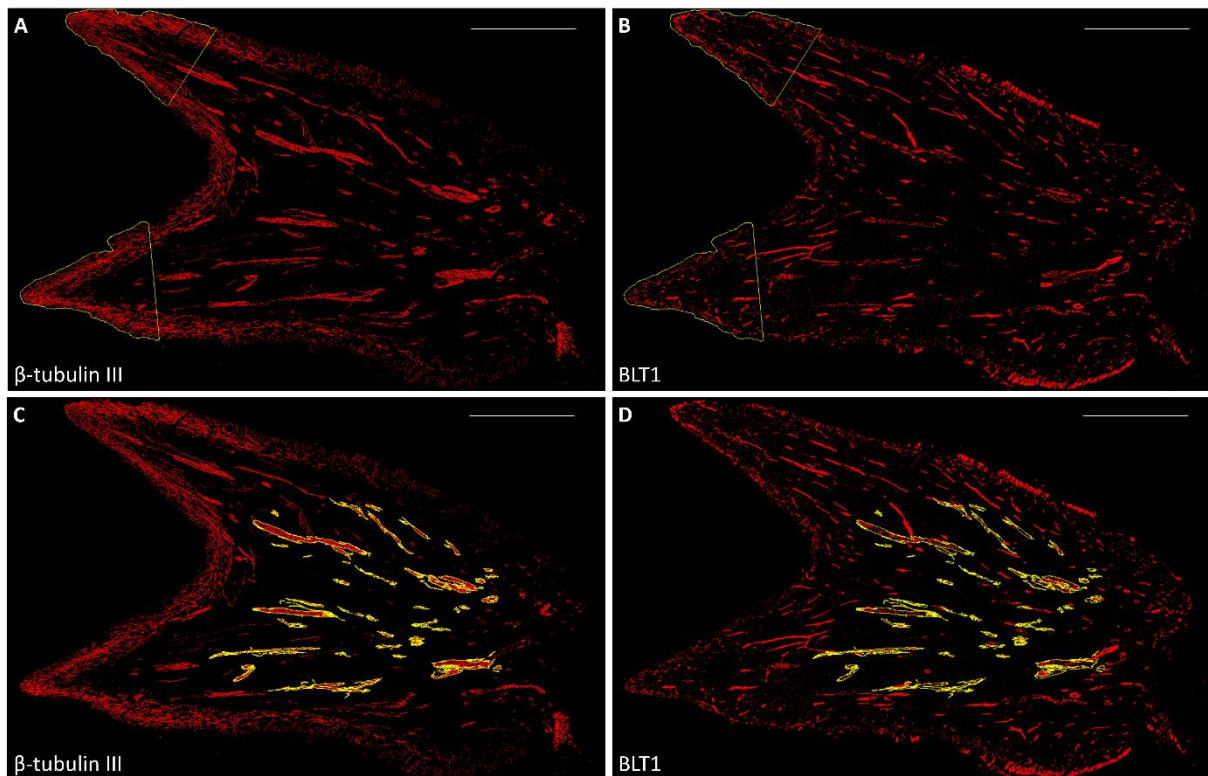


Figure 4.3. Quantification of the area of BLT1 and β -tubulin III labelling in the pulp horn (A – B) and central region (C – D) of the tooth pulp using Image J, based on the processed image that indicates positive β -tubulin III staining (A & C).

(A – B) Based on the processed image that indicates positive β -tubulin III staining (A), two areas of interest (outlined by yellow lines) around two-thirds of the pulp horns were drawn. These two areas of interest were copied onto the image of BLT1 labelling (B) so the same regions were analysed for the area of positive BLT1 and β -tubulin III labelling in the pulp horns.

(C – D) Based on the processed image that indicates positive β -tubulin III staining (C), areas of interest (outlined by yellow lines) were drawn around nerve bundles. These areas of interest were copied onto the image of BLT1 labelling (D) so the same regions were analysed for the area of positive BLT1 and β -tubulin III labelling in the central region of the tooth pulp.

Scale bar = 1000 μ m and refers to all images.

* The same method of quantification was also applied to quantify ChemR23 labelling associated with nerve fibres.

4.3. Results

To characterise the expression of resolvins receptors in human tooth pulp tissues, antibodies raised against BLT1, ChemR23, and GPR32 were used in immunohistochemistry assays. The specificity of these antibodies has been validated, as described in Chapter 3.

4.3.1. Characterisation of BLT1 expression in human tooth pulp tissues

Positive staining for BLT1, a receptor for RvE1 and E2, was found in human tooth pulp tissues, as indicated in Figures 4.4 – 4.12, and Figures 4.14 – 4.18. Positive labelling with the BLT1 antibody was present in all sections from all samples investigated from all three groups of teeth. Labelling was present throughout the entire pulp (Figures 4.7A, 4.9A, and 4.11A), but appeared to be localised to specific structures. To validate the specificity of the BLT1 antibody in this tissue, it was pre-absorbed with its corresponding blocking peptide for approximately 18 hours prior to performing the immunohistochemistry staining assay, as detailed in section 2.2.2.1 in the methods. Following pre-absorption of the BLT1 antibody, no positive staining was observed (Figures 4.4D and 4.5D).

4.3.1.1. Preliminary staining to identify structures with BLT1

BLT1 expression was found in structures that have a similar morphology to nerve fibres and blood vessels (Figures 4.4 and 4.5 respectively).

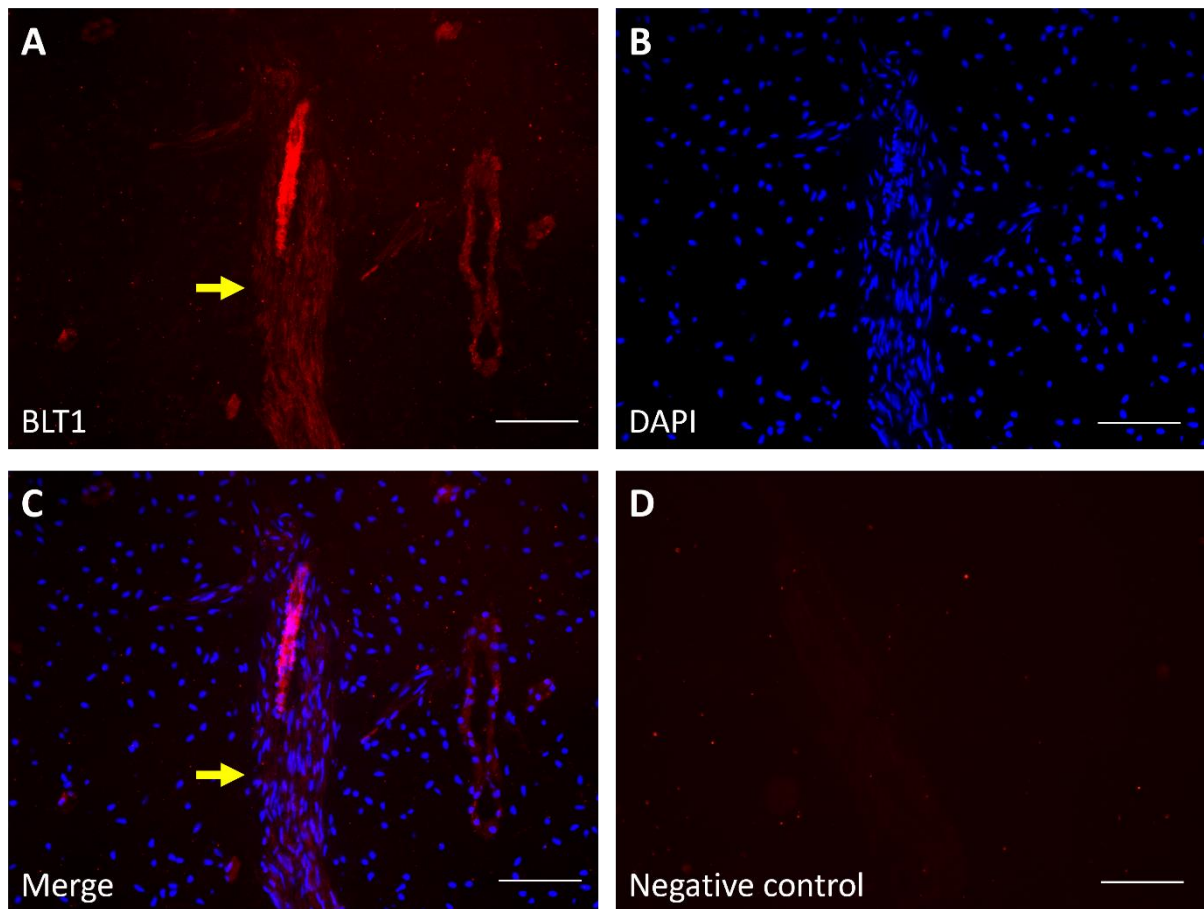


Figure 4.4. BLT1 expression in structures with nerve fibre-like morphology.

(A) Positive staining with the antibody against BLT1, showing BLT1 expression in a structure with nerve fibre-like morphology (indicated by a yellow arrow). (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with BLT1 (red) and DAPI (blue). (D) Pre-absorption with the blocking peptide for the antibody against BLT1. Scale bar = 100 μ m and refers to all images.

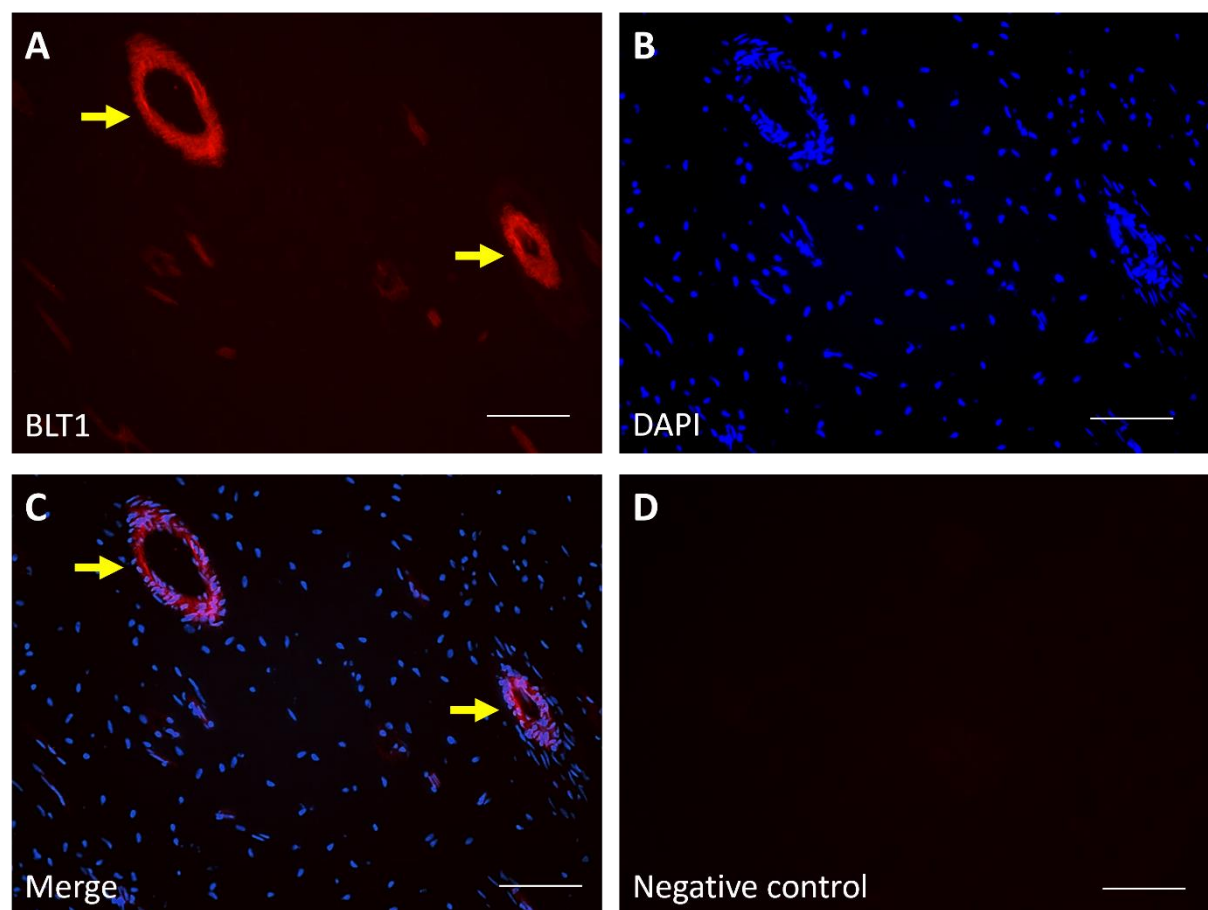


Figure 4.5. BLT1 expression in structures with blood vessel-like morphology.

(A) Positive staining with the antibody against BLT1, showing BLT1 expression in structures with blood vessel-like morphology (indicated by yellow arrows). (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with BLT1 (red) and DAPI (blue). (D) Pre-absorption with the blocking peptide for the antibody against BLT1. Scale bar = 100 μm and refers to all images.

4.3.1.2. Investigation of BLT1 expression associated with nerve fibres

β -tubulin III has been widely regarded as a neuronal marker (Geisert and Frankfurter, 1989), and thus was used to investigate if BLT1 expression was associated with nerve fibres. BLT1 was shown to associate with nerve fibres as there was clear BLT1 expression in regions labelled with β -tubulin III (Figure 4.6).

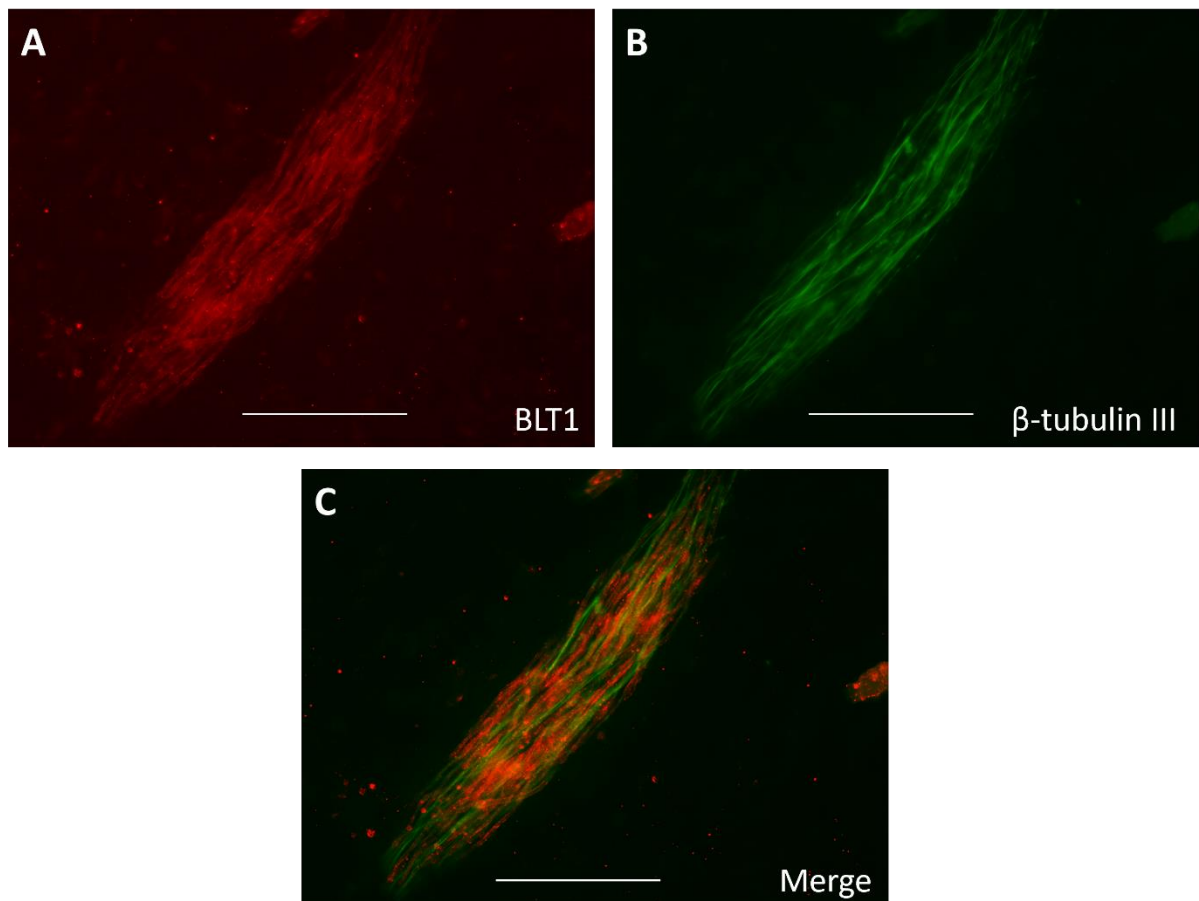


Figure 4.6. BLT1 expression associated with nerve fibres in human tooth pulp.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Double staining with BLT1 (red) and β -tubulin III (green), showing BLT1 expression within regions of nerve fibres. Scale bar = 100 μ m and refers to all images.

4.3.1.3. Characterisation of BLT1 labelling associated with nerve fibres

To characterise BLT1 expression associated with nerve fibres in all three groups of human tooth pulp tissues, images of whole tooth pulp sections were taken from tooth pulp samples from carious painful (Figure 4.7), carious non-painful (Figure 4.9), and non-carious teeth (Figure 4.11) that had been dual labelled using BLT1 and β -tubulin III antibodies. These images show that BLT1 and β -tubulin III were present throughout the entire pulp.

Therefore, confocal imaging was used to examine the association between BLT1 and β -tubulin III expression in all three groups of human tooth pulp tissues by acquiring images of nerve bundles. These images show that within a few fibres (marked by white arrows in Figures 4.8E, 4.10E, and 4.12E), BLT1 labelling appeared to overlap with β -tubulin III.

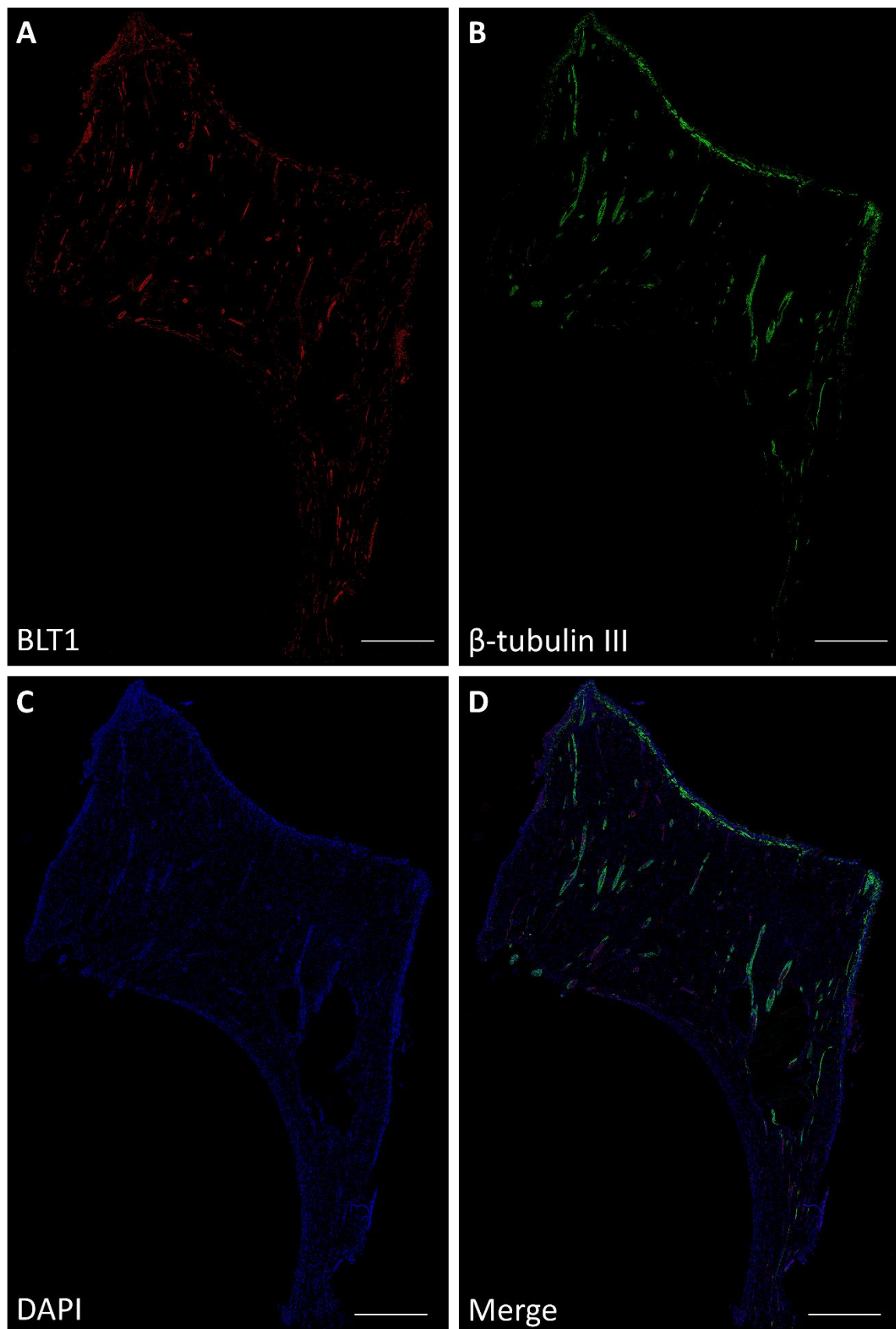


Figure 4.7. Whole section images showing BLT1 expression associated with nerve fibres in human tooth pulp tissues from carious painful teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

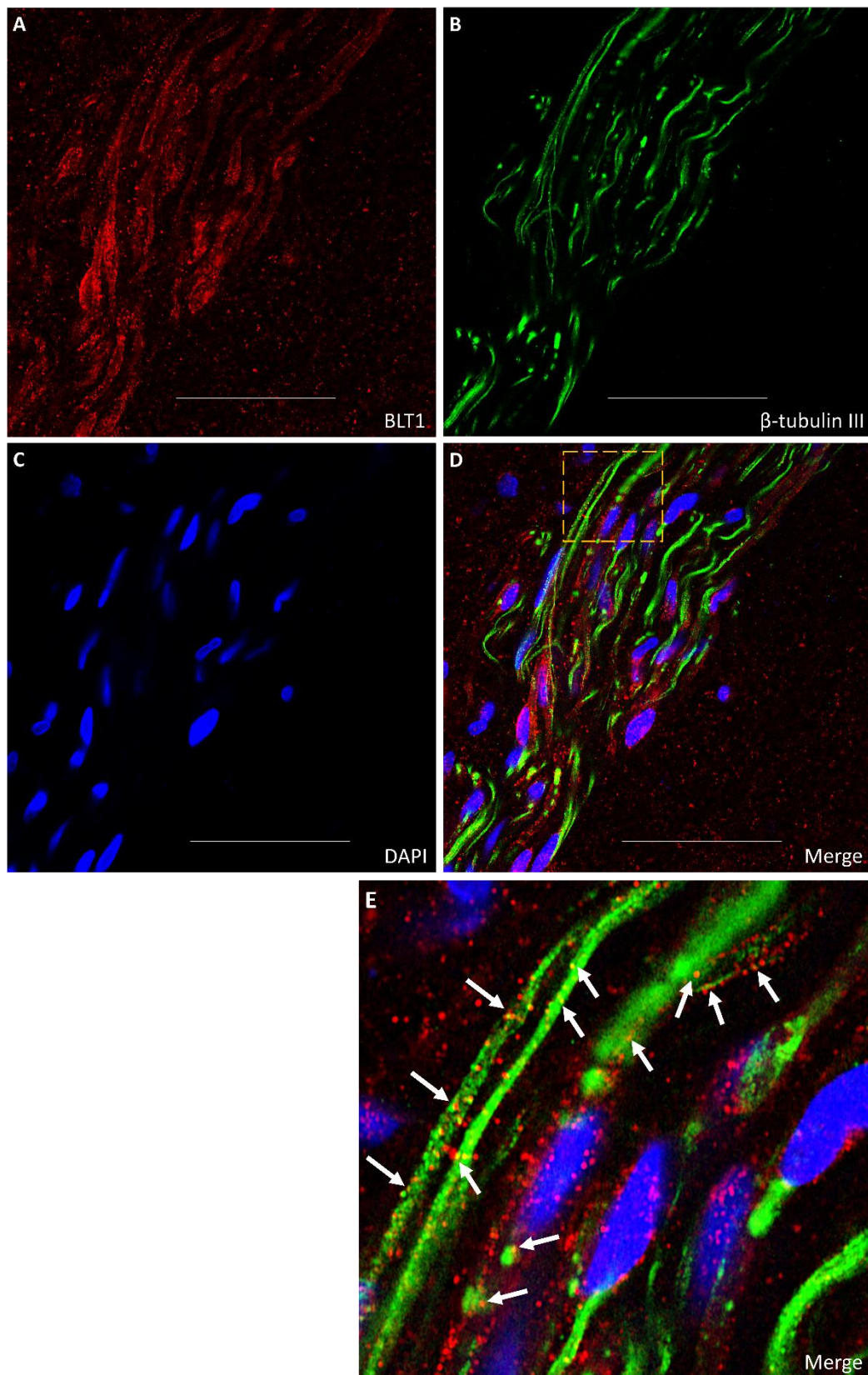


Figure 4.8. Confocal microscopy images showing an association between BLT1 and β -tubulin III in human tooth pulp tissues from carious painful teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing some colocalisation between BLT1 and β -tubulin III within a few fibres (marked with white arrows). Scale bar = 50 μ m and refers to A – D.

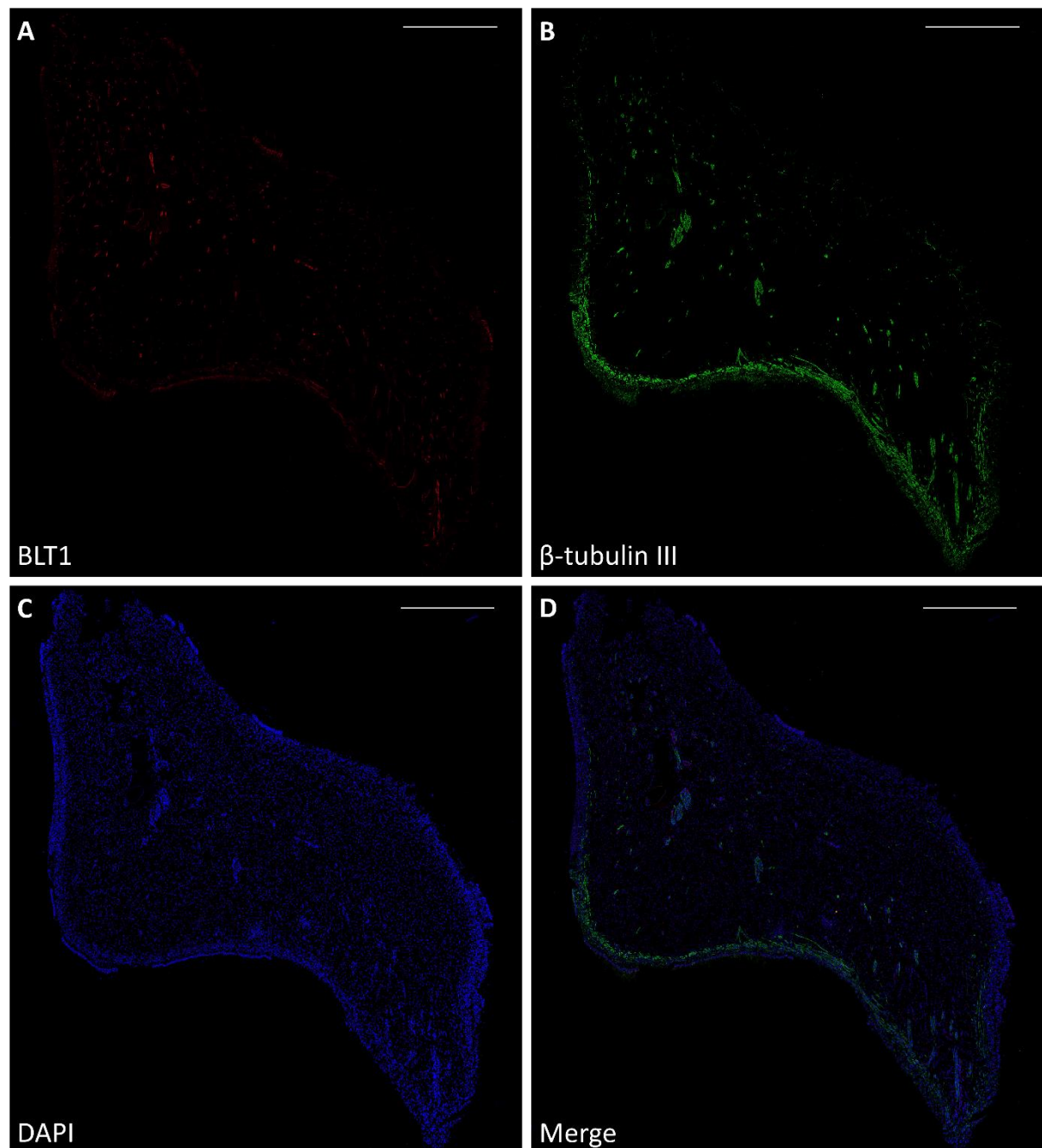


Figure 4.9. Whole section images showing BLT1 expression associated with nerve fibres in human tooth pulp tissues from carious non-painful teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

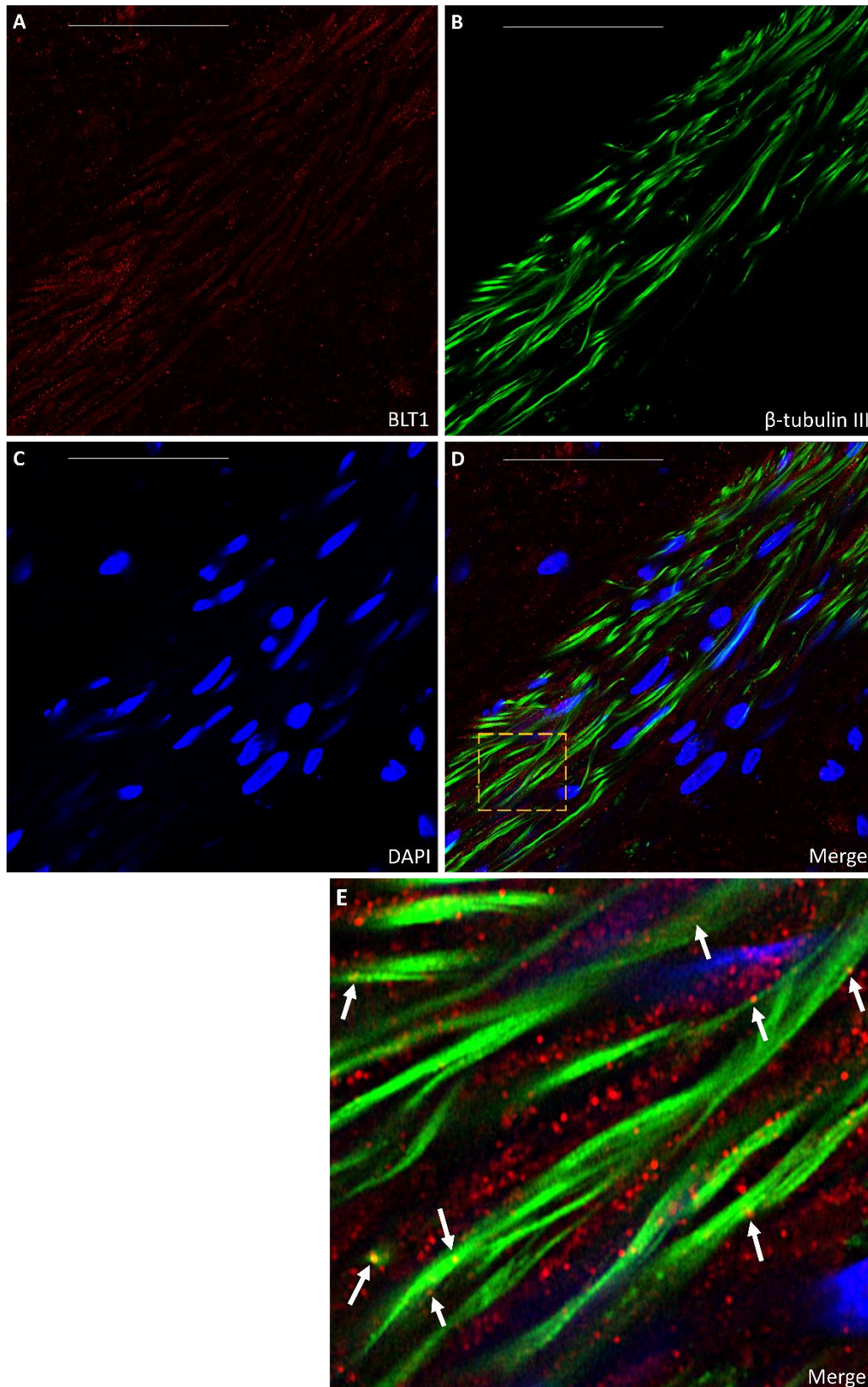


Figure 4.10. Confocal microscopy images showing an association between BLT1 and β -tubulin III in human tooth pulp tissues from carious non-painful teeth. (A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing some colocalisation between BLT1 and β -tubulin III within a few fibres (marked with white arrows). Scale bar = 50 μ m and refers to A – D.

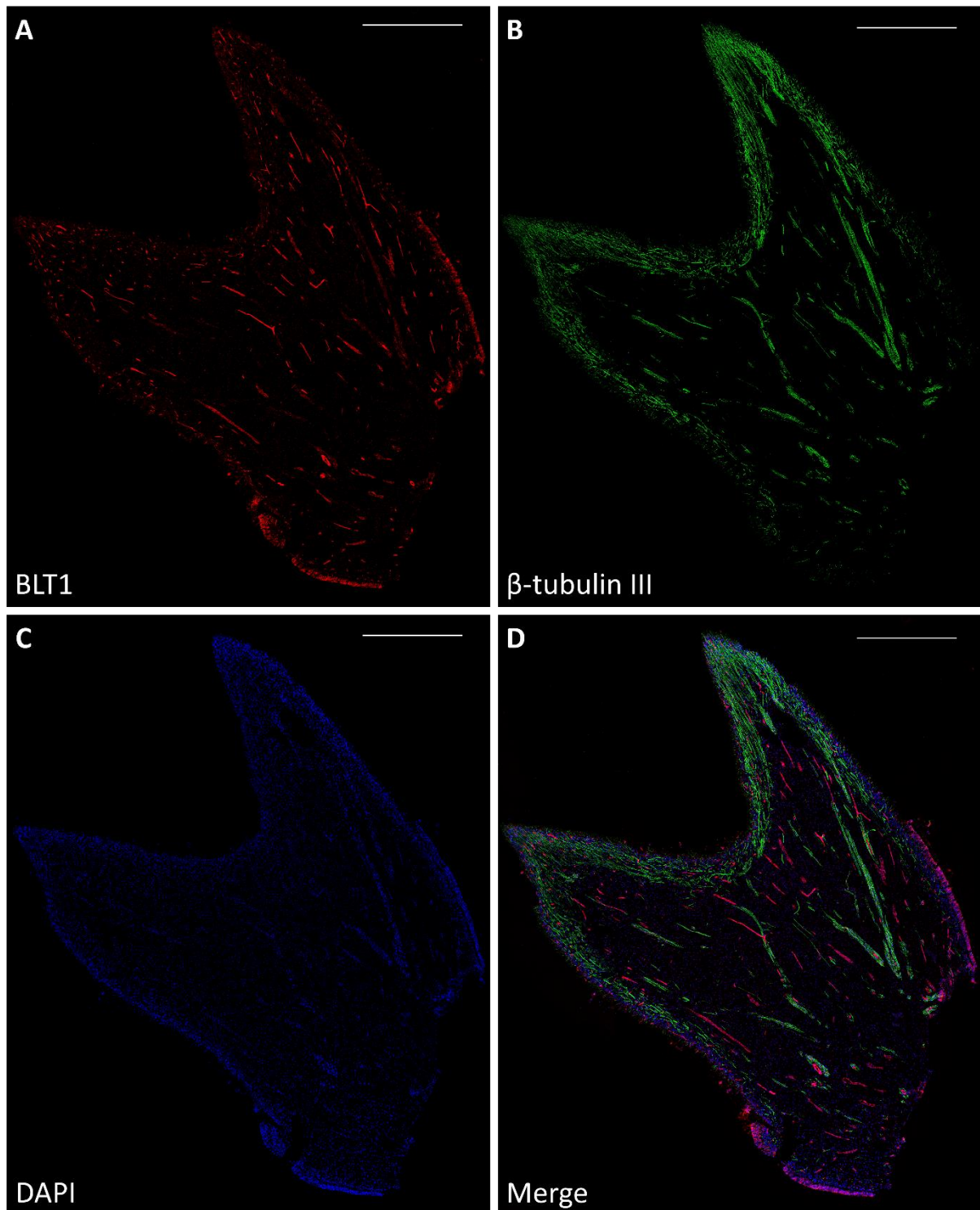


Figure 4.11. Whole section images showing BLT1 expression associated with nerve fibres in human tooth pulp tissues from non-carious teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

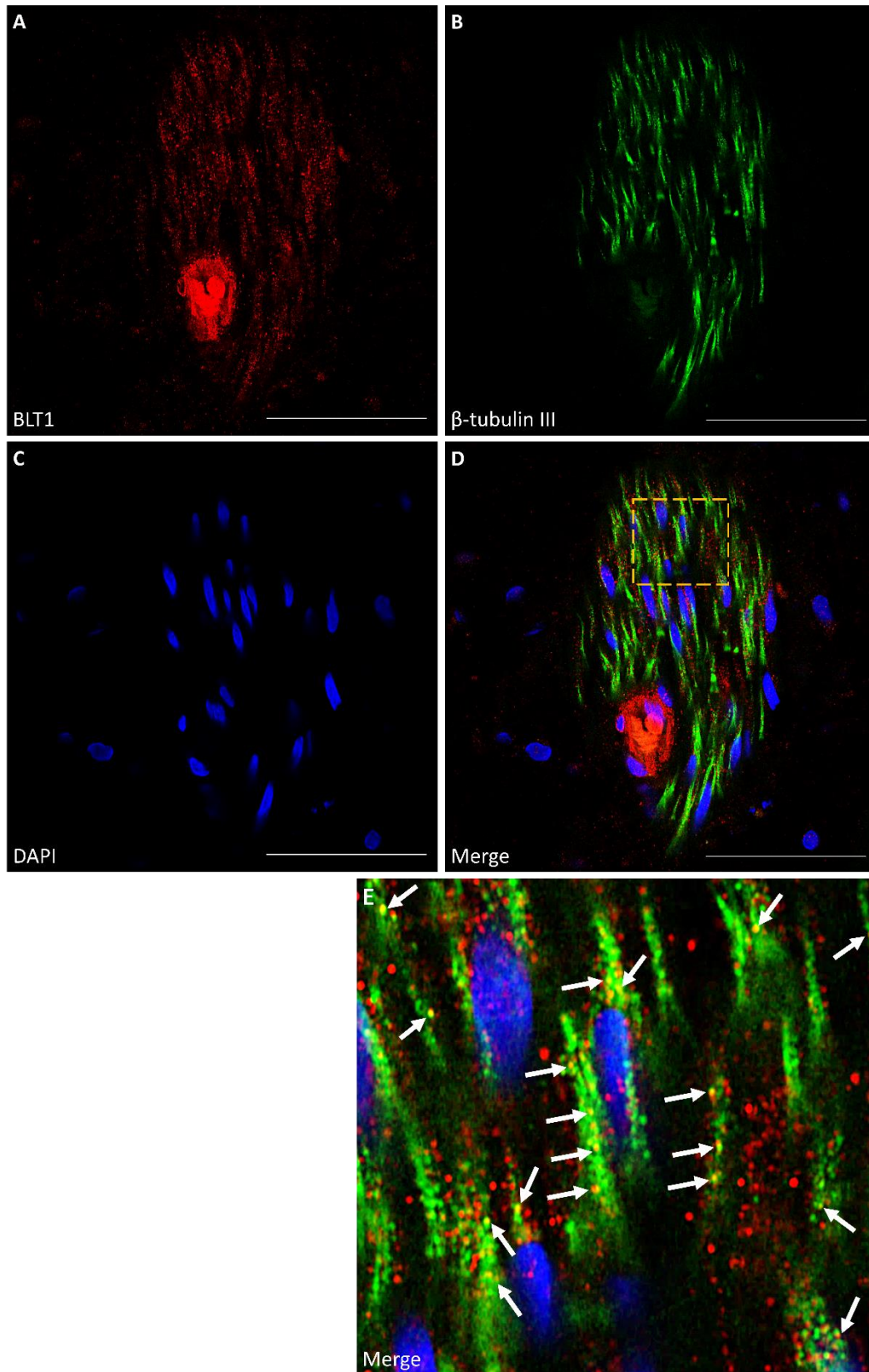


Figure 4.12. Confocal microscopy images showing an association between BLT1 and β -tubulin III in human tooth pulp tissues from non-carious teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing some colocalisation between BLT1 and β -tubulin III within a few fibres (marked with white arrows). Scale bar = 50 μ m and refers to A – D.

4.3.1.4. Quantification of BLT1 labelling associated with nerve fibres

BLT1 expression associated with nerve fibres in tissues from carious painful, carious non-painful, and non-carious teeth was analysed by quantifying the area of positive BLT1 and β -tubulin III staining in the whole tooth pulp sections, pulp horn and central region of the tooth pulp (quantification protocol is described in section 4.2). All analyses were conducted with the investigator blinded to the category of the tooth pulp samples to avoid investigator bias. The percentage ratio of BLT1 to β -tubulin III was calculated by the formula:

$$\% \text{ ratio} = \frac{\text{Area of BLT1 labelling}}{\text{Area of } \beta\text{-tubulin III labelling}} \times 100.$$

No significant difference in the area of positive staining or percentage ratio of BLT1/ β -tubulin III between the three groups of samples was found using ANOVA tests (Figure 4.13).

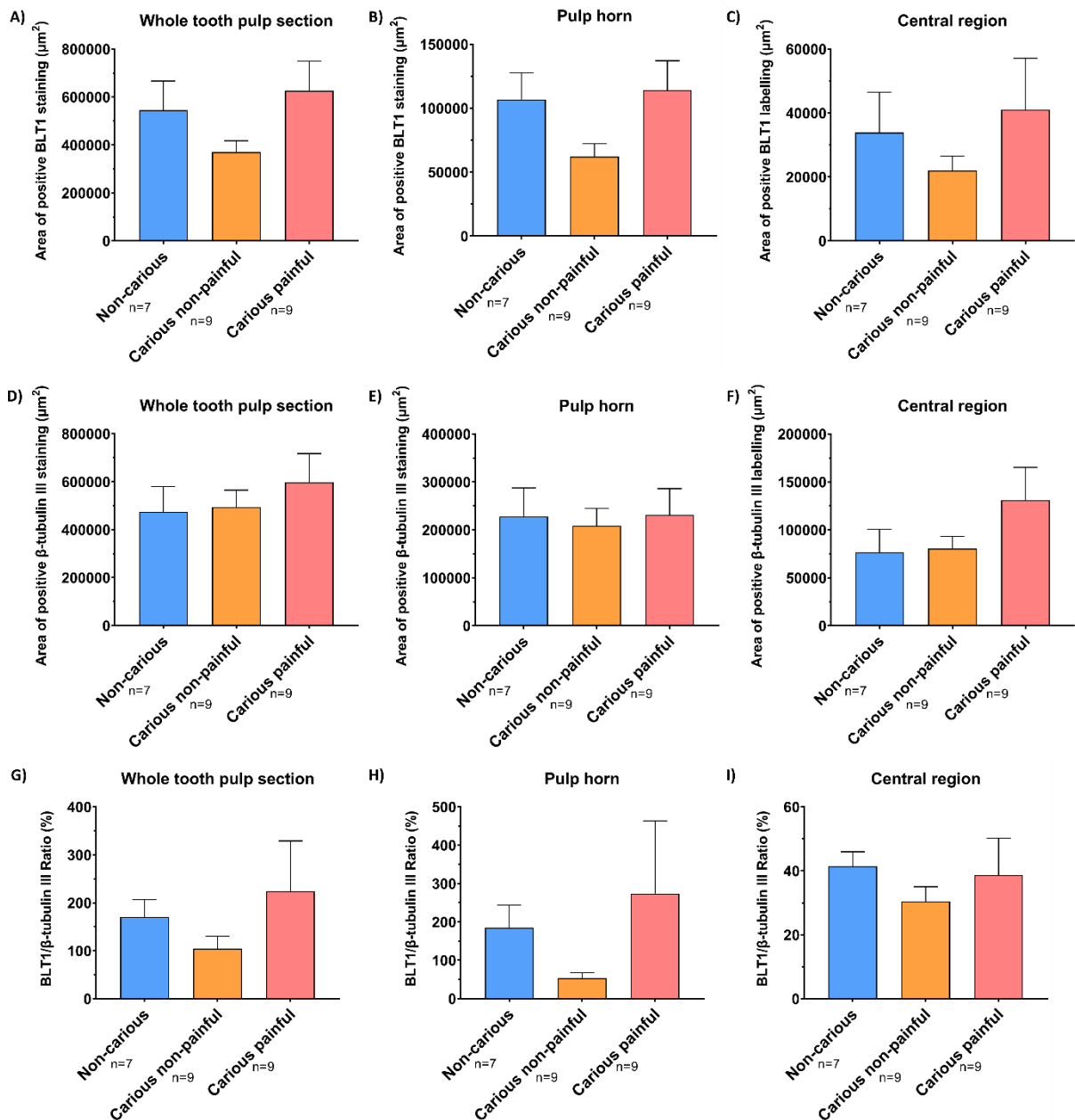


Figure 4.13. Quantification analysis of positive BLT1 and β -tubulin III staining in human tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth.

(A – C) Area of positive BLT1 staining in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. ANOVA tests (one-way ANOVA for whole tooth pulp section, Kruskal-Wallis ANOVA for pulp horn and central region) revealed no significant difference in the area of positive BLT1 staining between the three groups of samples. Error bars represent mean $\pm$ SEM.

(D – F) Area of positive β -tubulin III staining in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. ANOVA tests (one-way ANOVA for whole tooth pulp section, Kruskal-Wallis ANOVA for pulp horn and central region) revealed no significant difference in the area of positive β -tubulin III staining between the three groups of samples. Error bars represent mean $\pm$ SEM.

(G – I) Percentage ratio of BLT1/ β -tubulin III in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. ANOVA tests (Kruskal-Wallis ANOVA for whole tooth pulp section and pulp horn, Brown-Forsythe and Welch ANOVA for central region) revealed no significant difference in the % ratio of BLT1/ β -tubulin III between the three groups of samples. Error bars represent mean $\pm$ SEM.

4.3.1.5. Investigation of BLT1 expression in blood vessels

Since positive BLT1 staining was observed in cells that showed blood vessel-like morphology (Figure 4.5), the vascular smooth muscle cell marker α -SMA (Van der Loop et al., 1997), was used to investigate if BLT1 expression was found in the smooth muscle component of the blood vessels. BLT1 was shown to be expressed in vascular smooth muscle cells of blood vessels as there was colocalisation between BLT1 and α -SMA in human tooth pulp tissues (Figure 4.14). In the negative control where the FITC-conjugated α -SMA antibody was omitted, no positive staining was identified (Figures 4.14D).

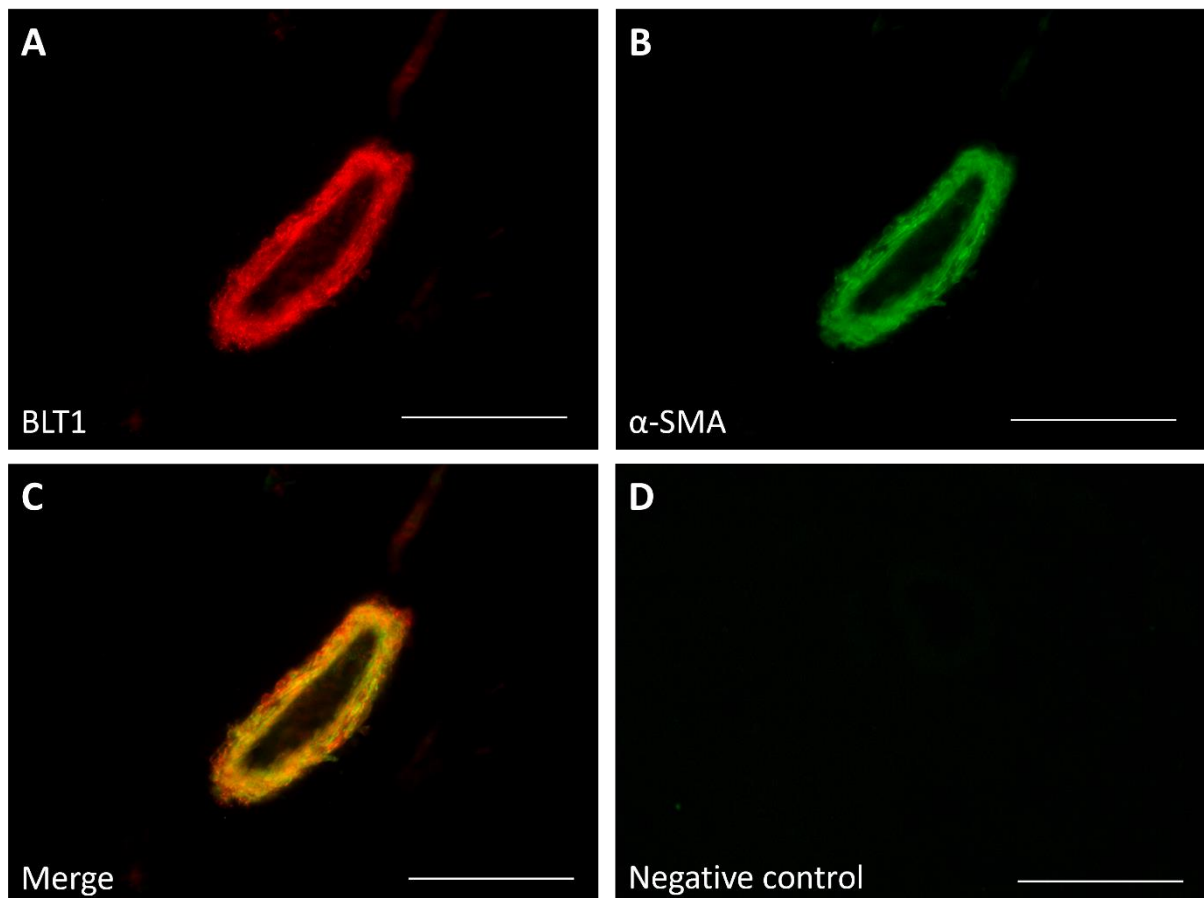


Figure 4.14. BLT1 expression in vascular smooth muscle cells of blood vessels in human tooth pulp.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against vascular smooth muscle cell marker α -SMA. (C) Dual labelling for BLT1 (red) and α -SMA (green). (D) Negative control by omitting the antibody against α -SMA. Scale bar = 100 μ m and refers to all images.

UEA I, a specific marker for human vascular endothelial cells (Roussel and Dalion, 1988) was then used to investigate if BLT1 expression was found in the endothelium of the blood vessels. Positive BLT1 staining overlapped with UEA I in the inner layer (endothelium) of blood vessels in human tooth pulp tissues (Figure 4.15). The specificity of UEA I was tested by using L-fucose to inhibit lectin binding, and the majority of the UEA I signal was eliminated (Figure 4.15D).

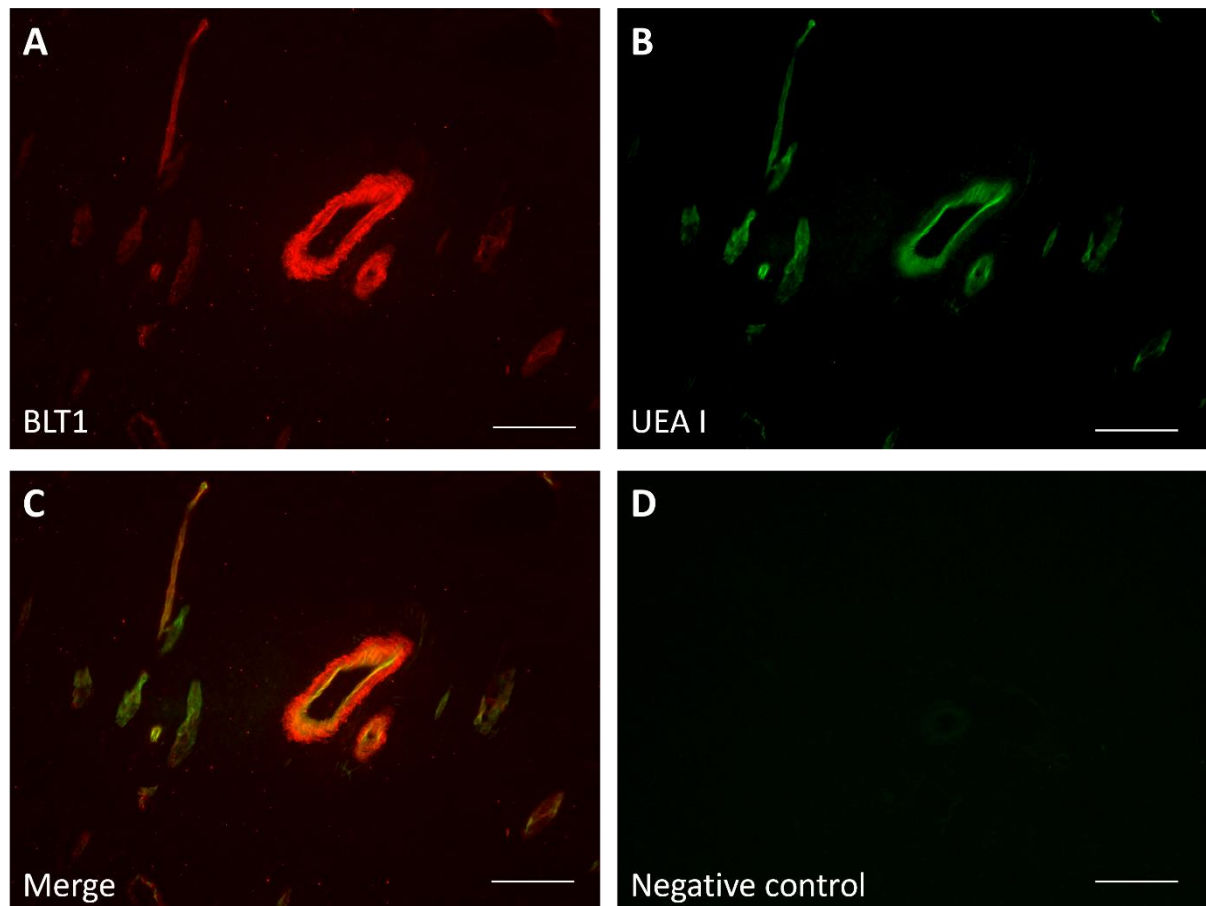


Figure 4.15. BLT1 expression in the vascular endothelium of blood vessels in human tooth pulp.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against endothelial cell marker UEA I. (C) Dual labelling for BLT1 (red) and UEA I (green). (D) UEA I pre-absorbed with L-fucose was used as a negative control for UEA I. Scale bar = 100 μ m and refers to all images.

4.3.1.6. Characterisation of BLT1 labelling in blood vessels

To characterise BLT1 expression in blood vessels in all three groups of human tooth pulp tissues, images were taken in areas rich in blood vessels from the pulp horn and central region of the tooth pulp from carious painful (Figure 4.16), carious non-painful (Figure 4.17), and non-carious teeth (Figure 4.18). BLT1 was dual labelled with smooth muscle cell marker α -SMA and endothelial cell marker UEA I to fully label the vasculature in the tooth pulp.

Interestingly, BLT1 labelling appeared to be weaker in some blood vessels (marked with yellow arrows in Figure 4.16 – 4.18) even though BLT1 was found in both vascular smooth muscle cells and endothelial cells in dual labelling studies.

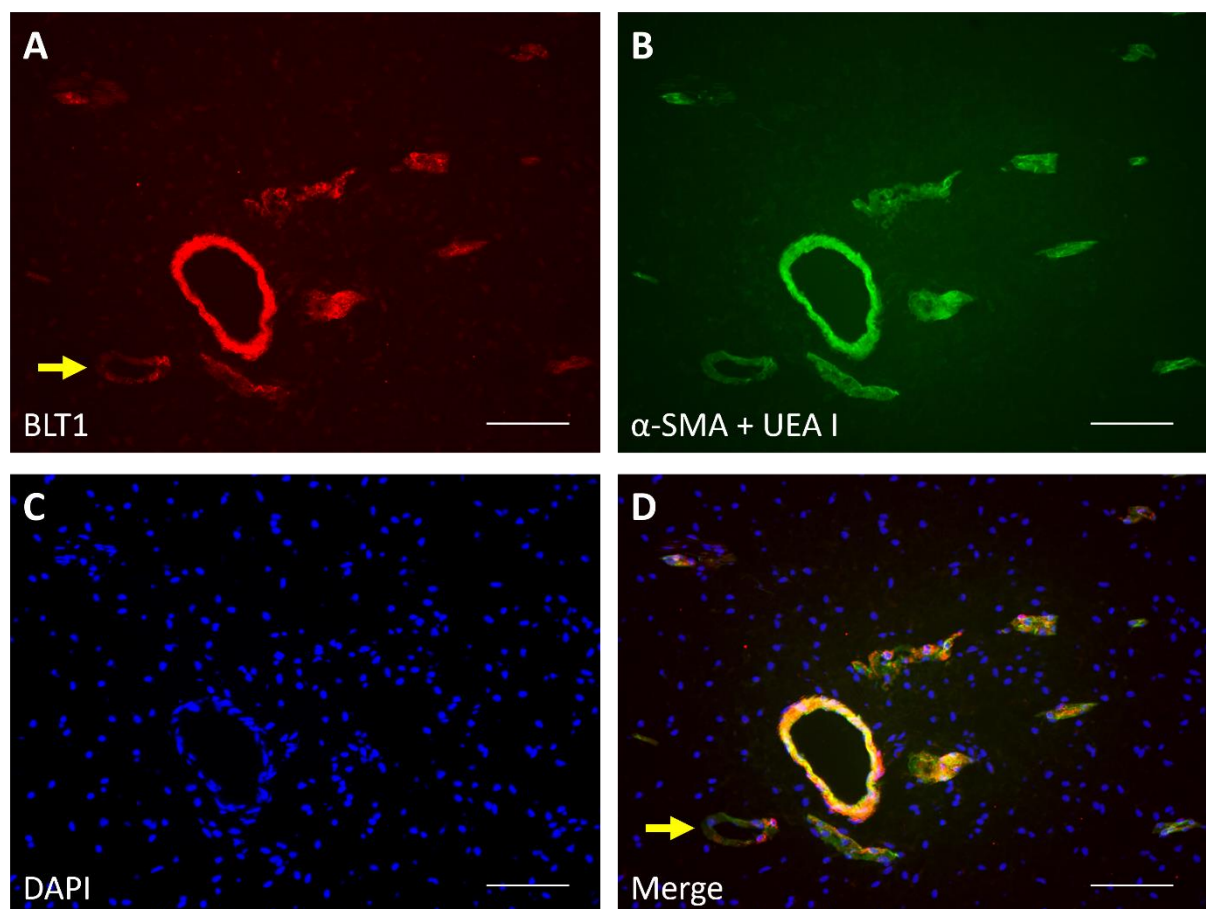


Figure 4.16. BLT1 expression in blood vessels in human tooth pulp tissues from carious painful teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against smooth muscle cell marker α -SMA and endothelial cell marker UEA I. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), α -SMA and UEA I (green), and DAPI (blue). Scale bar = 100 μ m and refers to all images.

* Blood vessel with lower intensity of BLT1 labelling is marked with a yellow arrow.

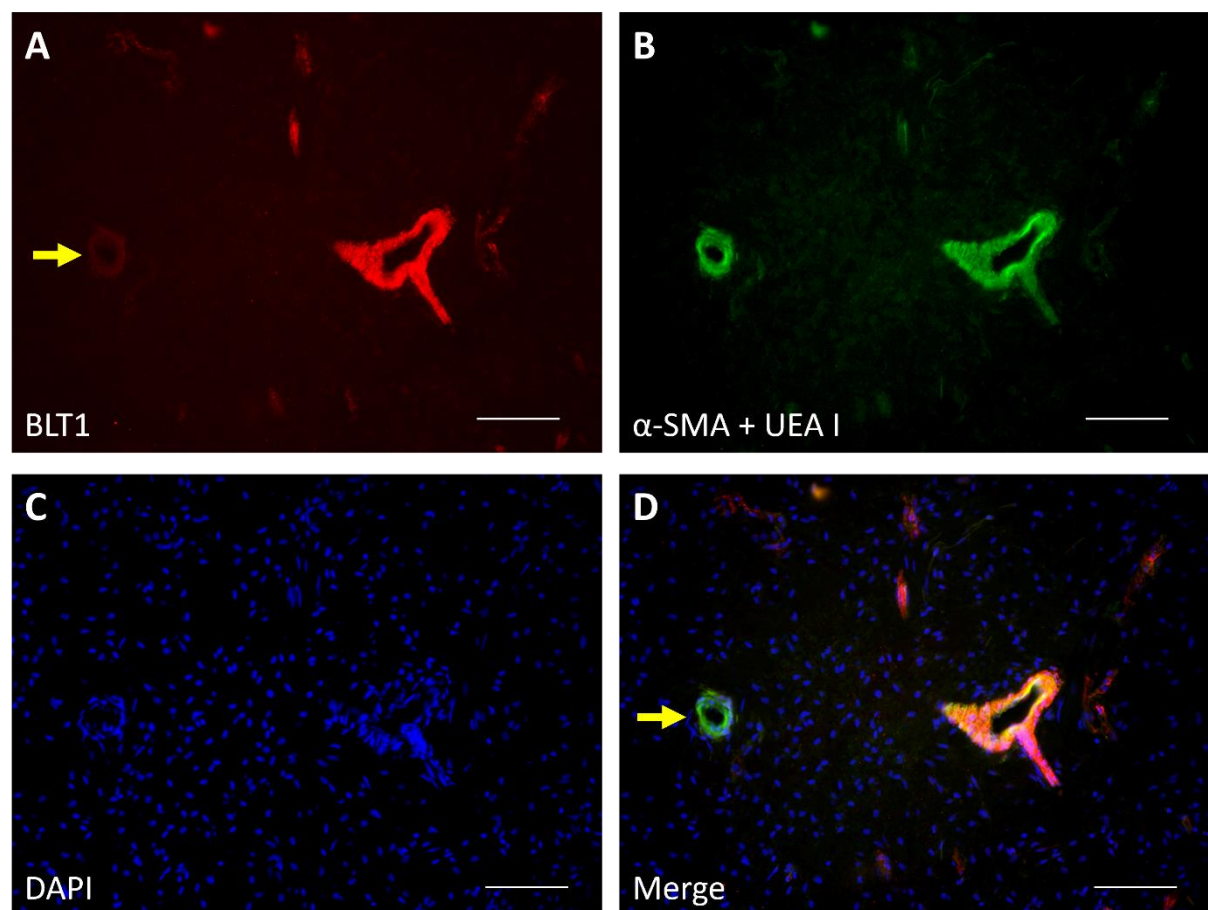


Figure 4.17. BLT1 expression in blood vessels in human tooth pulp tissues from carious non-painful teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against smooth muscle cell marker α -SMA and endothelial cell marker UEA I. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), α -SMA and UEA I (green), and DAPI (blue). Scale bar = 100 μ m and refers to all images.

* Blood vessels with lower intensity of BLT1 labelling are marked with yellow arrows.

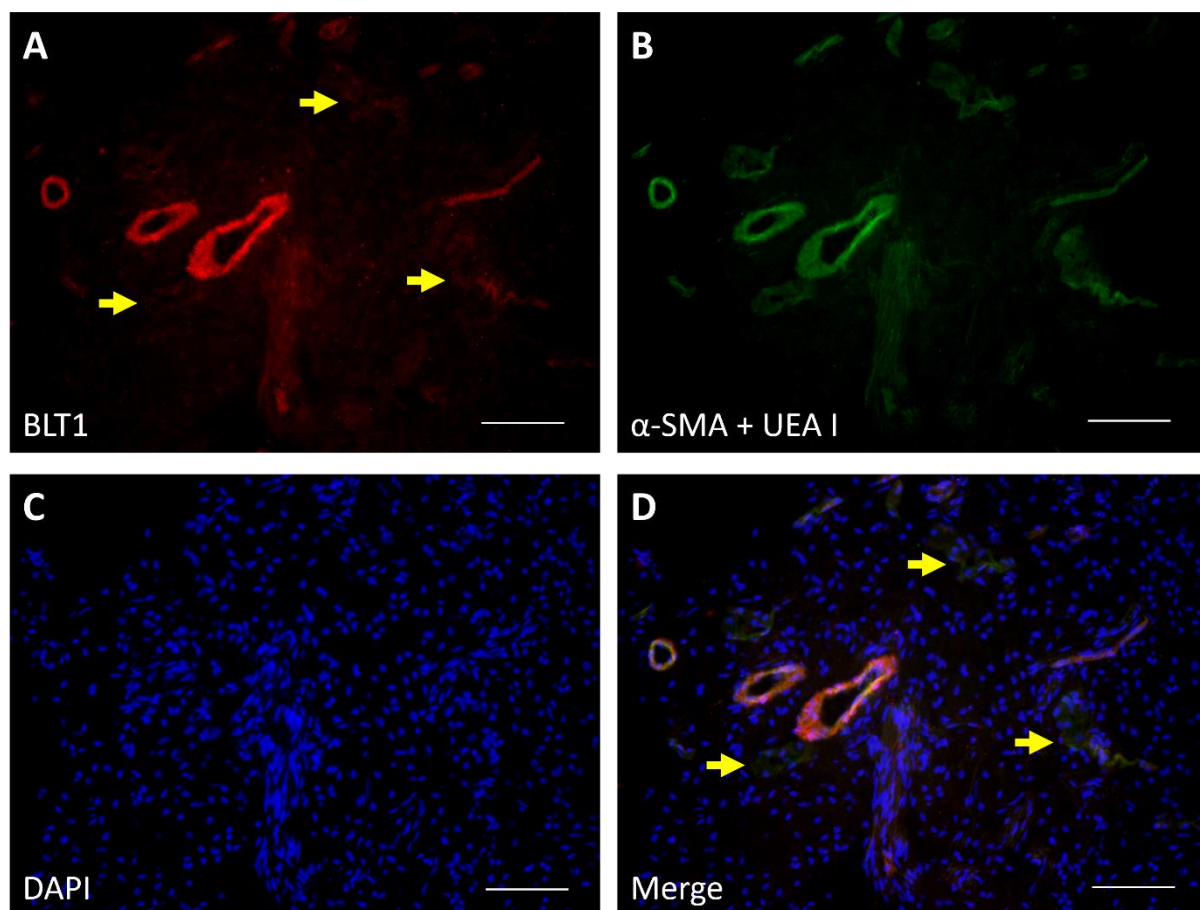


Figure 4.18. BLT1 expression in blood vessels in human tooth pulp tissues from non-carious teeth.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against smooth muscle cell marker α -SMA and endothelial cell marker UEA I. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), α -SMA and UEA I (green), and DAPI (blue). Scale bar = 100 μ m and refers to all images.

* Blood vessels with lower intensity of BLT1 labelling are marked with yellow arrows.

4.3.1.7. Quantification of BLT1 labelling in blood vessels

Images taken in areas rich in blood vessels from the pulp horn area and central region of tooth pulp tissues were selected for quantification. BLT1 expression in blood vessels in tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth was analysed by quantifying the area of positive BLT1 and FITC (α -SMA and UEA I) staining in the images selected. All analyses were conducted with the investigator blinded to the category of the tooth pulp samples to avoid investigator bias. The percentage ratio of BLT1 to FITC was calculated by the formula: $\% \text{ ratio} = \frac{\text{Area of BLT1 labelling}}{\text{Area of FITC labelling}} \times 100$.

In the central region of tooth pulp tissues, BLT1 expression in blood vessels in tissues from carious painful teeth was found to be significantly higher than that from carious non-painful teeth ($p=0.02$; one-way ANOVA with Tukey's post-hoc test). However, no significant difference was found in the area of positive FITC staining or percentage ratio of BLT1/FITC between the three groups of samples (Figure 4.19).

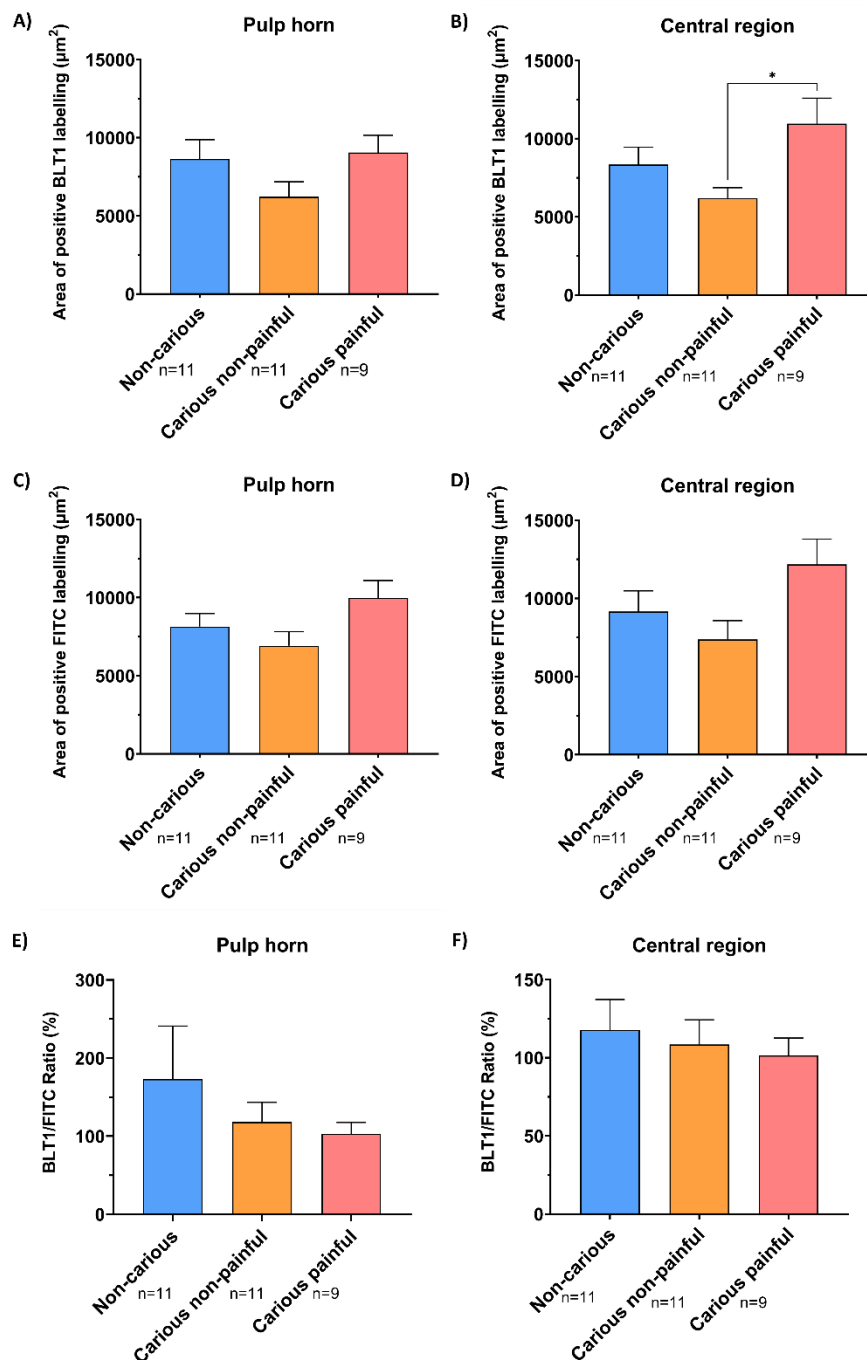


Figure 4.19. Quantification analysis of positive BLT1, α-SMA and UEA I staining in human tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth.

(A – B) Area of positive BLT1 staining in pulp horn (A) and central region (B) of the tooth pulp. One-way ANOVA followed by Tukey's post-hoc test revealed a significant difference in the area of positive BLT1 staining in the central region of the tooth pulp between tissues from carious painful teeth and carious non-painful teeth ($p=0.02^*$). Error bars represent mean $\pm$ SEM.

(C – D) Area of positive FITC (α-SMA and UEA I) staining in pulp horn (A) and central region (B) of the tooth pulp. Kruskal-Wallis ANOVA revealed no significant difference in the area of positive FITC staining between the three groups. Error bars represent mean $\pm$ SEM.

(E – F) Percentage ratio of BLT1/FITC (α-SMA and UEA I) in pulp horn (A) and central region (B) of the tooth pulp. ANOVA tests (Kruskal-Wallis ANOVA for pulp horn and one-way ANOVA for central region) revealed no significant difference in the % ratio of BLT1/FITC between the three groups. Error bars represent mean $\pm$ SEM.

4.3.2. Characterisation of ChemR23 expression in human tooth pulp tissues

Positive staining of ChemR23, another receptor for RvE1 and E2, was also found in human tooth pulp tissues, as indicated below in Figures 4.20 – 4.28. Positive labelling with the ChemR23 antibody was present in all sections from all samples investigated from all three groups of teeth. Labelling was present throughout the entire pulp (Figures 4.23A, 4.25A, and 4.27A), but appeared to be localised to specific structures. In the negative control where the ChemR23 antibody was omitted, no positive staining was identified (Figures 4.20D and 4.21D).

4.3.2.1. *Preliminary staining to identify structures with ChemR23*

ChemR23 expression was found in structures that have a similar morphology to nerve fibres (Figure 4.20) and blood vessels (Figure 4.21). ChemR23 labelling was also found in some cell nuclei (marked by white arrows in Figures 4.20 and 4.21). This nuclear labelling appeared largely restricted to the pulpal stroma and is largely absent in nuclei associated with nerve bundles or blood vessels.

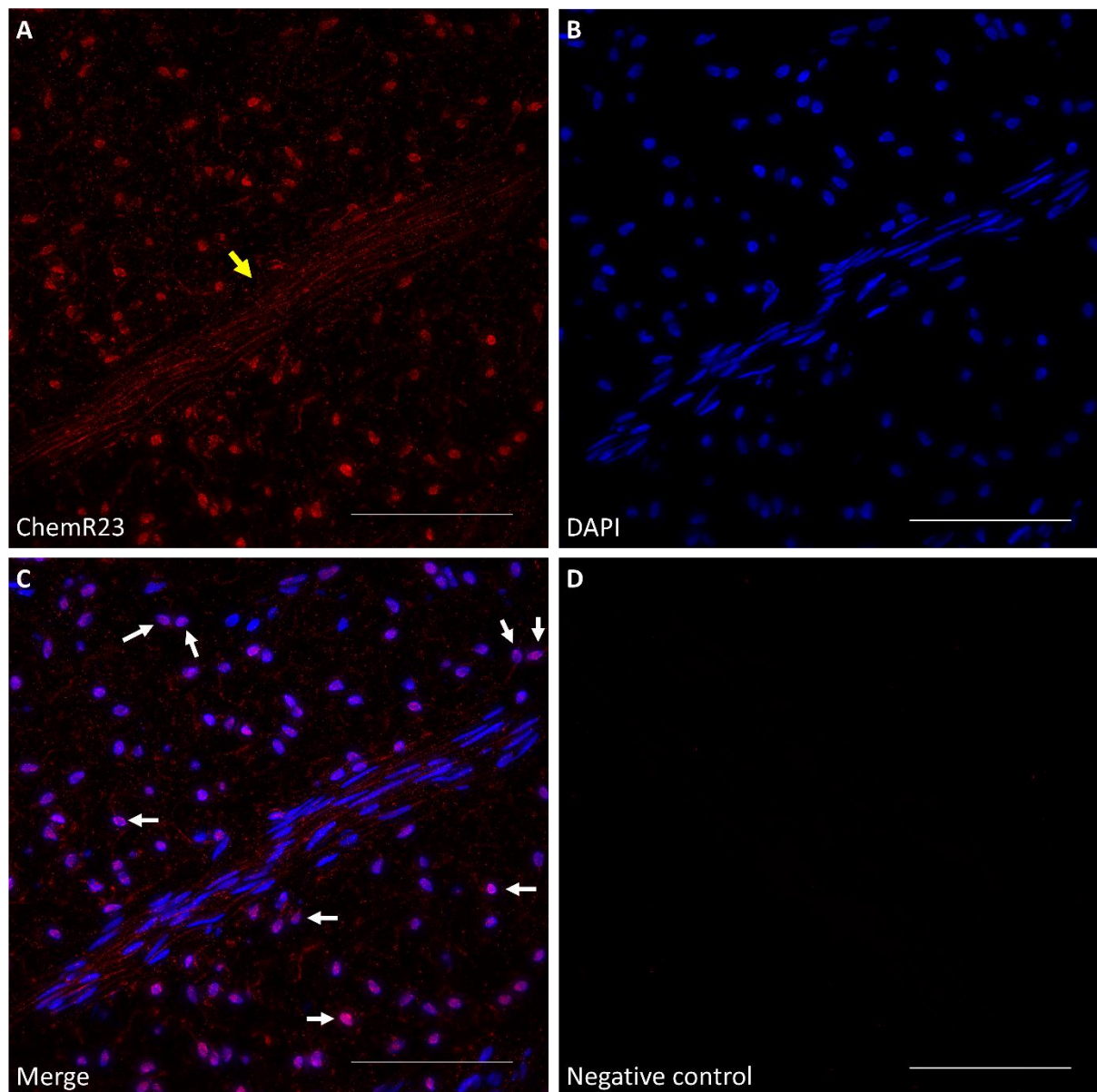


Figure 4.20. ChemR23 expression in structures with nerve fibre-like morphology.

(A) Positive staining with the antibody against ChemR23, showing ChemR23 expression in a structure with nerve fibre-like morphology (indicated by a yellow arrow) (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with ChemR23 (red) and DAPI (blue), showing ChemR23 labelling in some nuclei (marked by white arrows). (D) Negative control by omitting the antibody against ChemR23. Scale bar = 100 μ m and refers to all images.

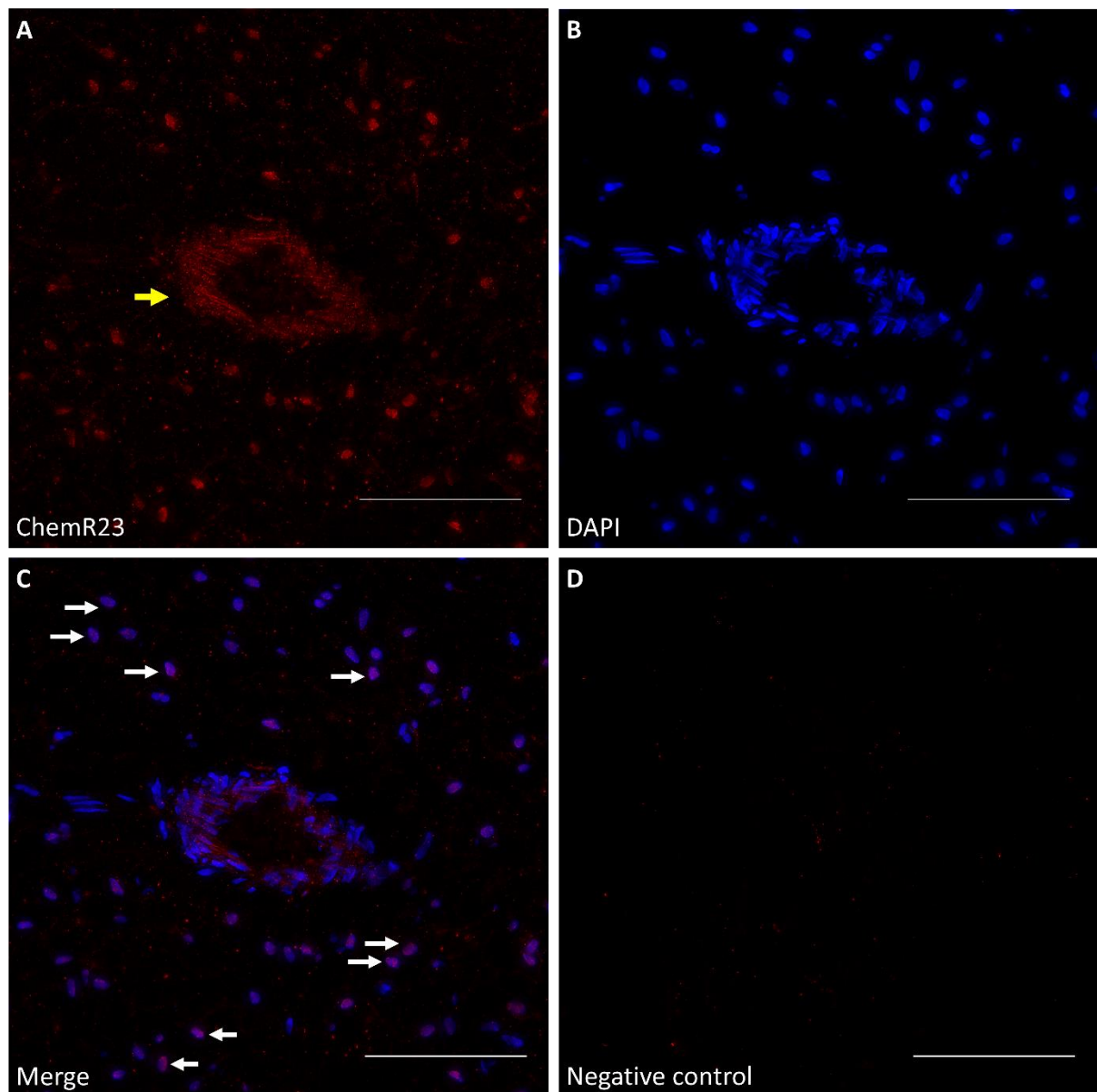


Figure 4.21. ChemR23 expression in structures with blood vessel-like morphology.

(A) Positive staining with the antibody against ChemR23, showing ChemR23 expression in a structure with blood vessel-like morphology (indicated by a yellow arrow). (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with ChemR23 (red) and DAPI (blue), showing ChemR23 labelling in some nuclei (marked by white arrows). (D) Negative control by omitting the antibody against ChemR23. Scale bar = 100 μm and refers to all images.

4.3.2.2. Investigation of ChemR23 expression associated with nerve fibres

Since ChemR23 appeared to be present in cells that showed nerve fibre-like morphology, dual labelling with neuronal marker β -tubulin III was conducted. ChemR23 labelling overlapped with β -tubulin III in a number of axons, indicating colocalisation in axons (marked by white arrows in Figure 4.22), which suggests that it is expressed in nerve fibres.

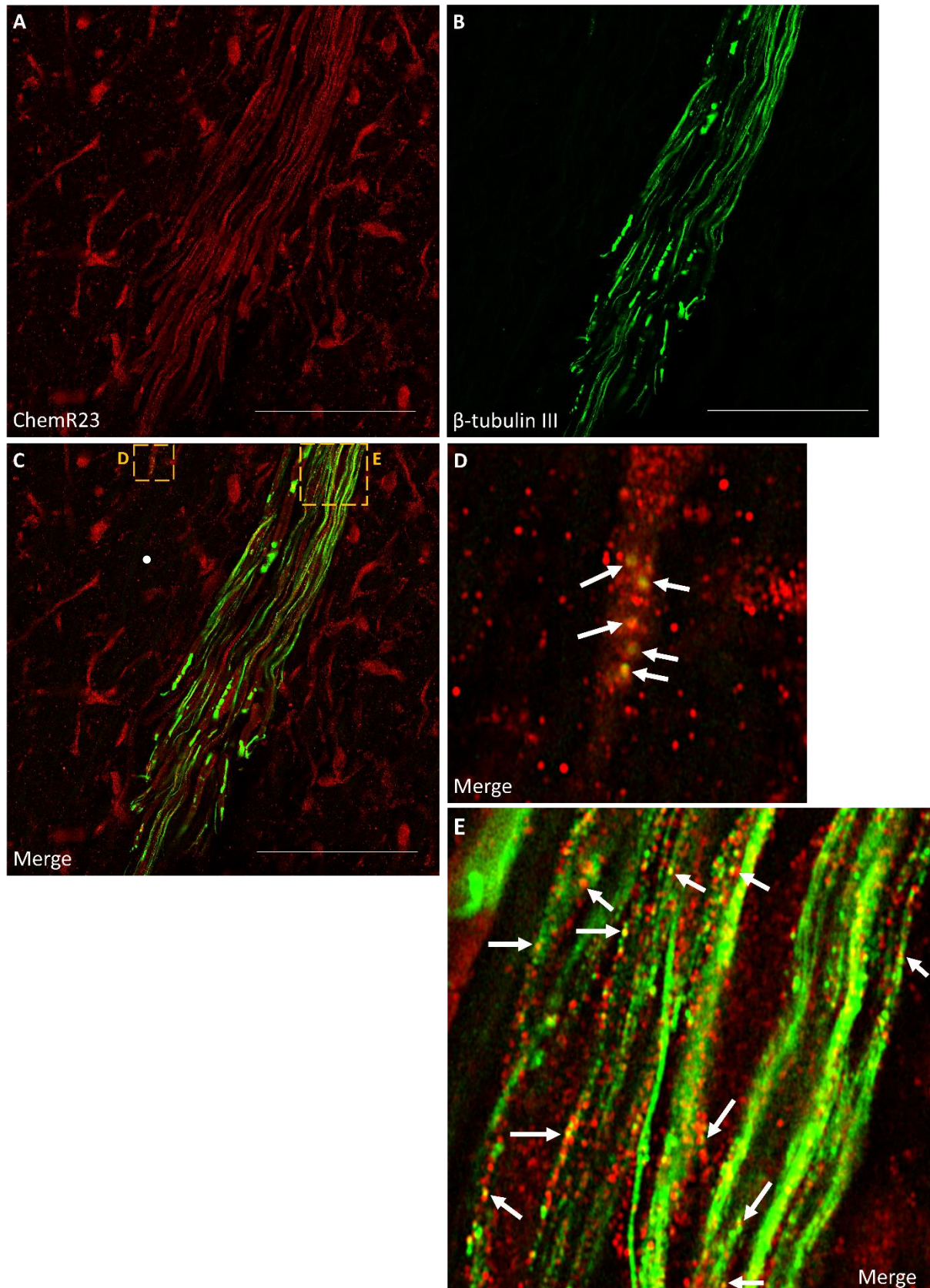


Figure 4.22. ChemR23 expression in nerve fibres in human tooth pulp.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Double staining with ChemR23 (red) and β -tubulin III (green). (D – E) Enlargement of the areas marked by the boxes in (C), showing colocalisation between ChemR23 and β -tubulin III in several axons (marked by white arrows). Scale bar = 50 μ m and refers to A – C.

4.3.2.3. *Characterisation of ChemR23 labelling associated with nerve fibres*

To characterise ChemR23 expression in nerve fibres in all three groups of human tooth pulp tissues, images of whole tooth pulp sections were taken from tooth pulp samples from carious painful (Figure 4.23), carious non-painful (Figure 4.25), and non-carious teeth (Figure 4.27) using ChemR23 antibody and neuronal marker β -tubulin III. These images show that ChemR23 and β -tubulin III were present throughout the entire pulp.

Therefore, confocal imaging was used to examine the association between ChemR23 and β -tubulin III expression in all three groups of human tooth pulp tissues by acquiring images of nerve bundles. These images show that ChemR23 labelling overlapped with β -tubulin III in several axons (indicated by white arrows in Figures 4.24E, 4.26E, and 4.28E).

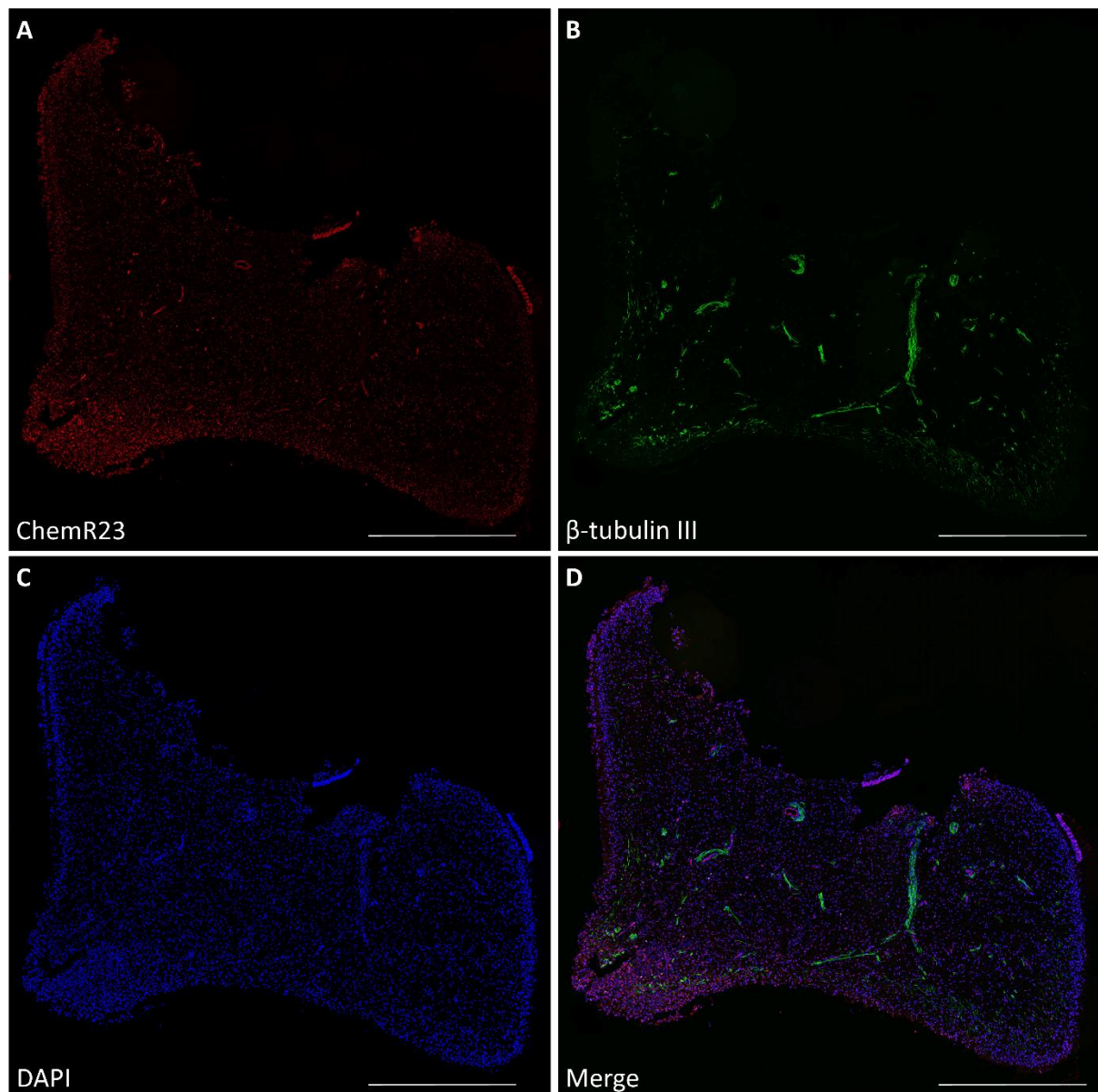


Figure 4.23. Whole section images showing ChemR23 expression associated with nerve fibres in human tooth pulp tissues from carious painful teeth.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

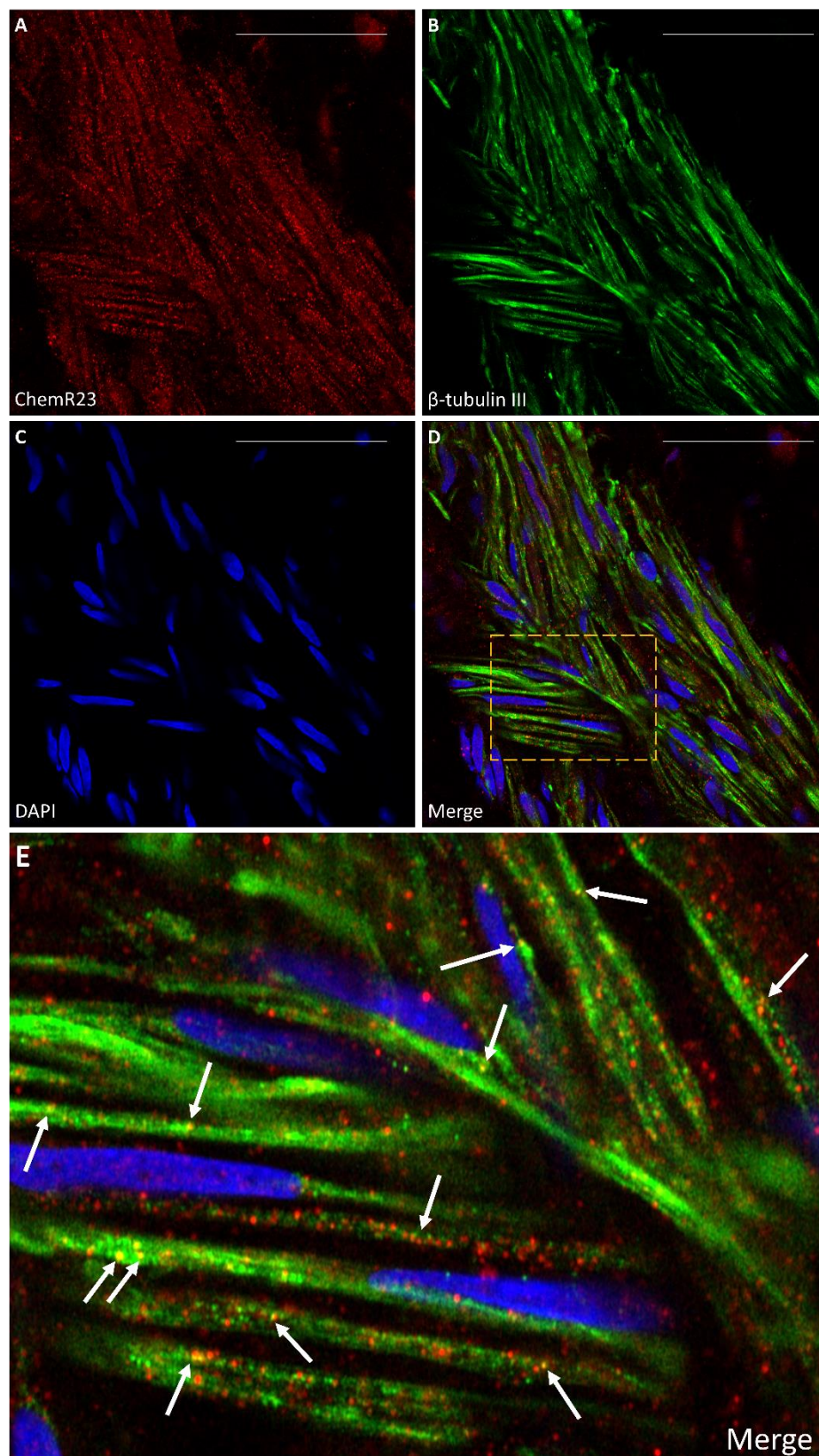


Figure 4.24. Confocal microscopy images showing an association between ChemR23 and β -tubulin III in human tooth pulp tissues from carious painful teeth.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing colocalisation between ChemR23 and β -tubulin III in several axons (marked by white arrows). Scale bar = 50 μ m and refers to A – D.

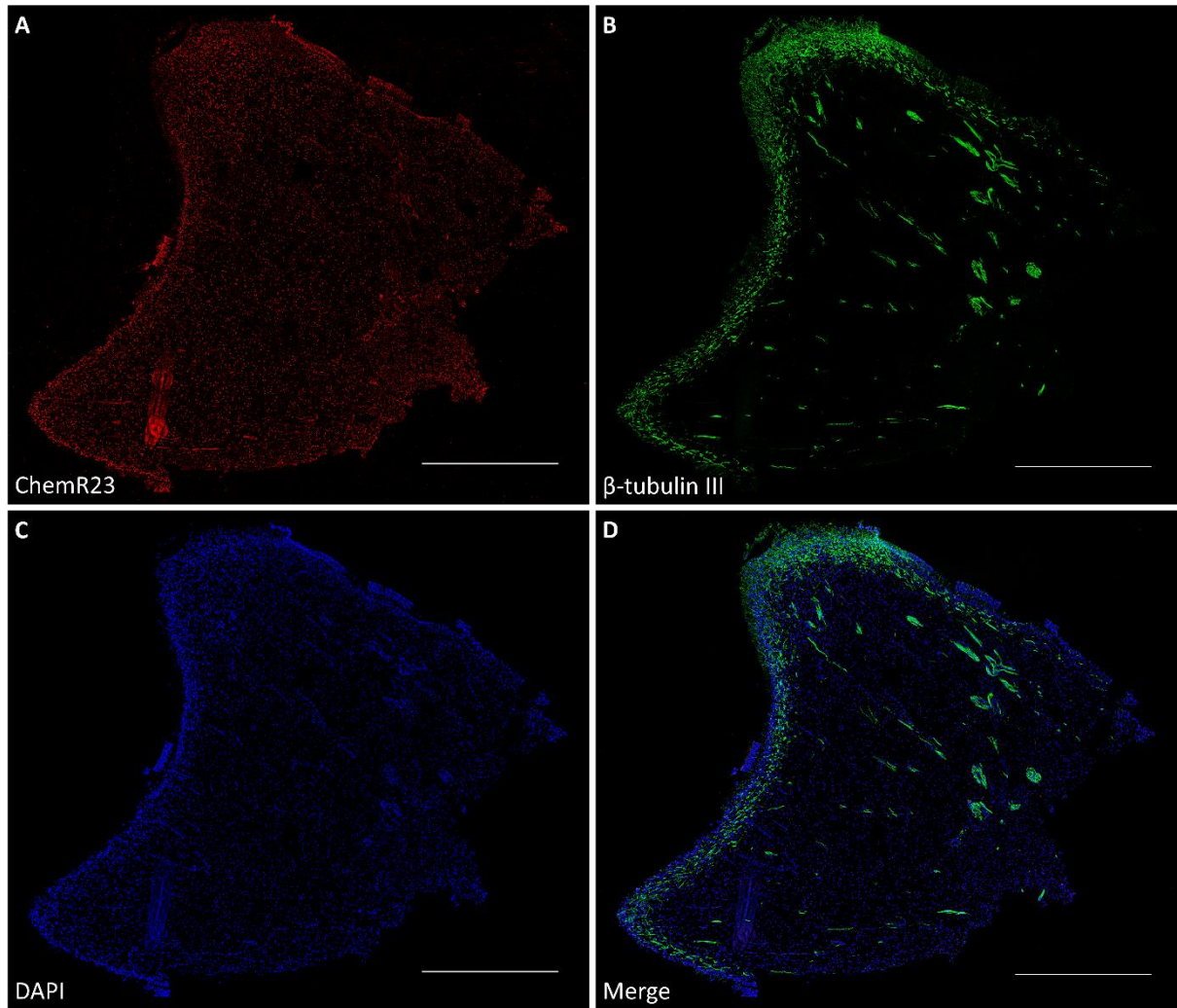


Figure 4.25. Whole section images showing ChemR23 expression associated with nerve fibres in human tooth pulp tissues from carious non-painful teeth.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

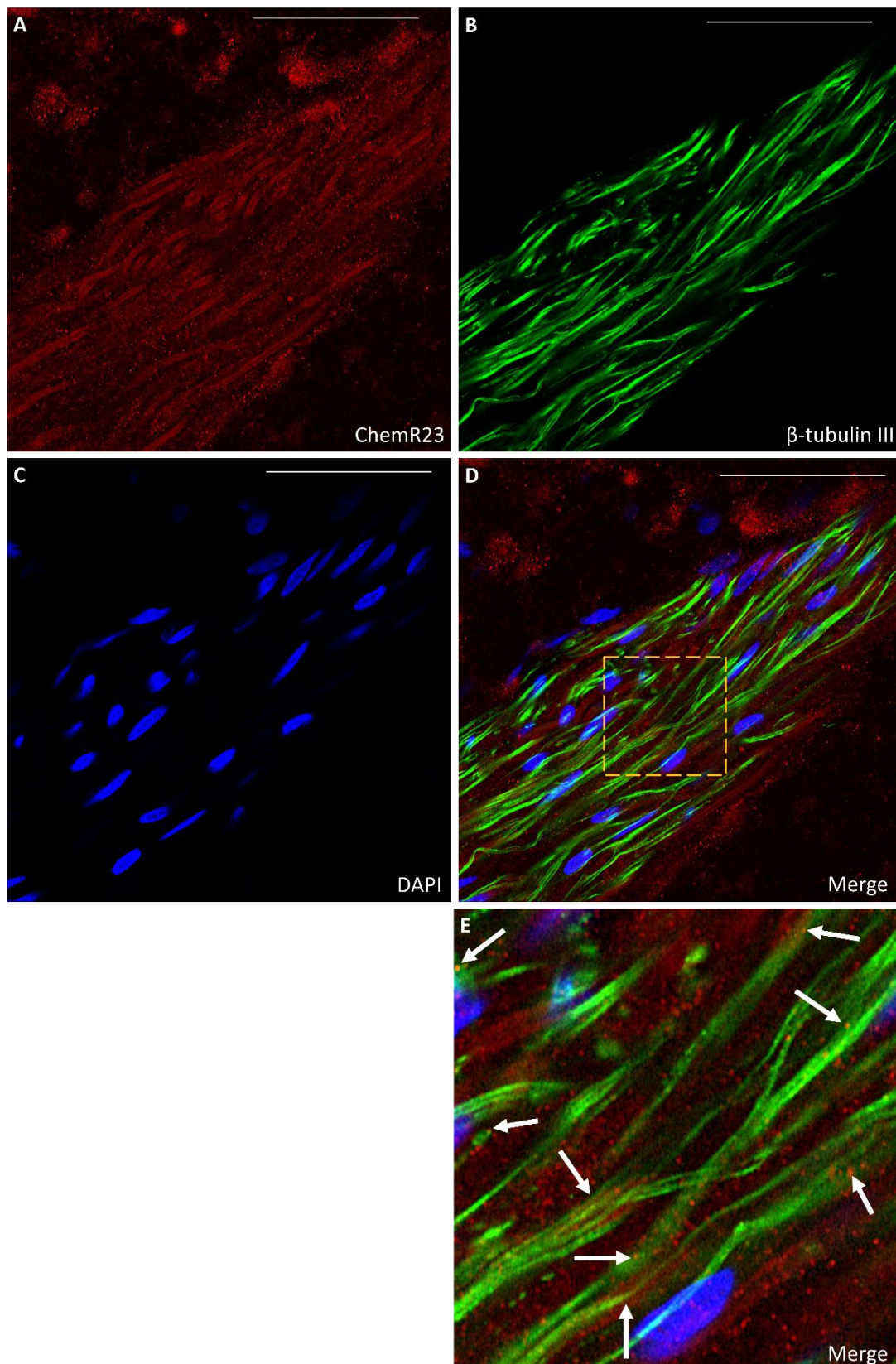


Figure 4.26. Confocal microscopy images showing an association between ChemR23 and β -tubulin III in human tooth pulp tissues from carious non-painful teeth. (A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing colocalisation between ChemR23 and β -tubulin III in several axons (marked by white arrows). Scale bar = 50 μ m and refers to A – D.

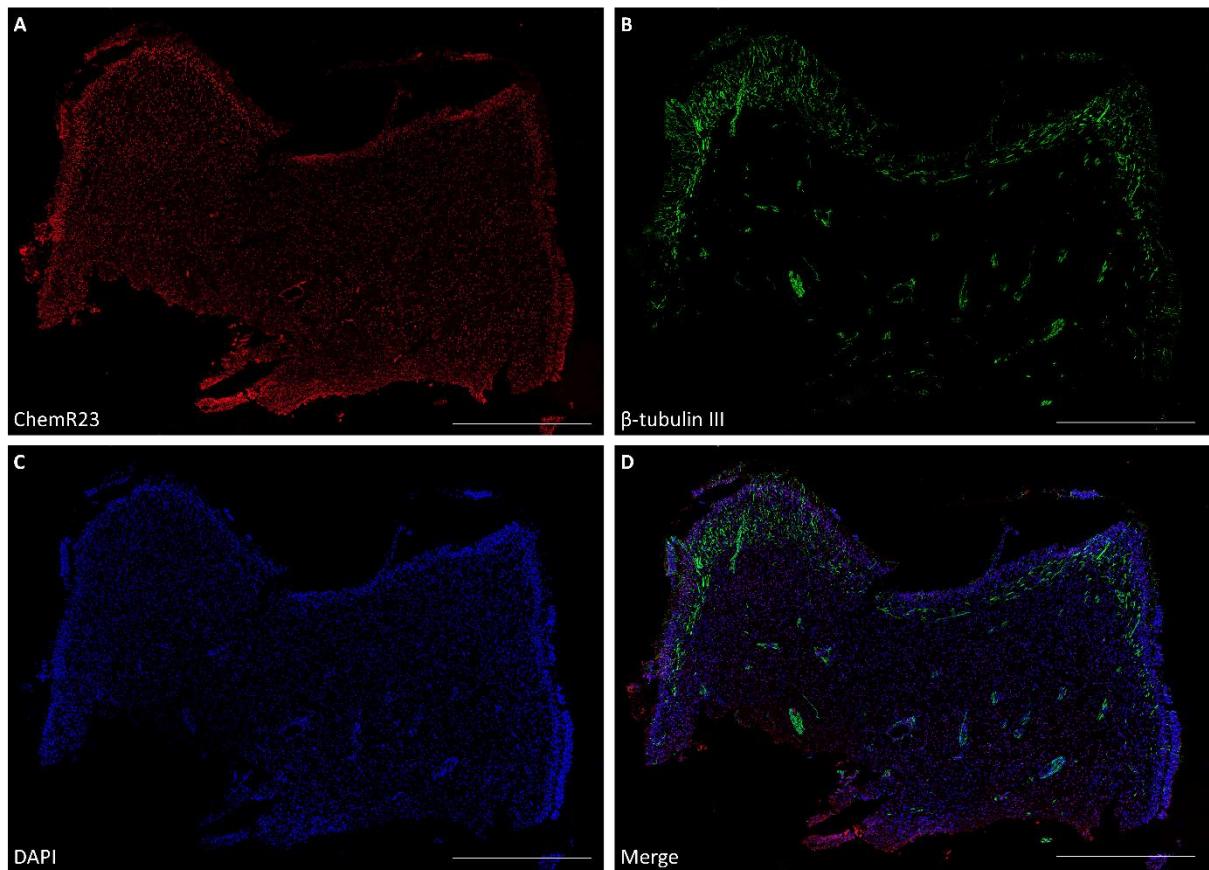


Figure 4.27. Whole section images showing ChemR23 expression associated with nerve fibres in human tooth pulp tissues from non-carious teeth.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). Scale bar = 1000 μ m and refers to all images.

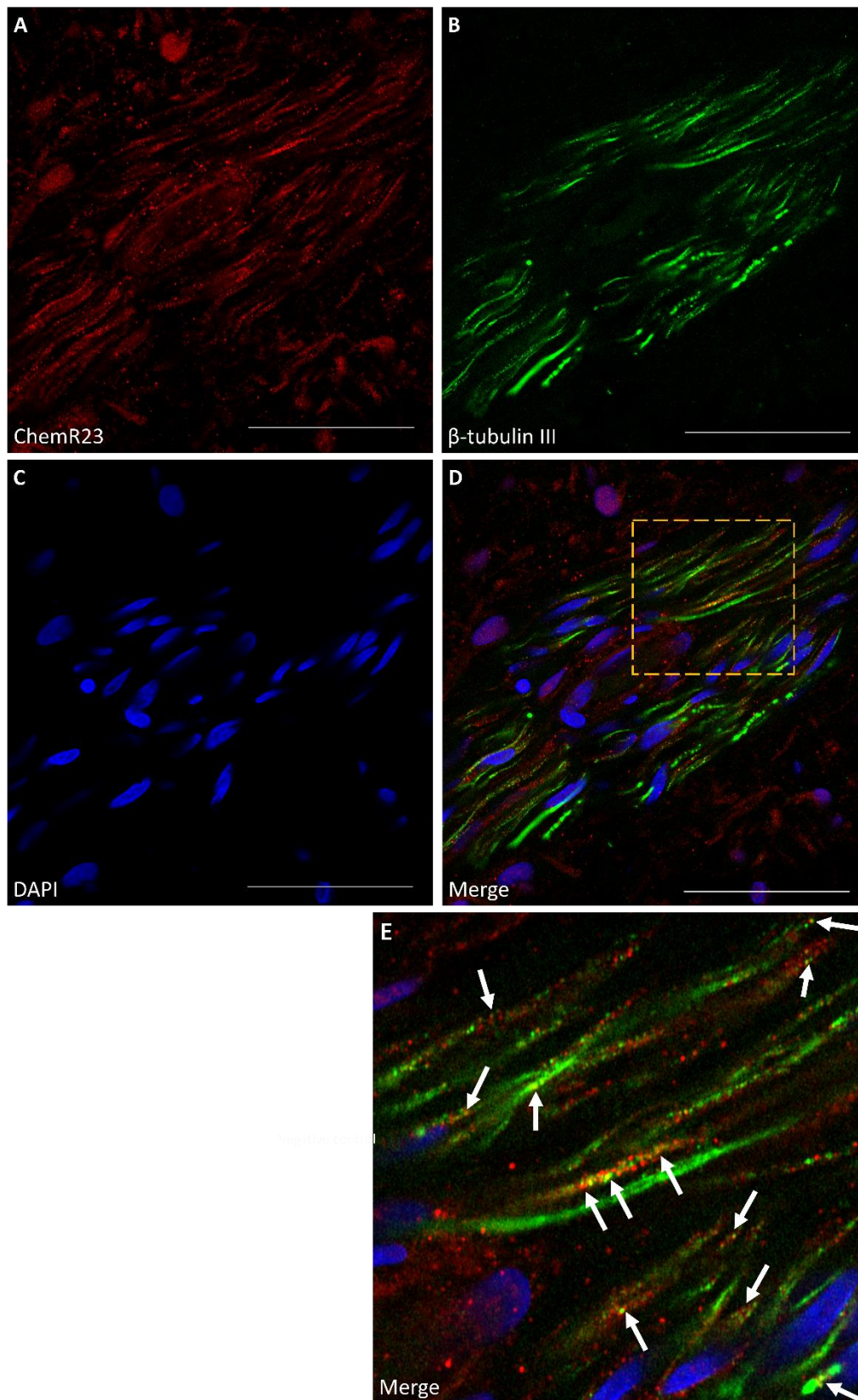


Figure 4.28. Confocal microscopy images showing an association between ChemR23 and β -tubulin III in human tooth pulp tissues from non-carious teeth.

(A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for ChemR23 (red), β -tubulin III (green), and DAPI (blue). (E) Enlargement of the area marked by the box in (D), showing colocalisation between ChemR23 and β -tubulin III in several axons (marked by white arrows). Scale bar = 50 μ m and refers to A – D.

4.3.2.4. Quantification of ChemR23 labelling associated with nerve fibres

ChemR23 expression in nerve fibres in tissues from carious painful, carious non-painful, and non-carious teeth was analysed by quantifying the area of positive ChemR23 and β -tubulin III staining in the whole tooth pulp sections, pulp horn and central region of the tooth pulp (quantification protocol is described in section 4.2). All analyses were conducted with the investigator blinded to the category of the tooth pulp samples to avoid investigator bias. The percentage ratio of ChemR23 to β -tubulin III was calculated by the formula:

$$\% \text{ ratio} = \frac{\text{Area of ChemR23 labelling}}{\text{Area of } \beta\text{-tubulin III labelling}} \times 100.$$

No significant difference in the area of positive staining or percentage ratio of ChemR23/ β -tubulin III between the three groups of samples was found using ANOVA tests (Figure 4.29).

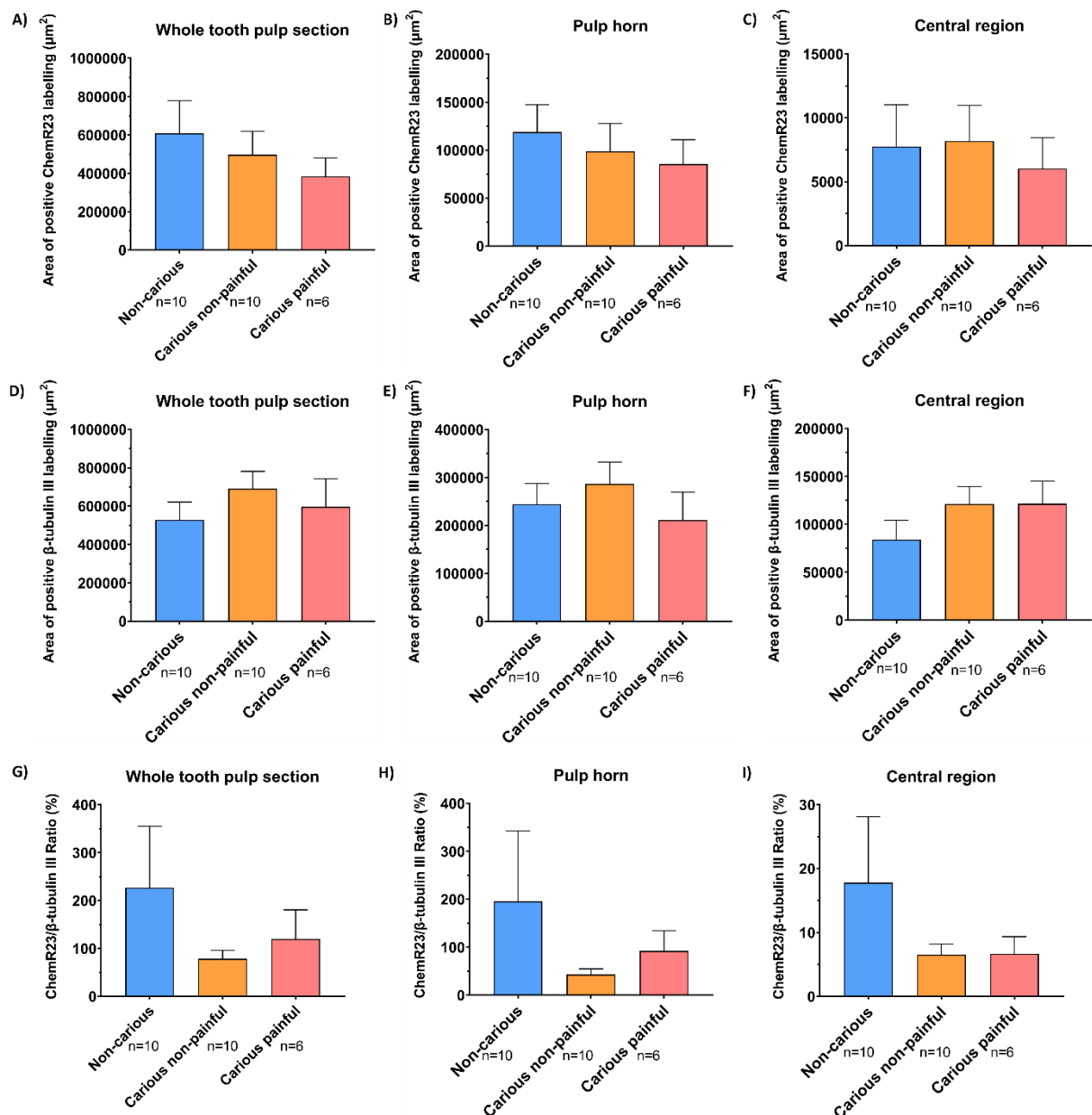


Figure 4.29. Quantification analysis of positive ChemR23 and β -tubulin III staining in human tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth.

(A – C) Area of positive ChemR23 staining in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. Kruskal-Wallis ANOVA revealed no significant difference in the area of positive ChemR23 staining between the three groups. Error bars represent mean $\pm$ SEM.

(D – F) Area of positive β -tubulin III staining in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. One-way ANOVA revealed no significant difference in the area of positive β -tubulin III staining between the three groups. Error bars represent mean $\pm$ SEM.

(G – I) Percentage ratio of ChemR23/ β -tubulin III in whole tooth pulp section (A), pulp horn (B) and central region (C) of the tooth pulp. Kruskal-Wallis ANOVA revealed no significant difference in the % ratio of ChemR23/ β -tubulin III between the three groups. Error bars represent mean $\pm$ SEM.

4.3.2.5. ChemR23 labelling in putative blood vessels and nuclei

As described above in section 4.3.2.1, ChemR23 is found in structures with similar morphology to blood vessels and stromal cells. However, ChemR23 expression in these structures was not explored further with dual labelling due to time constraints.

Interestingly, in some blood vessels, ChemR23 labelling appeared to be found in cells with similar morphology to endothelial cells (indicated by a yellow arrow in Figure 4.30), as ChemR23 labelling appeared to be largely associated with the endothelium in this blood vessel.

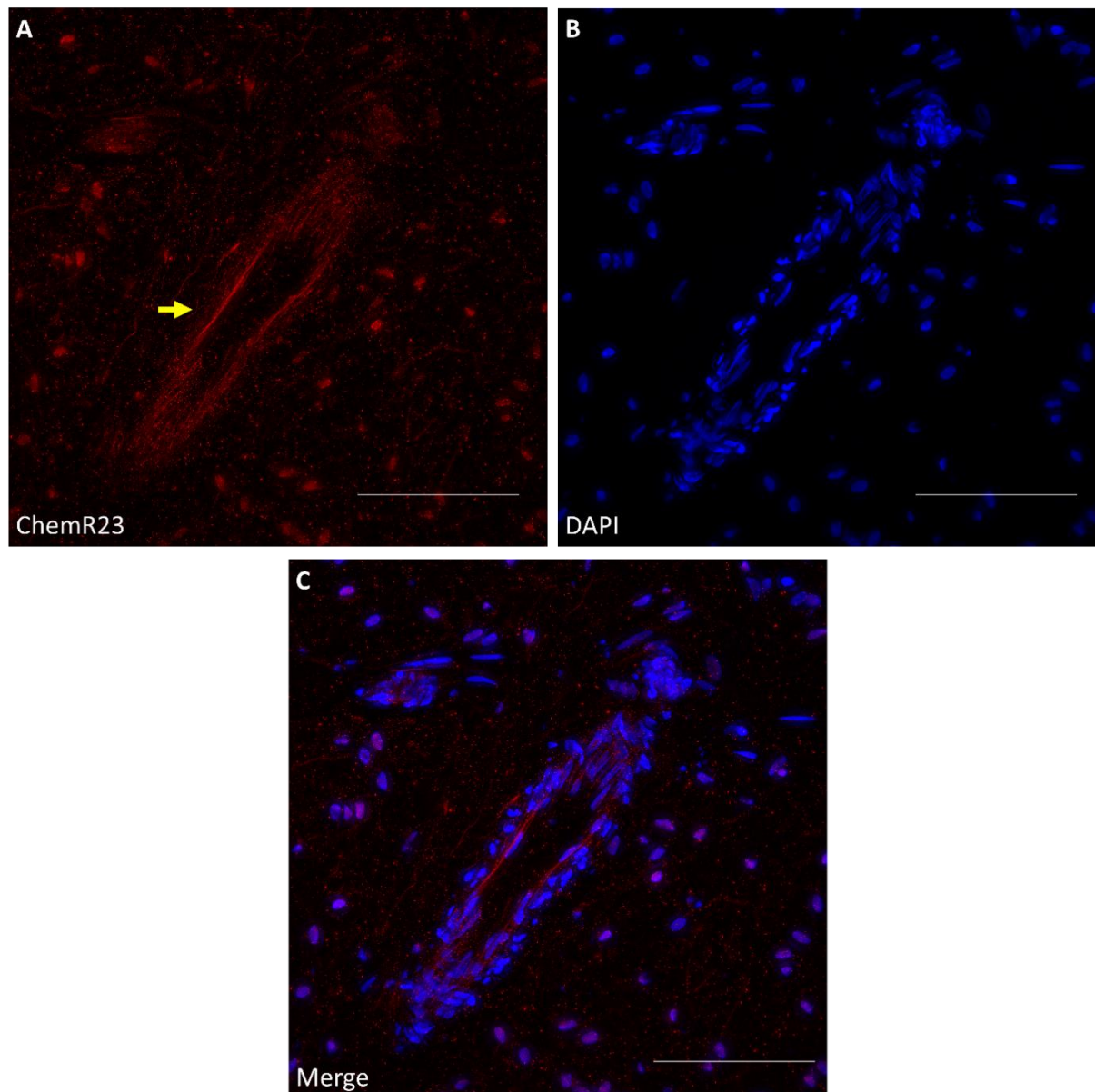


Figure 4.30. ChemR23 expression in structures with similar morphology to endothelial cells of blood vessels.

(A) Positive staining with the antibody against ChemR23, showing ChemR23 expression in structures with similar morphology to endothelial cells of blood vessels (indicated by a yellow arrow). (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with ChemR23 (red) and DAPI (blue), showing ChemR23 labelling in some nuclei (marked by white arrows). Scale bar = 100 μm and refers to all images.

ChemR23 is also found in nuclei and cytoplasm of a subset of cells within the pulp, this appeared to be largely restricted to the mesenchymal stromal cells. This is seen in the image taken at higher magnification with confocal microscopy in Figure 4.31, with putative stromal cell labelling marked by white arrows.

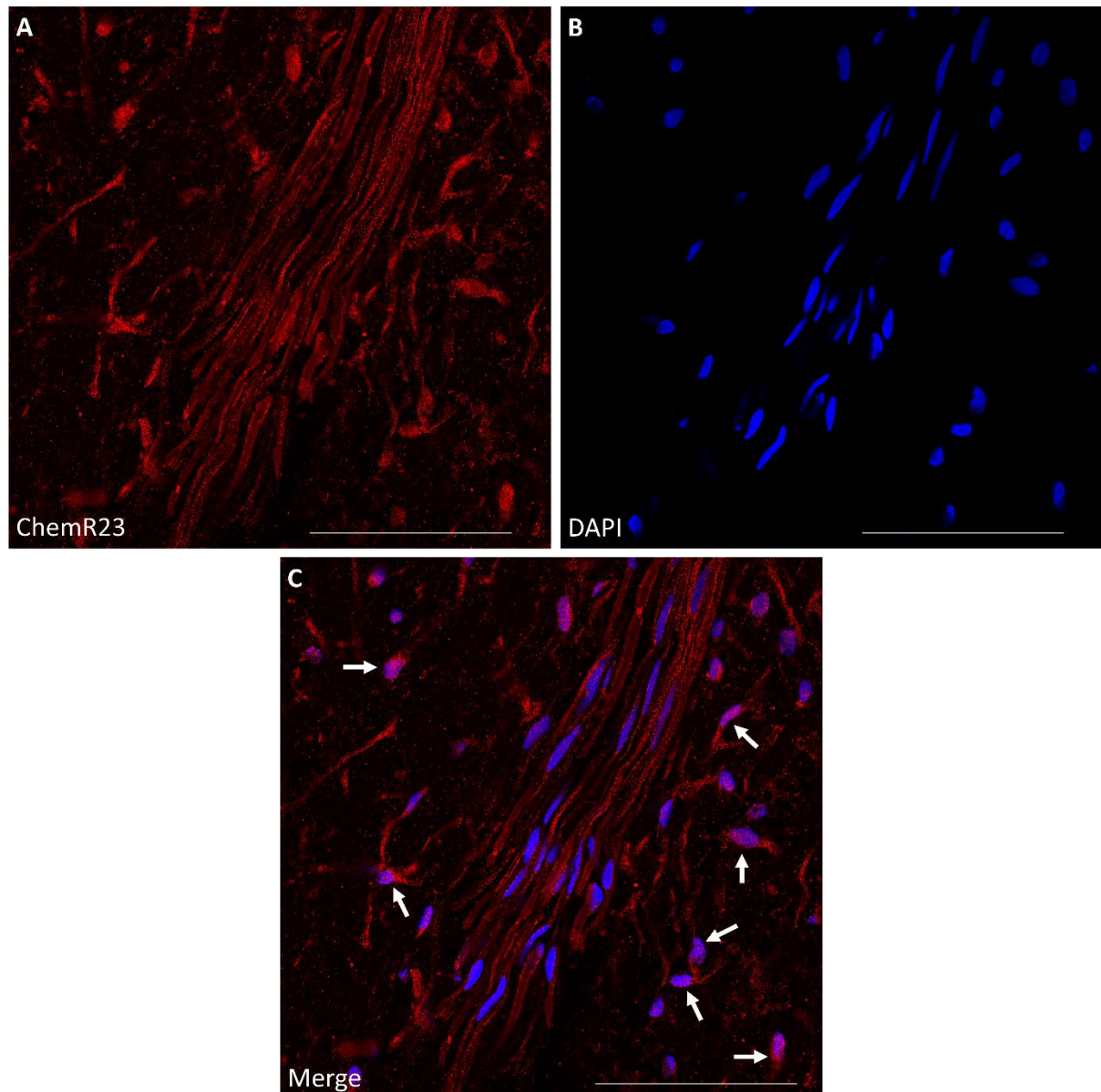


Figure 4.31. Confocal microscopy images showing ChemR23 expression in cell nuclei (A) Positive staining with the antibody against ChemR23. (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with ChemR23 (red) and DAPI (blue), showing ChemR23 labelling in nuclei (marked with white arrows). Scale bar = 50 μ m and refers to all images.

4.3.3. Characterisation of GPR32 expression in human tooth pulp tissues

GPR32, a receptor for RvD1, D3, and D5, was found to be expressed in human tooth pulp tissues, as indicated below in Figures 4.32 – 4.34. In the negative control where the GPR32 antibody was omitted, no positive staining was identified (Figures 4.32D and 4.33D).

4.3.3.1. Preliminary staining to identify structures with GPR32

GPR32 expression was found in structures that have a similar morphology to blood vessels (Figure 4.32) and nerve fibres (Figure 4.33).

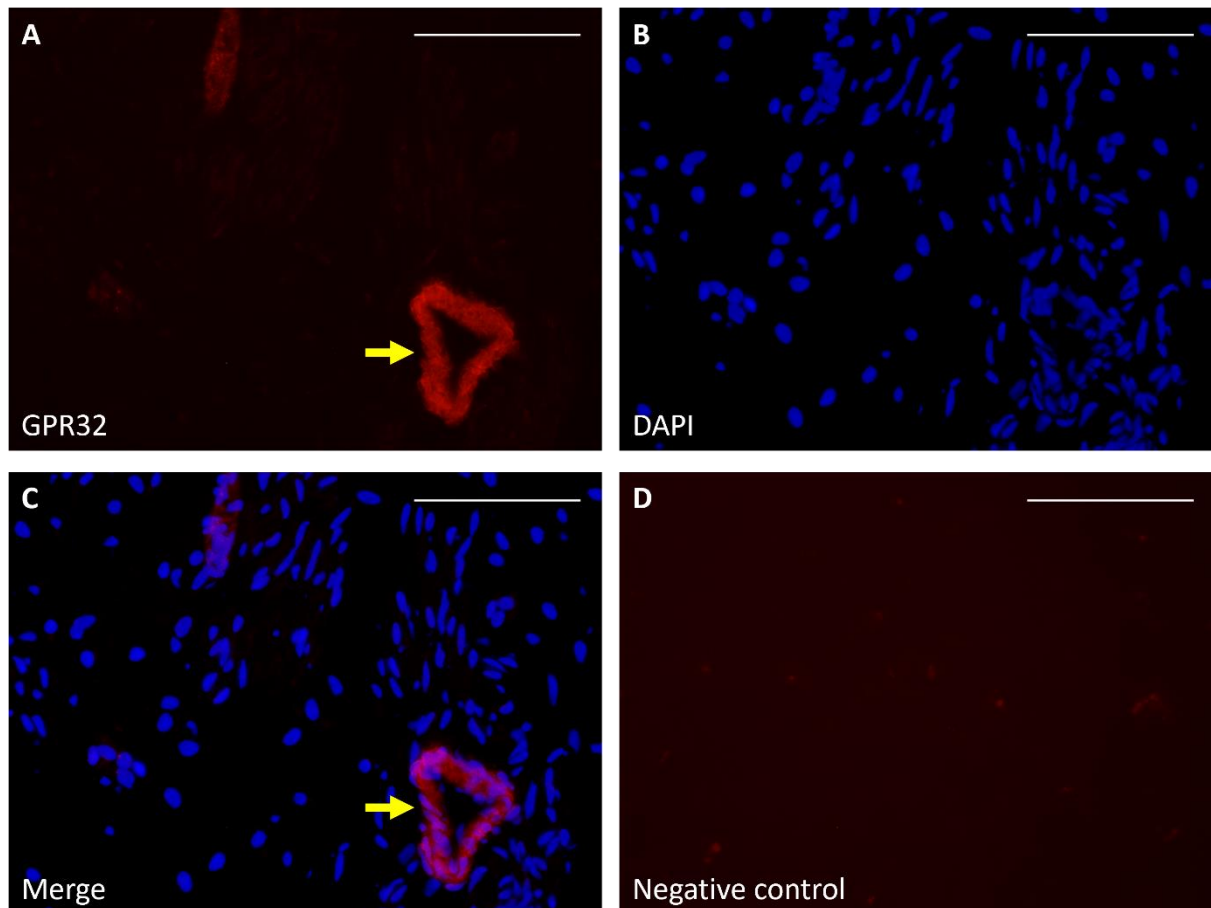


Figure 4.32. GPR32 expression in structures with blood vessel-like morphology. (A) Positive staining with the antibody against GPR32, showing GPR32 expression in structures with blood vessel-like morphology (indicated by a yellow arrow) (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with GPR32 (red) and DAPI (blue). (D) Negative control by omitting the antibody against GPR32. Scale bar = 100 μ m and refers to all images.

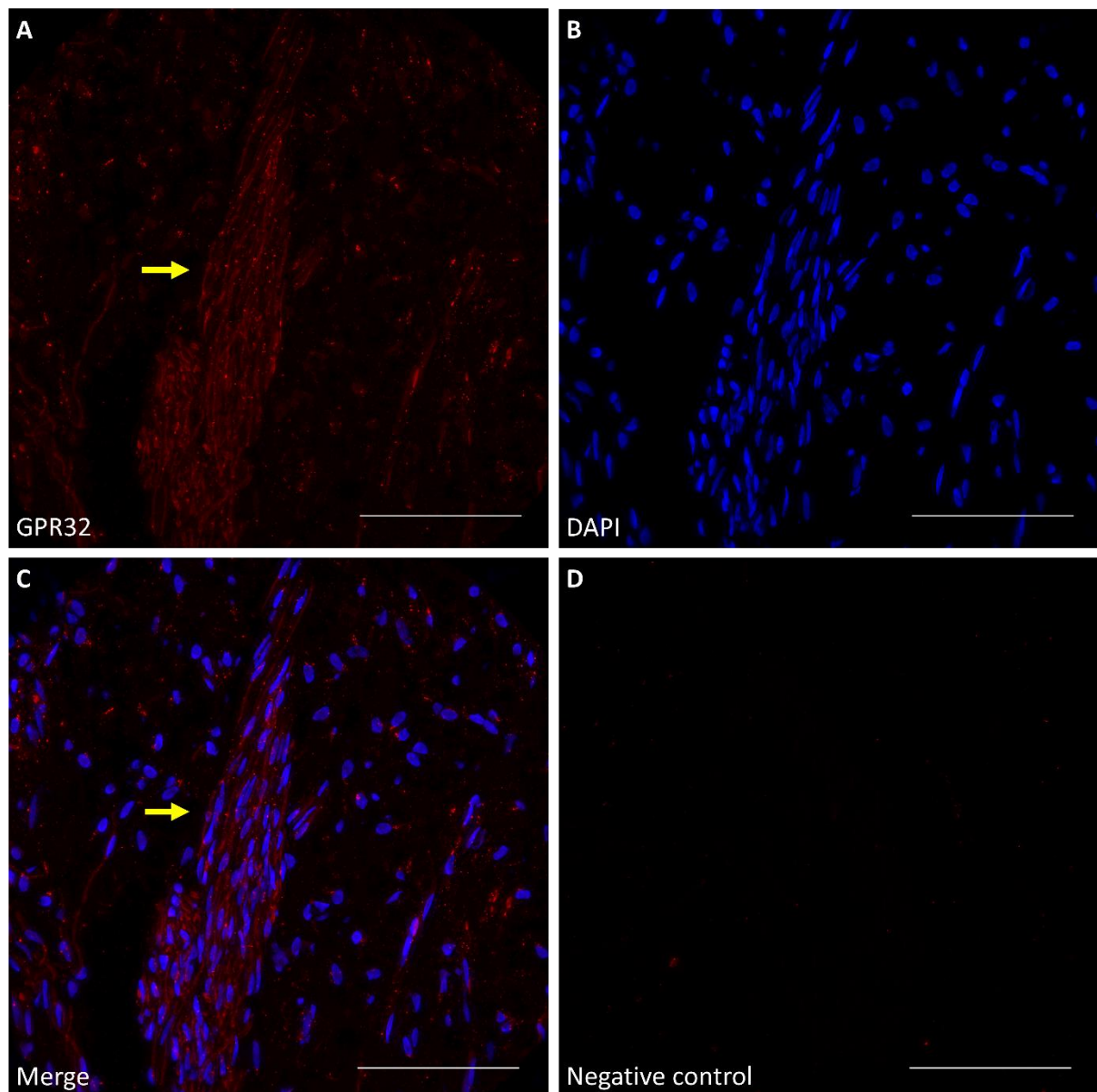


Figure 4.33. GPR32 expression in structures with nerve fibre-like morphology.

(A) Positive staining with the antibody against GPR32, showing GPR32 expression in a structure with nerve fibre-like morphology (indicated by a yellow arrow) (B) Positive staining with the antibody against cell nuclei marker DAPI. (C) Double staining with GPR32 (red) and DAPI (blue). (D) Negative control by omitting the antibody against GPR32. Scale bar = 100 μm and refers to all images.

4.3.3.2. Investigation of GPR32 expression associated with nerve fibres

Since GPR32 appeared to be present in cells that showed nerve fibre-like morphology, dual labelling with neuronal marker β -tubulin III was conducted. There was some overlap between GPR32 and β -tubulin III labelling (indicated by arrows in Figure 4.34D), indicating that it is expressed in nerve fibres.

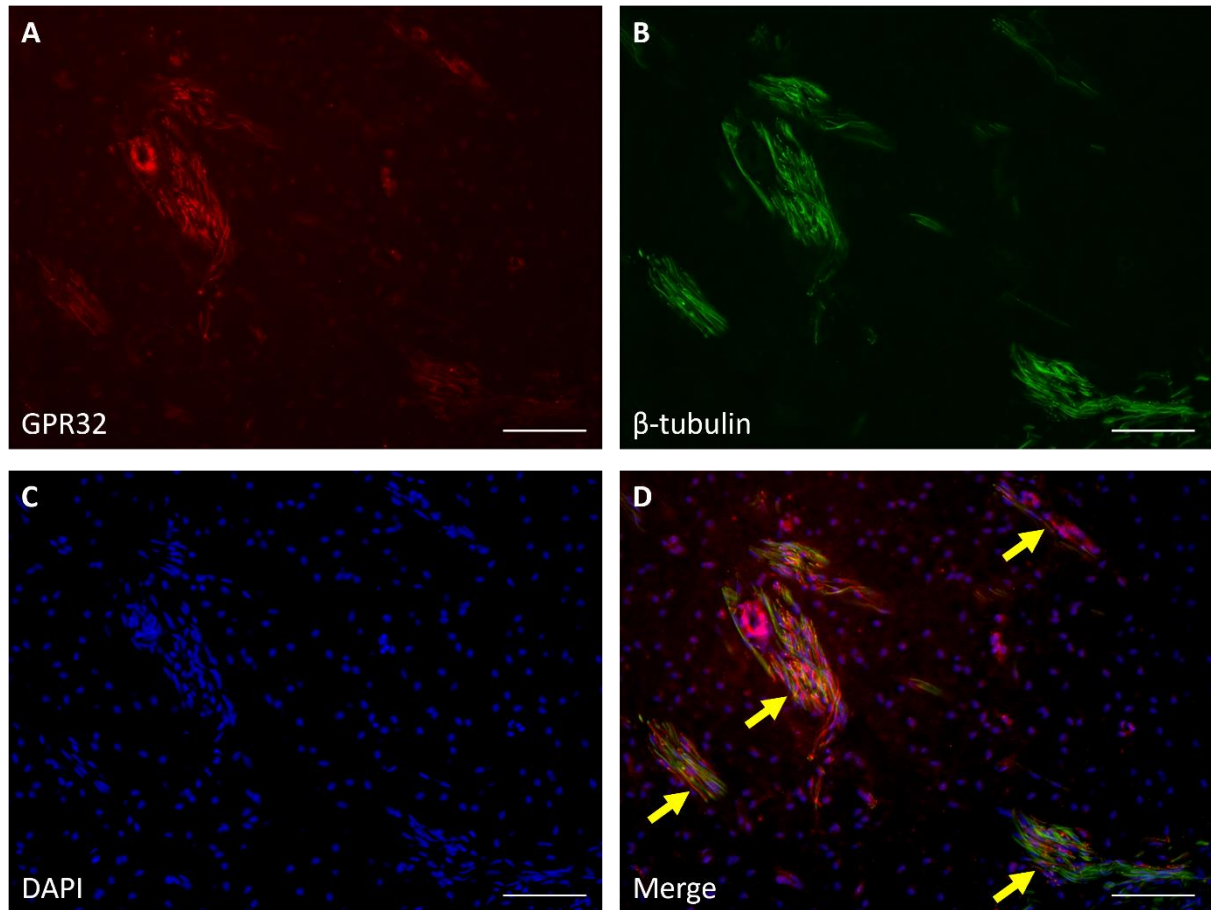


Figure 4.34. GPR32 expression in nerve fibres in human tooth pulp.

(A) Positive staining with the antibody against GPR32. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for GPR32 (red), β -tubulin III (green), and DAPI (blue), showing some colocalisation between GPR32 and β -tubulin III (indicated by yellow arrows). Scale bar = 100 μ m and refers to all images.

4.4. Discussion

Expression of resolvins receptors in human tooth pulp tissues was investigated to explore their relevance in inflammatory pain. Data obtained from this study suggest that BLT1, ChemR23, and GPR32 were found in human tooth pulp tissues, specifically in nerve fibres and blood vessels. To the best of my knowledge, this is the first study that reports resolvins receptor expression in human tooth pulp.

BLT1

BLT1, a receptor for RvE1 and RvE2, appeared to be closely associated with nerve fibres in tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth. This is shown in the representative confocal microscopy images (Figures 4.8, 4.10, and 4.12), where BLT1 staining appeared to overlap slightly with neuronal marker β -tubulin III. This is consistent with findings from a previous preliminary study in our laboratory, where BLT1 staining was found to overlap with another nerve fibre marker PGP 9.5 in human lingual nerve neuroma tissues (Tavares Ferreira, 2017). These data suggest that BLT1 is present in or closely associated with peripheral nerve fibres in man, and provide the first description of BLT1 expression in human peripheral nerves to the best of my knowledge. Based on publicly available spatial transcriptomic data from human DRG (<https://sensoryomics.shinyapps.io/RNA-Data/>), gene expression of BLT1 has been identified within C low-threshold mechanoreceptors, as well as several nociceptor subtypes in human DRG neurons, most notably within A β nociceptors which are characterised by their high expression of sodium voltage-gated channel alpha subunit 10 (SCN10A)/Na_v1.8 (Tavares-Ferreira et al., 2022). Additionally, based on publicly available single-nucleus RNA sequencing data from human trigeminal ganglion (TG) (<https://painseq.shinyapps.io/tg-painseq/>), BLT1 gene expression has also been identified within C low-threshold mechanoreceptors, as well as peptidergic, non-peptidergic, and TRPM8+ nociceptors in TG neurons (Yang et al., 2022). Pulpal sensory nerves are primary afferent nerve fibres of the trigeminal nerve with cell bodies in the TG. Since BLT1 is present in several subtypes of nociceptors in human TG and DRG neurons, it is likely that BLT1 may also be expressed in nociceptors in the tooth pulp, and thus contribute to pain sensitisation. In future studies, this could be explored further by dual labelling, for example with Na_v1.8 antibody, peptidergic neuronal marker CGRP, non-peptidergic neuronal marker isolectin B4 (IB4) (Barabas et al., 2012), and TRPM8 antibody.

While the area of positive BLT1 staining associated with nerve fibres appeared to be higher in tissues from carious painful teeth than that from carious non-painful teeth, no significant difference was identified between the three groups of samples used in the present study, indicating that BLT1 is not differentially expressed in the peripheral nerves in the

presence of pain or inflammation. Interestingly, data from bulk RNA sequencing of human DRG samples from male and female patients with and without pain found that BLT1 gene expression in human DRG is higher in male patients with pain than without pain, whereas the opposite was found with female patients (North et al., 2019). Since the tooth pulp samples collected from patients were not grouped further according to sex, the possible sex-specific difference in BLT1 expression in human DRG may explain why BLT1 is not differentially expressed within pulpal nerves in the presence of pain or inflammation in this present study. More importantly, in the present study, BLT1 expression that was found within the region of nerve fibres was quantified. Although some colocalisation between BLT1 and β -tubulin III was seen in all three groups of tooth pulp tissues, much of the labelling did not appear to be colocalised. It is possible that BLT1 may be found in other cell types in the nerve bundles, such as Schwann cells, elements of the vasculature, or elements of endoneurium. Since axonal BLT1 expression was not directly quantified specifically in this study, it is not known if axonal BLT1 expression differs in the presence of pain and inflammation, and thus it may not be appropriate to make a direct comparison with findings from DRG cell bodies. In future studies, axonal BLT1 expression could be examined more closely with further confocal imaging studies, so a more detailed quantification that specifically analyses the colocalisation between BLT1 and β -tubulin III in nerve fibres could be conducted. Nevertheless, findings from the present study indicate that BLT1 appeared to be present in nerve axons so they may potentially influence neuronal activity in the tooth pulp.

Similarly, even though the area of positive β -tubulin III staining in the central region of the tooth pulp from carious painful teeth appeared to be higher than that from both carious non-painful and non-carious teeth, no significant difference was found between the three groups using the quantification methods for this study. This may appear somewhat inconsistent with what was observed in a previous study where it was found that the degree of caries has a significant effect on neural density (Rodd and Boissonade, 2001). However, a different quantification method was used in the previous study, in which the % area of neural staining was quantified from different regions of the tooth pulp section (Rodd and Boissonade, 2001). Additionally, a large sample size per group was used in the previous study (20 samples per group) (Rodd and Boissonade, 2001), which increases the statistical power and the likelihood to detect a statistical difference. A smaller sample size per group was used in the present study as it was limited by the availability of samples in the archive of human tooth pulp tissues from the School of Clinical Dentistry.

Expression of BLT1 was also identified in blood vessels in tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth. This is shown in the representative images showing BLT1 expression in blood vessels (Figures 4.16 – 4.18), where BLT1 staining overlapped with both vascular smooth muscle cell marker α -SMA and

endothelial cell marker UEA I. It is important to note that BLT1 labelling appeared to vary between blood vessels. Some blood vessels were strongly BLT1-positive, but some blood vessels were only weakly stained with BLT1 (see Figures 4.16 – 4.18), and thus it is possible that BLT1 may have a functional role in a subset of blood vessels only. Further immunostaining was also carried out to examine BLT1 expression in individual components of blood vessels. Co-localisation of BLT1 and UEA I was found in the inner layer of blood vessels (Figure 4.15), which is lined by endothelial cells. Additionally, BLT1 expression also overlapped with α -SMA in blood vessels (Figure 4.14), indicating that BLT1 is expressed in the two major cell types in blood vessels. This supports findings from previous literature where BLT1 expression is found in endothelial cells and vascular smooth muscle cells in human vascular tissues (Back et al., 2005), as well as isolated human coronary smooth muscle cells (Back et al., 2005) and human umbilical vein endothelial cells (Qiu et al., 2006). It is reported that resolvins can be biosynthesised locally from omega-3 PUFAs by blood vessels (Chatterjee et al., 2017), and both cultured human endothelial and vascular smooth muscle cells are capable of producing resolvins from their precursors (Chatterjee et al., 2017), which suggests that resolvins generated in blood vessels may act locally via their receptors in the vasculature.

In the central region of tooth pulp tissues, the area of positive BLT1 staining in blood vessels in tissues from carious painful teeth was found to be significantly higher than that from carious non-painful teeth, indicating that BLT1 expression in pulpal vessels was upregulated in pain. While no significant difference in the area of positive FITC (α -SMA and UEA I) expression was found between the three groups of samples, the pattern in the levels of positive FITC labelling (Figure 4.19D) is very similar to the levels of BLT1 labelling (Figure 4.19B), where the area of positive FITC labelling appeared to be highest in carious painful teeth and lowest in carious non-painful teeth. This suggests that there may potentially be a small difference in the area of blood vessels between carious painful and carious non-painful teeth, but not enough to be statistically significant. Interestingly, this trend is lost after calculating the percentage ratio of BLT1/FITC, which are relatively similar across the three groups of samples. These findings suggest that the upregulation in BLT1 expression in areas rich in blood vessels within the central region of the tooth pulp may reflect an increase in the number of blood vessels, but it is possible that there may also be an increase in BLT1 expression in individual blood vessels. To identify if BLT1 expression is upregulated in individual blood vessels, in future studies areas of interest could be drawn around individual blood vessels to better quantify colocalisation. This was not conducted in the present study due to time constraints.

Results from this study suggest that the upregulation of BLT1 expression in blood vessels in tooth pulp is associated with pain. LTB₄-BLT1 signalling was previously shown to

be involved in the mechanism of inflammatory pain (Asahara et al., 2015), and thus it is possible that high levels of LTB₄ could act on the upregulated BLT1 and contribute to the progression of inflammatory pain (Asahara et al., 2022). Furthermore, a previous study suggested that LTB₄ contributes to the development of vasodilation (Larkin et al., 1995). Therefore, it is possible that the increased BLT1 expression in the vasculature may play a role in vasodilation in the tooth pulp. Since the tooth pulp is enclosed in the pulp chamber and surrounded by hard tissues, vasodilation in this confined space would lead to increased pressure in the pulp chamber, compressing interdental nerves around the tooth pulp, thus leading to an increase in pain sensation. It is plausible that limiting LTB₄-BLT1 signalling in the vasculature with RvE1 and RvE2 may counteract the effects of LTB₄ in blood vessels, and thus provide an analgesic effect, but this would require further investigation using animal models of pain.

ChemR23

ChemR23, another receptor for RvE1 and RvE2, is found in nerve fibres in tooth pulp tissues from carious painful, carious non-painful, and non-carious teeth. This is shown in the representative confocal microscopy images (Figures 4.24, 4.26, and 4.28), where ChemR23 staining appeared to colocalise with neuronal marker β -tubulin III in some axons. This suggests that ChemR23 is present in or closely associated with peripheral nerve fibres in man. Data from the present study is the first description of ChemR23 expression in human peripheral nerves to the best of my knowledge. Based on publicly available spatial transcriptomic data from human DRG (<https://sensoryomics.shinyapps.io/RNA-Data/>), gene expression of ChemR23 has been identified within several nociceptor subtypes in human DRG neurons, most notably within two clusters of C-nociceptors that are enriched in proenkephalin (PENK) and pruritogen receptors (Tavares-Ferreira et al., 2022). These two clusters of C-nociceptors are characterised by high expression of PENK and natriuretic peptide B (NPPB) respectively (Tavares-Ferreira et al., 2022). Additionally, based on publicly available single-nucleus RNA sequencing data from human TG (<https://painseg.shinyapps.io/tg-painseg/>), notable ChemR23 gene expression has also been identified within peptidergic nociceptors in TG neurons (Yang et al., 2022). Therefore, it is likely that ChemR23 may also be expressed in nociceptors in the tooth pulp, and thus contribute to pain sensitisation. In future studies, this could be explored further by dual labelling, for example with peptidergic neuronal marker CGRP and antibodies raised against PENK and NPPB.

Even though the area of positive ChemR23 staining in nerve fibres seemed to be lower in tissues from carious painful teeth than that from non-carious teeth, no significant difference was found between the three groups of samples, which suggests that ChemR23 is

not differentially expressed within peripheral nerves in the presence of pain or inflammation. Previous studies have shown upregulation of ChemR23 expression in response to peripheral inflammation. For example, a previous study reported upregulation of ChemR23 gene expression in the L4-L6 spinal cord dorsal horn after injection of carrageenan in the carrageenan model of inflammatory pain (Meesawatson et al., 2016). Another study using the MIA model of osteoarthritis also reported that ChemR23 gene expression in the L3-L5 spinal cord dorsal horn is higher in MIA-treated rats compared with saline-treated rats at a later time point (Huang et al., 2017). However, these studies used qPCR to investigate changes in ChemR23 expression in the spinal cord dorsal horn, while the present study analysed ChemR23 expression within nerve fibres, and thus direct comparison cannot be drawn. On the other hand, data from bulk RNA sequencing of human DRG samples from male and female patients with and without pain found that ChemR23 gene expression is higher in patients with pain than without pain (both male and female) (North et al., 2019). This also differs from the findings of the present study. However, nerve fibres in DRG innervate much wider structures and thus some nerve fibre subtypes do not exist in the tooth pulp. This can therefore contribute to the discrepancy in ChemR23 expression within nerve fibres seen in tooth pulp and DRG. Moreover, axonal ChemR23 expression was not specifically quantified in the present study, and thus it is not known if axonal ChemR23 expression differs in the presence of pain and inflammation. Therefore, it may not be appropriate to directly compare findings from the present study with findings from DRG cell bodies. In future studies, axonal ChemR23 expression could be examined more closely with further confocal imaging studies, so a more detailed quantification that specifically analyses the colocalisation between ChemR23 and β -tubulin III in nerve fibres could be conducted.

Additionally, although the area of positive β -tubulin III staining in the central region of the tooth pulp from both carious painful and non-painful teeth appeared to be higher than that from non-carious teeth, no significant difference was found. This indicates that neural density is not affected by pulpal inflammation or pain, which is similar to what was found in the analysis of BLT1 and β -tubulin III immunostaining described above. Furthermore, while the percentage ratio of ChemR23 to β -tubulin III in tissues from non-carious teeth appeared to be higher than that from both carious painful and non-painful teeth, no significant difference was found between them. Overall, results from this study indicate that ChemR23 is present in nerve axons, so they may potentially have a role in influencing neuronal activity in the tooth pulp.

Expression of ChemR23 also appeared to be found in blood vessels (Figures 4.21 and 4.30) in tooth pulp tissues. However, further colocalisation studies using markers for blood vessels (UEA I and α -SMA) are required to verify this, which has not been carried out due to time constraints. ChemR23 expression was also found in cell nuclei that appeared to

be restricted to mesenchymal stromal cells (Figure 4.31). Mesenchymal stromal cells are thought to have a role in modulating inflammation (Andruxhov et al., 2019), and thus it would be of interest to explore this further by dual labelling, for example with a mesenchymal stromal cell marker STRO-1 (Shi and Gronthos, 2003).

GPR32

GPR32, a receptor for RvD1, D3, and D5, was found to be associated with nerve fibres in tooth pulp tissues, as indicated in Figure 4.34 where GPR32 labelling appeared to overlap with neuronal marker β -tubulin III. GPR32 expression also appeared to be found in blood vessels in tooth pulp tissues (Figure 4.32), which will need to be verified with further double immunofluorescence assays using β -tubulin III. Further characterisation of GPR32 expression in human tooth pulp samples with a clinical history of pain is also needed to establish the functional relevance of GPR32 in pain and pulpal inflammation.

Conclusion and future work

In summary, expression of BLT1, ChemR23, and GPR32 was identified in nerve fibres and blood vessels of human tooth pulp tissues using immunohistochemistry.

Expression levels of BLT1 in nerve fibres and blood vessels, and ChemR23 in blood vessels in non-inflamed tooth pulp tissues from non-carious teeth and inflamed tooth pulp tissues from carious painful and non-painful teeth were also quantified and analysed to study their functional relevance in pain and pulpal inflammation. Using confocal imaging, BLT1 and ChemR23 were identified in several axons from all three groups of tooth pulp tissues, which suggests that they may have a role in influencing neuronal activity in the tooth pulp. BLT1 expression in blood vessels was found to be upregulated in tooth pulp tissues from carious painful teeth in comparison to carious non-painful teeth, indicating that BLT1 expressed in the vasculature may have a functional role in inflammatory pain. Further studies characterising ChemR23 and GPR32 expression in the vasculature in human tooth pulp samples with a clinical history of pain should therefore be conducted to investigate if these resolvins might have a functional role in pain. It would also be of interest to measure the levels of resolvins in pulpal tissues from patients to establish if there is any correlation with the expression of resolvins receptors. This was not conducted in the present study as the archive of tooth pulp sections available to our laboratory was not suitable for the measurement of resolvins levels. Nevertheless, this may help to interpret their functional significance in inflammatory pain, allowing the identification of potential targets for inflammatory pain treatment.

BLT1 gene expression has been identified in human TG neurons and satellite glial cells using single-nucleus RNA sequencing (Yang et al., 2022). Therefore, the signalling

cascades of E-series resolvins, RvE1 and RvE2, in TG cells will be explored in the next chapter.

CHAPTER 5: INTRACELLULAR SIGNALLING CASCADES OF RESOLVINS IN SENSORY NEURONS

5.1. Introduction

BLT1 is a G-protein coupled receptor (GPCR) activated by its full agonist LTB₄, which is a pro-inflammatory mediator that acts by recruiting neutrophils and macrophages to the site of inflammation (Yokomizo et al., 1997), and could enhance pain sensation through the release of inflammatory mediators by the recruited immune cells (Bisgaard and Kristensen, 1985). Both RvE1 and RvE2 exhibit similar potency for BLT1 (Oh et al., 2012). In the literature, some studies claimed that RvE1 acts as a partial agonist for BLT1 and can counteract the effects of LTB₄ (Arita et al., 2007). However, some studies claimed that they act as antagonists for BLT1 (Sawada et al., 2018, Archambault et al., 2019), as RvE1 can abolish the effects of LTB₄ to a similar extent as a BLT1 antagonist (Sawada et al., 2018). Despite these conflicting claims, it can be established that RvE1 and RvE2 may mediate the resolution of inflammation by competing with LTB₄ and dampening LTB₄-BLT1 signalling. This is demonstrated by a previous study, where it was shown that the increase in IL-23 production by dendritic cells induced by LTB₄ is reversed by RvE1 (Sawada et al., 2018).

G proteins are classified into four families according to their constituent G α subunit: Gs, Gi/o, Gq/11, and G12/13 (Kim et al., 2020). Each of the four families of G proteins activates different signalling cascades. For example, Gs proteins activate adenylyl cyclase, leading to an increased intracellular level of cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA). On the contrary, Gi/o proteins inhibit adenylyl cyclase activity, resulting in reduced production of intracellular cAMP (Calebiro et al., 2021). Gq protein activates phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Sanchez-Fernandez et al., 2014). IP₃ triggers the release of Ca²⁺ from the intracellular stores such as the endoplasmic reticulum (ER) into the cytosol, whereas DAG is necessary for the activation of protein kinase C (PKC).

Based on various studies in the literature, BLT1 appears to be coupled to both Gi and Gq types of G α protein, leading to inhibition of adenylyl cyclase and calcium mobilisation respectively (Yokomizo et al., 2001). However, the mechanism by which RvE1 and RvE2 interact with BLT1, particularly in sensory neurons, remains unclear. Therefore, the aim of the experiments in this chapter was to investigate the downstream signalling pathways of BLT1 activated upon binding of RvE1 and RvE2. The trigeminal ganglion (TG) is involved in the orofacial pain transmission pathway. It contains clusters of cell bodies of sensory neurons from the trigeminal system, which are responsible for transmitting sensory information to the CNS (Sessle, 2000). Cultured rat TG cells were therefore used to study

how RvE1 and RvE2 interact with BLT1. Since activation of Gq-coupled signalling cascades results in the elevation of intracellular calcium levels, and activation of Gi-coupled signalling cascades leads to decreased levels of intracellular cAMP, the intracellular calcium and cAMP responses induced by application of RvE1 and RvE2 were examined to establish if BLT1 is coupled to Gq and Gi protein in TG cells.

5.2. Results

5.2.1. Characterisation of BLT1 expression in cultured TG cells

Expression of BLT1 in cultured TG cells from rats was first tested with immunocytochemistry. Positive BLT1 staining was identified in rat TG cells (Figures 5.1 – 5.3). In the negative control where the BLT1 antibody was omitted, no positive staining was identified (Figure 5.1E).

To visualise BLT1 expression in specific cell types within the cultured TG cells, colocalisation studies were performed using neuronal marker β -tubulin III, myelinated neuron marker NF160/200, and satellite glial cell marker glutamine synthetase. Positive BLT1 staining overlapped with β -tubulin III in a neuronal cell body and its processes (Figure 5.1), indicating that BLT1 is expressed in TG neurons. Similarly, positive BLT1 staining overlapped with NF160/200 in a neuronal cell body and its processes (Figure 5.2), indicating that BLT1 is found in large-diameter TG neurons. Positive BLT1 labelling also overlapped with glutamine synthetase (Figure 5.3), indicating that BLT1 is present in satellite glial cells that surround the neuronal cell bodies in the TG.

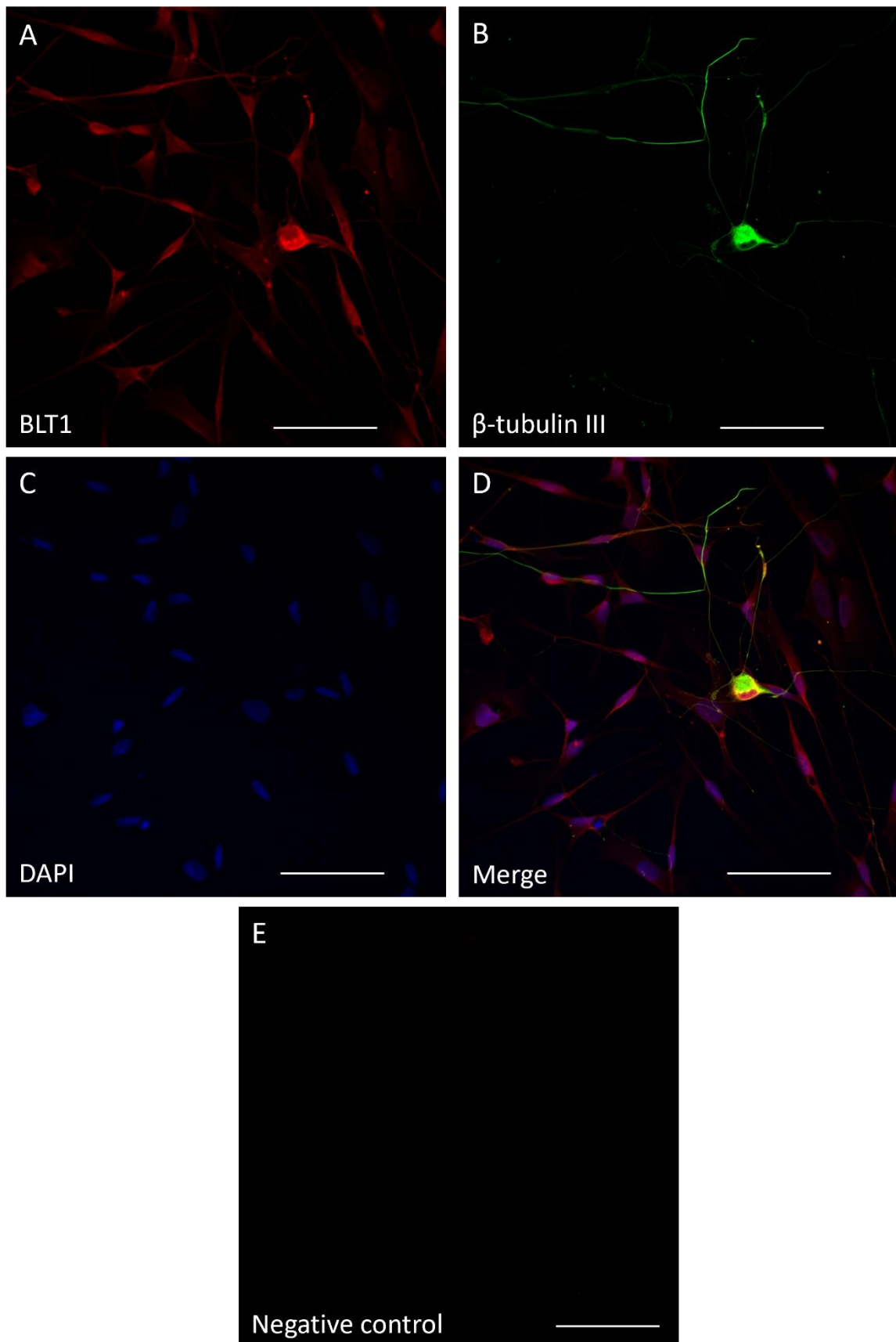


Figure 5.1. BLT1 expression in rat TG neurons.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against neuronal marker β -tubulin III. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), β -tubulin III (green), and DAPI (blue), showing BLT1 expression in a neuronal cell body and its processes. (E) Negative control by omitting the antibody against BLT1. Scale bar = 50 μ m and refers to all images.

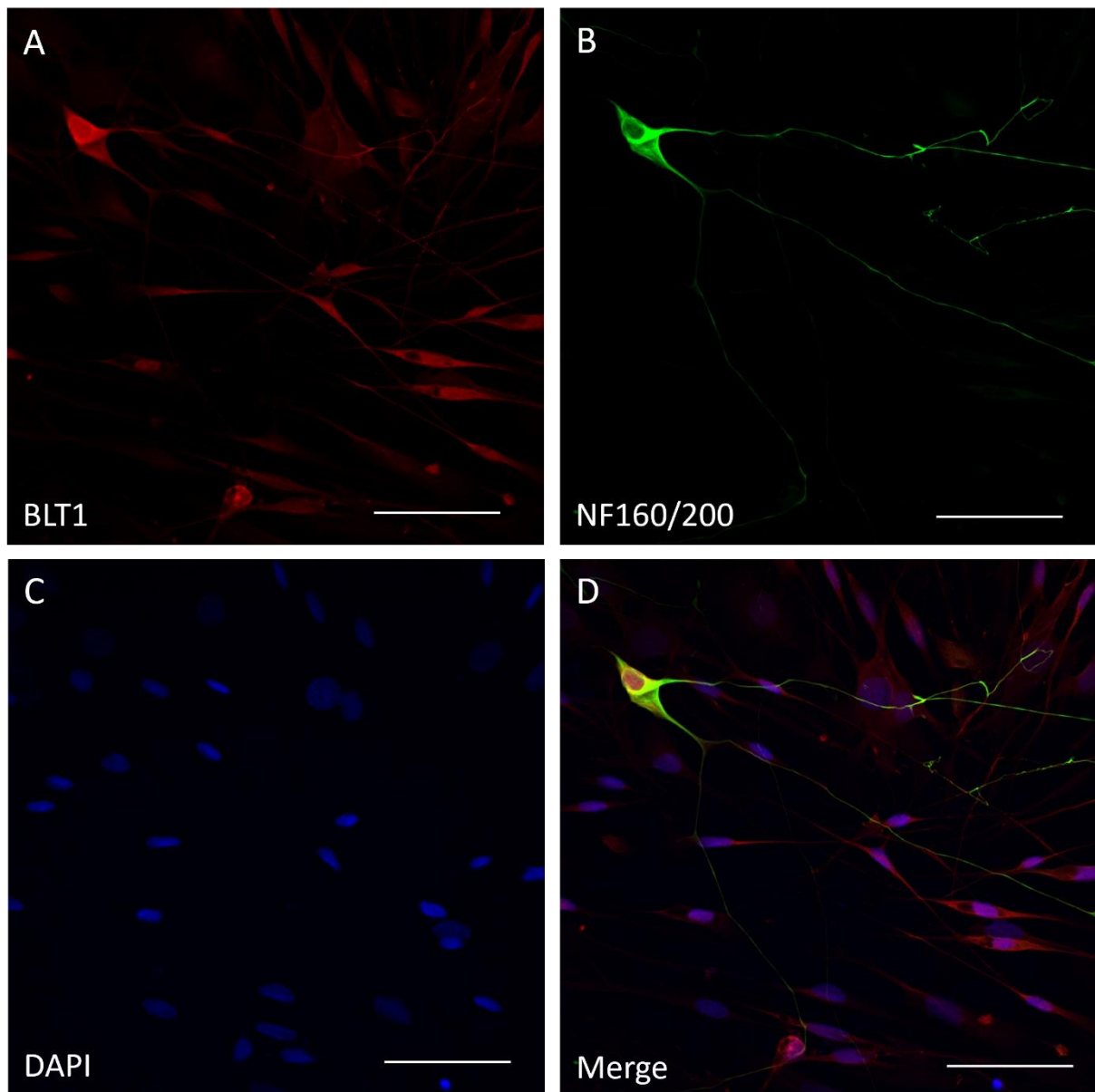


Figure 5.2. BLT1 expression in rat TG neurons.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against myelinated neuron marker NF160/200. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), NF160/200 (green), and DAPI (blue), showing BLT1 expression in a neuronal cell body and its processes. Scale bar = 50 μm and refers to all images.

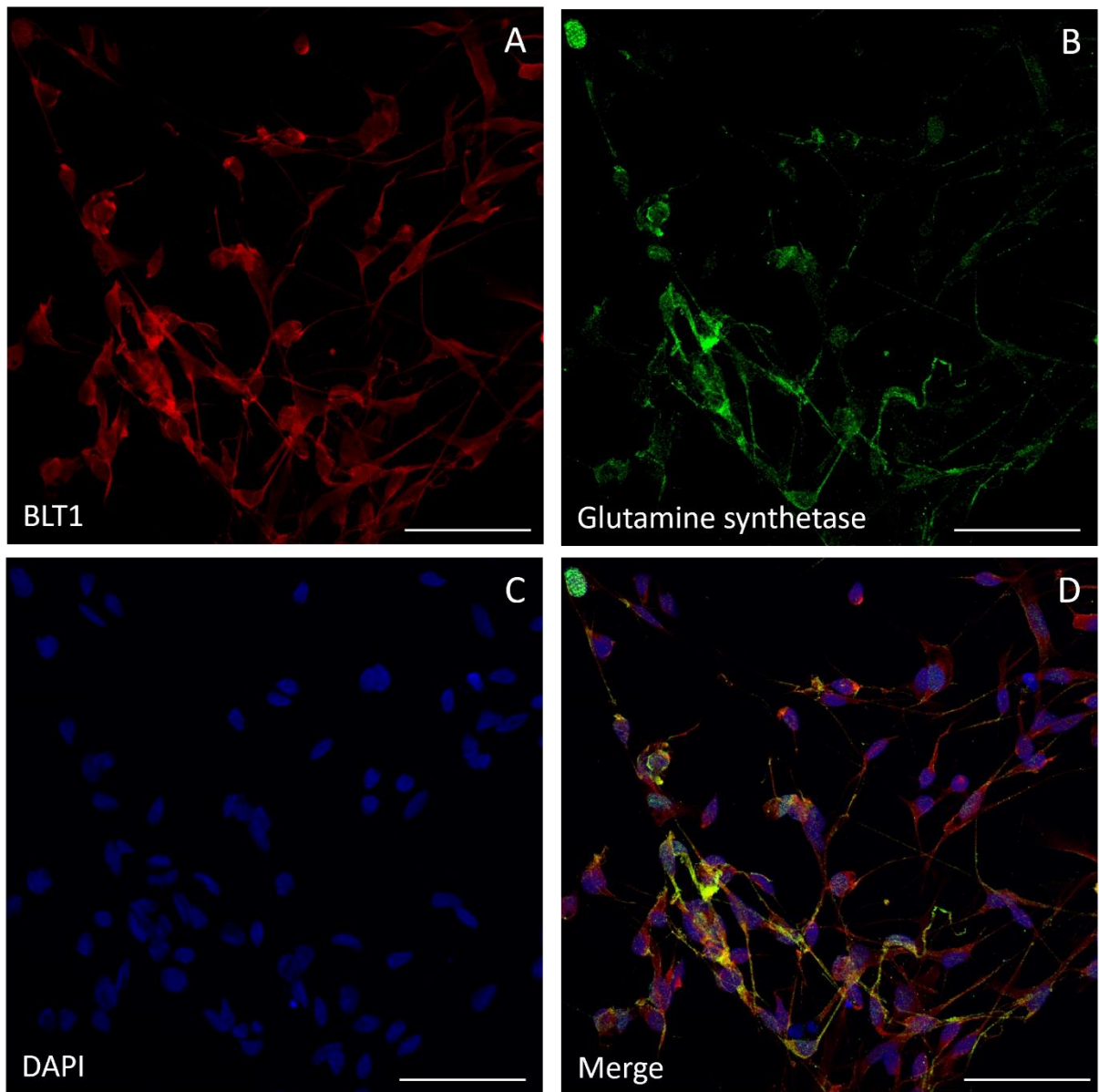


Figure 5.3. BLT1 expression in rat TG glial cells.

(A) Positive staining with the antibody against BLT1. (B) Positive staining with the antibody against satellite glial cell marker glutamine synthetase. (C) Positive staining with the antibody against cell nuclei marker DAPI. (D) Triple labelling for BLT1 (red), glutamine synthetase (green), and DAPI (blue). Scale bar = 50 μm and refers to all images.

5.2.2. Effects of application of resolvins on calcium signalling in cultured TG cells

Activation of Gq protein-coupled receptors results in calcium release from intracellular stores via the PLC-IP₃ signalling pathway (Sanchez-Fernandez et al., 2014). To establish if BLT1 was coupled to Gq protein in TG cells, calcium imaging was used to monitor changes in intracellular calcium levels in cultured rat TG cells by measuring changes in Fluo-4 fluorescence intensity over time following application of RvE1, RvE2, and selective BLT1 agonist 20-OH-LTB₄ (Yokomizo et al., 1997) via time-lapse confocal microscopy. The TG cells were loaded with a fluorescent calcium indicator Fluo-4 AM, which would exhibit an increase in fluorescence upon binding of calcium (Gee et al., 2000).

To distinguish neurons from non-neuronal cells, a solution containing 47.5 mM KCl (equimolar substitution of NaCl; 'high K⁺ solution') was applied (as detailed in section 2.3.2 in the methods), which depolarised neurons and led to the release of calcium via the voltage-gated calcium channels, so the cells that responded to high K⁺ solution were identified as neurons.

Additionally, in order to establish if the responses induced by RvE1, RvE2, and selective BLT1 agonist 20-OH-LTB₄ in TG cells are mediated by BLT1, they were tested in the presence of a selective BLT1 antagonist U-75302 (Lundeen et al., 2006).

5.2.2.1. Effects of application of RvE1 on calcium signalling in TG cells

Both TG neurons and non-neuronal cells responded to application of RvE1 with Ca^{2+} transients (measured as the rise in total cell fluorescence intensity, $\Delta F/F_0$), as shown in Figure 5.4A and 5.4B respectively.

In both TG neurons and non-neuronal cells, Ca^{2+} transients rose rapidly in response to application of RvE1, and thus a comparison between the amplitudes of Ca^{2+} transients in TG neurons and non-neuronal cells was made (Figure 5.4C). The average maximal amplitude of RvE1-induced Ca^{2+} transients in TG non-neuronal cells was significantly higher than that in TG neurons ($p=0.007$; unpaired t-test).

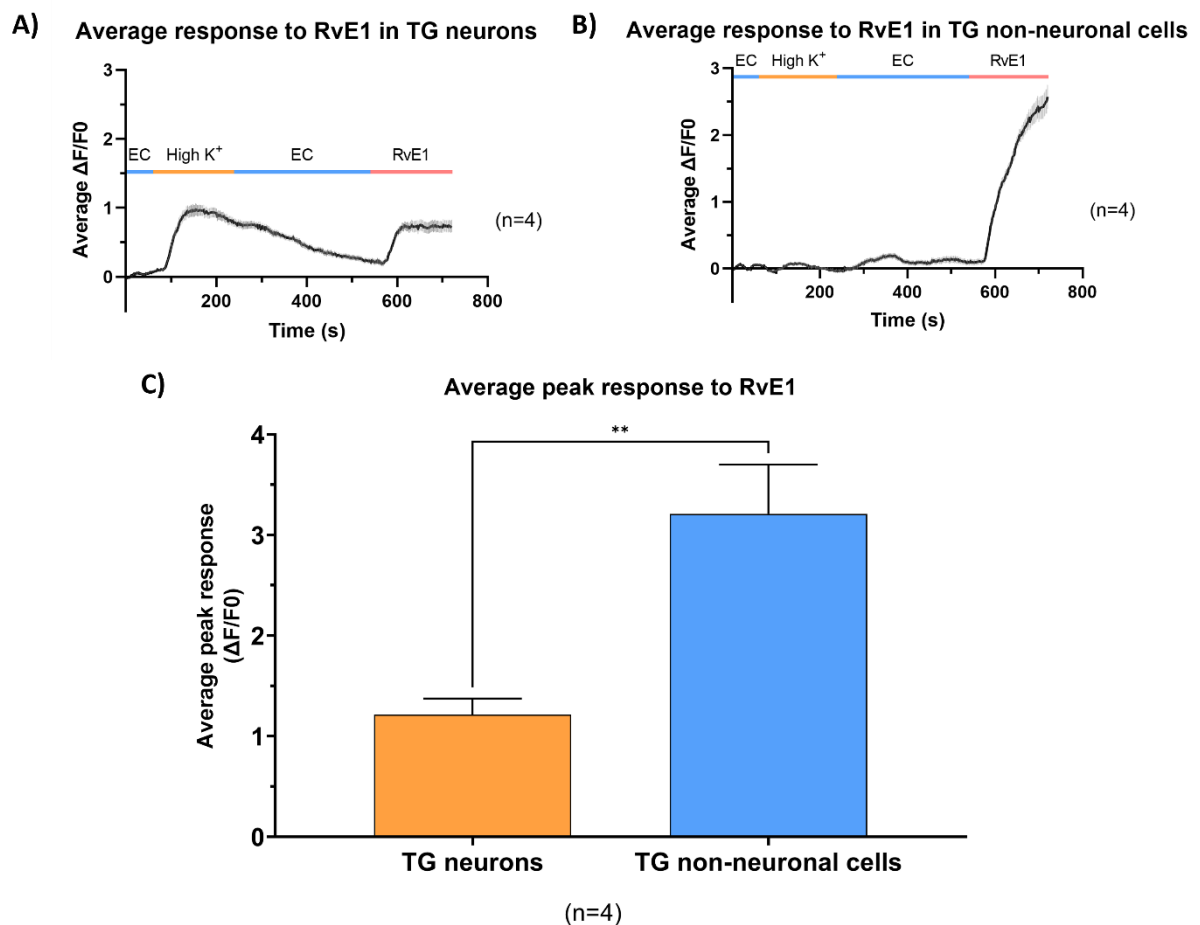


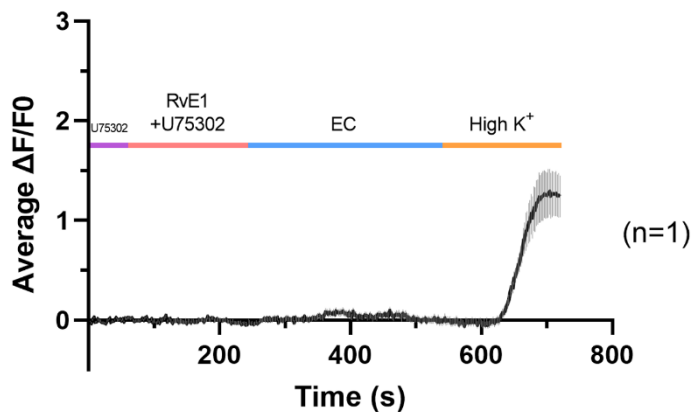
Figure 5.4. Effects of RvE1 application on intracellular calcium signals in TG cells

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE1. The shaded area represents SEM (Mean of 66 TG neurons and 119 TG non-neuronal cells across 4 animals).

(C) Average peak response to RvE1 in TG neurons and non-neuronal cells. Unpaired t-test revealed a significant difference in the average maximal amplitudes of RvE1-induced Ca^{2+} transients between TG neurons and non-neuronal cells ($p=0.007^{**}$). Error bars represent mean $\pm$ SEM.

In the presence of the selective BLT1 antagonist U-75302, the responses induced by RvE1 in both TG neurons and non-neuronal cells were mostly abolished (Figures 5.5), indicating that the responses induced by RvE1 were mediated by BLT1.

A) Average response to RvE1 in TG neurons in the presence of BLT1 antagonist U-75302



B) Average response to RvE1 in TG non-neuronal cells in the presence of BLT1 antagonist U-75302

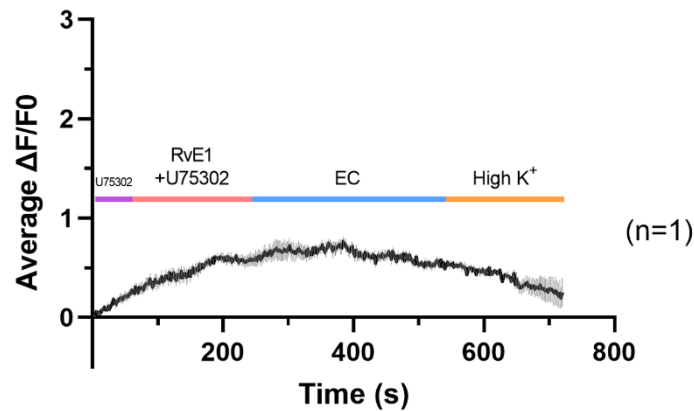


Figure 5.5. Effects of RvE1 application on intracellular calcium signals in the presence of BLT1 antagonist U-75302 in TG cells

Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE1 in the presence of selective BLT1 antagonist U-75302. The shaded area represents SEM (Mean of 10 TG neurons and 3 TG non-neuronal cells in 1 animal).

To eliminate the possibility that RvE1 may directly activate plasmalemmal Ca^{2+} -permeable channels (such as TRP channels), the cells were incubated in calcium-free extracellular solution (CaCl_2 was removed and 1 mM EGTA was added to chelate extracellular Ca^{2+}) before applying RvE1. Both TG neurons (Figure 5.6A) and non-neuronal cells (Figure 5.6B) still responded to RvE1 with Ca^{2+} transients in calcium-free conditions. In calcium-free conditions, no significant difference in the maximal amplitudes of RvE1-induced Ca^{2+} transients was found between TG neurons and non-neuronal cells (Figure 5.6C).

A comparison was also made between the amplitudes of RvE1-induced Ca^{2+} transients in the presence and absence of extracellular Ca^{2+} . In presence of extracellular Ca^{2+} , the average maximal amplitude of RvE1-induced Ca^{2+} transients in TG non-neuronal cells was significantly higher than that observed in calcium-free condition ($p=0.02$; unpaired t-test) (Figure 5.6E), indicating that TG non-neuronal cells responded to RvE1 with a larger Ca^{2+} influx in the presence of extracellular Ca^{2+} . On the other hand, no significant difference was found between the average maximal amplitudes of RvE1-induced Ca^{2+} transients in TG neurons in the presence and absence of extracellular Ca^{2+} (Figure 5.6D).

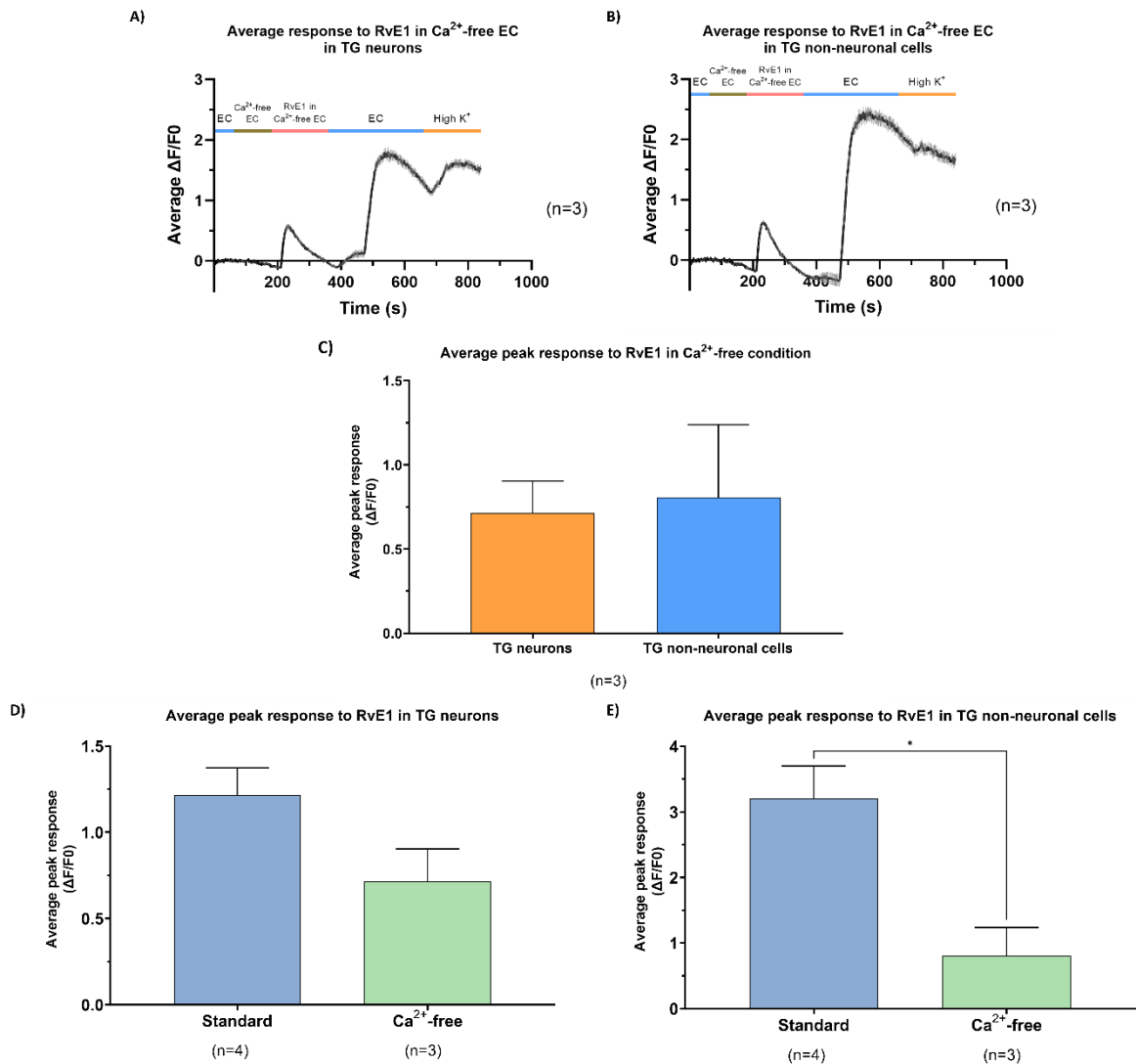


Figure 5.6. Effects of RvE1 application on intracellular calcium signals in TG cells in Ca^{2+} -free conditions

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE1 in Ca^{2+} -free extracellular solution. The shaded area represents SEM (Mean of 175 TG neurons and 94 TG non-neuronal cells across 3 animals).

(C) Average peak response to RvE1 in TG neurons and non-neuronal cells in calcium-free conditions. Unpaired t-test revealed no significant difference in the average maximal amplitudes of RvE1-induced Ca^{2+} transients between TG neurons and non-neuronal cells. Error bars represent mean $\pm$ SEM.

(D – E) Comparison between the average peak response to RvE1 in TG neurons (D) and non-neuronal cells (E) in the presence and absence of extracellular Ca^{2+} . Unpaired t-test revealed a significant difference in the average maximal amplitudes of RvE1-induced Ca^{2+} transients in TG non-neuronal cells ($p=0.02^*$). Error bars represent mean $\pm$ SEM.

5.2.2.2. Effects of application of RvE2 on calcium signalling in TG cells

RvE2 generally produced effects similar to RvE1 in TG cells. Both TG neurons and non-neuronal cells responded to application of RvE2 with Ca^{2+} transients, as shown in Figure 5.7A and 5.7B respectively.

In both TG neurons and non-neuronal cells, Ca^{2+} transients rose rapidly in response to application of RvE2, and thus a comparison between the amplitudes of Ca^{2+} transients in TG neurons and non-neuronal cells was made (Figure 5.7C). The average maximal amplitude of RvE2-induced Ca^{2+} transients in TG non-neuronal cells was significantly higher than that in TG neurons ($p=0.008$; unpaired t-test).

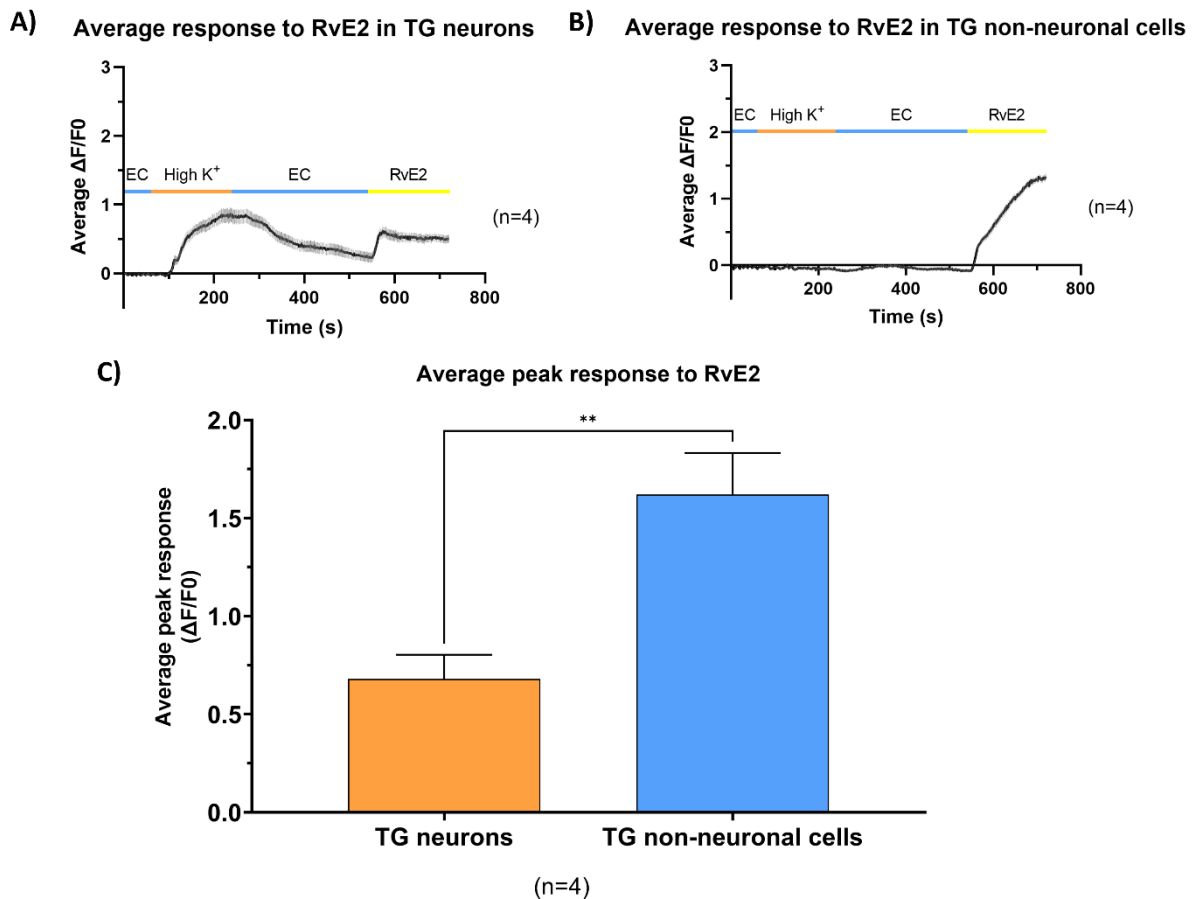


Figure 5.7. Effects of RvE2 application on intracellular calcium signals in TG cells

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE2. The shaded area represents SEM (Mean of 45 TG neurons and 111 TG non-neuronal cells across 4 animals).

(C) Average peak response to RvE2 in TG neurons and non-neuronal cells. Unpaired t-test revealed a significant difference in the average maximal amplitudes of RvE2-induced Ca^{2+} transients between TG neurons and non-neuronal cells ($p=0.008^{**}$). Error bars represent mean $\pm$ SEM.

In the presence of the selective BLT1 antagonist U-75302, the responses induced by RvE2 in both TG neurons and non-neuronal cells were mostly abolished (Figures 5.8), indicating that the responses induced by RvE2 were mediated by BLT1.

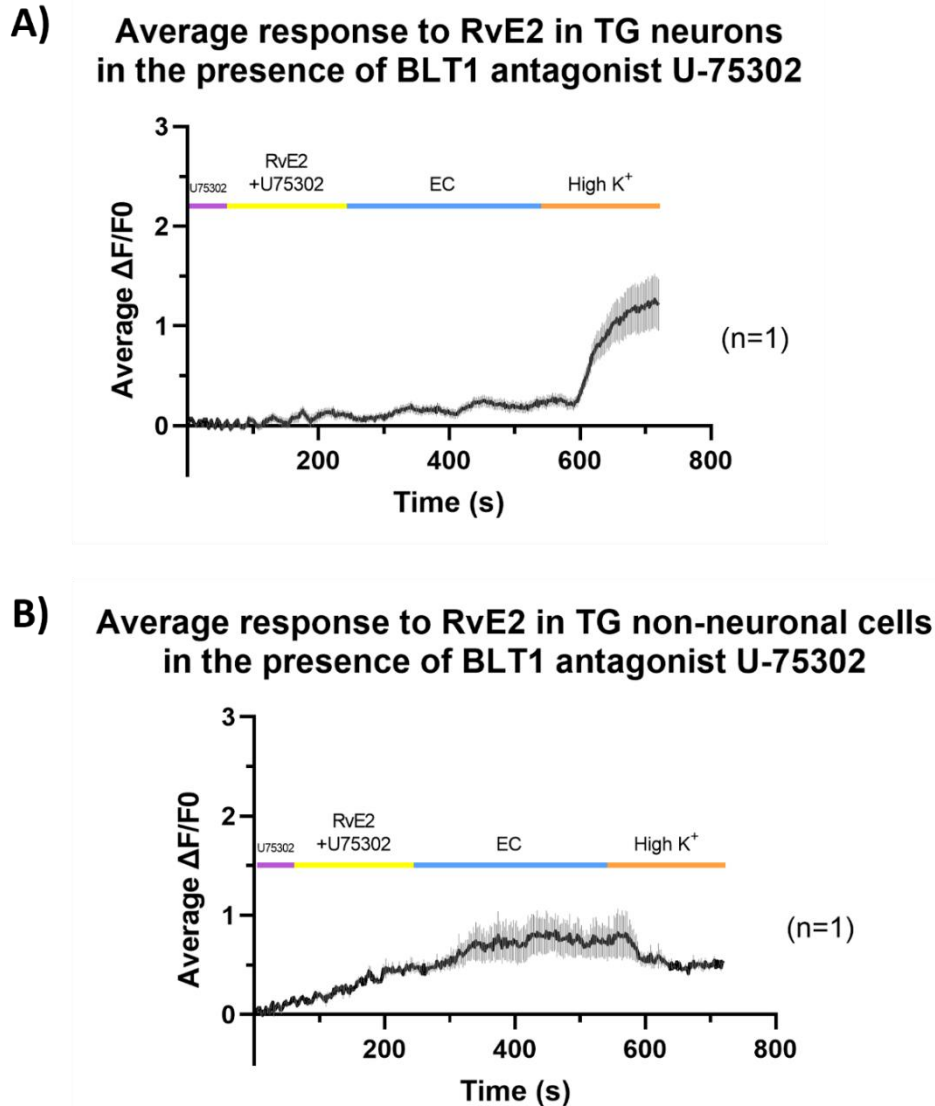


Figure 5.8. Effects of RvE2 application on intracellular calcium signals in the presence of BLT1 antagonist U-75302 in TG cells

Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE2 in the presence of selective BLT1 antagonist U-75302. The shaded area represents SEM (Mean of 11 TG neurons and 2 TG non-neuronal cells in 1 animal).

To eliminate the possibility that RvE2 may directly activate plasmalemmal Ca^{2+} -permeable channels (such as TRP channels), the cells were incubated in calcium-free extracellular solution (CaCl_2 was removed and 1 mM EGTA was added to chelate extracellular Ca^{2+}) before applying RvE2. Both TG neurons (Figure 5.9A) and non-neuronal cells (Figure 5.9B) still responded to RvE2 with Ca^{2+} transients in calcium-free conditions. However, in calcium-free conditions, no significant difference in the maximal amplitudes of RvE2-induced Ca^{2+} transients was found between TG neurons and non-neuronal cells (Figure 5.9C).

A comparison was also made between the amplitudes of RvE2-induced Ca^{2+} transients in the presence and absence of extracellular Ca^{2+} , and no significant difference in the average maximal amplitudes of RvE2-induced Ca^{2+} transients was found in both TG neurons and non-neuronal cells (Figures 5.9D and 5.9E).

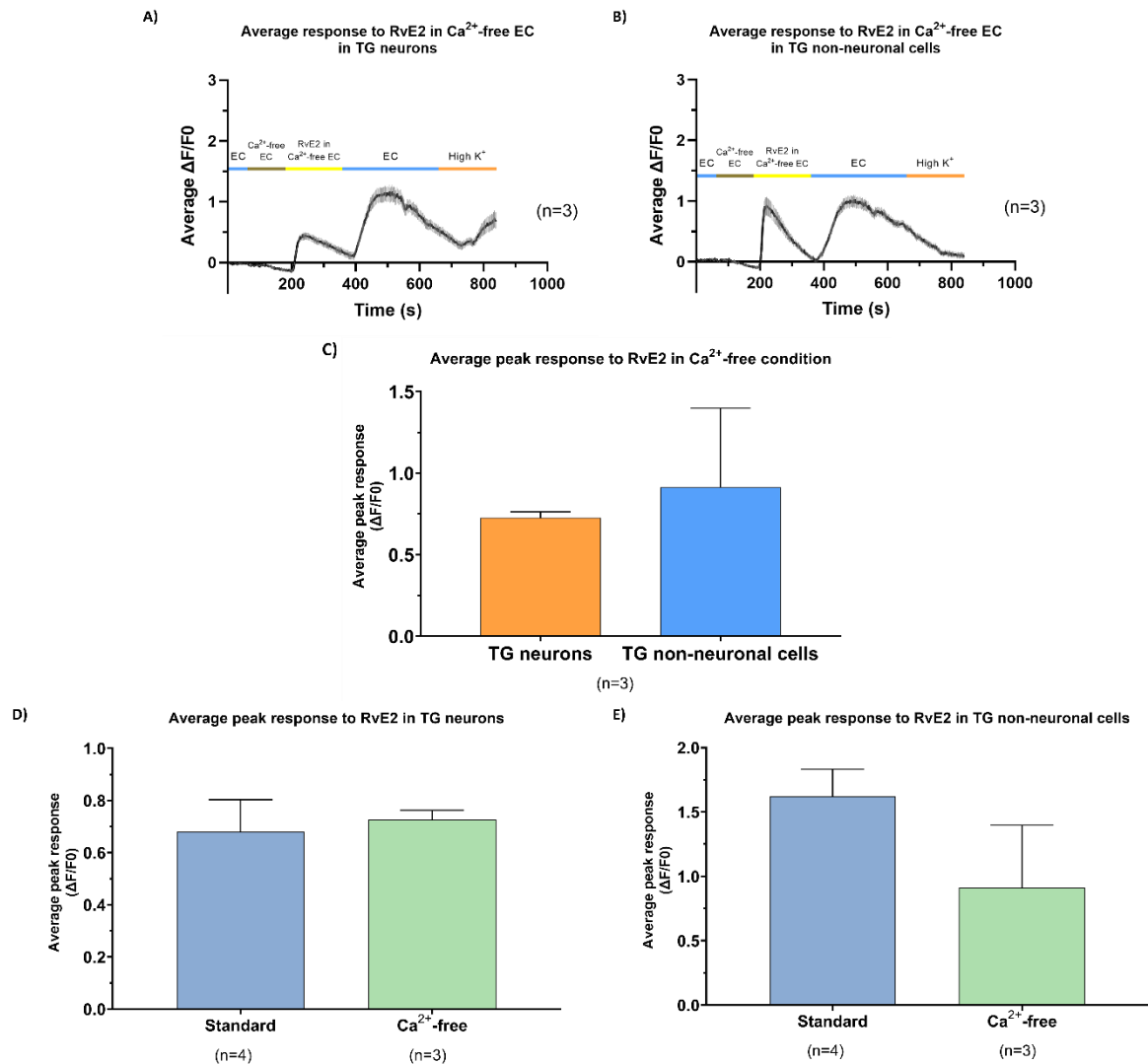


Figure 5.9. Effects of RvE2 application on intracellular calcium signals in TG cells in Ca^{2+} -free conditions

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of RvE2 in Ca^{2+} -free extracellular solution. The shaded area represents SEM (Mean of 59 TG neurons and 41 TG non-neuronal cells across 3 animals).

(C) Average peak response to RvE2 in TG neurons and non-neuronal cells in calcium-free conditions. Welch's t-test revealed no significant difference in the average maximal amplitude of RvE2-induced Ca^{2+} transients between TG neurons and non-neuronal cells. Error bars represent mean $\pm$ SEM.

(D – E) Comparison between the average peak response to RvE2 in TG neurons (D) and non-neuronal cells (E) in the presence and absence of extracellular Ca^{2+} . Unpaired t-test revealed no significant difference in the average maximal amplitudes of RvE2-induced Ca^{2+} transients in TG neurons and non-neuronal cells. Error bars represent mean $\pm$ SEM.

5.2.2.3. Effects of application of 20-OH-LTB₄ on calcium signalling in TG cells

To explore if the calcium signalling induced by RvE1 and RvE2 in TG cells was mediated by BLT1, a selective BLT1 agonist 20-OH-LTB₄ was used. Generally, 20-OH-LTB₄ produced similar effects as RvE1 and RvE2 in TG cells. Both TG neurons and non-neuronal cells responded to application of 20-OH-LTB₄ with Ca²⁺ transients, as shown in Figure 5.10A and 5.10B respectively.

In both TG neurons and non-neuronal cells, Ca²⁺ transients rose rapidly in response to application of 20-OH-LTB₄, and thus a comparison between the amplitudes of Ca²⁺ transients in TG neurons and non-neuronal cells was made (Figure 5.10C). The average maximal amplitude of RvE1-induced Ca²⁺ transients in TG non-neuronal cells was significantly higher than that in TG neurons ($p=0.008$; unpaired t-test).

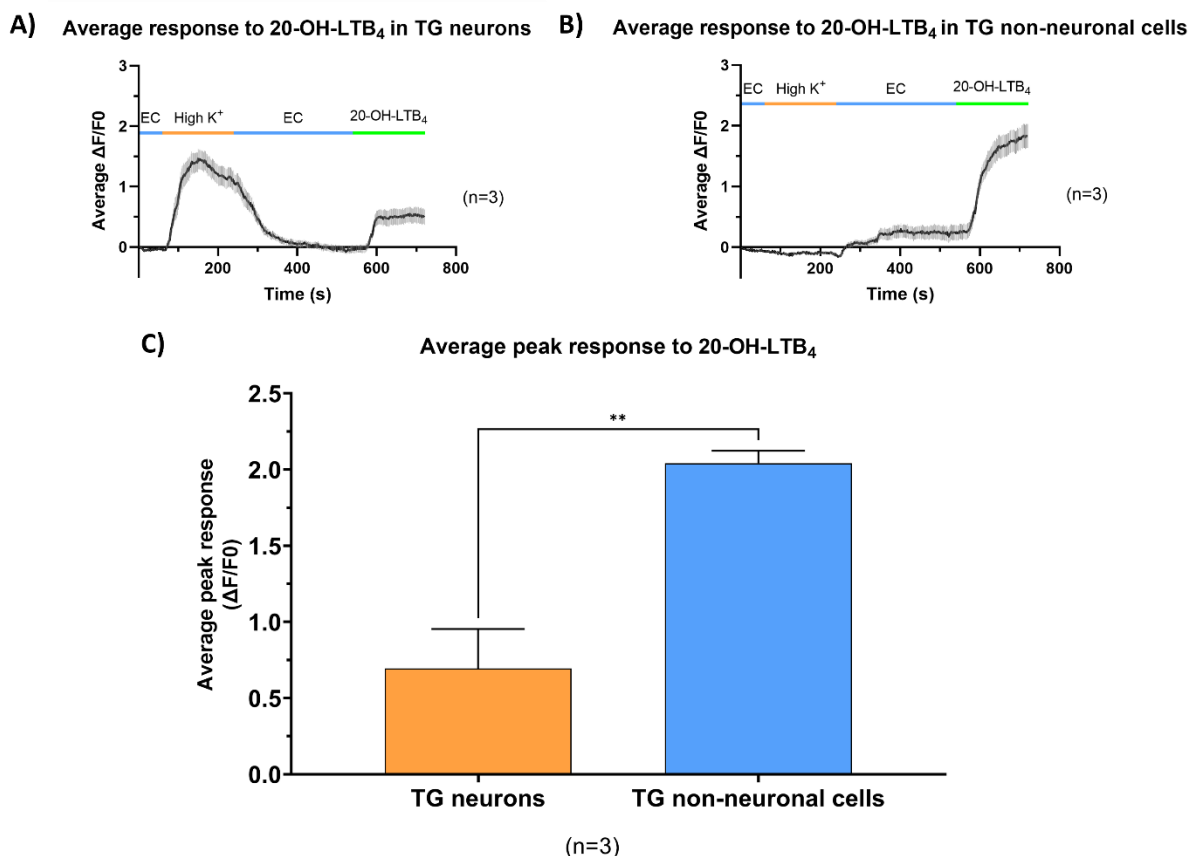


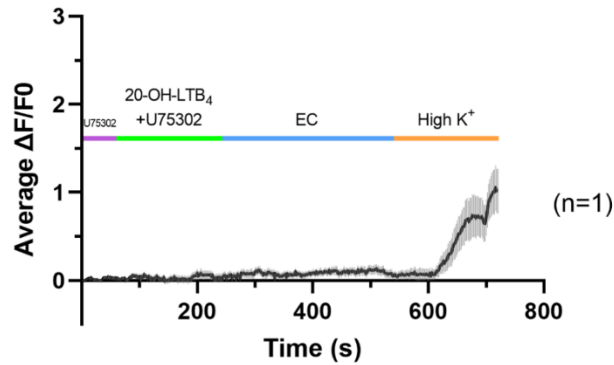
Figure 5.10. Effects of 20-OH-LTB₄ application on intracellular calcium signals in TG cells

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of selective BLT1 agonist 20-OH-LTB₄. The shaded area represents SEM (Mean of 18 TG neurons and 58 TG non-neuronal cells across 3 animals).

(C) Average peak response to 20-OH-LTB₄ in TG neurons and non-neuronal cells. Unpaired t-test revealed a significant difference in the average maximal amplitudes of 20-OH-LTB₄-induced Ca²⁺ transients between TG neurons and non-neuronal cells ($p=0.008^{**}$). Error bars represent mean $\pm$ SEM.

In the presence of the selective BLT1 antagonist U-75302, the responses induced by 20-OH-LTB₄ in both TG neurons and non-neuronal cells were mostly abolished (Figures 5.11), indicating that the responses induced by 20-OH-LTB₄ were mediated by BLT1.

A) Average response to 20-OH-LTB₄ in TG neurons in the presence of BLT1 antagonist U-75302



B) Average response to 20-OH-LTB₄ in TG non-neuronal cells in the presence of BLT1 antagonist U-75302

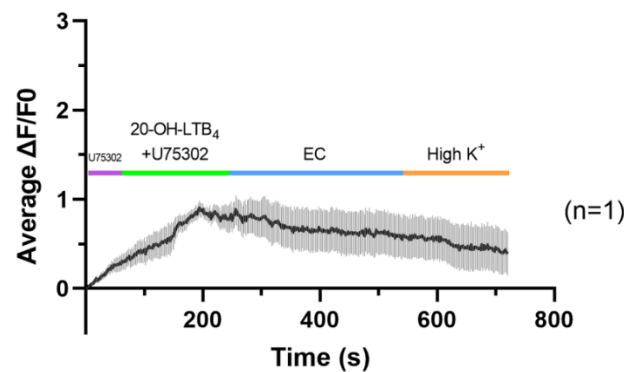


Figure 5.11. Effects of 20-OH-LTB₄ application on intracellular calcium signals in the presence of BLT1 antagonist U-75302 in TG cells

Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of selective BLT1 agonist 20-OH-LTB₄ in the presence of selective BLT1 antagonist U-75302. The shaded area represents SEM (Mean of 11 TG neurons and 4 TG non-neuronal cells in 1 animal).

To eliminate the possibility that 20-OH-LTB₄ may directly activate plasmalemmal Ca²⁺-permeable channels (such as TRP channels), the cells were incubated in calcium-free extracellular solution (CaCl₂ was removed and 1 mM EGTA was added to chelate extracellular Ca²⁺) before applying 20-OH-LTB₄. Both TG neurons (Figure 5.12A) and non-neuronal cells (Figure 5.12B) still responded to 20-OH-LTB₄ with Ca²⁺ transients in calcium-free conditions. However, in calcium-free conditions, no significant difference in the maximal amplitudes of 20-OH-LTB₄-induced Ca²⁺ transients was found between TG neurons and non-neuronal cells (Figure 5.12C).

A comparison was also made between the amplitudes of 20-OH-LTB₄-induced Ca²⁺ transients in the presence and absence of extracellular Ca²⁺. In presence of extracellular Ca²⁺, the average maximal amplitude of 20-OH-LTB₄-induced Ca²⁺ transients in TG non-neuronal cells was significantly higher than that observed in calcium-free condition ($p=0.002$; unpaired t-test) (Figure 5.12E), indicating that TG non-neuronal cells responded to RvE1 with a larger Ca²⁺ influx in the presence of extracellular Ca²⁺. On the other hand, no significant difference was found between the average maximal amplitudes of 20-OH-LTB₄-induced Ca²⁺ transients in TG neurons in the presence and absence of extracellular Ca²⁺ (Figure 5.12D).

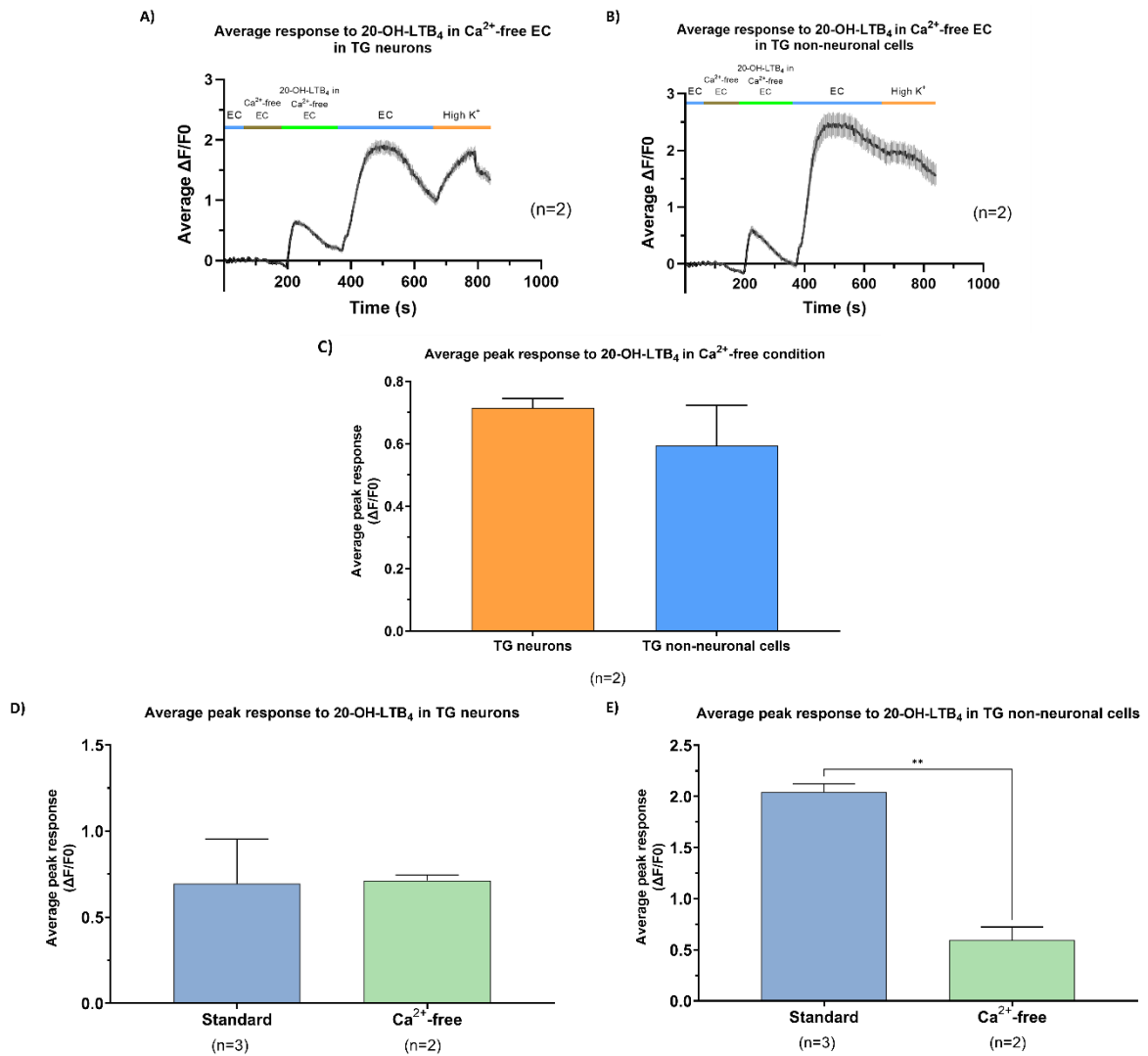


Figure 5.12. Effects of 20-OH-LTB₄ application on intracellular calcium signals in TG cells in Ca²⁺-free conditions

(A – B) Traces showing mean calcium responses in TG neurons (A) and non-neuronal cells (B) loaded with Fluo-4 AM following application of 20-OH-LTB₄ in Ca²⁺-free extracellular solution. The shaded area represents SEM (Mean of 95 TG neurons and 22 TG non-neuronal cells across 2 animals).

(C) Average peak response to 20-OH-LTB₄ in TG neurons and non-neuronal cells in calcium-free conditions. Unpaired t-test revealed no significant difference in the average maximal amplitude of 20-OH-LTB₄-induced Ca²⁺ transients between TG neurons and non-neuronal cells. Error bars represent mean ± SEM.

(D – E) Comparison between average peak response to 20-OH-LTB₄ in TG neurons (D) and non-neuronal cells (E) in the presence and absence of extracellular Ca²⁺. Unpaired t-test revealed a significant difference in the average maximal amplitudes of 20-OH-LTB₄-induced Ca²⁺ transients in TG non-neuronal cells ($p=0.002^{**}$). Error bars represent mean ± SEM.

5.2.2.4. Comparison of Ca^{2+} transients induced by RvE1, RvE2, and 20-OH- LTB_4 in TG cells

Application of RvE1, RvE2, and 20-OH- LTB_4 all induce calcium signalling in TG cells. A comparison was then made between the Ca^{2+} transients induced by these test compounds. The maximal amplitude of Ca^{2+} transients induced by RvE1 was found to be significantly higher than that induced by RvE2 in TG non-neuronal cells ($p=0.01$; one-way ANOVA with Tukey's post-hoc test). On the other hand, in calcium-free conditions, no significant difference in the amplitudes of Ca^{2+} transients induced by RvE1, RvE2, and 20-OH- LTB_4 were found in both TG neurons (Figure 5.13C) and non-neuronal cells (Figure 5.13C and 5.13D respectively).

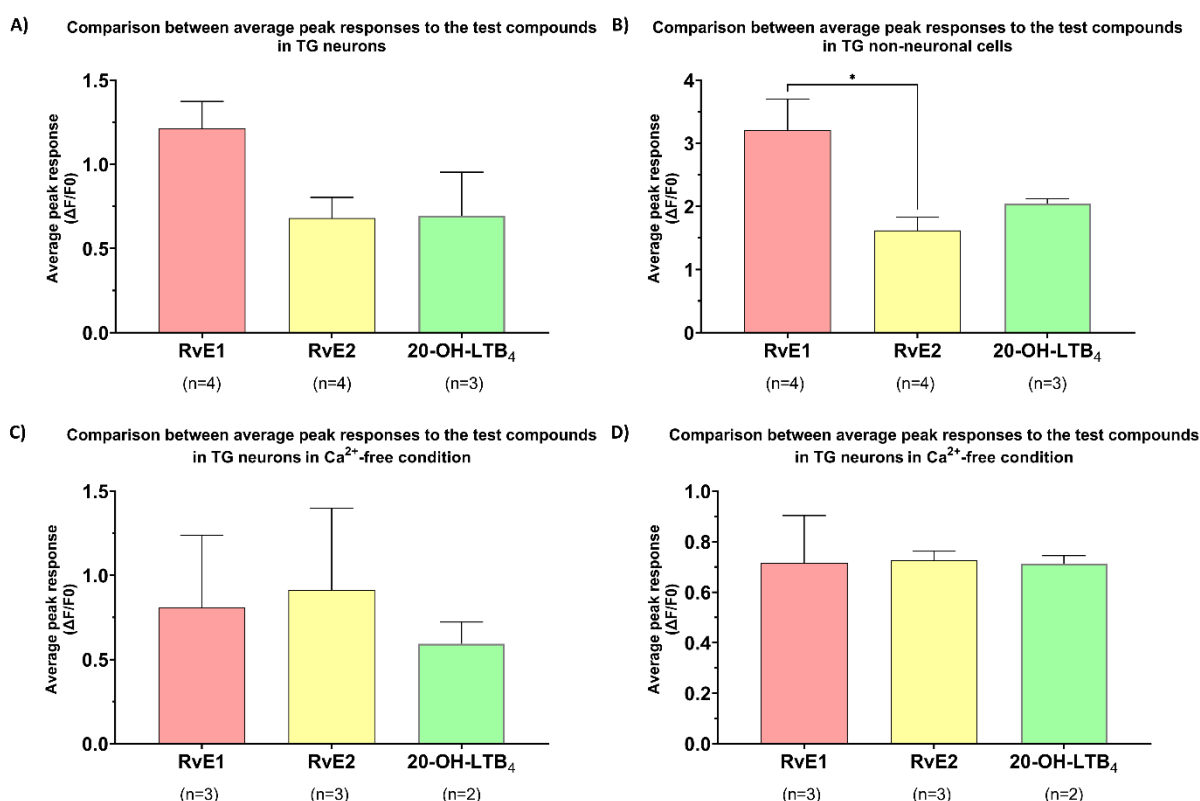


Figure 5.13. Comparison of Ca^{2+} transients induced by RvE1, RvE2, and 20-OH- LTB_4 in TG cells

(A – B) Average peak response to the test compounds (RvE1, RvE2, and 20-OH- LTB_4) in TG neurons (A) and non-neuronal cells (B). One-way ANOVA followed by Tukey's post-hoc test revealed a significant difference in the average maximal amplitude of Ca^{2+} transients induced by RvE1 and RvE2 in TG non-neuronal cells ($p=0.01^*$). Error bars represent mean $\pm$ SEM.

(C – D) Average peak response to the test compounds (RvE1, RvE2, and 20-OH- LTB_4) in TG neurons (C) and non-neuronal cells (D) in calcium-free conditions. One-way ANOVA revealed no significant difference in the average maximal amplitude of Ca^{2+} transients induced by the test compounds in TG cells. Error bars represent mean $\pm$ SEM.

5.2.3. Effects of applications of resolvins on cAMP responses in cultured TG cells

Activation of Gi/o protein-coupled receptors results in inhibition of adenylyl cyclase and the subsequent reduction in intracellular levels of cAMP. In order to establish if BLT1 was coupled to Gi/o GPCR signalling cascades, changes in cAMP levels in cultured rat TG cells were detected by measuring changes in fluorescence intensity of a genetically encoded cAMP biosensor, cADDIs, over time following application of RvE1, RvE2, and selective BLT1 agonist 20-OH-LTB₄ via time-lapse confocal microscopy. The cells were transduced with a cADDIs, a fluorescent protein that changes in fluorescence intensity in response to changes in cAMP level. A downward sensor was used, and thus an increase in fluorescence intensity is indicative of a reduction in the level of cAMP (Tewson et al., 2018).

Forskolin, which directly activates adenylyl cyclase and increases the level of cAMP (Seamon et al., 1983), was first applied to (i) test the reliability of cAMP level reporting by cADDIs and (ii) to raise the basal cAMP level in cells such, that the potential activation of Gi/o-coupled signalling cascade following application of RvE1, RvE2, and 20-OH-LTB₄ could be observed. In the experiments presented in Figures 5.14 – 5.16, a decrease of the cADDIs fluorescence corresponds to raising cAMP levels, while an increase in fluorescence is indicative of a decline in cytosolic cAMP. The proof of principle experiment is shown in Figure 5.14. Forskolin application produced a robust drop in cADDIs fluorescence, indicating adenylyl cyclase activation. I noted however, that after the washout of forskolin, the level of cAMP appeared to reduce gradually (manifested in the recovery of cADDIs fluorescence), which occurred in the absence of test compounds (extracellular solution only) (Figure 5.14).

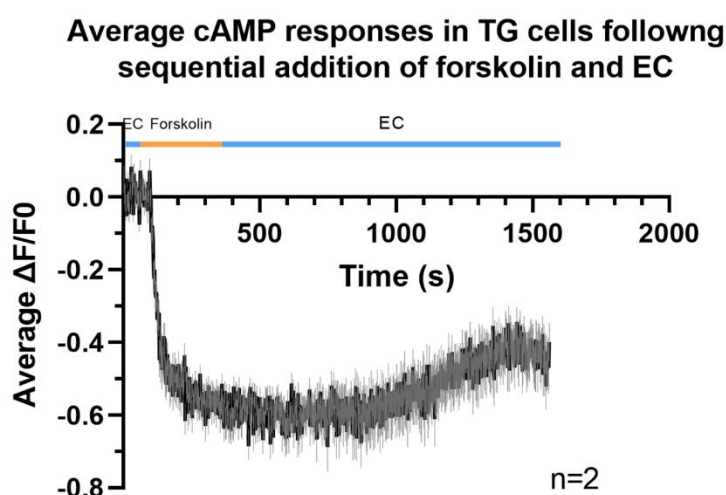


Figure 5.14. Recovery of cAMP level in the absence of test compounds following forskolin-stimulated production of cAMP in TG cells

Traces showing mean cAMP responses in TG cells transduced with a cADDIs cAMP sensor following application of forskolin and extracellular solution. The shaded area represents SEM (Mean of 19 TG cells across 2 animals).

In the second proof of principle experiment, the TG cells were also transduced with a Gi-coupled receptor, hD2 receptor. Quinpirole, a D2 receptor agonist, was thus used as a positive control as it could stimulate Gi signalling. To evaluate the validity of the G_{ai} assay used, cAMP responses induced by quinpirole were compared with responses induced in the absence of test compounds (extracellular solution only). Quinpirole produced a much faster recovery of cADDis fluorescence after forskolin-induced quenching, as compared with the slow recovery seen in the absence of test compounds (Figure 5.15).

Comparison of cAMP responses to quinpirole or EC in TG cells following addition of forskolin

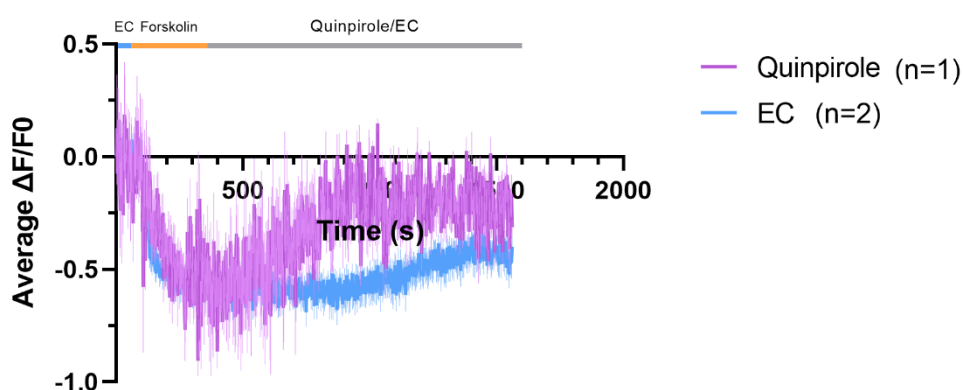


Figure 5.15. Reversal of forskolin-stimulated production of cAMP by application of quinpirole in TG cells

Traces comparing mean cAMP responses in TG cells transduced with a cADDis cAMP sensor induced by application of quinpirole and extracellular solution only. The shaded area represents SEM (Quinpirole: Mean of 4 TG cells in 1 animal; EC: Mean of 19 TG cells across 2 animals).

cAMP responses induced by RvE1, RvE2, and 20-OH-LTB₄ were therefore compared with responses induced in the absence of test compounds to evaluate if Gai signalling was activated. RvE2 (Figure 5.16B) and 20-OH-LTB₄ (Figure 5.16C) produced much faster cADDIs fluorescence recovery, as compared with that induced in the absence of test compounds, while the responses induced by RvE1 (Figure 5.16A) appeared to mirror closely with that induced in the absence of test compounds. Time limitations prevented me from obtaining sufficient repeats for these experiments, but the data do indicate activation of Gi/o signalling cascade by RvE2 and 20-OH-LTB₄.

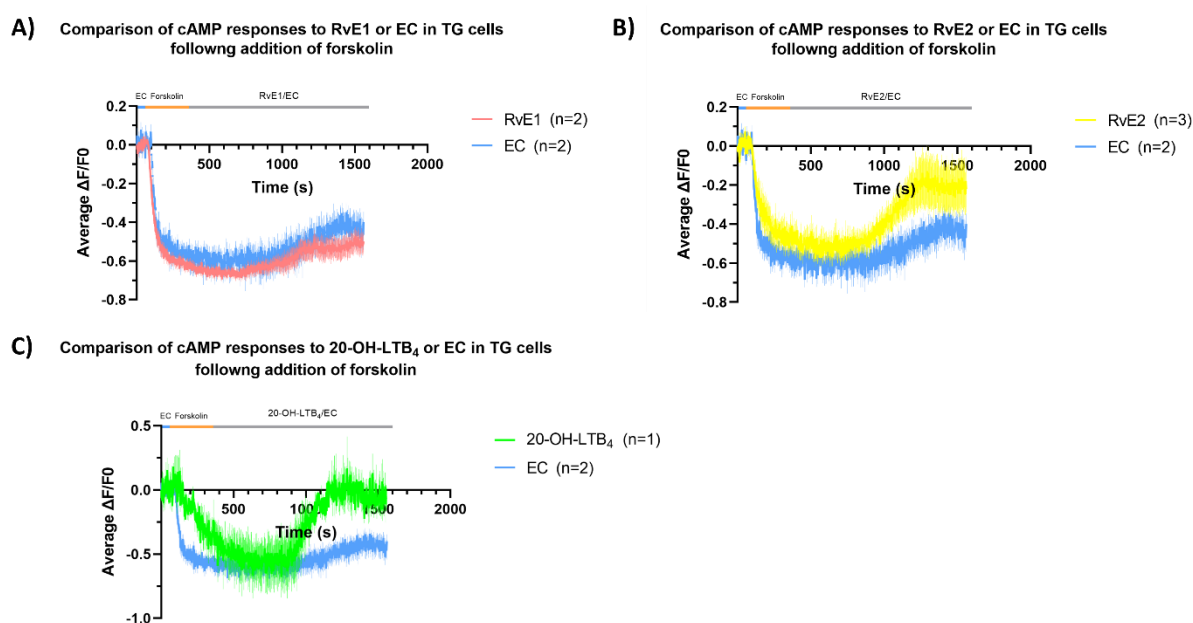


Figure 5.16. Reversal of forskolin-stimulated production of cAMP by application of RvE1, RvE2, and 20-OH-LTB₄ in TG cells

- A) Traces comparing mean cAMP responses in TG cells transduced with a cADDIs cAMP sensor induced by application of RvE1 and extracellular solution only. The shaded area represents SEM (RvE1: Mean of 18 TG cells across 2 animals; EC: Mean of 19 TG cells across 2 animals).
- B) Traces comparing mean cAMP responses in TG cells transduced with a cADDIs cAMP sensor induced by application of RvE2 and extracellular solution only. The shaded area represents SEM (RvE2: Mean of 14 TG cells across 3 animals; EC: Mean of 19 TG cells across 2 animals).
- C) Traces comparing mean cAMP responses in TG cells transduced with a cADDIs cAMP sensor induced by application of 20-OH-LTB₄ and extracellular solution only. The shaded area represents SEM (20-OH-LTB₄: Mean of 2 TG cells in 1 animal; EC: Mean of 19 TG cells across 2 animals).

5.3. Discussion

In this study, the intracellular signals transduced upon binding of RvE1 and RvE2 to BLT1 in TG cells were examined to establish the possible downstream signalling cascades triggered in TG. Results obtained from this study suggest that BLT1 may be coupled to Gq protein in TG neurons and non-neuronal cells, and gave some indication that RvE1 and selective BLT1 agonist 20-OH-LTB₄ may also directly activate Ca²⁺-permeable channels on the plasma membrane in TG non-neuronal cells. The findings also suggest that RvE2 and 20-OH-LTB₄ may also activate Gi/o-linked signalling in TG cells, as they could induce a reduction in the level of cytosolic cAMP. To the best of my knowledge, this is the first study that investigates the intracellular signalling cascades activated by RvE1 and RvE2 in TG cells.

5.3.1. BLT1 expression in TG cells

BLT1, a receptor for RvE1 and RvE2, was identified in rat TG neuronal cell body and its processes. This is shown in the confocal images where BLT1 staining overlapped with neuronal marker β -tubulin III (Figure 5.1) and myelinated neuron marker NF160/200 (Figure 5.2). This suggests that BLT1 is found in large-diameter myelinated A-fibres such as A β - and A δ - fibres. However, additional studies are required to further characterise BLT1 expression in TG neurons by dual labelling, for example with the peptidergic neuronal marker CGRP and the non-peptidergic neuronal marker isolectin B4 (IB4) (Barabas et al., 2012). BLT1 expression was also identified in rat TG glial cells. This is shown in the representative confocal image where BLT1 staining overlapped with satellite glial cell marker glutamine synthetase (Figure 5.3). A blocking peptide corresponding to this BLT1 antibody was not available, but no positive staining was identified in the absence of the BLT1 antibody (Figure 5.1E), indicating that there is no non-specific binding by the secondary antibody. Taken together, these findings provide evidence that BLT1 is present in the major cell types, neuronal cells and glial cells, that are present in the cultured TG cells (Bender, 2008). To the best of my knowledge, this is the first description of BLT1 expression in rat TG cells. This is consistent with findings from human TG using single-nucleus RNA sequencing (Yang et al., 2022), where BLT1 gene expression is also identified in TG neurons and satellite glial cells (based on publicly available single-nucleus RNA sequencing data from human TG <https://painseq.shinyapps.io/tg-painseq/>).

5.3.2. Ca²⁺ transients induced by RvE1, RvE2, and 20-OH-LTB₄ in TG cells in the presence of extracellular Ca²⁺

Application of RvE1, RvE2, and selective BLT1 agonist 20-OH-LTB₄ was found to result in an increase in intracellular calcium levels in TG neurons and non-neuronal cells (Figures 5.4, 5.7, and 5.10 respectively), and the average maximal amplitudes of Ca²⁺

transients induced by RvE1, RvE2, and 20-OH-LTB₄ was found to be significantly higher in TG non-neuronal cells than TG neurons. This might be due to higher expression of BLT1 in TG non-neuronal cells, but this would need to be examined with more detailed immunocytochemistry experiments to characterise BLT1 expression in TG neurons and non-neuronal cells in future studies.

Extracellular Ca²⁺ is transported into the cytosol when Ca²⁺-permeable channels on the plasma membrane are activated (Taylor et al., 2009). In the present study, the average maximal amplitudes of Ca²⁺ transients induced by RvE1 and 20-OH-LTB₄ in TG non-neuronal cells are significantly higher in the presence of extracellular Ca²⁺ than that induced in Ca²⁺-free conditions ((Figures 5.6 E and 5.12E respectively). This indicates that the increase in intracellular calcium induced by application of RvE1 and 20-OH-LTB₄ is induced by both the entry of extracellular Ca²⁺ via plasmalemmal Ca²⁺-permeable channels and the release of calcium from intracellular stores. Therefore, it is possible that RvE1 and 20-OH-LTB₄ might directly activate Ca²⁺-permeable channels on the plasma membrane such as TRP channels in TG non-neuronal cells. This might also explain why the maximal amplitude of RvE1-induced Ca²⁺ transients was significantly higher than RvE2-induced Ca²⁺ transients in TG non-neuronal cells (Figure 5.13B), while no significant difference was found between the maximal amplitudes of Ca²⁺ transients induced by RvE1, RvE2, and 20-OH-LTB₄ in TG neurons (Figure 6.13A). Previous studies demonstrated that RvE1 can inhibit the activities of TRPV1 and TRPA1 in DRG neurons (Xu et al., 2010, Park et al., 2011, Pyo et al., 2022), but these studies did not explore the effects of RvE1 on TRPV1 and TRPA1 activities in non-neuronal cells. In future studies, RvE1 can be applied in TG cells in the presence of TRP channel antagonists to investigate the potential interactions between RvE1 and TRP channels in TG non-neuronal cells. This can also help to establish if RvE1 and 20-OH-LTB₄ activate other Ca²⁺-permeable channels on the plasma membrane.

5.3.3. BLT1 may be coupled to Gq protein in TG cells

The Ca²⁺ transients induced by RvE1, RvE2, and 20-OH-LTB₄ in both TG neurons and non-neuronal cells were mostly abolished in the presence of selective BLT1 antagonist U-75302 (Figures 5.5, 5.8, and 5.11 respectively), indicating that calcium signalling in both TG neurons and non-neuronal cells are mediated by BLT1. However, since RvE1 and RvE2 also interact with ChemR23 (Deyama et al., 2018), additional studies using a selective ChemR23 antagonist are required to eliminate the possibility that the calcium signalling induced by RvE1 and RvE2 in TG cells is also mediated by ChemR23.

Application of RvE1 (Figure 5.6), RvE2 (Figure 5.9), and 20-OH-LTB₄ (Figure 5.12) in extracellular Ca²⁺-free condition resulted in an increase in intracellular calcium in TG neurons and non-neuronal cells, indicating that the increase in cytosolic calcium levels is

predominantly caused by the release of intracellular calcium from the intracellular store, such as the endoplasmic reticulum. This is what is expected from a Gq-type GPCR activation, as these activate PLC, release IP₃ and trigger the ER Ca²⁺ release in cells (Sanchez-Fernandez et al., 2014). In Ca²⁺-free condition, no significant difference was found between the amplitudes of Ca²⁺ transients induced by RvE1, RvE2, and selective BLT1 agonist 20-OH-LTB₄ (Figures 5.13C and 5.13D), which suggests that RvE1 and RvE2 are likely to act as agonists for BLT1 in TG cells. This is consistent with findings from previous studies in human and rat conjunctival goblet cells (Yang et al., 2020) and human peripheral blood mononuclear cells (Arita et al., 2007). Furthermore, it has previously been shown that calcium signalling induced by application of LTB₄ in CHO cells transfected with BLT1 are unaffected by pre-treatment with a Gi inhibitor pertussis toxin (PTX) (Yokomizo et al., 1997, Yokomizo et al., 2001), which suggests that the LTB₄-dependent calcium responses are not mediated by the Gi-Gβγ-PLCβ-Ca²⁺ pathway (Zeng et al., 2003). Therefore, it can be established that BLT1 is coupled to Gq protein in TG neurons and non-neuronal cells. Nevertheless, this would need to be confirmed in future experiments by pre-treating TG cells with PTX to explore if calcium signalling induced by RvE1 and RvE2 via BLT1 is sensitive to the blockade of the Gi-Gβγ pathway.

Activation of BLT1 by RvE1 and RvE2 likely leads to the release of intracellular calcium via the PLC signal transduction pathway. IP₃ can bind to IP₃ receptor in the endoplasmic reticulum, resulting in the release of stored calcium into the cytoplasm. However, further experiments are needed to demonstrate the association between binding of RvE1 and RvE2 to BLT1 and activation of the PLC signalling pathway in TG cells. In future experiments, TG cells can be pre-treated with the PLC inhibitor U-73122 (Macmillan and McCarron, 2010) prior to administration of RvE1 and RvE2 to investigate if the calcium responses induced by RvE1 and RvE2 are dependent on PLC. In a previous study, it was shown that PLC inhibitor U-73122 could completely abolish calcium responses induced by LTB₄ in HeLa HF1 cells transfected with BLT1 (Sabirsh et al., 2004). However, another study using cultured rat goblet cells found that pre-incubation with PLC inhibitor U-73122 does not affect RvE1-induced calcium signalling (Lippestad et al., 2018), and thus it is possible that the signalling pathways associated with BLT1 may vary in different cells. Therefore, it would be of interest to establish if inhibiting PLC in TG cells can abolish responses to RvE1 and RvE2 to further establish the downstream signalling pathways activated by binding of RvE1 and RvE2 to BLT1 in TG cells.

5.3.4. BLT1 may be coupled to Gi protein in TG cells

It is not uncommon that one GPCR can signal via more than one type of Gα protein (Fonin et al., 2019). Therefore, a Gai assay that could detect real-time Gai-mediated reduction in cAMP level (Tewson et al., 2018) was used to investigate if BLT1 is also coupled

to Gi protein. The probe faithfully responded to forskolin-induced cAMP elevation. However, after the washout of forskolin, the level of cAMP appeared to reduce gradually in the absence of any test compounds (Figure 5.14). This suggests that cAMP might be gradually hydrolysed to AMP by cAMP phosphodiesterase (Sassone-Corsi, 2012) within the 20-minute period when changes in fluorescence intensity were recorded in the presence of extracellular solution only.

To establish the validity of the G α i assay, quinpirole, a D2 receptor agonist that acts on the Gi-coupled hD2 receptor transduced in the TG cells, was used as a positive control to stimulate Gi signalling. Application of quinpirole led to a faster increase in cADDis fluorescence that is indicative of a reduction in cytosolic cAMP, as compared with the gradual reduction in cAMP seen in the absence of test compounds (Figure 5.15). This indicates that the G α i assay can detect G α i-mediated reduction in adenylyl cyclase activity, and the reduction in cAMP level in TG cells is predominantly caused by application of quinpirole, rather than conversion to AMP by cAMP phosphodiesterase.

Application of RvE2 and selective BLT1 agonist 20-OH-LTB₄ led to a faster increase in cADDis fluorescence that is indicative of a reduction in cytosolic cAMP in TG cells, as compared with the gradual reduction in cAMP seen in the absence of test compounds (Figure 5.16B and 5.16C). This suggests that the reduction in cAMP level in TG cells is predominately elicited by administration of RvE2 and 20-OH-LTB₄, rather than the gradual conversion to AMP. This provides some indication that BLT1 may, in addition to G α q, also couple to Gi signalling. Nevertheless, it is important to note that it is not known if BLT1 is intrinsically promiscuous and can couple to multiple G α proteins in all TG cells, or if BLT1 is coupled to G α q protein only in some subsets of cells and couples to Gi/o protein in other cells. On the other hand, the responses induced by application of RvE1 were indistinguishable from that induced in the absence of test compounds, indicating that the gradual reduction in cAMP level observed might be caused by the conversion to AMP by cAMP phosphodiesterase. It came as a surprise as this contradicts findings from a previous study, where RvE1 application was shown to reverse the production of cAMP stimulated by forskolin in HEK293 cells transfected with BLT1 (Arita et al., 2007). Additional repeats, which could not be conducted due to time constraints, are ideally needed to reach a better conclusion on whether RvE1 could activate Gi/o signalling in TG cells.

A recent study has revealed the cryo-electron microscopy structure of the LTB₄-bound human BLT1 in complex with a Gi protein in an active conformation (Wang et al., 2022a), and thus it is likely that BLT1 is coupled to Gi protein in TG cells, but further experiments are needed to demonstrate this. In future experiments, RvE1, RvE2 and 20-OH-LTB₄ can be administered with selective BLT1 antagonist U-75302 to determine if the Gi

signalling activated are mediated by BLT1 only. Additionally, TG cells can be pre-treated with a Gi inhibitor PTX (Mangmool and Kurose, 2011), which may potentially block the reversal of forskolin-activated cAMP responses induced by RvE2 and 20-OH-LTB₄, and thus can help to further establish that if BLT1 is coupled to Gi protein in TG cells.

5.3.5. Conclusion and future work

Results from this study showed that both RvE1 and RvE2 could induce calcium signalling via BLT1 in TG cells. Both RvE1 and RvE2 could activate the PLC-IP₃ signalling pathway to induce the release of calcium from intracellular stores in TG neurons and non-neuronal cells, indicating that BLT1 is coupled to Gq protein. In addition to this, RvE1 appeared to be able to directly activate Ca²⁺-permeable channels on the plasma membrane in TG non-neuronal cells to induce Ca²⁺ influx into the cytosol. On the hand, there was also some indication that application of RvE2 could lead to a reduction in cytosolic cAMP levels in TG cells, which suggests that they could activate the Gi/o signalling cascade. Taken together, these findings suggest that BLT1 may be coupled to both Gq and Gi proteins in TG cells, and the potential intracellular signalling cascades of RvE1 and RvE2 in TG cells are depicted in Figure 5.17. However, further experiments are needed to determine the validity of the proposed pathways, as well as investigate their role in pain.

As I was only able to spend three months at the University of Leeds to carry out the experiments described in this chapter, the number of animals used to prepare the cultured TG cells used in different experiments were limited by the time constraints and were relatively small, particularly in calcium imaging experiments using the BLT1 antagonist U-75302 and experiments using TG cells transduced with the cADDis cAMP sensor. In future experiments, a larger sample size can be used to improve the reliability of the results. Furthermore, to the best of my knowledge, this was the first time that the Gai assay was used in TG cells and thus the protocol might need to be further optimised for TG cells, which unfortunately was not done in the present study due to time constraints. Additional experiments are ideally needed to determine the optimal cAMP sensor volume using a titration series.

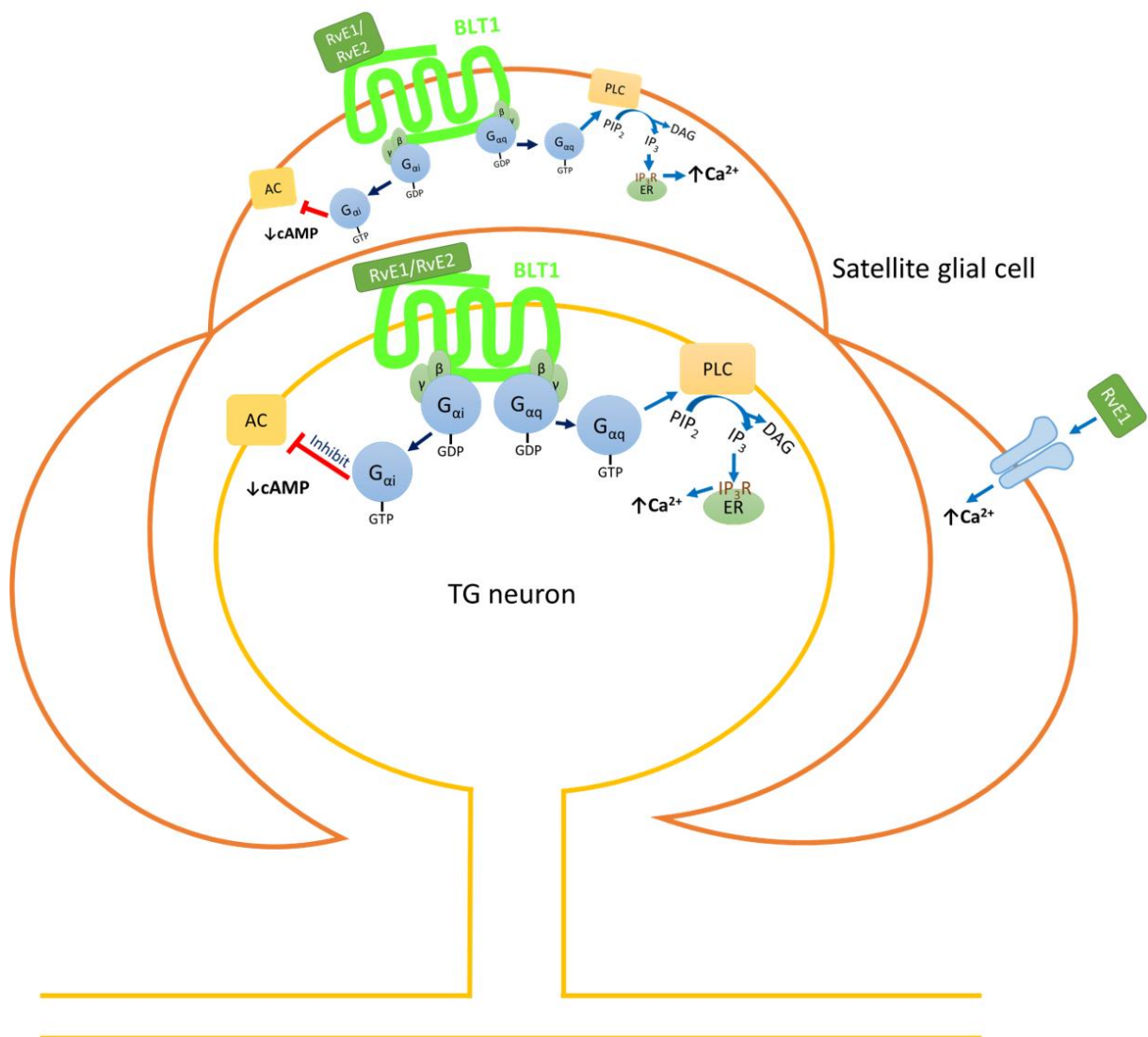


Figure 5.17. Proposed intracellular signalling cascades of RvE1 and RvE2 in TG cells. BLT1 is a GPCR that is likely to be coupled to both Gq and Gi proteins in TG neurons and non-neuronal cells such as satellite glial cells. Upon activation of BLT1 by RvE1 and RvE2, GDP-GTP exchange occurs, leading to dissociation of the heterotrimeric G protein, releasing α -GTP and $\beta\gamma$ subunits in their active forms. G α_q -GTP activates PLC, which hydrolyses PIP₂ to produce DAG and IP₃. IP₃ binds to its receptor, resulting in the release of calcium from the endoplasmic reticulum (ER) into the cytosol. On the other hand, G α_i -GTP inhibits the adenylyl cyclase (AC) signalling pathway and reduces the level of cAMP. In addition, RvE1 can also directly activate Ca²⁺-permeable channels to induce Ca²⁺ influx into the cytosol in TG non-neuronal cells such as satellite glial cells.

CHAPTER 6: EFFECT OF LINGUAL NERVE INJURY ON DRINKING BEHAVIOUR

6.1. Introduction

Several animal models have been used to investigate behavioural, physiological, and molecular changes linked with neuropathic pain. These models include a partial injury to the sciatic nerve induced by chronic constriction injury (CCI), first described by Bennett and Xie (1988). In this model, four sutures are loosely ligated around the sciatic nerve, thus constricting the nerve. This causes oedema to form around the injured portion of the nerve and is associated with a range of behavioural changes such as altered withdrawal thresholds to thermal and mechanical stimuli. Vos et al. (1994) then adapted this model for use in the orofacial system by carrying out CCI of the infraorbital nerve and demonstrated that behavioural changes such as face-grooming activity occurred after the surgery. The experiments described in this chapter aimed to evaluate the validity of CCI of the rat lingual nerve to investigate neuropathic pain associated with lingual nerve injury, with a view to using this model to investigate the effects of resolvins on pain-like behaviour change following lingual nerve injury.

Since the lingual nerve innervates the tongue, its receptive field is extremely difficult to access using conventional behavioural assessments (e.g., testing for withdrawal thresholds to thermal and mechanical stimuli using the plantar test and von Frey test respectively). Additionally, in recent years there has been a shift in the assessment of pain behaviour in animals, moving away from the predominant reliance on reflex responses (such as withdrawal reflexes). Therefore, the Orofacial Stimulation Test was adapted in this model (described in section 2.4.2 in the methods) to evaluate the effect of lingual nerve CCI on drinking behaviour. As described in section 2.4.2, in this test the rat voluntarily inserts the snout through an opening and uses the tongue to access a reward (chocolate milk) from a bottle. Animals have a choice between receiving the reward and avoiding tongue contact with the drinking bottle. Avoidance of drinking the reward, therefore, provides an indication of altered pain threshold to mechanical stimuli, linked with allodynia-like symptoms found in neuropathic pain.

This pre-clinical model was previously established in our laboratory (Tavares-Ferreira et al., 2019) and used to investigate the relationship between altered drinking behaviour and the expression of microRNAs in injured lingual nerve. The model allows quantification of drinking behaviour following CCI of the lingual nerve. Our previous work (using chromic catgut for the constriction) revealed a significant change in drinking behaviour; however, the behavioural change was less marked than those reported following sciatic nerve CCI (Medeiros et al., 2021). Many studies of sciatic CCI have used chromic catgut sutures for

constriction (Gabay and Tal, 2004, Medeiros et al., 2021), but there are several reports of similar behavioural changes using different sutures, including black silk (Kukkar et al., 2013) and cotton thread (De Vry et al., 2004), which are non-resorbable. In some cases, more extensive and longer-term changes are reported when using non-resorbable sutures (polypropylene sutures) as compared to resorbable chromic catgut sutures (Kupers et al., 1992). However, in other studies, chromic catgut ligatures appeared to induce more long-lasting and robust behavioural symptoms compared to silk ligatures (Robinson and Meert, 2005). In addition, at the time of these experiments, chromic catgut sutures were not readily available to purchase. Therefore, the aim of this study was to compare behavioural changes following lingual nerve CCI using chromic catgut and black silk sutures and determine their relative effectiveness in inducing pain-related behavioural changes; with a view to establishing a model to investigate the effects of resolvins on pain-like behaviour change following lingual nerve injury.

6.2. Results

6.2.1. Training period

As described in section 2.4.2 in the methods, all animals were trained to develop an interest in the reward (chocolate milk) for five days consecutively prior to surgery to establish a baseline on the day of surgery (day 0), as illustrated in Figure 6.1.

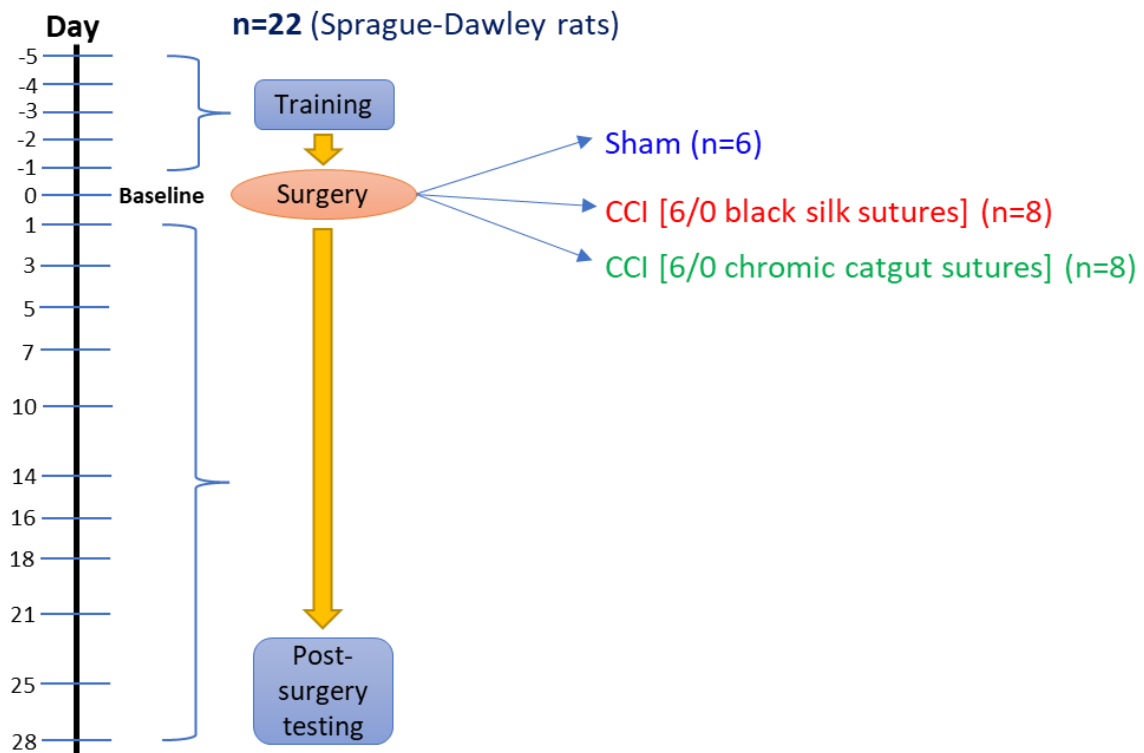


Figure 6.1. Time points of training and post-surgery behaviour testing.

Animals were trained to use the equipment for five days consecutively prior to surgery. Behavioural testing was then performed on the day of the surgery (day 0), and on days 1, 3, 5, 7, 10, 14, 16, 18, 21, 25, and 28 after the surgery.

The total number and duration of drinking attempts over the test period of 10 minutes were recorded and analysed automatically by the ORO software, as depicted in Figure 6.2A. The blue lines correspond to the interruption of the infrared barrier traversing the opening to the reward bottle, and therefore represent the duration and number of attempts to access the reward. During the training period, animals developed an increased interest in the reward, indicated by the increase in total time spent drinking over the 5-day training period, as shown in Figure 6.2B.

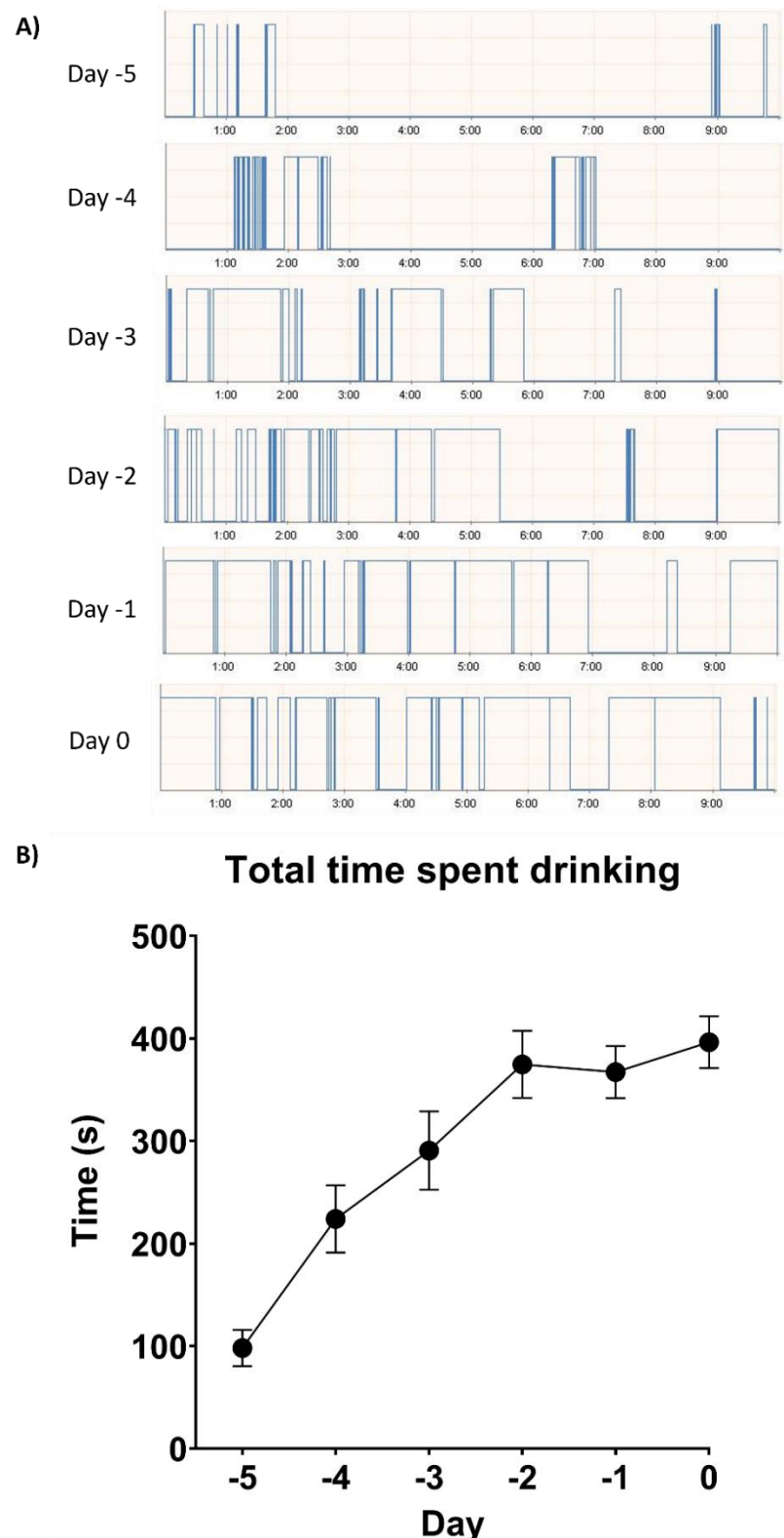


Figure 6.2. Development of interest towards the reward during the training period.

A) Example traces showing the automatic recording of the drinking behaviour of an animal over the 5-day training period (Day -5 to Day -1), and on the day of the surgery (day 0). The blue lines correspond to the duration of interruption of the infrared barrier traversing the opening to the reward bottle, and therefore represent the duration and number of attempts to access the reward.

B) Line graph showing total time spent drinking over the training period (Day -5 to Day -1) and on the day of the surgery (Day 0) in all animals ($n=22$). Error bars represent mean $\pm$ SEM.

6.2.2. Time spent drinking post-surgery

As described in section 2.4.2 in the methods, behavioural testing was conducted at regular intervals over a period of 28 days after the surgery (see Figure 6.1). The representative traces showing automatic recording of drinking behaviour by the ORO software during this period are shown in Figure 6.3. The blue lines correspond to the duration of interruption of the infrared barrier traversing the opening to the reward bottle, and therefore represent the duration and number of attempts to access the reward. This allows the total duration of the attempts to access the reward within the sham, CCI silk, and CCI catgut group to be further analysed.



Figure 6.3. Automatic recording of drinking behaviour by the ORO software.

Representative traces showing the automatic recording of the drinking behaviour of animals within the sham (left panel), CCI silk (middle panel), and CCI catgut group (right panel) over the test period of 10 minutes pre-surgery (Day 0), and on days 1, 3, 5, 7, 10, 14, 16, 18, 21, 25, 28 post-surgery. The blue lines correspond to the duration of interruption of the infrared barrier traversing the opening to the reward bottle, and therefore represent the duration and number of attempts to access the reward.

The total time spent drinking over the test period of 10 minutes post-surgery was adjusted to the percentage of baseline to take account of the variability between each animal. As shown in Figure 6.4, the total time spent drinking on days 1 and 3 post-surgery reduced in comparison to the baseline taken pre-surgery, with a greater reduction within the CCI catgut group especially seen on day 3 post-surgery. However, no significant difference between the sham, CCI silk, and CCI catgut group was found using two-way ANOVA repeated measures.

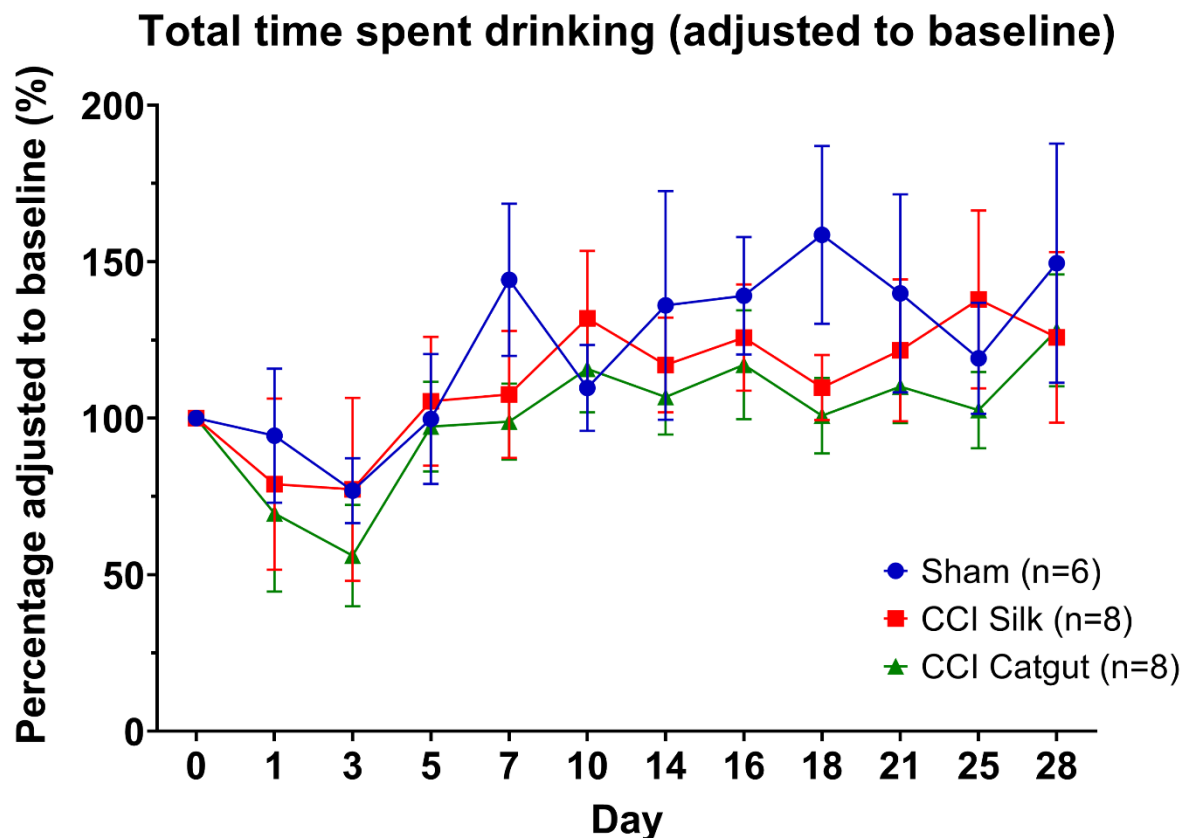


Figure 6.4. Total time spent drinking over the 28-day post-surgery test period. Line graph showing total time spent drinking (adjusted to the percentage of baseline) in sham, CCI silk, and CCI catgut group. Two-way ANOVA repeated measures revealed no significant difference in total time spent drinking between groups. Error bars represent mean $\pm$ SEM.

The effect of surgery on the total time spent drinking just within each group at individual post-surgery time points against the baseline obtained pre-surgery was also evaluated. Using a paired two-tailed t-test, the time spent drinking at each post-surgery time point was compared to the baseline obtained pre-surgery. A significant reduction in time spent drinking was found three days after lingual nerve injury within the CCI catgut group (Figure 6.5C) in comparison with the baseline ($p=0.004$). Within the CCI silk and sham group, however, no significant difference was found on day 3 following surgery in comparison with the baseline, as shown in Figure 6.5B and 6.5A respectively.

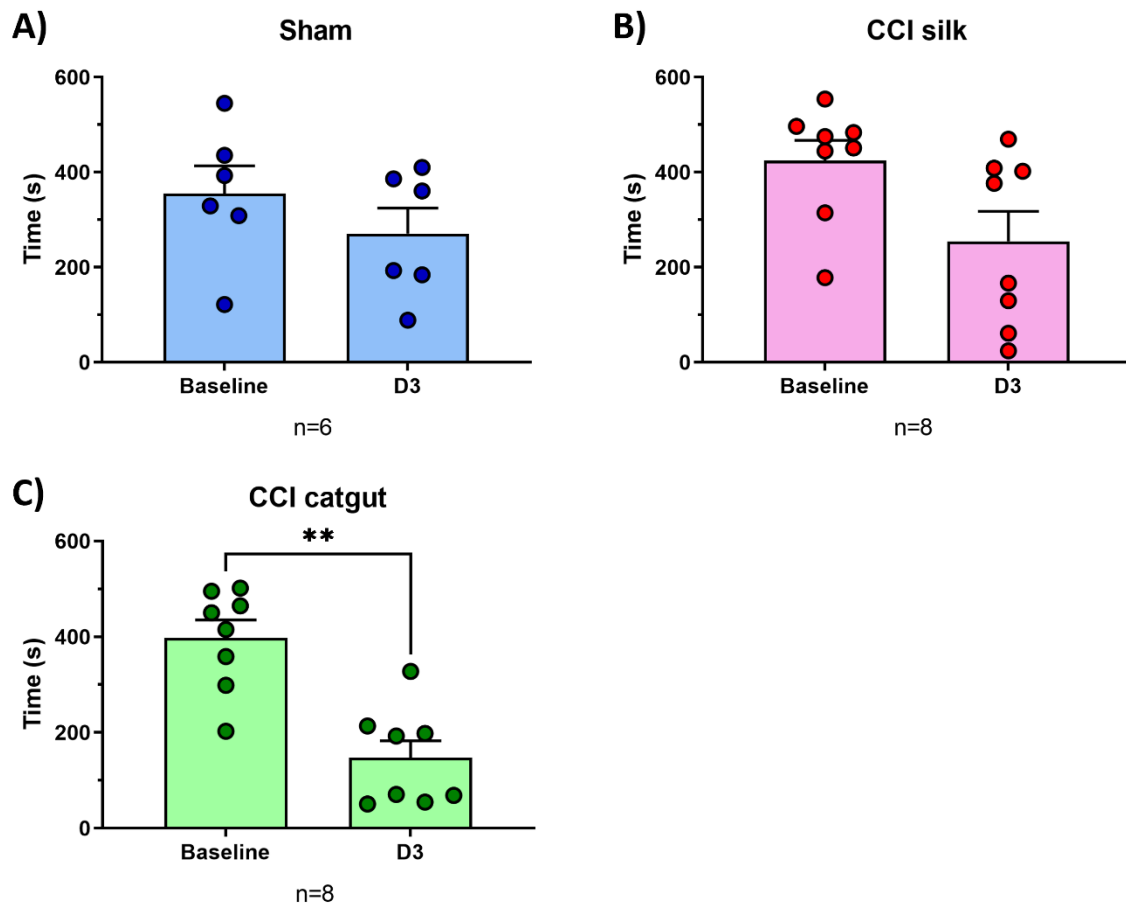


Figure 6.5. Lingual nerve injury in rats induced by chronic constriction using chromic catgut sutures reduced their total time spent drinking three days post-surgery.

A) Total time spent drinking within the sham group represented as mean $\pm$ SEM. Paired two-tailed t-test revealed no significant difference in total time spent drinking three days after the surgery (D3) in comparison with the baseline. Filled circles represent the total time spent drinking from each animal.

B) Total time spent drinking within the CCI silk group represented as mean $\pm$ SEM. Paired two-tailed t-test revealed no significant difference in total time spent drinking three days after CCI (D3) in comparison with the baseline. Filled circles represent the total time spent drinking from each animal.

C) Total time spent drinking within the CCI catgut group represented as mean $\pm$ SEM. Paired two-tailed t-test revealed a significant reduction in total time spent drinking three days after CCI (D3) in comparison with the baseline ($p=0.004^{**}$). Filled circles represent the total time spent drinking from each animal.

6.3. Discussion

In this chapter, the validity of the lingual nerve injury model of neuropathic pain in rats was evaluated. This pre-clinical model was previously developed in the laboratory to mimic the clinical condition of neuropathic pain resulting from lingual nerve injury in patients (Tavares-Ferreira et al., 2019). In addition to chromic catgut sutures, black silk was also used as suture material to evaluate if it could be a suitable replacement as chromic catgut sutures were not readily available to purchase at the time of planning this experiment.

As per the previous study in the laboratory, animals were first trained to develop an interest in the reward for five consecutive days prior to the surgery, and then underwent 28 days of behavioural testing after the surgery. The total time spent drinking within the 10-minute test period was analysed, and the values were adjusted to the percentage of baseline to account for variability between each animal. No significant difference in total time spent drinking was found between the sham, CCI silk, and CCI catgut group over 28 days (Figure 6.4). However, a clearer effect of surgery could be observed when the total time spent drinking was compared within each group on day 3. Within the group of animals that received lingual nerve CCI using chromic catgut sutures, the total time spent drinking chocolate milk was significantly reduced three days after CCI in comparison with the baseline (Figure 6.5C). However, there was no significant difference in the total time the sham-operated rats spent drinking on day 3 in comparison with the baseline (Fig. 6.5A). This suggests that surgical trauma alone was insufficient to induce changes in their drinking behaviour, indicating the changes in drinking behaviour within the CCI catgut group were due to lingual nerve injury. Since the tongue is innervated by the lingual nerve and tongue contact with the drinking bottle was required to access the reward, this behavioural change was indicative of increased sensitivity to touch stimuli and the presence of neuropathic pain within the CCI catgut group on day 3.

Within the group of animals that received lingual nerve CCI using black silk sutures, there was no significant difference in total time spent drinking on day 3 compared to the baseline (Figure 6.5B). This suggests that black silk sutures might not be a suitable replacement for chromic catgut in this animal model, as they failed to induce a similar behavioural change observed in animals within the CCI catgut group on day 3. This appears inconsistent with previous studies using CCI and other nerve injury models in the sciatic nerve, where silk sutures have produced significant and long-lasting behavioural changes (Kukkar et al., 2013). Nevertheless, previous studies directly comparing silk and chromic catgut for their effectiveness in inducing neuropathic pain behaviour in rodents suggest that suture materials could influence the stability of behavioural symptoms (Robinson and Meert, 2005, van der Wal et al., 2015). In a partial sciatic nerve ligation model in rats, while both suture materials could lead to sensory deficits after ligation, chromic catgut ligatures

appeared to induce more long-lasting and robust behavioural symptoms compared to silk ligatures, whereas the sensory deficits induced by silk ligatures diminished overtime at around 21 to 28 days after surgery (Robinson and Meert, 2005). This could be due to some of the chemical agents in chromic catgut as a previous study comparing the effects of chromic catgut, plain gut, and silk sutures in a sciatic nerve CCI model in rats observed that chromic catgut sutures could elicit a greater local inflammation in the constricted nerve while silk sutures could only incite a minor inflammatory reaction (Maves et al., 1993). The suggestion that silk sutures might be less effective in inducing neuropathic pain behaviour in rats might therefore contribute to the large variability in total time spent drinking between each animal in the CCI silk group on day 3 (Figure 6.5B).

It is important to note that the total time animals spent drinking within all 3 groups returned to baseline levels from day 5 after the surgery (Figure 6.4). However, this was not seen in the previous study conducted in our laboratory. In that study, the total time spent drinking within the CCI group did not return to the baseline, and two-way ANOVA repeated measures revealed a significant difference in the total time drinking between CCI and sham groups (Tavares Ferreira, 2017). Additionally, in the present study, a significant difference in total time spent drinking within the CCI catgut group was only observed on day 3 in comparison with the baseline (Figure 6.5C). However, in the previous study conducted in our laboratory, comparison to the baseline at individual time points showed significant differences at days 1 – 7, and 14 – 18 as well. It is unclear why there were such differences between the present study and the previous study conducted in our laboratory, but it may be attributed to a different surgeon conducting the surgery or a different recipe for the chocolate milk (even though the same brand was used). Nevertheless, despite these differences, the change in drinking behaviour on day 3 was consistent between the studies. Interestingly, a previous study investigating the generation of ectopic neural activity following lingual nerve CCI in ferrets demonstrated that the proportion of spontaneously active units and mechanically sensitive units in the filament were highest three days after injury, and the level of spontaneous activity reduced between three days and three weeks after injury (Yates et al., 2000). This could explain why drinking behaviour in all experimental groups returned to baseline levels from day 5. A similar electrophysiological study investigating ectopic activity after lingual nerve injury in rats could help to establish if the time course of spontaneous activity and mechanical sensitivity found in lingual nerve correlates with changes in drinking behaviour.

While a significant change in drinking behaviour was observed following CCI of the rat lingual nerve three days after injury, the behavioural change observed was less pronounced than those observed following CCI of the sciatic nerve, where pain-like behavioural changes such as a significant reduction in mechanical withdrawal thresholds to

non-noxious stimuli (that can be interpreted as allodynia) are sustained throughout the 28-day observation period (Medeiros et al., 2021). A previous study studied the effects of eating on digastric and neuronal responses to tooth pulp stimulation in cats, and found that these responses reduce when the animals are eating (Boissonade and Matthews, 1993). It is possible that pain responses in animals may be suppressed when they are actively eating, which was also observed in another study exploring the analgesic effects associated with chocolate consumption in rats injected with lipopolysaccharide (an inflammatory pain model) (Foo and Mason, 2009). Since animals could have free access to chocolate milk during behavioural testing, it might explain why the pain-like behavioural change observed in this model was less robust.

Based on the data from this study, chronic constriction injury of the lingual nerve using chromic catgut sutures affected drinking behaviour in rats, whereby a significant reduction in total time spent drinking was observed three days after injury. This reduction in time spent drinking might be indicative of hypersensitivity to touch stimuli applied to the tongue (innervated by the lingual nerve) via the drinking bottle, and thus the presence of neuropathic pain. Therefore, it was originally planned to test the effects of resolvins administration using this lingual nerve injury model of neuropathic pain to establish whether resolvins can alleviate neuropathic pain induced by lingual nerve injury. Plans were made to investigate if 17(R)-HDHA, a precursor of D-series resolvins with a longer-lasting effect (Huang et al., 2017), could reverse the behavioural changes that occurred on day 3. The effects of the 17(R)-HDHA would also be compared against the saline vehicle (as negative control) and gabapentin (as a positive control), a drug which is used clinically for treating peripheral neuropathic pain (Patel and Dickenson, 2016), and has been used to reduce behavioural signs of neuropathic pain in other animal models (Griggs et al., 2015, Christensen et al., 2001). Unfortunately, this planned experiment could not be conducted due to time constraints.

CHAPTER 7: GENERAL DISCUSSION

7.1. Summary of findings

In the studies reported in this thesis, expression of resolvins receptors BLT1, GPR32, and ChemR23 was characterised in human tooth pulp tissues that are relevant to inflammatory pain. To the best of my knowledge, this study is the first report that describes the distribution of these receptors in the human tooth pulp. The signalling pathways by which resolvins modify activity in trigeminal ganglion (TG) neurons were also investigated.

Based on data from a previous pilot study in our laboratory and the small number of studies that evaluated resolvins receptor expression in human tissues in the literature, antibodies that were raised against BLT1, ChemR23, and GPR32 were selected. A series of experiments were then conducted to validate the specificity of these antibodies (Chapter 3) using HUVECs, a human cell line that was previously shown to express these receptors via qPCR and western blot in the literature (Qiu et al., 2006, Krishnamoorthy et al., 2010b, Hao et al., 2015). The specificity of the antibodies was then further validated by selectively knocking down resolvins receptor expression in HUVECs via siRNA transfection. This study demonstrates that the antibodies raised against BLT1, ChemR23, and GPR32 could specifically label the relevant proteins, and were suitable for use in immunohistochemistry assays on human tooth pulp tissues.

These antibodies were then used to evaluate the expression of resolvins receptors in human tooth pulp tissues via immunohistochemistry to identify their relevance in inflammatory pain (Chapter 4). Expression of BLT1, ChemR23, and GPR32 was identified in nerve axons and blood vessels in human tooth pulp tissues. In particular, BLT1 expression in the vasculature was found to be significantly higher in inflamed tooth pulp tissues from carious painful teeth in comparison to inflamed tooth pulp tissues from carious non-painful teeth and non-inflamed human tooth pulp tissues from non-carious healthy teeth. This suggests that BLT1 activation may have a role in inflammatory pain.

The signalling pathways by which resolvins modify activity in rat TG cells were also investigated to explore the responses initiated by the resolvins-BLT1 axis (Chapter 5). To the best of my knowledge, this is the first study that investigates the intracellular signalling cascades of resolvins in TG cells. BLT1 was identified in TG neurons and satellite glial cells, and it was found that RvE1 and RvE2 could induce calcium mobilisation in TG neurons and non-neuronal cells via BLT1. Both RvE1 and RvE2 could activate the PLC-IP₃ signalling pathway to induce the release of calcium from intracellular stores in TG neurons and non-neuronal cells, indicating that BLT1 is coupled to Gq protein in TG cells. Additionally, RvE1 appeared to be able to directly activate Ca²⁺-permeable channels on the plasma membrane in TG non-neuronal cells to induce Ca²⁺ influx into the cytosol. There was also some

indication that application of RvE2 and selective BLT1 agonist 20-OH-LTB₄ could lead to a reduction in the level of cytosolic cAMP, which suggests that they can activate the Gi/o signalling cascade. Taken together, these findings indicate that BLT1 may be coupled to both Gq and Gi proteins in TG cells.

Additionally, the validity of the lingual nerve injury model of neuropathic pain in rats which was previously developed in our laboratory was evaluated (Chapter 6). It was found that chronic constriction injury of the lingual nerve affected drinking behaviour in rats, as indicated by a reduction in the time spent accessing the reward three days after injury. This suggests that this model may be used in future studies to investigate if resolvins administration could reverse or reduce the effects of lingual nerve injury on drinking behaviour in rats.

7.2. BLT1 expression in human pain-related tissues

To the best of my knowledge, this is the first study demonstrating that the resolvin receptor BLT1 is expressed in the nerve axons in the human tooth pulp. Due to the very limited availability of human TG tissues, rodent TG cells are a viable alternative to study the cell bodies of the pulpal afferent neurons located in the TG. In the present study, BLT1 is found within the rat TG neurons and in satellite glial cells that surround the neuronal cell bodies using immunocytochemistry. This is consistent with findings from human TG using single-nucleus RNA sequencing data (Yang et al., 2022), where BLT1 gene expression is also detected in TG neurons and satellite glial cells. Specifically, within human TG neurons, notable BLT1 gene expression is detected within peptidergic, non-peptidergic, and TRPM8+ nociceptors (based on publicly available single-nucleus RNA sequencing data from human TG (<https://painseq.shinyapps.io/tg-painseq/>)). Pulpal sensory nerves are primary afferent nerve fibres of the trigeminal nerve with cell bodies in the TG. Since BLT1 is present in several subtypes of nociceptors in human TG neurons, BLT1 is likely to also be expressed in nociceptors in the tooth pulp, and thus contribute to pain sensitisation. However, this would need to be investigated in future studies by dual labelling with peptidergic neuronal marker CGRP, non-peptidergic neuronal marker IB4, and TRPM8 antibody.

3D imaging of the whole tooth pulp shows that almost all blood vessels within the tooth pulp receive some degree of neural innervation along their path through the pulp (Franca et al., 2019). This indicates that there is a functional relationship between the vasculature and pulpal nerves. BLT1 is found in blood vessels in tooth pulp tissues, and its expression on the vasculature is upregulated in the inflamed tooth pulp tissues from patients with pain. Interestingly, a similar pattern of expression levels was also seen with TRPV1, as a previous study found that TRPV1 expression is significantly higher in carious painful samples in the central region of the tooth pulp compared to carious non-painful and healthy

samples (Morgan et al., 2005). Therefore, it is possible that the upregulated TRPV1 expression in carious painful tissues may be related to upregulated BLT1 expression. Previous studies in the literature found that RvE1 can suppress TRPV1 activity in DRG neurons (Xu et al., 2010, Park et al., 2011, Jo et al., 2016, Pyo et al., 2022). Jo et al. (2016) also suggested that RvE1-induced TRPV1 inhibition is dependent on GPCR signalling pathways, as the inhibitory effects of RvE1 on TRPV1 are abolished by GDP β S, a G protein inhibitor. Therefore, it is possible that RvE1 may indirectly inhibit TRPV1 activity via BLT1, as illustrated in Figure 7.1. However, the detailed molecular mechanisms by which resolvins inhibit TRP channels via BLT1 are still unknown and thus requires further investigation.

While the significance of the upregulated BLT1 in the vasculature in relation to pain is still unknown, it could also be related to the role of LTB₄ in inflammatory pain and vasodilation. LTB₄ is a pro-inflammatory mediator that acts as a full agonist for BLT1. The LTB₄-BLT1 axis was thought to be involved in inflammatory pain as blockade of LTB₄-BLT1 signalling reduces formalin-induced inflammatory pain in mice (Asahara et al., 2015). Additionally, a previous study indicated that LTB₄ may contribute to the development of vasodilation (Larkin et al., 1995). Therefore, it is plausible that the increased BLT1 expression in the vasculature may have a role in vasodilation in the tooth pulp. Since the tooth pup is enclosed in the pulp chamber and surrounded by hard tissues, vasodilation in this confined space would lead to increased pressure in the pulp chamber, compressing interdental nerves around the tooth pulp, thus leading to an increase in pain sensation.

E-series resolvins such as RvE1 can counter-regulate the action of LTB₄ by interacting with BLT1 and preventing the binding of LTB₄ to BLT1 (Yang et al., 2020). In a previous study, RvE1 can inhibit the LTB₄-induced IL-23 production in dendritic cells to a similar extent as a BLT1 antagonist (Sawada et al., 2018). Therefore, it is thought that RvE1 may mediate the resolution of inflammation by competing with LTB₄ for binding to BLT1, and thus dampens LTB₄-BLT1 signalling. However, a previous study reported that the levels of RvD1 and RvE1 in serum from patients with rheumatoid arthritis are significantly lower than that from healthy individuals (Ozgul Ozdemir et al., 2020), which raises the possibility that there may be a defect in the synthesis or catabolism of resolvins in patients with chronic pain. As a result of insufficient production of resolvins, the efficiency of the resolution of inflammation and pain may be compromised. Therefore, it is possible that limiting LTB₄-BLT1 signalling in the vasculature with RvE1 and RvE2 may counteract the effects of LTB₄ in blood vessels, and thus provide an analgesic effect. However, further studies are required to investigate if treatment with E-series resolvins (RvE1 and RvE2) can reduce the vasodilative effects of LTB₄ (Larkin et al., 1995). Additional studies are also needed to examine if the level of LTB₄ in the tooth pulp correlates with BLT1 expression and pain. In the present study, the levels of resolvins in the tooth pulp tissues could not be measured using the archive of tooth

pulp sections available to our laboratory. Further studies to measure the levels of resolvins and LTB₄ in tooth pulp tissues are therefore needed to interpret the functional significance of BLT1 in pain.

7.3. Responses mediated by BLT1 in TG cells

We have also shown that E-series resolvins, RvE1 and RvE2 can modify activity in TG cells via BLT1. As detailed in Chapter 5, application of RvE1 and RvE2 in TG cells can induce calcium signalling. Based on data reported in Chapter 5, RvE1 and RvE2 can activate the PLC-IP₃ signalling cascades to induce the release of calcium from intracellular stores in TG neurons and non-neuronal cells, indicating that BLT1 is coupled to Gq protein in TG cells. Previous studies suggested that RvE1 can indirectly inhibit the activities of TRPV1 and TRPA1 via GPCR-mediated signalling pathways (Xu et al., 2010, Park et al., 2011, Jo et al., 2016, Pyo et al., 2022). Additionally, it is thought that GPCRs can regulate TRP channel activity via their downstream signalling pathways including the PLC pathway (Roh et al., 2020). Based on publicly available single-nucleus RNA sequencing data from human TG (<https://painseq.shinyapps.io/tg-painseq/>), gene expression of BLT1 is found to colocalise with TRPV1 and TRPA1 in TG neurons (Yang et al., 2022). Taken together, these findings suggest that downstream signalling of resolvins-BLT1 may be linked to TRPV1 and TRPA1. Based on this proposed theory, it is possible that interactions between resolvins and BLT1 in nociceptive neurons may play a role in peripheral sensitisation via modulation of TRPV1 and TRPA1, as shown in Figure 7.1. However, further studies are needed to establish that RvE1 and RvE2 can attenuate TRPV1 and TRPA1 activities via BLT1 in TG cells, as their actions were mostly studied in DRG neurons in the literature (Pyo et al., 2022). Additionally, further experiments are needed to investigate the molecular mechanisms by which resolvins inhibit TRP channels via their receptors. Nevertheless, data from the studies presented in this thesis support the potential of BLT1 as a novel therapeutic target for chronic pain.

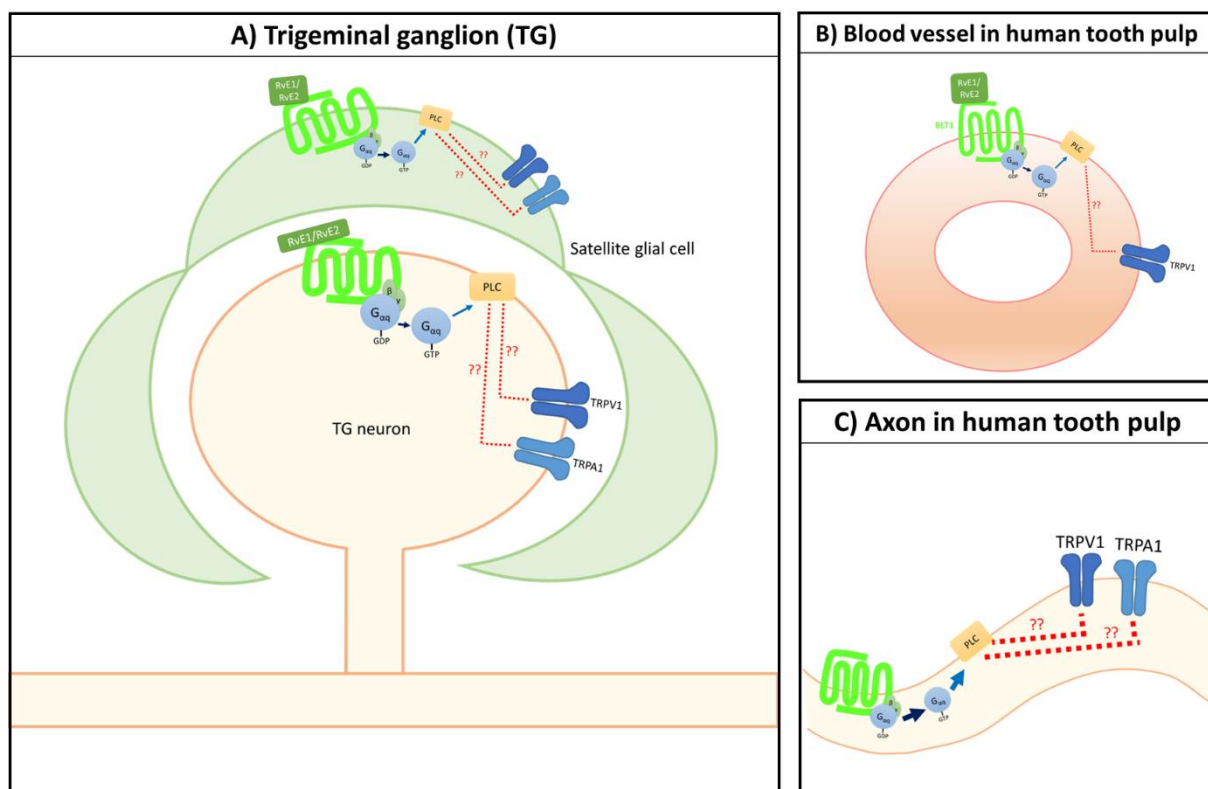


Figure 7.1. Proposed mechanism by which E-series resolvins (RvE1 and RvE2) may modulate TRPV1 and TRPA1 activity via BLT1, and potentially influence pain from human tooth pulp.

Based on the findings reported in this thesis, BLT1 expression was identified in rat TG neurons and satellite glial cells (A). In the human tooth pulp, BLT1 was found in blood vessels (B) and axons (C). It is possible that RvE1 and RvE2 may indirectly inhibit the activities of TRPV1 and TRPA1 via downstream signalling pathways mediated by Gq protein-coupled BLT1. However, the molecular mechanisms are still unknown and require further investigation.

7.4. Limitation of studies

After establishing the specificity of the antibodies raised against resolvin receptors, indirect immunofluorescence assays were conducted to examine the expression of resolvin receptors in human tooth pulp tissues. This is a well-established method that has been used to identify and assess receptor or protein expression in human tooth pulp tissues (Rodd and Boissonade, 2000, Rodd and Boissonade, 2001, Rodd and Boissonade, 2003, Morgan et al., 2005, Rodd et al., 2007) and lingual nerve neuromas (Morgan et al., 2009, Bird et al., 2013) in our laboratory. This technique enables the detection of the presence and localisation of resolvin receptors within the tooth pulp tissues. Dual labelling with known cellular markers also identifies cells that express the receptors, which may provide insights into the possible contributions of resolvin receptors in pain. However, the process of selecting a suitable threshold during image quantification can be subjective, as the threshold would need to be adjusted so it matches the positive labelling in the acquired image. In the present study, this was circumvented by selecting a suitable automated thresholding algorithm that applied to all images, so the data obtained would be consistent and replicable. Quantification was also repeated to check that the measurements were consistent and reproducible. Additionally, colocalisation between resolvin receptors and cellular markers was interpreted visually in the present study and thus might be subjective. In future studies, the level of colocalisation could be quantified by analysing colocalisation within individual pixels, so the level of colocalisation between BLT1/ChemR23 and β -tubulin III could be determined. This would help to establish whether axonal expression of BLT1 and ChemR23 alter with inflammation and pain.

Similar to the previous study in our laboratory that established the pre-clinical model of lingual nerve injury (Tavares Ferreira, 2017), only male rats were used in the present study. However, in previous studies, sex-related differences are observed in male and female rats with chronic constriction injury (CCI) of the sciatic nerve (Fonseca-Rodrigues et al., 2021). While both male and female rats with CCI develop behavioural signs of pain (e.g., allodynia to mechanical and thermal stimuli), only male rats show signs of recovery after three weeks which is not seen in female rats even after five weeks (Fonseca-Rodrigues et al., 2021). Sex-specific pain differences are also seen in human. It was reported that chronic pain is more prevalent in women, and they experience pain symptoms that are more intense and longer-lasting (Melchior et al., 2016). In future studies, both male and female rats should be included so findings may be more clinically relevant.

In the experiments presented in this thesis, sample sizes were mainly determined based on previous studies conducted in our laboratory. A total of 12 animals were used to establish the pre-clinical model of lingual nerve injury previously in our laboratory, with six animals randomly assigned to two groups: experimental CCI group and sham-control group (Tavares Ferreira, 2017). A similar number of animals was also used in the present study

(n=8 for the two experimental CCI groups and n=6 for the sham-control group) to establish the validity of this pre-clinical model. On the other hand, the number of animals used for calcium imaging and cAMP imaging of TG cells was limited by time constraints as these experiments had to be completed within three months. Additionally, the number of human tooth pulp samples per group was limited by the availability of samples in the archive available to our laboratory, particularly for the carious painful group where there were only six tooth pulp samples available.

7.5. Conclusions and future studies

In conclusion, findings from this study demonstrate that BLT1 expression in the vasculature is upregulated in inflamed tooth pulp tissues from patients with pain and show that RvE1 and RvE2 can modify activity via BLT1 in TG neuronal cell bodies. Taken together, these findings suggest BLT1 may be a promising target for chronic pain. To build on the findings described in this thesis, it would be of interest to measure the levels of resolvins in tooth pulp samples to establish if there is a correlation with pain and inflammation, as well as the expression of resolvin receptors. This may help to establish the functional role of resolvin receptors in inflammatory pain. Due to time constraints, only the signalling cascades of RvE1 and RvE2 via BLT1 were explored in the studies presented in this thesis. RvE1 and RvE2 also act via ChemR23, and thus it would be of interest to study their interactions with ChemR23 in TG cells as well. Further experiments could also be conducted to study the interactions between resolvins and TRP channels in TG cells to establish if resolvin receptors such as BLT1 are involved in the modulation of TRP channels, which may help to understand the underlying mechanisms by which resolvins regulate pain. The lingual nerve injury drinking behaviour model has also been further investigated in this study, demonstrating an effect of lingual nerve injury on drinking behaviour in rats. Further studies assessing resolvin receptor expression in tissues collected from animals in the sham and CCI groups three days after lingual nerve surgery can also be conducted. This may help to establish if there is a correlation between the expression of resolvin receptors and behavioural changes linked with lingual nerve injury in rats. The pre-clinical model of lingual nerve injury may also be used in future studies to assess if resolvins can reduce or reverse the behavioural change related to lingual nerve injury, however, the model is variable and it seems that consistent drinking behaviour changes are only present at early time points following nerve injury. Together, these may help to demonstrate the therapeutic potential of resolvins in neuropathic pain.

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