

**Investigation of the molecular
mechanism of VGF-derived peptides
in the pain pathway**

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Abstract

Pain is the major reason patients seek for medical care. Neuropathic pain is a form of chronic pain associated with neuropathy, which remains an area of considerable therapeutic need. The lack of effective therapeutic treatment for neuropathic pain is largely due to the fact that the underlying mechanism is incompletely understood.

Recent studies revealed *vgf* is the commonly upregulated gene in different animal models of neuropathic pain. In addition, VGF-derived peptides, TLQP-21, LQEQ-19 and TLQP-62 have recently been demonstrated to modulate pain pathways when applied *in vivo*. However, the precise underlying mechanisms of VGF-derived peptides in neuropathic pain and the molecular identity of the receptor for VGF-derived peptides have not yet been investigated. Here, I demonstrated that TLQP-21, but not LQEQ-19 nor TLQP-62, acts on the primary macrophage in a calcium-dependent and dose-dependent manner. The peptide induces release of calcium from the intracellular stores, and repetitive treatment of TLQP-21 desensitises the calcium response in the macrophages. In addition, intraplantar injection of TLQP-21-treated macrophages to the rat hind paw is demonstrated to elicit mechanical hypersensitivity. I have also verified GC1qR, the globular heads of the C1q receptor, is the potential receptor for TLQP-21 on macrophages by neutralising antibody and siRNA knock-down techniques. Moreover, application of the GC1qR neutralising antibody to partial sciatic nerve ligation model rats is shown to potentially delay the onset of mechanical hypersensitivity *in vivo*. Furthermore, I demonstrated TLQP-21 treatment induces differential gene expression in the primary macrophages by microarray. The results presented in this thesis highlighted the importance of VGF-derived peptide, TLQP-21 and its receptor, GC1qR, in the neuropathic pain development through, at least partly, activation of macrophages. The findings furthered our current understanding of the peripheral mechanism of neuropathic pain development, thus suggesting new potential target, and shed lights, for new neuropathic pain analgesic development.

Declaration of originality

I hereby declare that the data described in this thesis are original and they are the result of my own work, with exceptions indicated. All collaborations have been acknowledged in the appropriate places. Information derived from the published and unpublished work of others has been specified in the text and a list of references is given.

This work has been carried out at the Imperial College London and funded by the Wellcome Trust, UK.

A handwritten signature in dark ink, reading 'YaChun Chen' in a cursive script.

Ya-Chun Chen

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Abbreviation

5-HT	5-hydroxytryptamine (serotonin)
5HT-3R	5-HT receptor subtype 3
AGRP	Agouti-related peptide
ALS	Amyotrophic lateral sclerosis
Arg1	Arginase 1
ATP	Adenosine triphosphate
BAMBI	Bone morphogenetic protein and activin membrane-bound inhibitors
BDNF	Brain-derived neutrophic factor
CCI	Chronic constriction injury
CCR-3	CC chemokine receptor-3
CGC	Cerebellar granule cells
CGRP	Calcitonin gene-related peptide
ClCs	Voltage-gated chloride channels family
CNS	Central nervous system
Cox-2	Cyclooxygenase 2
CRE	Cyclic AMP responsive element
DRG	Dorsal root ganglia
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
FST	Forced swim test
FXII	Factor XII
GC1qR	The globular head of complement C1q receptor
GDFs	Growth and differentiation factors
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescence protein
GLUR5	Kainite receptor
GPCR	G protein-coupled receptors

GRKs	GPCR kinases
HCV	Hepatitis C virus
HEK293T	Human embryonic kidney 293T cell
HIV	Human immunodeficiency virus
HK	High molecular weight kininogen
IASP	International Association for the Study of Pain
IB ₄	α -D-galactosyl-binding lectin
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-18R	Interleukin-18 receptor
IL-1 β	Interleukin-1 β
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase/
IP ₃	Inositol 1,4,5-trisphosphate
IRAK	IL-1 receptor-related protein-activating kinase
JNK	c-Jun N-terminal Kinase
KCC2	K ⁺ -Cl ⁻ co-transporter isoform 2
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
M1	Classical activated macrophages
M2a	Alternatively activated macrophages
M2c	Anti-inflammatory macrophages
MAbs	Monoclonal antibodies
MCM	Macrophage conditioned media
MCP-1	Chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
NfatC2	T-cells calcineurin-dependent 2
NF κ B	Nuclear factor kappa B

NGF	Nerve growth factor
NGF	Nerve growth factor
NHS	N-hydroxysuccinimide
NIK	Nuclear factor kappa B -inducing kinase
NK1	Neurokinin 1
NKCC1	$\text{Na}^+ \text{-K}^+ \text{-2Cl}^-$ co-transporter
NpY	Neuropeptide Y
NT-3	Neurotrophin 3
PAG	Periaqueductal grey
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PKC	Protein kinase C
PNS	Peripheral nervous system
POD	Post-operative day
POMC	Pro-opiomelanocortin
PPC	Positive PCR Control
PSNL	Partial sciatic nerve ligation
PVDF	Polyvinylidene fluoride
PWT	Paw withdrawal threshold
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RIN	RNA integrity number
RMA	Robust multi-array analysis
rRNA	Ribosomal RNAs
RTC	Reverse Transcription Control
RVM	Rostral ventromedial medulla
RyR	Ryanodine receptors
ScrTLQP-21	Scrambled TLQP-21
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SNI	Spared nerve injury
SNL	Spinal Nerve Ligation
SNT	Sciatic Nerve Transection
SP	Substance P
SP-SAP	Substance P and saporin conjugate

SSRI	Selective serotonin reuptake inhibitor
STZ	Streptozotocin
TGF- β 3	Transforming growth factor β 3
TNF- α	Tumor necrosis factor- α
TRAF-6	Tumor necrosis factor receptor-associated factor-6
Tregs	Regulatory T cells
TrkA	High-affinity NGF tyrosine kinase receptor
TrpV1	Transient receptor potential vanilloid receptor-1
TST	Tail-suspension test
TTX	Tetrodotoxin
VGF	Plate V nerve Growth Factor inducible gene
VZV	Varicella Zoster Virus
WDR	Wide dynamic range

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CHAPTER 1:

GENERAL INTRODUCTION

1.1. PAIN

1.1.1. The problem of pain

Pain is a commonality among humans. According to the International Association for the Study of Pain (IASP), pain is a phenomenon described as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey 1994). The sensation of pain is an unique and subjective experience, which could be dependent on an individual’s past experience of pain, sex, personality, emotion and genetic predisposition. Due to all these contributory factors, the pain intensity perceived by different individuals, or even the same individual on different occasions, is more than often not in a fixed relationship to the same harmful or “noxious” stimulus. With so many variables and its underlying mechanism being incompletely understood, the pain assessment and management continues to challenge physicians and scientists.

Transient and acute pain, which exists for a limited duration, has evolved as a protective mechanism that is necessary for survival. It allows one to detect noxious stimuli, body injuries or diseases, which leads to the avoidance of the hazardous situation and other needed actions to stop it. The other benefit of such pain is that it forces one to rest and protect the wounds while repairing occurs, thereby enhances the body’s healing processes. Memories of earlier pain experiences also prevent re-exposure to the hazardous situations. Despite its many valuable functions, the molecular mechanism of pain still remains incompletely understood. It is currently known that pain perception process involves the detection of noxious stimuli by specialised sensory neurons (i.e. nociceptors). Upon encountering noxious stimuli, nociceptors respond by membrane depolarisation leading to subsequent events of action potential generation and signal transmission. Nociceptive signals are transmitted from the periphery to the spinal cord and, through an ascending pathway, to the brain. The brain could exert inhibitory controls over transmission in the dorsal horn and hence modulate the spinal output signals through a descending control system. The mechanism triggers the response of a body to injuries is extremely sensitive and sophisticated. Upon tissue damage, acute pain is typically reported, followed by an

increase in pain sensitivity to noxious stimuli (a phenomenon known as hyperalgesia) and pain response to innocuous stimuli (a phenomenon known as allodynia). Such transient acute pain serves as a protective mechanism that allows one to detect noxious stimuli and body injuries, and hence subsequently avoiding the dangerous situations while repair occurs.

However, when pain outlasts its usefulness and persists after the wound is healed (or exist without any identifiable causes), it becomes problematic. When pain becomes “chronic”, it is becoming a syndrome on its own rather than a by-product of injuries or diseases. Unlike acute pain that often goes away once the primary cause is resolved, chronic pain often persists for a long time or even life-long. Indeed, in a 4-year follow-up study of 2184 individuals, Elliott *et al.* revealed that the average annual incident rate of chronic pain in Grampian (UK) is about 8.3%, and only about 5% of the patients make a recovery (Elliott, Smith et al. 2002). Furthermore, chronic pain is problematic also in terms of the secondary psychological symptoms incurred. Secondary psychological symptoms such as anxiety and depression can profoundly affect patients’ physical activities and the quality of life. Indeed, a World Health Organisation collaborative survey of about 26,000 primary care patients across 15 centres around the world (including Asia, Africa, Europe and Americas) reported that 22% of the primary care patients (age between 18-65) suffer from persistent pain that lasts more than 6 months. It is also demonstrated that these pain sufferers are more prompt to psychological disorders such as depression and anxiety distress compared to the patients without pain. Moreover, some of these pain patients also reported physical disability that can affect their work, and often perceive themselves in moderate or even poor health (Gureje, Von Korff et al. 1998).

Neuropathic pain is a subtype of chronic pain that often associate with neuropathy. It is defined as “pain arising as direct consequence of a lesion or disease affecting the somatosensory system” (Geber, Baumgartner et al. 2009). Neuropathic pain affects a large proportion of patients suffering from a wide range of peripheral or central nervous system diseases. These include the direct nerve trauma, diabetes, viral infection such as human immunodeficiency virus (HIV) or Varicella Zoster Virus (VZV), cancer, spinal cord injury and as a side effect of anti-viral or anti-cancer treatments. Unlike effective treatment available for acute pain, the available treatments for neuropathic pain are

inadequate, and more than often, patients' conditions are not completely relieved after the treatments. Determining the treatment for individual patients and special populations (such as child) remains challenging. Knowledge of the molecular and pathophysiological mechanism underlying neuropathic pain may help the development of more specific, mechanisms-based analgesic for neuropathic pain, and hence shed the light for better neuropathic pain therapy and management. Thus, the aim and focus of this thesis is to further the understanding of the peripheral mechanism(s) contributing to the initiation and/or maintenance of neuropathic pain development.

1.1.2. Involvement of the primary sensory afferents in sensory transmission

Pain sensation is part of the somatosensory system. Indeed, the somatosensory system can be broadly divided into two major components: the mechanosensory system that detects mechanical stimulations (such as pressure, light touch, vibration, and physical distortion including bending and stretching) and the nociceptive system that is responsible for the detection of pain arising from tissue injuries or noxious thermal, mechanical, and chemical stimuli. Together, these two systems allow animals to sense the surrounding environment and events in their bodies, and most importantly, to evaluate these events for self-protection, survival and reproduction.

Initiation of nociception, as well as mechanoreception, requires the activation of the primary sensory neurons that innervate the stimulated regions. Such primary sensory fibres differ in sizes, peptide contents, and electrophysiological properties. All the sensory fibres that innervate regions of body arise from cell bodies in the dorsal root ganglia (DRG) and can be roughly divided into two groups, A fibres and C fibres, according to the myelination status and the corresponding axon diameters. The A fibres are subdivided into three subgroups, designated as A α , A β , A δ , in the order of decreasing in sizes (figure 1.1). These primary afferent fibres propagate action potentials at different conduction velocities, which are directly related to their axonal diameter and the extent of myelination (Djoughri, Bleazard et al. 1998). The myelinated, large-diameter A α / β fibres transduce fast action potential conductivity, and are involved in mechanoreception or proprioception (perception of the positions of limbs and other body parts in space). The sensory neurons involved in touch sensation are collectively known as low-threshold mechanoreceptor, because weak stimulus intensity

is sufficient to trigger action potentials in these fibres.

Sensory neurons that can be activated by the pain-producing stimuli are known as the nociceptors. Most of the nociceptors are small or medium in sizes, including the unmyelinated C-fibres that confer slow conductivity and thinly myelinated A δ fibres with moderate conductivity. It is worth noting that, some nociceptive neurons do exhibit fast conduction range of A α/β fibres (Lawson 2002). In general, nociceptors are duller than the mechanoreceptors and are referred to be the high-threshold sensory afferents, as the stimulation must be intense enough to activate these neurons and resulting in the subsequent perception of pain. C fibres are suggested to be the most abundant of the nociceptive fibres (Lawson 2002). Most, but not all, C-fibres are responsive to noxious thermal, mechanical and chemical stimulations, and hence are known as 'polymodal'. Some C-fibres respond only to the noxious thermal stimulation and some are only mechanically sensitive. Moreover, a small number of C-fibres are known as the 'silent' nociceptors as they only become responsive when sensitised by tissue injury (Schmidt, Schmelz et al. 1995). The C-fibres can be differentiated into two main groups by their neurochemistry, the peptidergic and the non-peptidergic population. The peptidergic C fibres express peptide neurotransmitter including substance P (SP) and calcitonin gene-related peptide (CGRP). Moreover, they are nerve growth factor (NGF) responsive as they express TrkA high-affinity NGF tyrosine kinase receptor. The peptidergic C fibres terminate superficially in the lamin I and outer lamina II in the spinal horn (Snider and McMahon 1998). The non-peptidergic C fibres, on the other hand, express α -D-galactosyl-binding lectin IB₄, P₂X₃ (a subtype of ATP receptor) and receptors for glial cell line-derived neurotrophic factor (GDNF). In addition, they terminate in the inner lamina II of the spinal horn (Snider and McMahon 1998).

The primary afferent inputs have been demonstrated to be pivotal in neuropathic pain by pharmacological evidences. AM1241 is a selective agonist for CB2 cannabinoid receptor, which is not expressed in the neurons of the central nervous system. Administration of AM1241 systemically has been shown to relief mechanical and thermal hyperalgesia in the Spinal Nerve Ligation (SNL) animal model of neuropathic pain (Ibrahim, Deng et al. 2003), suggesting a peripheral mechanism and the involvement of peripheral primary sensory afferents in the neuropathic pain

development. Moreover, intrathecal administration of antisense oligodeoxynucleotides targeting voltage-gated sodium channel $\text{Na}_v 1.8$, which is exclusively expressed in the small-diameter peripheral sensory afferents, results in reversal of mechanical hyperalgesia in the SNL animals (Porreca, Lai et al. 1999). Together, it is suggested that the peripheral sensory neuronal input plays an important role in, at least partly, the initiation and/or maintenance of neuropathic pain.

Nevertheless, the subsequent molecular mechanism of neuropathic pain has not been fully puzzled. It is generally believed that there is an initiation process and a maintenance step of neuropathic pain development occurring peripherally and centrally. Indeed, neuropathic pain normally develops gradually, which implies the onset and the maintenance of neuropathic pain is a complicated process. The current understanding of the molecular mechanisms of neuropathic pain can be divided into the peripheral mechanism and the central mechanism. It is currently believed both mechanisms play a pivotal role in initiating and maintaining neuropathic pain, which will be introduced in the following sessions.

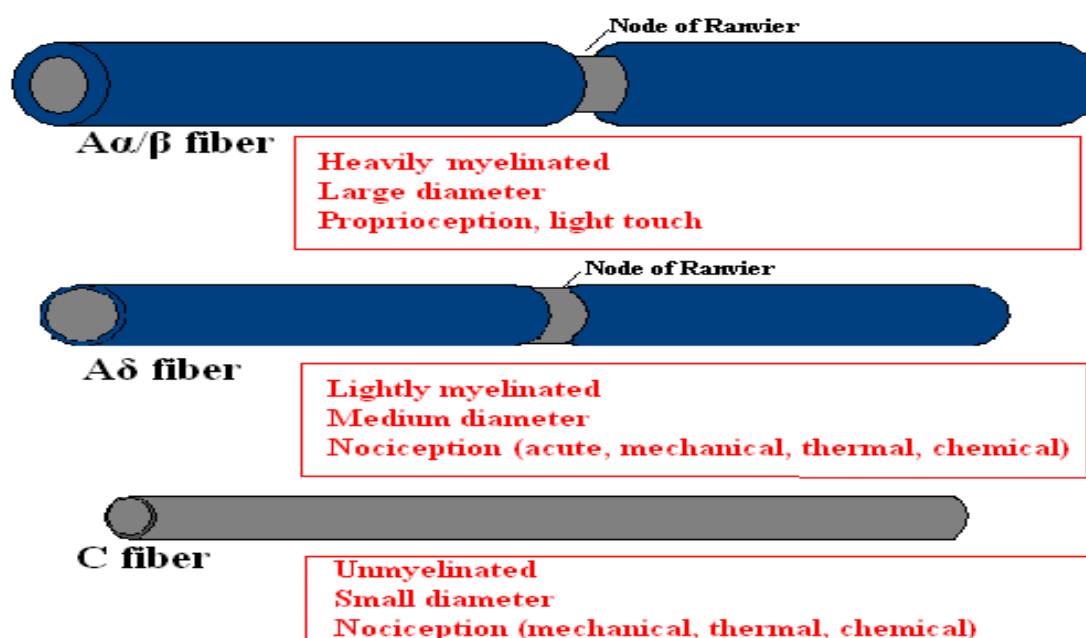


Figure 1. 1 Schematic illustration of different type of primary afferent neurons.

Aα/Aβ fibres are the heavily myelinated sensory fibres with large diameter axons. They confer conduction velocity up to 120 ms^{-1} (Aα fibre: $80\text{-}120 \text{ ms}^{-1}$, Aβ fibre: $35\text{-}75 \text{ ms}^{-1}$). Aδ fibre is thinly myelinated and possesses medium-size axon diameter, which propagate action potential at a velocity of $5\text{-}30 \text{ ms}^{-1}$. In contrast to the A fibres, C fibres are not myelinated and have a small axon diameter, which conduct at a velocity ranging from $0.5 - 2 \text{ ms}^{-1}$.

1.1.3. Peripheral mechanism of neuropathic pain

The existing understanding of peripheral mechanism of neuropathic pain can be roughly divided into two broad mechanisms, the neuronal mechanism of peripheral sensitisation and the peripheral non-neuronal mechanism of neuropathic pain.

Peripheral neuronal mechanism of neuropathic pain

Abnormal transduction

Upon nerve injuries, aberrant nociceptive signal transduction typically occurs. Such abnormal transduction can originate either from the injured neuron itself or the non-injured intact sensory neuron. Injured nociceptors produce spontaneous activity, which may play an important role in neuropathic pain (Matzner and Devor 1987). Indeed, the action potential propagation has been evident to be aberrant in the injured sensory afferents in the pathological condition, which change from regular firings to irregular bursts with increased spike duration, reduced threshold and slow rise- and fall-time kinetics (Liu, Michaelis et al. 2000). Moreover, clinical evidence also shown that blockade of the injured nerves by local anaesthetic attenuated spontaneous and evoked pain in patients with peripheral nerve injury (Arner, Lindblom et al. 1990). Study of the neuropathic pain animal model also suggested anaesthetic treatment of the L5 DRG relieved the mechanical hypersensitivity behaviour in SNL model (Sukhotinsky, Ben-Dor et al. 2004). Although the spontaneous and increased activities of injured nociceptors are believed to contribute to the enhanced nociception in the neuropathic condition, it has been demonstrated that activation of nociceptors is not the only way to trigger pain in the pathological condition. Low threshold sensory fibres that normally convey signals for light touch and other innocuous stimuli also start to elicit pain in the neuropathic condition. This is evident by enhanced spontaneous activity in the A fibre afferents recorded from the L5 dorsal root after spinal nerve injury (Liu, Wall et al. 2000). In addition, it is possible that A fibres could undergo phenotype switch, e.g. in terms of altered neurochemistry (Noguchi, Dubner et al. 1994), thus enhancing nociceptive transmission in the neuropathic condition.

The non-injured/intact nociceptor adjacent to the injured nerve has also been proposed to account for certain aspects of the aberrant nociceptive signalling upon peripheral

nerve injury. Removal of the injured afferents in the SNL animal by L5 ganglionectomy was demonstrated to still develop neuropathic pain, suggesting a role of the intact afferents in the initiation and maintenance of neuropathic pain (Sheth, Dorsi et al. 2002). In addition, it was demonstrated that after lesion to the L5 spinal nerve in the SNL model, spontaneous activity was observed in most of the C fibre afferents of the non-injured L4 spinal nerve within 1 day post-surgery, which is hypothesised to be resulted from the process of Wallerian degeneration (Wu, Ringkamp et al. 2001). It is thus speculated that the intact nociceptors that terminate around the same region as the injured nerve fibres may innervate the territory of the injured fibres and develop sensitisation and spontaneous activity, which contribute to neuropathic pain development. Indeed, sensitisation has been suggested in the intact nociceptors of the SNL model, in which an increase in responsiveness to heat and mechanical stimuli was observed in the intact nociceptor (Shim, Kim et al. 2005). Moreover, it has been shown, in the same animal model of neuropathic pain, that the intact nociceptor displays increase sensitivity to tumor necrosis factor- α (TNF- α) by single-fibre recording (Schafers, Lee et al. 2003).

Altered expression of key molecules in the nociceptors

Nerve injuries are often associated with neuroma formation at the injured nerve ending, which can elicit spontaneous activity and hypersensitivity to mechanical, thermal and chemical stimuli (Breward and Gentle 1985, Seltzer, Paran et al. 1991, Burchiel, Johans et al. 1993). It is revealed that large numbers of proteins are altered in expressions in the injured nerves, which are postulated to contribute to the hyperexcitability of the neuroma upon injury (Huang, Cendan et al. 2008). Thus, altered protein expressions in the injured neurons are speculated to profoundly change the threshold, excitability and transmission properties of the nociceptors, which may account for, at least partly, the hypersensitivity and the persistent nociception.

Indeed, altered expression or trafficking of voltage-gated sodium channels on the injured nociceptors has been suggested to cause the membrane hyperexcitability and subsequent spontaneous generation of action potentials. This resultant neuronal hyperexcitability is associated with ectopic nociceptor activities in the neuropathic condition. Among all members of voltage-gated sodium channels, altered expressions of Na_v1.3, Na_v1.7, Na_v1.8 and Na_v1.9 have been associated with neuropathic pain,

which will be briefly covered here.

Voltage-gated sodium channel $\text{Na}_v1.3$ is traditionally thought to be one of the sodium channels involved in embryonic nervous system development (Felts, Yokoyama et al. 1997). The expression of $\text{Na}_v1.3$ in the adult DRG is typically only observed at very low level in about 3% of the DRG neuronal population, which is shown to markedly increase in expression in a larger population (up to 21%) after peripheral nerve injury (Kim, Oh et al. 2001). In addition, the $\text{Na}_v1.3$ mRNA and protein expression in the DRG neurons has been shown to be significantly upregulated in a number of neuropathic pain animal models including axotomy (Waxman, Kocsis et al. 1994, Xiao, Huang et al. 2002), SNL (Kim, Oh et al. 2001), spared nerve injury (SNI) (Lindia, Kohler et al. 2005), chronic constriction injury (CCI) (Dib-Hajj, Fjell et al. 1999) and diabetic neuropathy model of neuropathic pain (Hong, Morrow et al. 2004). $\text{Na}_v1.3$ facilitates a tetrodotoxin (TTX)-sensitive current. The channel exhibits fast activation and inactivation kinetics with rapid repriming ability. The differential expression of $\text{Na}_v1.3$ in the neuropathic condition has been associated with the new emerged rapid-repriming TTX-sensitive current in the injured nociceptors (Kim, Oh et al. 2001). The plasticity and the increased rapid-repriming TTX-sensitive current in the injured nerve are postulated to account for the hyperalgesia of neuropathic pain (Dib-Hajj, Fjell et al. 1999). However, the hypothesis of the upregulated $\text{Na}_v1.3$ in the DRG after nerve injury plays an important role in development of neuropathic pain remains controversial. As it is demonstrated in the $\text{Na}_v1.3$ knock out mice that robust ectopic activity from the injured nociceptors are still observed in the absence of $\text{Na}_v1.3$, and the mechanical hypersensitivities are unaltered in both the $\text{Na}_v1.3$, conditional and global knock out. Thus, Nassar *et al.* proposed that $\text{Na}_v1.3$ is neither sufficient nor important for the development of nerve injury-induced neuropathic pain (Nassar, Baker et al. 2006).

$\text{Na}_v1.7$ is predominately expressed on the myelinated A-fibres. The expression of $\text{Na}_v1.7$ in the DRGs of the injured nerves is noticeably decreased following segmental spinal nerve ligation (Kim, Oh et al. 2002). Paradoxically, the $\text{Na}_v1.7$ expression and phosphorylation of the channel are shown to increase in the DRGs of the diabetic neuropathy models of neuropathic pain (Hong, Morrow et al. 2004). Such differential expression and channel phosphorylation are proposed to play roles in the neuropathic

pain associated with diabetic neuropathy. However, this concept is shown to contradict with the $\text{Na}_v1.7$ conditional knock out studies, in which the mechanical hyperalgesia associated with neuropathic pain developed normally in mice lacking $\text{Na}_v1.7$ or $\text{Na}_v1.7$ and $\text{Na}_v1.8$ (Nassar, Stirling et al. 2004, Nassar, Levato et al. 2005).

One of the other member of the voltage-gated sodium channel, $\text{Na}_v1.8$, has been proposed to be essential for the generation of spontaneous activity in the injured sensory neurons and is suggested to account for the development of aberrant mechano-hypersensitivity in the neuropathic condition (Roza, Laird et al. 2003). Unlike $\text{Na}_v1.7$, $\text{Na}_v1.8$ is mainly expressed on the unmyelinated nociceptive C-fibres. Surprisingly, when the expression pattern of this channel is examined in the neuropathic condition, a reduced expression of $\text{Na}_v1.8$ is observed in the DRGs of injured nerves in animal models of neuropathic pain (Boucher, Okuse et al. 2000, Decosterd, Ji et al. 2002, Wang, Sun et al. 2002). Such reduction in $\text{Na}_v1.8$ expression is also observed in the DRG of human patients (Coward, Plumpton et al. 2000). Although it is once suggested that an increase in $\text{Na}_v1.8$ expression is observed in the DRGs of non-injured nerves in the SNL model (Boucher, Okuse et al. 2000), Decosterd *et al.* showed that the $\text{Na}_v1.8$ expression in the non-injured nerves are not altered in the SNI, SNL, CCI and sciatic axotomy models of neuropathic pain (Decosterd, Ji et al. 2002). Therefore, the hypothesis of enhanced $\text{Na}_v1.8$ expression in the non-injured neurons, upon peripheral nerve injury, contributes to neuropathic pain still remains contrary and requires further investigation. Nevertheless, it has been claimed that $\text{Na}_v1.8$ undergoes redistribution in the axons of non-injured nerves without much affecting the expression in the DRG, and antisense oligodeoxynucleotides targeting $\text{Na}_v1.8$ prevents the channel redistribution along the axon and attenuates neuropathic pain *in vivo* (Gold, Weinreich et al. 2003).

$\text{Na}_v1.9$ is another voltage-gated sodium channel that is predominately expressed in the unmyelinated nociceptive C-fibres. $\text{Na}_v1.9$ expression has been shown to be significantly downregulated in DRGs in a number of models of neuropathic pain (Dib-Hajj, Tyrrell et al. 1998, Boucher, Okuse et al. 2000, Wang, Sun et al. 2002) and human patients in pain states (Coward, Plumpton et al. 2000). However, it is suggested by $\text{Na}_v1.9$ knock out mice that the channel contributes little to the mechanical and thermal hypersensitivity in the neuropathic condition (Priest, Murphy

et al. 2005). Nevertheless, it was demonstrated in the same study that inflammatory mediators could potentially modulate the function of Na_v1.9, and hence contribute to the inflammation-associated neuronal hyperexcitability.

Although some evidences are still controversial, the current literatures suggest that ectopic neuronal activity of the injured nerves, and possibility the non-injured afferents, is enhanced after neuropathy possibility through remodeling of sodium channel conductance. The alteration in sodium channel conductance can be resultant from changes in sodium channel trafficking, redistribution of the sodium channel and the differential expressions of the sodium channels upon nerve injury. Thus resulting in an increase in membrane hyperexcitability and reduced in firing threshold in the injured afferents and the subsequent neuropathic hypernociception. However, it is worth noting that voltage-gated sodium channel is merely one of the families that exhibit altered expressions upon nerve injury. Indeed, Hung *et al.* has demonstrated that about 200 proteins display changes in expression level at the injured nerve upon peripheral nerve injury. It is identified that various cytoskeletal proteins, metabolic proteins, redox proteins and protein chaperones are dysregulated after injury (Huang, Cendan et al. 2008). Moreover, other ion channels have also been shown to be dysregulated in gene expression upon nerve injury. Voltage-gated potassium channels (including K_v1.4, K_v4.3 and K_v9.3), kainite receptor GLUR5, serotonin receptor 5-HT₃R and nicotinic acetylcholine receptor α 3 are evident to downregulate in expression in the DRGs of SNL model (Wang, Sun et al. 2002). Whilst voltage-gated calcium channel α 2 δ subunit, together with a number of ion transporters, neuropeptides and signalling molecules, are shown to be upregulated in the DRGs of SNL animals (Luo, Chaplan et al. 2001, Wang, Sun et al. 2002).

The dysregulation process of differential gene expressions upon nerve injury and the exact mechanisms of how they contribute to the onset and maintenance of neuropathic pain are likely to be more complicated than one could comprehend. Nevertheless, the changes in the ion channels expressions in the injured nerves appear to suggest a potential modulatory mechanism to the injured nerve conductance, and the resultant ectopic activity subsequently contributes, at least partly, to the phenotypes of neuropathic pain.

Peripheral non-neuronal mechanism of neuropathic pain

It is now widely accepted that neuron is not the only cell type that is involved in the neuropathic pain development (Scholz and Woolf 2007). Schwann cells, satellite cells, glial cells and a number of immune cells including mast cells, neutrophils, macrophages and T cells have been implicated in the peripheral mechanism of neuropathic pain development.

Activation and infiltration of non-neuronal cells at the site of injury and DRG

Immediately after nerve injury, a cascade of signalling often occurs, which includes activation of resident mast cells, infiltration of neutrophils and macrophages to the site of injury, T cell activation and increased expression/release of proinflammatory cytokines (Olsson 1967, Perry, Brown et al. 1987, Kim and Moalem-Taylor 2011).

Indeed, peripheral resident mast cells are, among all, the first to be activated. The activated mast cells undergo degranulation at the site of injury, releasing histamine, proteases and cytokines (Zuo, Perkins et al. 2003). Degranulation of mast cells has been suggested to play an important role in initiating neuropathic pain development in the partial sciatic nerve ligation (PSNL) model of neuropathic pain. It is demonstrated inhibiting mast cell degranulation significantly attenuated neuropathic pain development in PSNL animals and the number of subsequent infiltrated neutrophils and macrophages are reduced (Zuo, Perkins et al. 2003). Moreover, denervated Schwann cells at the site of injury release chemoattractants such as leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1) to recruit resident macrophages to the lesion site (Sugiura, Lahav et al. 2000, Tofaris, Patterson et al. 2002). Consequently, both activated macrophages and denervated Schwann cells release matrix metalloproteases that acts on the basal lamina of endoneurial blood vessels, leading to blood-nerve barrier interruption.

Blood circulating neutrophils subsequently adhere to the vascular endothelium, followed by rolling and transendothelial migration to the site of injury (Witko-Sarsat, Rieu et al. 2000). A significant number of neutrophils are observed in the site of injury after peripheral nerve damage, which is often not found in the intact nerves (Perkins and Tracey 2000). It has been evident that neutrophils further infiltrate to the DRG

seven days after nerve injury. The number of neutrophils in the DRG ipsilateral to the side of injury is shown to increase by three folds. In addition, the isolated neutrophils from the peripheral nerve injured animals 7 days post surgery exhibit enhanced activity of superoxide production by 170% (Morin, Owolabi et al. 2007). Moreover, ablation of circulating neutrophils prior to nerve injury is demonstrated to alleviate the peripheral hypersensitivity in vivo. However, when neutrophils are depleted at day 8 post-injury, the peripheral hypersensitivity is developed normally (Perkins and Tracey 2000). This suggests a role for neutrophil in the early stage, possibly the initiation, of neuropathic pain development.

Increased activation of resident macrophages and enhanced peripheral macrophages infiltration to the injured nerve and the ipsilateral DRG have also been observed following peripheral nerve injury (Perry, Brown et al. 1987, Hu and McLachlan 2002, Hu, Bembrick et al. 2007, Isami, Haraguchi et al. 2013). Intriguingly, it is suggested that different subtypes of macrophages are differentially recruited to the injured nerve and the DRG. The M1 classically activated macrophages are predominantly recruited to the injured nerve after nerve injury. On the other hand, the M2 alternatively activated macrophages infiltrate the DRG ipsilateral to the injury on day 2 post-injury (Komori, Morikawa et al. 2011). In addition, it is recently evident that macrophage infiltration further proceeds to the central nervous system. Infiltration of peripheral macrophages into the spinal cord is observed 14 days after peripheral injury (Isami, Haraguchi et al. 2013). Furthermore, systemic depletion of macrophages prior to nerve injury has been demonstrated to alleviate peripheral nerve injury-induced thermal hypersensitivity, suggesting a role of macrophage in the development of neuropathic hypersensitivity following peripheral nerve injury (Liu, van Rooijen et al. 2000).

Following peripheral nerve injury, infiltration of T cells to the injured nerve and the ipsilateral DRG has also been reported (Hu and McLachlan 2002, Moalem, Xu et al. 2004, Hu, Bembrick et al. 2007). Infiltration of T cells to the injured nerve is first observed on day 3 post-peripheral nerve injury and peaks at day 21 (Moalem, Xu et al. 2004). The number of T cells is also observed to drastically increase in the ipsilateral DRG 3 days after sciatic nerve lesions, which is sustained up to 11 weeks post-injury (Hu and McLachlan 2002). It is hence suggested that T cells may contribute to the development of neuropathic pain. Indeed, it is demonstrated that rats lacking mature T

cells develop significantly less mechanical and thermal hypersensitivity after CCI surgery (Moalem, Xu et al. 2004). Although a role is suggested for T cell in the development of neuropathic pain, it is recently demonstrated that a subpopulation of T cells, regulatory T cells (Tregs), alleviate mechanical hypersensitivity after sciatic nerve injury. Depletion of this subpopulation of T cells is demonstrated to exacerbate hypersensitivity to mechanical stimulus following partial sciatic nerve ligation (Austin, Kim et al. 2012). Thus, it is possible that different subpopulations of T cells could contribute to the development of neuropathic pain differently.

Ectopic discharge of inflammatory mediators

As discussed, peripheral nerve injury is often accompanied by a dense cellular infiltration to the site of injury and DRGs ipsilateral to the injury. The infiltrated cells secrete many cytokines, chemokines and inflammatory mediators, which are postulated to contribute to the initiation of neuropathic pain. These inflammatory mediators include bradykinin (Janig, Levine et al. 1996), prostaglandin E2 (PGE2)(Ma and Eisenach 2003), nerve growth factor (NGF)(Boucher and McMahon 2001), serotonin, adenosine triphosphate (ATP)(Toulme, Tsuda et al. 2010), histamine, and endothelin-1 (Campbell and Meyer 2006). Proinflammatory cytokines such as IL-1 β (Okamoto, Martin et al. 2001), IL-6(Wei, Na et al. 2013), IL-18 (Miyoshi, Obata et al. 2008), interferon- γ (Racz, Nadal et al. 2008), tumor necrosis factor- α (TNF- α)(Wagner and Myers 1996, George, Schmidt et al. 1999), and leukemia inhibitory factor (LIF)(Tofaris, Patterson et al. 2002) have also been evident to play roles in the development of neuropathic pain, possibly by exacerbating the axonal damage (Keswani, Polley et al. 2003) and modulating nociceptor hyperexcitability (Wolf, Gabay et al. 2006) and stimuli hypersensitivity (Schafers, Lee et al. 2003) in the neuropathic condition.

The ectopic discharge of inflammatory mediators from the infiltrated cells play a role in the peripheral sensitisation of the nociceptors, and consequently account for, at least partly, the maintenance of neuropathic pain. Indeed, many of these inflammatory mediators rapidly excite primary sensory neurons in the peripheral nervous system and increase their excitability and ectopic firing. Such hyperexcitability of the peripheral nociceptors is fundamental to the neuropathic pain development.

For instance, Serotonin acts on the 5-HT₃ receptor that is expressed on sensory neurons. Activation of this ligand-gated ion channel causes sodium ions influx and neuronal

excitation upon binding. In addition, TNF- α is shown to induce ectopic activity in nociceptive primary afferent neurons (Sorkin, Xiao et al. 1997) and is evident to induce hyperalgesia upon endoneurial administration (Wagner and Myers 1996). Furthermore, ATP released at the extracellular space is evident to enhance neuronal activity through activation of its receptors. One of which, P2X₃, is shown to be selectively expressed at high level in the nociceptive sensory neurons (Chen, Akopian et al. 1995), and is implicated in neuropathic and chronic inflammatory pain conditions.

1.1.4. Mechanism of neuropathic pain: Central mechanism

In addition to the changes described at the periphery for neuropathic pain development, a diverse range of events also occur at the central nervous system upon nerve injury, which are believed to play important roles in the maintenance of neuropathic pain.

The nociceptive input is typically relayed from the periphery to the central locus via the nociceptive primary afferents that terminate in different laminae of the spinal dorsal horn. Here, various convergences and modulations can occur through input from, for example, the secondary nociceptive spinal neurons, wide dynamic range (WDR) excitatory neurons and inhibitory interneurons. The modulated nociceptive signal is subsequently transmitted to the brain via an ascending pathway. Secondary modulation of the nociceptive signals can also occur during descending signal transduction from the brain back to the spinal cord, and via neuronal plasticity within the spinal cord and higher structures. Modulations at these sites generally enhance neuronal excitability and disinhibition, and hence contributing to neuropathic pain development.

Spinal and supraspinal mechanisms

Neurotransmitters that are released from the central terminals of peripheral afferent fibres after nerve injury, such as SP and CGRP, are believed to induce amplification and prolongation of the postsynaptic response of spinal dorsal horn neurons to the nociceptive inputs (Budai and Larson 1996). This phenomenon is known as wind up, which is a form of long term plasticity of dorsal horn neurons. The enhanced activity is resulted from introduction of prolonged slow depolarization of the neurons by the neurotransmitters, and the subsequent activation of NMDA receptors. NMDA receptor

activation is evident to play a pivotal role in enhancing nociceptive signalling and resulting in hyperalgesia in the neuropathic conditions (Price, Mao et al. 1994, Sindrup and Jensen 1999). The output from the dorsal horn is transmitted by spinal projection neurons along the ascending pathway. Vast majority of these projection neurons are found at superficial dorsal horn in laminae I. About 80% of these neurons express the neurokinin 1 (NK1) receptor for SP. It is widely believed that NK1-positive cells transmit signals on to the brain regions such as thalamus, the periaqueductal grey (PAG), and parabrachial area (PB) (Todd 2002). In addition, these cells also project into brainstem areas such as rostral ventromedial medulla (RVM). Interestingly, RVM has been shown to be one of the regions where descending projection back to the dorsal horn occurs. The NK-1 expressing cells hence modulate spinal processing via this spino-bulbo-spinal loop, which first projects up from the spinal cord to the brain as described, via brainstem structure to modify the signals, and then descend back down to the spinal cord. Ablation of projections of NK1-expressing cells by substance P and saporin conjugate (SP-SAP) has shown to reduce excitability of deep dorsal horn WDRs, reverse neuropathy pain and plasticity in dorsal horn neurons (Suzuki, Morcuende et al. 2002). It has also been evident these changes are mediated, at least in part, by serotonin (5HT) acting on facilitatory 5HT₃ receptors.

Central immune mechanism and disinhibition of GABAergic neurons

There is growing evidence suggesting the central immune mechanisms also play a role in central sensitization. Microglia is a population of resident macrophages in the CNS, consisting about 5-12% of all cells in the CNS. Resting microglia have small soma with fine processes, which allow them to sense changes in the microenvironment. Once activated by stimuli such as trauma, ischemia or inflammation, they adapt an amoeboid shape and function as phagocytes in CNS (Vilhardt 2005). Activation of microglia promotes release of cytokines and chemokines such as IL-1 β , TNF- α and free radicals and increase in major histocompatibility complex (MHC) expression (McMahon, Cafferty et al. 2005). The MHC on the microglia in turn presents antigen to the T lymphocytes and causes their subsequent release of proinflammatory cytokines upon activation. Moreover, astrocytes in the CNS are also shown to be activated in response to nerve injury. There is growing evidence suggesting that astrocytes, along with microglia, contribute to neuropathic pain development (Colburn and DeLeo 1999,

Watkins, Milligan et al. 2001, McMahon, Cafferty et al. 2005). They are activated by peripheral nerve injury, and inhibition of their activations has been shown to attenuate the development of hyperalgesia and allodynia in many animal models of neuropathic pain (Raghavendra, Tanga et al. 2003, Ledeboer, Sloane et al. 2005).

Several inflammatory mediators play important roles in activating astrocyte and microglia in CNS, which include ATP, bradykinin, substance P and prostaglandins. ATP acts through one of its receptors, P2X₄, which is selectively expressed on the active microglia in response to the nerve injury and is suggested to be required for neuropathic pain development (Tsuda, Shigemoto-Mogami et al. 2003). Indeed, it is proposed that ATP induces a microglia-dependent disinhibition mechanism on GABAergic neurons, which results in aberrant excitatory signalling of the inhibitory neurons. The resultant aberrant signalling in turn contributes to the hypernociception in the neuropathic state (Tsuda, Shigemoto-Mogami et al. 2003, Coull, Beggs et al. 2005, De Koninck 2007). An increase in synthesis and activation of P2X₄ receptor is observed in hyperactive microglia after nerve injury through an ATP-dependent pathway, which stimulates the release of Brain-derived neurotrophic factor (BDNF) from the microglia. The secreted BDNF, in turn, acts on tyrosine kinase B (TrkB) receptors on the GABAergic neurons and results in downregulation of K⁺-Cl⁻ co-transporter isoform 2 (KCC2) (Rivera, Li et al. 2002, Rivera, Voipio et al. 2004) and upregulation of Na⁺-K⁺-2Cl⁻ co-transporter (NKCC1). The homeostasis of Cl⁻ in these neurons is altered as the Cl⁻ accumulates, which disrupts in the transmembrane chloride gradient and results in anion flux in the inverted direction when activated (Coull, Beggs et al. 2005). Thus, activation of these neurons becomes excitatory and the GABAergic neurons are ‘disinhibited’, which is proposed to be one of the central mechanisms that underlies neuropathic pain development (Coull, Boudreau et al. 2003) (figure 1.2). Interfering this disinhibition mechanism has been shown to alleviate neuropathic pain, in which inhibition of P2X₄ receptor is demonstrated to inhibit allodynia associated with neuropathy (Nagata, Imai et al. 2009).

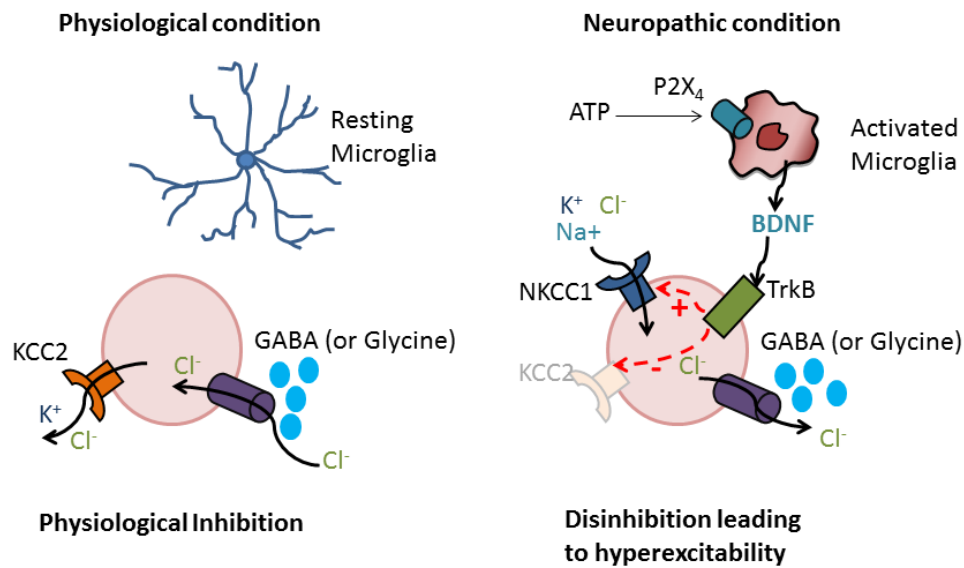


Figure 1. 2 Schematic diagrams illustrating the central mechanism of disinhibition in the pathophysiological condition compared to the normal inhibition at the physiological condition.

1.1.5. Therapeutic treatment of neuropathic pain and obstacles

Neuropathic pain therapy, unlike therapy available for acute and inflammatory pain, remains an area of considerable therapeutic need. Neuropathic pain often develops gradually, and it is believed rarely can one single mechanism be responsible for initiating and maintaining the symptoms. The process of generating and maintaining the symptoms is complicated and not fully understood. Despite large pharmaceutical interests in developments of analgesic for neuropathic pain, therapeutic treatment for neuropathic pain remains difficult. Currently, there is no single treatment that can be employed to treat all conditions of neuropathic pain, and more often than not, certain treatment could work on a subgroup of patients but not others with the same neuropathic symptom. It is also observed that drugs from the same categories can have completely opposite effect in pain relief.

The current treatments to neuropathic pain can be broadly divided into five drug classes, antidepressants, anticonvulsants, opioids, NMDA antagonists, and miscellaneous (Finnerup, Otto et al. 2005). Tricyclic antidepressants such as amitriptyline (other related drugs include imipramine, desipramine, venlafaxine and paroxetine) has been shown to relieve central post-stroke pain (Leijon and Boivie 1989) post-herpetic neuralgia (Watson, Evans et al. 1982, Kishore-Kumar, Max et al. 1990), painful

diabetic and non-diabetic polyneuropathy (Young and Clarke 1985, Sindrup, Ejlertsen et al. 1989, Sindrup, Gram et al. 1990, Sindrup, Bach et al. 2003) and post-mastectomy pain syndrome (Kalso, Tasmuth et al. 1996). However, the same class of drug has been shown to be ineffective in relieving spinal cord injury (Cardenas, Warms et al. 2002), phantom limb pain (Robinson, Czerniecki et al. 2004) and HIV-associated neuropathic pain (Kiebertz, Simpson et al. 1998) The fact that effect of amitriptyline on pain relief in certain types of neuropathic pain but not others and the efficacy of pain reliefs differed for different types of pain underlies the complicated mechanism involved in neuropathic pain.

Unlike antidepressants that have been shown to be ineffective in relieving pain associated with Phantom limb pain and HIV-associated neuropathic pain, another class of drug, anticonvulsants (such as gabapentin and lamotrigine), has been demonstrated to exhibit positive effect in some small trials (Simpson, Olney et al. 2000, Bone, Critchley et al. 2002). Anticonvulsant drugs also reduce pain associated with painful diabetic neuropathy (Eisenberg, Lurie et al. 2001) post-stroke pain (Vestergaard, Andersen et al. 2001), post-herpetic neuralgia (Rowbotham, Harden et al. 1998, Rice, Maton et al. 2001, Sabatowski, Galvez et al. 2004), spinal cord injury (Levendoglu, Ogun et al. 2004). It is worth noticing that anticonvulsant drug such as Topiramate could induce adverse side effects, and hence its frequent high withdrawal rate in most of the clinical trials.

The third class of drugs used to treat neuropathic pain is the Opioids. This class of drug is often combined with others to achieve better analgesic effect in clinic. For example, morphine has been shown to increase pain relief at lower doses when combined with gabapentin for treatments to painful diabetic neuropathy and postherpetic neuralgia when compared to single agent treatment (Gilron, Bailey et al. 2005). Morphine has also been shown to exhibit profound effect on pain relief for patients with phantom limb pain (Huse, Larbig et al. 2001). In addition, other opioids drug such as oxycodone also exhibits effects on reducing pain associated with post-herpetic neuropathy (Watson and Babul 1998) and diabetic-associated neuropathy (Watson, Moulin et al. 2003), and the effect is as good as to that of morphine.

One of the other drug classes that has been extensively studied and used in clinical trial is the NMDA antagonists. This class of drug (such as dextromethorphan, riluzole and

memantine) have been applied in a number of small neuropathic pain trials. However, the outcome of the studies showed there is no or little pain relieving effect. Nevertheless, two trials of high doses of dextromethorphan have been shown to be effective to treat pain associated with diabetic polyneuropathy (Nelson, Park et al. 1997, Sang, Booher et al. 2002).

Other treatments for neuropathic pain that does not fall into any of the classes described have been classified into the Miscellaneous class (for example, lidocane patch, capsaicin and cannabis). Cannabinoids from this group has been shown to alleviate pain in multiple sclerosis (Svendsen, Jensen et al. 2004) and central neuropathic pain from brachial plexus avulsion (Berman, Symonds et al. 2004), which has not been demonstrated by other drug classes listed.

Despite the growing evidence for effective treatments of neuropathic pain, current therapy for neuropathic pain is still inadequate. The current best available therapies only offer about 50% pain relief in one in 2-3 patients treated. In addition, the available drugs currently in use for treatments of neuropathic pain often exhibit dose-limiting adverse side effects and limited efficacy. Regardless of the great interest and efforts in neuropathic pain drug development, most compounds predicted to be effective from the animal models show no or limited effect alleviating pain in the clinical trials. This failure in translation into the clinical trials may be due to the fact that animal models of neuropathic pain are often restricted to physical damage to the nerve. It is hence hard to reflect the broad range of clinical neuropathic pain diseases, in particular those originated from causes other than nerve trauma.

In the hope to overcoming this issue, there is a growing interest in the pain research field recently to identify the common genes, which are implicated in different types of neuropathic pain derived from different origins. Microarray studies of differential gene expression in the DRGs of different animal models and subsequent comparison have been heavily employed to achieve this. This approach allowed us to elucidate genes that are commonly upregulated, in the neuropathic condition, among different animal models. This dissects out the genes that are truly related to neuropathic pain development as opposed to those associated with other aspects of pathophysiological response to a nerve lesion (e.g. regeneration and repair). As a result of this, *vgf* is found

to be one of the common genes that is upregulated in different animal models of neuropathic pain, and is hypothesised to play a pivotal role in the initiation and/or maintenance of neuropathic pain (Costigan, Befort et al. 2002, Xiao, Huang et al. 2002, Maratou, Wallace et al. 2009).

1.2. *vgf*: plate V nerve Growth Factor inducible gene

1.2.1. Identification of *vgf* and its selective inducibility

Vgf is the non-acronymic abbreviation for “plate V nerve Growth Factor inducible gene”. It was originally identified as a nerve growth factor (NGF)-inducible gene in PC-12 rat pheochromocytoma cells by Levi *et al.* in 1985 (Levi, Eldridge et al. 1985). In response to NGF stimulation, PC-12 cells are able to differentiate into mature sympathetic neurons. During differentiation, *vgf* has been characterised as one of the many genes that is profoundly upregulated in expression, and the increase in mRNA level is observed at the ‘delayed-early’ stage (Levi, Eldridge et al. 1985, Cho, Skarnes et al. 1989, Salton 1991). The cell line has, therefore, been employed as a model of neuronal differentiation and extensively used for the investigations of neurotrophin-induced mechanisms and regulation mechanism of *vgf* expression (Greene and Tischler 1976, D’Arcangelo, Habas et al. 1996, Levi, Ferri et al. 2004).

Further to its response to NGF, *vgf* has been demonstrated to exhibit selective inducibility to a diverse range of stimuli *in vitro* and *in vivo*, which include neurotrophins, antidepressants and several other physical activities. Indeed, *vgf* expression is also robustly induced by BDNF, GDNF and neurotrophin 3 (NT-3) (Eagleson, Fairfull et al. 2001, Alder, Thakker-Varia et al. 2003, Cerchia, Duconge et al. 2005). Intriguingly, such robust induction of *vgf* mRNA is restricted to a subset of growth factors only, as it is demonstrated that epithelial growth factor (EGF) and fibroblast growth factor (FGF) could only induce trivial *vgf* expression in PC12 cells (Hawley, Scheibe et al. 1992, Possenti, Di Rocco et al. 1992).

Apart from neurotrophin inductions, *vgf* expression has also been shown to be modulated by selective serotonin reuptake inhibitor (SSRI) antidepressants. SSRI

antidepressants, such as fluoxetine and paroxetine, profoundly increase *vgf* mRNA expression in astrocytes (Allaman, Fiumelli et al. 2011). The induction *vgf* upregulation by SSRI antidepressants is suggested to be independent from the serotonin-mechanism (Allaman, Fiumelli et al. 2011). The finding here presents two surprises. The first surprise is the specific inducibility of SSRI antidepressants on *vgf* expression, as it is demonstrated that another class of antidepressants, tricyclic antidepressants (such as desipramine) is unable to alter *vgf* expression. The second surprise is that VGF is expressed in the cortical astrocytes, whereas previous work demonstrated that it is not expressed in the spinal astrocytes (Moss, Ingram et al. 2008). Although there is a possibility that the neurochemistry of cortical astrocytes and spinal astrocytes could be different, it is intriguing that SSRI antidepressants may play a role in initiating and upregulating *vgf* expression in astrocytes upon stimulation.

In addition *vgf* is also shown to be induced by physical conditions such as fasting (Hahm, Mizuno et al. 1999, Hahm, Fekete et al. 2002), circadian rhythmicity (Wisor and Takahashi 1997), seasonal changes (Ebling and Barrett 2008), oestrous cycle (Ferri, Gaudio et al. 1995), consumption of salt (Mahata, Mahata et al. 1993), learning (Bozdagi, Rich et al. 2008) and sleep deprivation (Cirelli and Tononi 2000)

1.2.2. Structure and tissue expression of *vgf* gene

Following to the identification of *vgf*, cloning of the *vgf* cDNA from the PC12 cells revealed a 2.7kb fragment (Levi, Eldridge et al. 1985). The human *vgf* gene was subsequently isolated from a genomic library, and chromosomal mapping revealed that *vgf* gene is situated in human chromosome 7q22 (Canu, Possenti et al. 1997). The gene is demonstrated to be a single-copy gene spanning approximately 6 kb of the genomic sequence. The organisation of the *vgf* gene is fairly simple, which is consisted of three exons flanking two introns in between. The entire VGF protein sequence is encoded by exon 3, while the 5' untranslated sequence contains two introns (figure 1.3) (Salton, Fischberg et al. 1991).

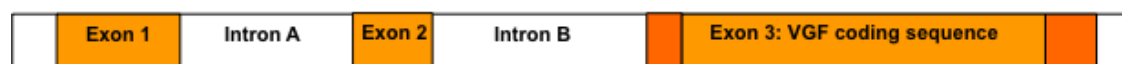


Figure 1. 3 Schematic diagram illustrating the organisation of *vgf* gene.

Vgf is a single copy gene with fairly simple organisation. The entire protein coding sequence is harboured by exon 3.

Vgf exhibits high sequence conservation between human and rodent. The evolutionary conserved sequence can be found not only in the coding region, but also within the untranslated region and the promoter region. Indeed, the promoter sequences between human and rat display over 80% homology (Canu, Possenti et al. 1997). The structural organisation of the mouse gene is similar to that of human *vgf* gene, and is revealed to locate in the chromosome 5 (Hahm, Mizuno et al. 1999). The rodent *vgf* gene has been shown to produce two mature mRNA forms by alternative splicing mechanism. The two spliced variants differ in the untranslated sequence, in which exon 2 is omitted in the shorter form of mature *vgf* mRNA, while the coding sequences remain unaffected (Hawley, Scheibe et al. 1992). Nonetheless, the functional consequences for such splicing event still remain unclear.

The expression of *vgf* is highly regulated developmentally. *In situ* hybridisation experiment on prenatal and postnatal rat demonstrated that *vgf* mRNA expression is first observed in the primordial of the cranial, dorsal root and sympathetic ganglia as early as E11.5. The distinct expression in regions of the brain starts as early as E13.5 during embryogenesis, and by E19.5, the thalamic nuclei, telencephalon, pituitary, spinal cord, sensory and autonomic ganglia, and most of the diencephalon and brainstem are stained labelled positive *vgf* expression (Snyder, Pintar et al. 1998). Interestingly, expression of *vgf* is temporally correlated with the neuronal differentiation, as seen in, among many others, the developing hippocampal neurons (Snyder, Pintar et al. 1998). The *vgf* mRNA expression is found to be expressed at high levels in most part of the brain, spinal cord, adrenal, stomach and pancreas by birth (Ferri, Levi et al. 1992, Snyder, Pintar et al. 1998, Snyder, Peng et al. 2003). In the adult rat brain, *vgf* mRNA is abundantly expressed in the olfactory system, hippocampus, septal, thalamus, amygdaloid, brainstem and cerebellum (van den Pol, Decavel et al. 1989, van den Pol, Bina et al. 1994, Snyder and Salton 1998).

Summing the existing *vgf* expression localisation data, *vgf* mRNA is believed to be distributed throughout the nervous system including the central nervous system (most

part of the brain, spinal cord and brainstem) and the peripheral nervous system (dorsal root and sympathetic ganglia). It is worth noting though, the expression is only extensively found in the neurons of the nervous system, the white matter is typically unlabelled. Moreover, *vgf* mRNA expression is shown to express at high level in the neuroendocrine cells, including that of adrenal medulla, pituitary, pancreas and gut. More recently, and unexpectedly, *vgf* mRNA is found to be expressed in the precursor cells of oligodendrocytes. The expression is shown to diminished after differentiation (Dugas, Tai et al. 2006). Moreover, it is also suggested that *vgf* expression is upregulated in the astrocytes following to SSRI antidepressant treatment (Allaman, Fiumelli et al. 2011). Thus, the traditional classification of *vgf* being a neuro-endocrine specific gene is recently challenged, and the expression of the gene in other cell types may implicate their roles in other cellular functions.

1.2.3. Regulatory mechanism of *vgf* expression

The regulatory mechanism of *vgf* expression during the development and/or maintenance of traumatic- or disease-induced neuropathic pain is largely unknown. Nevertheless, using PC12 cells as a neuronal model, a number of studies revealed the potential regulatory elements within the promoter region of *vgf*.

Vgf gene expression was first found to be largely impaired by mutation in the cyclic AMP responsive element (CRE) in PC12 cells in 1992 (Hawley, Scheibe et al. 1992). Since CRE binding proteins are mostly ubiquitous and respond to a variety of different stimuli, it was hence postulated other regulatory elements must be involved in conferring transcriptional regulation of *vgf* gene. A few studies have attempted to elucidate the regulatory mechanisms of *vgf* expression in response to neurotrophin in the PC12 cells. However, some of the findings from different groups are contradictory and the mechanism of *vgf* expression regulation in the PC12 cells still remains controversial.

D'Arcangelo *et al.* was the first group to show that at least three distinct promoter elements play a role in regulating *vgf* expression in the PC12 cells (D'Arcangelo, Habas et al. 1996). They confirmed CRE is one of the important elements regulating the expression of *vgf* in response to NGF stimulation in the PC12 cells. They also

further demonstrated CRE binds ATF-1, ATF-2 and CREB in the PC12 nuclear extract. In addition, a CCAAT box was found within the *vgf* promoter region and is required for NGF inducibility of *vgf* in the PC12 cells. CCAAT box often binds to transcription factors that belong to the C/EBP family member. In this study, they showed CCAAT box was able to bind proteins in nuclear extracts of PC12 cells. However, the complex formed did not contain a C/EBP family member, and hence it was proposed that a novel CCAAT-binding protein is required for the transcriptional activation of *vgf* expression upon NGF stimulation in the PC12 cells. Furthermore, it was also proposed a G(S)G element is required for the NGF-induced response in PC12 cells. Mutation of G(S)G element showed no significant change in *vgf* basal expression level but the NGF-induced response was reduced. In addition, it was suggested that NGF-induced transcription factor, NGF1-A, may bind to G(S)G element and amplify the transcriptional response. Lastly, they demonstrated an E box consensus core sequence found at position -56 did not alter *vgf* expression level in response to NGF induction after being mutated, and concluded this element is not an essential regulatory components for the induction of *vgf* expression in the PC12 cells (Figure 1.4.A for summary).

Contradictory to this story, Di Rocco et al published a study one year later showing two E-box elements (at position -56 and -170) confer weakly positive role in inducing *vgf* expression in response to NGF in the PC12 cells. More interestingly, they also showed the same E-boxes exhibit strongly inhibitory roles in the non-neuronal cell line - 3T3 cells. Mutation of either E-boxes relieved the low expression of *vgf* in the 3T3 cells (Di Rocco, Pennuto et al. 1997). However, the role of G(S)G elements in regulating *vgf* expression was not described in this study. Nevertheless, in agreeing with the previous study by D'Arcangelo et al, they also demonstrated the importance of CRE and CCAAT box in regulating *vgf gene expression* in PC12 cells. In 2001, the same group further published the roles of HEB and MASHI binding to the E box at position -170, suggesting its contribution towards neuron-specific expression of *vgf* (figure 1.4 C). In non-neuronal cells, HEB (a ubiquitously expressed transcription factor that belongs to the basic helix-loop-helix bHLH family) binds to both the E-box and CRE elements simultaneously, forming an inactive complex inhibiting transcription. On the other hand, another bHLH family transcription factor, MASH1 (found specifically in neurons), bind to -170 E box and CCAAT box forming an

activation complex that drives *vgf* transcription in the PC12 cells.

Although some parts of the studies of *vgf* regulatory mechanism remain controversial, it is clear that the *vgf* promoter is enrich in regulatory elements and that, at least in part, confer cell- and tissue-dependent expression of *vgf*. Much of what we know about the *vgf* expression regulating mechanism is restricted to the PC12 cell line investigations. Future study will need to be carried out to elucidate the regulatory mechanism of *vgf* expression in primary sensory neurons, in the context of neuropathic pain to further our understandings on this subject.

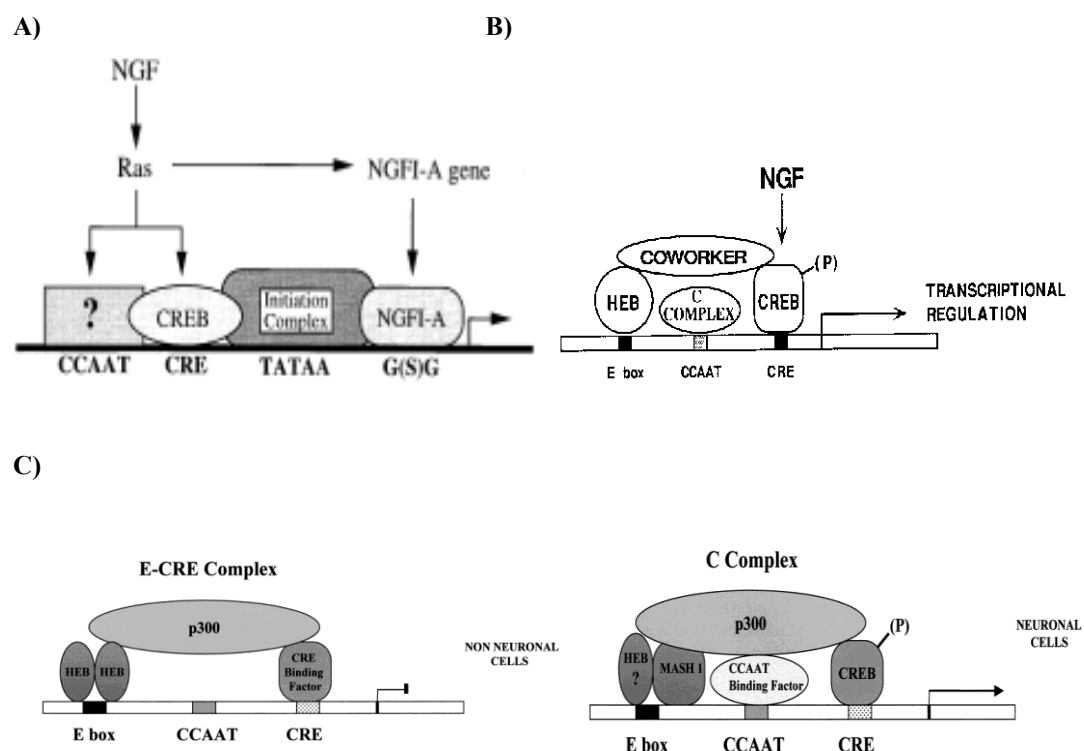


Figure 1. 4 Regulatory mechanisms of *vgf* expression in response to NGF stimulation in the PC12 cell line.

A) Diagram adopted from D'Arcangelo, Habas *et al.* 1996. NGF induced *vgf* expression in PC12 cells through a Ras-dependent pathway, which also induces expression immediate early gene NGFI-A that subsequently binds to G(S)G element in *vgf* promoter further induce *vgf* expression. CRE and CCAAT elements are shown to be play parts in the gene expression in response to NGF induction **B)** Diagram adopted from Di Rocco, Pennuto *et al.* 1997. First evident demonstrating roles of two E boxes in regulating *vgf* expression in the PC12 cells **C)** Diagram adopted from Mandolesi *et al.*, 2002. The first evident demonstrating the oppose effect of E boxes in the non-neuronal cell line, 3T3 cells. It is also suggested that CREB is phosphorylated on serine 133 in activated PC12 cells. In addition, bHLH family transcription factors (HEB and MASH1) confer neuron-specific expression of *vgf* gene.

1.2.4. VGF propeptide precursor and functional peptides

As mentioned previously, *vgf* is profoundly upregulated in the delayed early phase of NGF-induced response. The upregulated *vgf* gene encodes a polypeptide precursor comprising of 615 amino acids in human and 617 amino acids in rodents. The polypeptide sequence is demonstrated to display no homology with any known sequence (Salton, Fischberg et al. 1991). The N terminus of the synthesised propolypeptide is hydrophobic harbouring a 22 amino acid long secretory leader sequence, which is essential for endoplasmic reticulum translocation. Cleavage of the secretory leader sequence results in VGF polypeptide precursor with a calculated molecular weight of 68kDa and an isoelectric point (pI) of 7.1. Proline and Glutamic acid are prominent in the polypeptide, taking up 11% and 14% sequence identity, respectively, and are shown to cluster at the C terminus (Ferri and Possenti 1996).

The *vgf* polypeptide precursor is proteolytically processed into smaller peptide fragments. Indeed, analysis of neuroendocrine tissue using antiserum against the N-terminus of VGF by western blots typically reveals two polypeptide bands at 90 and 80 kDa, which corresponds to the polypeptide precursor. It is unexpectedly migrated at a higher molecular weight most likely due to the high content of proline in the sequence. However, smaller size bands down to 10kDa are prominent when using antiserum targeting the C-terminus, suggesting proteolytic processing of VGF polypeptide (Trani, Ciotti et al. 1995). The processed VGF peptides are stored in the secretory granules of neurons where it is released upon depolarisation (Possenti, Eldridge et al. 1989). To date, there are about 13 VGF-derived peptides that have been identified, which are named by the first four amino acids followed by the overall length of the peptide (figure 1.5).

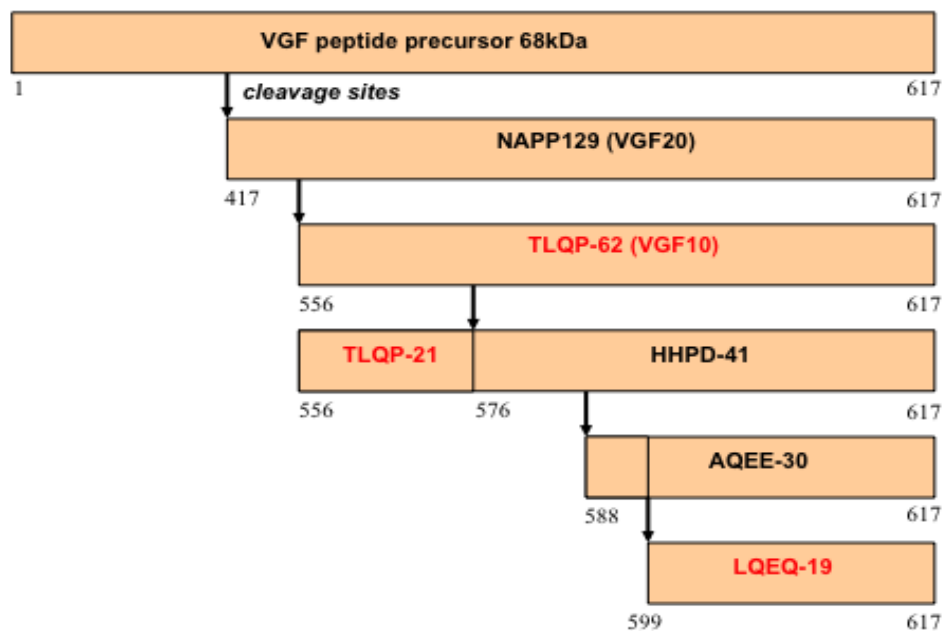


Figure 1. 5 Schematic diagram illustrating the processing of VGF peptide precursor into functional peptides.

VGF is synthesised as a 615 (human) and 617 (rodent) amino acids polypeptide precursor, which is proteolytically cleaved into smaller functional peptides that ultimately carry out functional activities (Picture modified from Jethwa and Ebling 2008).

1.2.5. Function of VGF

VGF knockout mice phenotype

Genetically engineered knockout mice inactivating *vgf* gene allow the assessment of *vgf* gene function by monitoring the consequences of the loss of gene activity, in terms of changes in the mouse phenotype. In 1999, Hahm *et al.* reported the first target deletion of the entire *vgf* coding sequence in mice by homologous recombination in the embryonic stem cells (Hahm, Mizuno et al. 1999). The study revealed a number of profound changes in the phenotype and suggested functional roles of VGF in a number of biological processes.

Firstly, the homozygous VGF mutant mice were observed to be smaller in the body size. Although the size of VGF-deficient pups were indistinguishable from its normal littermates at birth, the body weight of VGF mutants were 10-20% less by postnatal day 3 (P3), which dropped further to 40-60% less compared to the wild-type littermates by the third week. The body weight plateaus at 30-50% less than those of the wild-type mice after weaning. Interestingly, VGF deficiency was suggested to

prevent weight gain in the animal model of obesity and genetically obese mice, suggesting a functional role of *vgf* in obesity (Hahm, Fekete et al. 2002). Nonetheless, the survival rates of the VGF mutants were observed to be lower than that of the heterozygous and wild-type counterparts, especially during the time prior to weaning (Hahm, Mizuno et al. 1999).

VGF-mutant mice are also hypermetabolic. The basal metabolic rates of the mutants were significantly higher, as demonstrated with higher oxygen consumption and carbon dioxide output in these mice. Moreover, it was shown that the mutants exhibit an increase in the daily food intake per gram of the body. In addition, VGF mutant mice are also hyperactive as the activity (distance travelled per time) of these mice were shown to be about 5 times more than that of the wild type (Hahm, Mizuno et al. 1999).

Moreover, VGF-deficient mice have reduced fertility, suggesting a role of VGF in reproduction. Repetitive mating of the wild-type and heterozygous mice for four months demonstrated >90% of fertility. On the other hand, when the homozygous VGF mutants were mated, it was shown 85% of the male mutant mice and 90% of the female mutant mice were infertile. It was also observed that VGF mutant mice exhibit delayed in puberty and sexual maturation. In the mutant females, 50% display prolonged oestrous cycle and the other 50% failed to cycle. The ovaries, oviducts and uteri of these females were shown to be much smaller, comprising only 18-30% the weight of the normal mice. The VGF-deficient males exhibit abnormal sexual behaviours and have smaller testis and seminal vesicles (30% and 65% less in weight, respectively, when compared to the wild-type).

In terms of biochemistry, VGF mutants display reduced serum insulin and glucose levels (20% and 40% less than that in normal mice, respectively). The peripheral fat stores were also profoundly reduced in the mutants. The serum corticosterone level, on the other hand, was increased by 40% in the mutants when compared to that in the wild type. Moreover, VGF-deficient mice have reduced hormone contents (reduced in serum T4 thyroid hormone and pituitary thyroid-stimulating hormone). The neuropeptide expression levels were also altered in the mutant mice. Indeed, the mRNA expression level of neuropeptide Y and agouti-related peptide (AGRP) were shown to be increased by 6 and 8 folds in the hypothalamus, respectively. Whereas, the hypothalamic

pro-opiomelanocortin (POMC) expression was shown to be 75% lower than that of the wild type littermates. Furthermore, the leptin expression in the epididymal fat and the circulating leptin hormone were shown to be markedly reduced in the mutants.

Energy homeostasis and food intake

The VGF knock out mice exhibit high oxygen consumption and metabolic rate, suggested a role of VGF in inhibiting energy expenditure. Surprisingly, however, it was demonstrated by Bartolomucci et al. chronic intracerebroventricular injection (i.c.v.) of TLQP-21, in fact, increased the energy metabolism and rectal temperature in mice (Bartolomucci, La Corte et al. 2006). In addition, TLQP-21 i.c.v. injection prevented the high-fat diet-induced obesity in normal mice (Bartolomucci, La Corte et al. 2006) and fast weight gain mice (Bartolomucci, Bresciani et al. 2009). Further, the primary action of TLQP-21 was, controversially, suggested to decrease the food intake in Siberian hamster rather than increase energy expenditure (Jethwa, Warner et al. 2007). However, i.c.v. injection of another VGF-derived peptide, NERP-2, was shown to increase feeding and metabolism in rats (Toshinai, Yamaguchi et al. 2010). Although the data still remains controversial and the precise role yet to be characterised, VGF-derived peptides were demonstrated to play a role in the energy expenditure and food intake.

Depression

VGF has been implicated in depression. It has been demonstrated that the VGF protein level is markedly decreased in the hippocampi of animals subject to the depression paradigms (Thakker-Varia, Krol et al. 2007). Thakker-Varia *et al.* employed the forced swim test (FST) and learned helplessness test (LH), the two commonly used animal models of depression, and showed the VGF protein levels were decreased by more than 20% in the hippocampi, a brain region implicated in depression, of the animals exposed to FST and LH. In addition, it was also demonstrated that the effect of depression was reversible in the FST by intrahippocampal infusion of a VGF-derived peptide, TLQP-62, suggesting VGF elicits antidepressant-like effects when introduced to the depressed animals (Thakker-Varia, Krol et al. 2007). The antidepressant effect of VGF has been confirmed by the hippocampus transcription profiling in animals of the

exercise model (Hunsberger, Newton et al. 2007). It is suggested that exercise promotes antidepressant actions. Interestingly, *vgf* mRNA expression was found to be profoundly increased in the hippocampus of animals that exercised for 7 days. The VGF protein expression was also confirmed to be markedly induced in the mice hippocampus after exercise by immunohistochemistry. Behavioural assessments in FST and tail-suspension test (TST) also revealed VGF-derived peptide (AQEE) infusion to the hippocampus promoted antidepressant actions, which lead to reduction in the immobility time in both of the test. Furthermore, the VGF heterozygous null mice were shown to exhibit depression-like behaviour in the FST and TST, and the depression-like behaviour was shown to be unaltered after exercise in the VGF deficient mice, whereas exercised wild-type mice had an antidepressant-like response. The unaltered depression-like behaviour in the VGF deficient mice was proposed to be correlated to the lower VGF expression in the hippocampi of the VGF deficient mice (Hunsberger, Newton et al. 2007). Interestingly, the *vgf* mRNA levels in leukocytes of the drug-free depressed patients were found to be significantly lower than the normal population, and the expression could be restored in antidepressant responsive patients (Cattaneo, Sesta et al. 2010).

Reproduction

Despite the delayed puberty, delayed sexual maturation and infertility observed in the VGF knock out mice, the function role of VGF-derived peptide in reproduction was demonstrated by a number of other *in vivo* works. It was demonstrated hypothalamic Paraventricular Nucleus injection of C-terminal VGF-derived peptides induced rat penile erection. Four out of the five C-terminal VGF-derived peptides (HHPD-41, AQEE-30, LQEQ-19 and AQEE-10, but not TLQP-21) showed a positive effect in penile erection in a dose-dependent manner. It is proposed this process may be nitric-oxide mediated and the paraventricular oxytocinergic neurons are involved (Succu, Cocco et al. 2004, Succu, Mascia et al. 2005). Although TLQP-21 was shown to be ineffective in eliciting penile erection in rats, it was demonstrated it, in fact, modulates reproductive axis in male rats in various levels. Indeed, central administration of TLQP-21 was shown to modulate the gonadotropin response *in vivo*. In addition, TLQP-21 stimulated luteinizing hormone (LH) secretion from the

pituitaries and testosterone from the testicular tissue of pubertal male rats and adult male rats, respectively (Pinilla, Pineda et al. 2011). The multiple actions of the c-terminal VGF-derived peptides together revealed the role of VGF in reproduction.

Neuronal apoptosis/neurogenesis

VGF-derived peptide, TLQP-21 has been demonstrated to exhibit anti-apoptotic effect in rat cerebellar granule cells (CGCs) culture. Indeed, the apoptotic effect induced by serum and potassium deprivation in the CGCs culture was prevented by TLQP-21 treatment in a dose- and time-dependent manner. Such neuroprotective effect was evident to be protein kinase C(PKC)- and c-Jun N-terminal Kinase (JNK)- dependent (Severini, Ciotti et al. 2008). Moreover, the anti-apoptotic effect of VGF is also demonstrated in the in vitro model of Amyotrophic lateral sclerosis (ALS). Exogenous full-length *vgf* expression in the primary mixed spinal cord neuron cultures of the ALS animal model significantly attenuated excitotoxic injury-induced cell death (Zhao, Lange et al. 2008). Furthermore, Shimazawa et al. demonstrated SUN N8075 (a small molecule) treatment drastically upregulated the *vgf* mRNA expression in SH-SY5Y neuroblastoma cells, and pre-treatment of SUN N8075 prevented Tunicamycin and Thapsigargin-induced cell apoptosis in a dose-dependent manner. In addition, siRNA knockdown of *vgf* in the SH-SY5Y cells abolished the anti-apoptotic effect induced by SUN N8075. Such molecule has also been shown to attenuate the decrease of VGF protein expression in the ALS animal model and delay the disease progression. The results together suggested that SUN N8075-induced *vgf* expression prevents the cells from endoplasmic reticulum-induced cell death both in vitro and in vivo (Shimazawa, Tanaka et al. 2010), and hence implicating a role for *vgf* in neuroprotection, in terms of anti-apoptosis.

Pain signalling and modulation

Maratou *et al.* has recently reported an extensive study to search for the common gene that is upregulated in three different animal models of neuropathic pain, the peripheral nerve trauma - Sciatic Nerve Transection (SNT) model, the chemotherapy drug/viral protein-induced neuropathy - Gp120 + ddC model, and Varicella Zoster Virus infection – VZV infection model) (Maratou, Wallace et al. 2009). Gene expression profiling of

the L4 and L5 DRGs from these models were performed, and the comparison between the data revealed that only three genes were commonly upregulated among the three animal models. Two of which are the expressed sequence tags, and the third gene is *vgf*. Quantitative PCR study of DRGs from these three models also confirmed that *vgf* was commonly upregulated. In addition, they also showed Neuropeptide Y (NpY) and Pap/Reg2 were also commonly increased in expression in all three animal models by quantitative PCR. Furthermore, *vgf* is also found to be upregulated in other peripheral nerve injury animal models of neuropathic pain (SNT, SNL, SNI, and CCI models) by other groups (Costigan, Befort et al. 2002, Xiao, Huang et al. 2002, Valder, Liu et al. 2003, Moss, Ingram et al. 2008). The studies described collectively suggest that *vgf*, as a commonly upregulated gene in all animal models of neuropathic pain tested, may play a pivotal role in initiation and/or maintenance of neuropathic pain, rather than involving in other pathophysiological events upon nerve injury.

As described earlier, *vgf* is profoundly expressed in both sympathetic ganglia and dorsal root ganglia, and the expression is induced by neuronal lesion and neurotrophin stimulation (Levi, Ferri et al. 2004). One of the other evidence suggesting a possible role of VGF-derived peptides modulating pain signal transduction came from the study showing *vgf* expression was increased in the sensory neurons in response to NGF stimulation to the primary afferent (Ferri, Levi et al. 1992). In addition, it is also demonstrated that the increase VGF expression after traumatic nerve injury is also detected at the protein level. Indeed, VGF protein expression is increased and detected in 80% of the neurons of the L5 DRG that is ipsilateral to the injury site in SNL model. Comparatively, VGF protein expression is only observed in 37% of the sensory neurons in L5 DRG of the sham-operated animal and 20% of that in the naïve animals. (Riedl, Braun et al. 2009). This increase in VGF expression at protein level is shown to sustain for at least 7 days after SNL surgery. Interestingly, VGF-positive neurons in the ipsilateral L5 DRG immediately after nerve injury are the small and medium diameter neurons. Seven days after SNL surgery, the VGF protein expression in these groups of neurons is reduced back to the level that is similar to day 1 expression. Interestingly, at this time point, the number of large diameter, VGF-positive, neurons is increased. In addition, it is also demonstrated that VGF protein expression in the DRG neurons is colocalised with TrkA and P2X₃ receptors,

which have been implicated in the pain pathways. Indeed, about 52% of the VGF-positive neurons are shown to also express TrkA, and 52.3% of the VGF-expressing neurons are co-expressing P2X₃ (Riedl, Braun et al. 2009). Further to its increase in expression in the ipsilateral DRG, VGF expression is also shown to be upregulated in the spinal cord after nerve injury. Moreover, an increase in co-localisation of VGF with CGRP in the superficial dorsal horn ipsilateral to the nerve injury site is also reported (Riedl, Braun et al. 2009).

Besides the evidence of increases in VGF expression, at both mRNA and protein levels, after peripheral nerve injury, VGF-derived peptides have demonstrated functional properties *in vivo* and are functionally implicated in the pain pathways. One of the VGF peptide derivative, TLQP-21, is shown to induce a significant increase in the pain-related licking response in the second phase of formalin test following to the subcutaneous injection (Rizzi, Bartolomucci et al. 2008). Moreover, the same peptide alone at a dose of 4mM increases the nociceptive-like licking response. Other VGF peptide derivatives, such as AQEE-30 and LQEQ-19, have been shown to evoke thermal hypersensitivity in mice when injected intrathecally (Riedl, Braun et al. 2009). AQEE-30 injection induces thermal hypersensitivity to warm water tail immersion assay within 10 minutes, which peaks at 30 minutes and persists up to 90 minutes at high dose. LQEQ-19 contains the last 19 amino acids of AQEE-30. Intrathecal injection of the LQEQ-19 peptide also demonstrates a comparable thermal hypersensitivity with similar magnitude but a shorter duration. Interestingly, AQEE-11 (the first 11 amino acid of AQEE-30), on the other hand, shows no statistical significant effect on the thermal hypersensitivity. The LQEQ-19- and AQEE-30-evoked thermal hypersensitivity has recently been demonstrated to be reversible by p38 inhibitor SB202190, indicating the involvement of p38 MAPK in the signal transduction downstream to *vgf*. Indeed, p38 phosphorylation has been shown to increase, for more than 3 folds, in the spinal microglia after intrathecal injection of LQEQ-19 (Riedl, Braun et al. 2009). In consistence with this finding, a preliminary data also shows that the mechanical hypersensitivity is induced within 10 minutes upon intraplantar injection of LQEQ-19 (Unpublished data, Professor Andrew Rice, Chelsea and Westminster Hospital). Furthermore, an *in vivo* electrophysiological study also demonstrates the action potential amplitude of the nociceptive C-fibre components after LQEQ-19 application to the lumbar 5 and 6

DRGs is increased. However, such increase in the action potential amplitude is not observed in the A β - and A δ - fibres (unpublished data, Dr Istvan Nagy, Chelsea and Westminster Hospital).

Human diseases states

Alteration of vgf mRNA and protein expressions has been associated with a number of human diseases, particularly the psychiatric disorders. It was demonstrated the level of vgf mRNA was reduced in postmortem brain of patients with bipolar disorder, particularly in region of hippocampus and prefrontal cortex (Thakker-Varia, Jean et al. 2010). In addition, as mentioned, the vgf mRNA was also reduced in leucocytes of patients with depression disorder (Cattaneo, Sesta et al. 2010). In terms of VGF protein expression, VGF peptide is detectable in the cerebrospinal fluid of normal human (Yuan and Desiderio 2005), and a higher level of VGF peptide was reported in the cerebrospinal fluid samples of schizophrenia patients by mass spectrometry analysis, as well as in a few depressed and obsessive-compulsive disorder patients (Huang, Leweke et al. 2006). In addition, VGF peptide was also demonstrated to be reduced in the cerebrospinal fluid of patients with neurodegenerative diseases such as ALS (Ranganathan, Williams et al. 2005, Pasinetti, Ungar et al. 2006), Alzheimer's (Carrette, Demalte et al. 2003, Jahn, Wittke et al. 2011), Encephalopathy (Asano, Koizumi et al. 2011) and dementia (Ruetschi, Zetterberg et al. 2005). Due to the change in the relative abundance in the cerebrospinal fluid in disease pathological states, VGF has been proposed to be the biomarker for the mentioned neurological and psychiatric disorders (Bartolomucci, Pasinetti et al. 2010). However, the relative expression of VGF peptides in the cerebrospinal fluid of neuropathic pain patients has not yet been investigated and still awaits to be elucidated.

1.3. Aims

Although a number of VGF-derived peptides have been identified and some of their functions revealed in the pain pathway, the exact underlying mechanism(s) and the signalling pathway(s), in the context of pain, still remain largely unknown. The aim and emphasis of this thesis is to further the understanding of the peripheral mechanism(s) of action of VGF-derived peptides in the pain pathway, with particular focus on cells implicated in the neuropathic pain development.

The objectives of this project are to:

1. Establish an *in vitro* functional calcium assay for the investigation of effects of the VGF-derived peptides (LQEQ-19, TLQP-21 and TLQP-62) on cells that has been implicated in the peripheral mechanism of neuropathic pain (DRG neuron and macrophage).
2. Investigate the *in vivo* effect of intraplantar injection of the VGF-derived peptide (TLQP-21)-stimulated cells on nociception.
3. Verify the potential VGF receptor (GC1qR) as the receptor for VGF-derived peptide (TLQP-21) on primary macrophages by neutralising antibody and siRNA knock-down methodologies.
4. Investigate the effect of blocking the potential VGF receptor (GC1qR) *in vivo* on the mechanical sensitivity of neuropathic pain animal model (partial sciatic nerve ligation model).
5. Elucidate the downstream signalling events in the target cells after VGF-derived peptide application using quantitative real time PCR (qRT-PCR), PCR array and microarray.

CHAPTER 2:
THE MECHANISM OF ACTION OF THE VGF-DERIVED
PEPTIDES

Synopsis

In the general introduction I have outlined that *vgf* is the commonly up-regulated gene in different animal models of neuropathic pain and several VGF-derived peptides have been implicated in the pain pathway. In this chapter, the mechanism of actions of VGF-derived peptides, LQEQ-19, TLQP-21 and TLQP-62 (with a more in-depth study on TLQP-21) has been explored. A function assay, calcium imaging, has been developed for investigating the effects of the peptides on different cell types including the primary DRG neurons and untreated/polarised bone-marrow-derived primary macrophages. In addition, the effect of repeated treatment of peptides on its target cells has been investigated. Moreover, the origin of the calcium source and the effects of the VGF-derived peptide-conditioned media from the primary macrophages on the DRG neurons have been assessed. Lastly, an outline is presented for my data that explores the involvement of the VGF-derived peptide-stimulated macrophages in the *in vivo* study and finding is discussed in the context of pain.

2.1. Introduction

2.1.1. Investigating calcium signalling

Calcium is a simple ion that can be found ubiquitously. It is one of the most versatile and pluripotent ions that regulates a variety of physiological and pathological signalling events. Dynamic changes in the concentration of calcium need to be tightly regulated spatially, temporally and kinetically, in order to maintain its homeostasis and thus control various events of biological activities. In order to understand the role of calcium and elucidate its underlying regulatory mechanisms in any biological event, measurement and monitoring of intracellular calcium become essential. Due to this reason, there are a wide range of synthetic and biosynthetic calcium sensors developed, which allow quantification and visualisation of intracellular calcium in a spatio-temporal controlled manner. In this session, the commonly employed strategies for studying calcium signalling *in vitro* using optical techniques will be described, and that for studying calcium signalling *in vivo* will be briefly mentioned.

Strategies for studying calcium signalling in vitro

The spatio-temporal modulation of calcium concentrations initiates biological processes range from egg fertilisation, cell proliferation to cell death. Elucidating how calcium functions in cell physiology and investigation of its underlying mechanisms necessitates visualisation and/or measurements of the intracellular calcium. Here, the methods that are commonly coupled with optical techniques for studying calcium signalling *in vitro* are discussed.

Fluorescent chemical probe-based method

In the recent year, fluorescence-based visualisation becomes an influential method to detect and study calcium signalling *in vitro*. The method allows live cells to be examined and provides detailed information on spatio-temporal resolution and quantification of calcium concentration once the loading condition is optimised. All fluorescence-based methods rely on both calcium indicators and sophisticated detection technology to read out the emitted fluorescence signals, which will both be briefly discussed here.

The fluorescent-based calcium indicators available for studying calcium signalling can be broadly divided into two groups, ratiometric and non-ratiometric indicators. The ratiometric calcium indicators rely on ratio measurements of two fluorescence intensities. In general, ratiometric dye can work either by “excitation ratioing” or “emission ratioing”. For excitation ratio, the dye is excited by alternating two different wavelengths and the fluorescence emission of a single wavelength is captured. The ratio of emission at the excitation wavelengths correlates to the amount of intracellular calcium. For example, fura-2 ratiometric calcium dye is often used in excitation ratioing. The dye is excited at 340nm and 380nm wavelength and the emission is always measured at ~510nm. To correlate to the exact amount of calcium after calibration, the emission excited at 340nm is divided by the emission at 380nm. Should there be an increase in the intracellular calcium in the cell, an increase in 340/380 ratio will be observed. Fura-2 dye was employed *in vitro* to investigate the effect of VGF-derived peptide, TLQP-21, on cerebellar granule cells (CGCs) in calcium imaging. It was demonstrated that TLQP-21 elicited an increase in intracellular calcium level in the CGCs and exhibited neuroprotective effects (Severini, Ciotti et al. 2008).

Emission ratioing, on the other hand, works by exciting the dye at a single wavelength while capturing emission at two different wavelengths. Indo-1 is an example of emission ratioing ratiometric calcium dye. The dye is often excited at ~350nm wavelength, and the emission is measured at ~400nm and ~475nm simultaneously. The ratio between two emissions captured corresponds to the intracellular calcium concentration. The ratiometric quantification of calcium concentration is beneficial that it corrects the *in vitro* fluorescent artefacts due to photobleaching, changes in focus, uneven loading of the dye, or uneven thickness of the cells. Nevertheless, it exhibits drawbacks of more complicated data acquisition, measurement and processing. Moreover, not all microscopes are equipped with changeable excitation/emission wavelength and most of the ratiometric dyes, such as fura-2 and indo-2, require UV excitation that is often challenging to obtain.

The second class of fluorescent-based calcium dyes is the non-ratiometric calcium indicators. The free non-ratiometric calcium indicators often exhibit weak fluorescence, and a change in intensity, rather than a spectral shift, is observed upon binding with calcium. Therefore, the fluorescent intensity detected corresponds to the calcium level in the cells. Non-ratiometric calcium indicators generally have a longer excitation and emission wavelengths, and most of these indicators use fluorescein or rhodamine derivatives as the fluorophore (with the exception of Fura Red, which is one of the few long wavelength ratiometric calcium dye)(Minta, Kao et al. 1989). The main differences between different non-ratiometric calcium indicators are the differences in excitation and emission maxima, the brightness of the dye in calcium free conditions, the extent of fluorescence intensity change upon binding to calcium, their affinity to calcium and if they exhibit a tendency to accumulate in mitochondria. Non-ratiometric calcium indicators based on fluorescein derivatives include Fluo-3, Fluo-4, Calcium Green-1, Calcium Green-2, Oregon Green 488 BAPTA, and those based on Rhodamine derivatives include Rhod-2, X-Rhod-2, Calcium Orange, Calcium Crimson. Of all the long wavelength non-ratiometric indicators, Fluo-4 emerges to be the most assuring dye to be used in studying calcium signalling event. Fluo-4 has been shown to exhibit brighter fluorescence than other fluorescein-based derivatives because of the chloride to fluorine substitution on the fluorescein ring. This halogen substitution makes the dye 40% brighter than its structurally related dye, Fluo-3 (Gee, Brown et al. 2000). Moreover, Fluo-4, with a maximum excitation wavelength at 491nm, is suggested to be

better suited for the use with confocal microscopy that it is excited much more efficiently than Fluo-3 by a 488nm Argon laser. Furthermore, Fluo-4 also exhibits a better photostability than other indicators such as Oregon Green 488 BAPTA, which bleaches rapidly and possesses a lower dynamic range. Lastly, Fluo-4 also has an easier loading procedure when compared to other dyes including rhodamine-based indicators. The major advantages of non-ratiometric indicators over ratiometric indicators are that the increase in fluorescence signal can be directly correlated to the increase in calcium level, and changeable excitation/emission wavelengths are not required. However, it also exhibits a number of drawbacks, such as being prone to artefacts.

Both membrane permeable and membrane impermeable derivatives of ratiometric and non-ratiometric fluorescent chemical calcium dyes are commercially available. The most common detection methods for these dyes are the wide-field fluorescence imaging and the laser scanning confocal microscopy. The conventional wide-field fluorescence imaging often requires a xenon or mercury bulb to deliver excitation light. The excitation wavelength required for exciting the probe is controlled by optical filters. The fluorescence signals emitted from the cells are detected with a CCD digital camera or intensified video camera. Laser scanning confocal microscopy allows visualisation of the changes in the intracellular calcium level. The signal-to-noise ratio can be improved in confocal microscope by capturing the fluorescence emission that originates from the plane of focus only. Most of the confocal microscopy system is equipped with an argon-krypton light source, which produce excitation wavelength at 488nm, 568nm and 647nm. Calcium signals emitted from the cells can be recorded in one of the three possible imaging modes: xt-scanning mode, xyt-scanning mode or xyz-t-scanning mode. The images are subsequently acquired by a high sensitivity, high speed digital CCD.

Calcium sensitive protein –based method: Aequorin-/fluorescent protein-based measurement

Apart from the fluorescent chemical probes, fluorescent and luminescent proteins have also been utilised to monitor calcium signal in the biological events. Here, the applications offered by aequorin (a calcium sensitive protein) in the study of intracellular calcium signalling will be discussed, and a brief introduction to the new bioluminescent probes that couple aequorin to GFP emission will be mentioned.

The bioluminescence of the jellyfish *Aequorea Victoria* is a result of two proteins, GFP and aequorin. The aequorin was isolated from the jellyfish in 1962 (Shimomura, Johnson et al. 1962) and has been widely employed in studying intracellular calcium since the late 1960s (Ridgway and Ashley 1967, Allen and Blinks 1978). Aequorin is a 22kDa protein that contains four helix-loop-helix EF-hand domains, and three of those are able to bind to calcium. Binding of calcium to the EF-hand domains induces a conformational change that results in the oxidation of Coelenterazine and the subsequent release of CO₂ and blue light. Since the protein itself is not cell permeable, aequorin was initially used in studying changes in calcium in larger cells by microinjection procedures. Thanks to the advancement in molecular biology, recombinantly expressed aequorin can now be generated and transfected to different cell types. The transfected clone synthesises apo-aequorin that can be reconstituted to an active probe by adding Coelenterazine to the culture medium (which diffuse freely across membrane and bind to the polypeptide once inside the cells). The detection of the signal is classically performed in the dark by a low-noise photomultiplier. After adding stimulus to the cells, photons are emitted from the aequorin upon calcium binding, and an amplifier generates pulses that can be detected by a photon counting board connected to a computer.

One of the major advantages using recombinantly expressed aequorin over fluorescent chemical probe in the calcium detection is that it is possible to localise the photoprotein to different cellular compartments by introducing appropriate leader sequence to the aequorin coding sequence. This has been widely utilised to monitor calcium levels in the nucleus (Brini, Murgia et al. 1993), mitochondria (Rizzuto, Simpson et al. 1992), plasma membrane (Marsault, Murgia et al. 1997), Golgi apparatus (Pinton, Pozzan et al. 1998), endoplasmic reticulum and sarcoplasmic reticulum (Robert, De Giorgi et al. 1998). Another advantage of the use of photoprotein is that aequorin enables detection of calcium in the higher micromolar range that may occur, for example, in neurons upon excitation. The detection of such high concentration using fluorescent chemical probes is generally not permitted because most of the probes are saturated at high micromolar concentration. Moreover, aequorin is not toxic to cells, can be expressed in a variety cell types and efficiently folds into functional protein upon synthesis. The expressed fully functional protein is not secreted, exported, nor sequestered within cells thus allowing monitoring of the changes in the calcium levels over a relatively longer

period of time. However, aequorin-based calcium visualisation has a big limitation to image single cell, because each aequorin molecule emits one photon only and hence the measurable signal is often very low in a cell. Even with the most sophisticated high-resolution camera, higher emission of the light is required to detect the change in the calcium level in the single cell imaging. Due to this limitation, the aequorin-based detection has mostly been replaced by using the new modified bioluminescent calcium probe in single cell imaging. To overcome the low signal limitation, aequorin is combined with the GFP. A fusion protein is generated with the calcium sensitive aequorin acting as a calcium sensor, which generates an intra-molecular bioluminescence resonance energy transfer (BRET) to the GFP acceptor upon calcium binding. The improved higher signal-to-noise ratio of the new bioluminescent calcium probe enables investigation of calcium in a single cell in real time with modified detection using a fluorescence microscope coupled with a sensitive camera (Baubet, Le Mouellic et al. 2000).

A similar-in-concept approach has been used to generate fluorescent protein-based calcium indicators such as Cameleons. Cameleons are the GFP-based calcium indicators generally containing two fluorescent proteins that emit fluorescence at different wavelengths. The first constructed Cameleons consists two fluorescent proteins (BFP and GFP) that are bridged by a calcium sensitive protein (*Xenopus laevis* calmodulin) and calcium sensitive protein binding protein (M13, a calmodulin-binding protein). Binding of calcium to the calcium sensitive protein induces a conformational change, which consequently brings the two fluorescent proteins closer and results in fluorescence resonance energy transfer (FRET). Thus, the increase in FRET between the two fluorescent proteins is directly correlated to the calcium concentration in the cells (Miyawaki, Llopis et al. 1997). Various forms of Cameleons have been developed to improve the brightness, photostability and signal-to-noise ratio by replacing the BFP/GFP to enhanced CFP/YFP. In contrast to Cameleons, other fluorescent protein-based calcium sensors such as Camgaroo, G-CaMP and Pericam have been developed based on the direct changes in fluorescent intensities upon calcium binding, instead of FRET. The binding of calcium results in electrostatic changes in the microenvironment of the fluorescent proteins, leading to a calcium sensitive change in structure of the fluorophore and subsequent increase in fluorescent emission. The increase in the fluorescence intensity of these sensors is hence directly correlated to the

increase in the calcium level (Zhang, Campbell et al. 2002). The fluorescent protein-based calcium sensors exhibit a number of advantages over aequorin-based calcium sensor. Firstly, the enhanced fluorescence of fluorescent protein-based sensors allows the use of simpler detection system and results in comparatively high temporal resolution. The second major advantage would be that the calcium-induced increase in fluorescent is reversible. Calcium binding to aequorin-based sensor irreversibly consumes the indicator, which can introduce an artefact that mimics lowering calcium concentration. This limitation is not presented when using fluorescent protein-based calcium sensor. Lastly, the use of fluorescent protein-based sensors does not require specific cofactors as aequorin-based sensors, which gives a simpler experimental procedure. However, on the downside, the fluorescence of the fluorescent proteins is sensitive to the pH, particularly with YFP (Kneen, Farinas et al. 1998), and hence the pH differences in the intracellular compartments of the cells could alter the fluorescence intensity detected.

Strategies for studying calcium signalling in vivo

As mentioned previously, the development of different calcium indicators opened the door for investigating intracellular calcium signal at high temporal and spatial resolution. Imaging calcium signals in isolated primary cells, cell lines and tissue slides *in vitro* has become routine in most of the laboratories today. However, it is essential to visualise calcium dynamics in the whole animal to better understand the signal flows between cells in a complex system. Advances in microscope and optical technologies allow this goal to be achieved to enhance our understandings of the complexity of cellular functions and relationship between different cell populations in a complicated biological event *in vivo*.

The calcium indicators used in *in vivo* calcium imaging are similar to those *in vitro*. However, the loading of the calcium indicators to cells for *in vivo* calcium imaging is trickier. The simplest way is by microinjecting the calcium indicator to a single cell (Helmchen, Svoboda et al. 1999). However, this limits the *in vivo* imaging to the loaded cell only. Thus, more complex cell-cell interactions and signalling within cell populations cannot be monitored. To overcome this limitation, bulk loading of a population of cells within tissue can be achieved by local electroporation (Nagayama,

Zeng et al. 2007), retrograde passive diffusion (Takahashi, Narushima et al. 2002) or by pressure ejection of the dye (e.g. multi-cell bolus loading technique)(Stosiek, Garaschuk et al. 2003). These loading options provide rapid staining of cells within a local area thus allowing real-time calcium imaging of cell population activity. Nonetheless, these methods do not provide cell-type specific staining as it often results in a homogenous staining of all cells within the stained area. Cell type specificity expression of calcium indicator can be achieved by using the transgenic animals carrying genetically encoded fluorescent protein-based indicators (Hirrlinger, Scheller et al. 2005), which enable investigation of calcium signalling in specific cell type *in vivo*.

2.1.2. Behavioural assessment of nociception

The *in vivo* experimental studies of the mechanisms underlying nociception necessitate the behavioural testing. It is inevitable to employ the appropriate stimuli to provoke the sensation. Moreover, it is of great importance that these stimuli and the methods utilised to monitor the changes in the animal behaviours are sensitive, quantifiable, reproducible and noninvasive.

Because pain cannot be directly measured from the animals, a number of different sensitive and quantifiable surrogates have been suggested. The most commonly exploited nociception assessments *in vivo* can be divided into two broad categories, the Mechanical and Thermal assessment. In this session, both types of nociception assessments will be briefly described with particular highlight on the von Frey Mechanical assessment, which is the assessment used in my study.

Assessment of Mechanical nociception in rodent

Allodynia and hyperalgesia are two of the many problematic clinical symptoms of chronic pain patients. The International Association for the Study of Pain (IASP) defines allodynia as pain evoked by a stimulus that does not normally produce pain, and hyperalgesia as an elevated response to a stimulus that is normally painful(Merskey and Bogduk 1994). The *in vivo* assessment using mechanical stimuli accesses the mechanical allodynia-like behaviours in animals. It is important to note

that, as pain cannot be monitored directly in animals, it is only estimated by measuring their response to mechanical stimuli. However, such responses do not necessarily mean there is a painful sensation. Therefore, the responses observed cannot be described as “mechanical allodynia” in animal, but rather an “allodynia-like hypersensitivity to mechanical stimuli”.

Von Frey filaments

The Von Frey filaments are Nylon monofilaments that give a calibrated amount of force when applied against tissue until they bend. The mechanical stimulus given by von Frey filaments is commonly employed for assessing the mechanical response threshold of an animal. The testing is simple, filaments of known graded mechanical force are applied to the hind paw of the animal for about one second, and a positive response is obtained if the animal lifts its paw during the application. In general, Von Frey testing is often conducted by applying a set of hair filaments, ranging from 0.6 to 44mN as described by Fuchs et al (Fuchs, Roza et al. 1999). Each filament is applied 5 times, with at least a 10 seconds interval between stimulation, and the positive responses are recorded. The frequency of paw withdrawal to stimulation given is then converted to percentage of positive responses for each filament, and the result is presented as percent response versus von Frey force given.

More recently, the electronic von Frey system has been introduced to simplify the procedure and minimise the variability between researchers when using conventional von Frey filaments. Nonetheless, the operating principle remains similar for electronic von Frey. The tip of the probe is applied to the plantar surface of the hindpaw of an animal, and the animal will indicate the evoked noxious sensation by withdrawing its paw. The pressure, quantified in grams, applied to the paw is recorded in real time and the noxious threshold is recorded as an abrupt decrease in the applied force. It has been recently demonstrated that the electronic von Frey is more reliable and rapid than the conventional von Frey filament test in examining mechanical pain thresholds (Tena, Escobar et al. 2012). The reproducibility of repeated examinations, both by the same and by different observers, is good when using electronic von Frey system, and the time taken to conduct an experiment is almost half as that when using the conventional von Frey.

Randall Sellitto apparatus

The Randall Sellitto testing allows detection of the changes in nociception by measuring the thresholds of animals' withdrawal reflexes to the nociceptive mechanical stimulation. Unlike Von Frey filament that normally can only apply to the intraplantar skin of the hindpaw, Randall Sellitto apparatus can be applied to the forepaw, hindpaw and the tail of an animal. Typically, the animal is restrained and the paw/tail is placed under a pusher with a rounded tip. The force is applied until the animal attempt to withdraw the paw/tail. The applied force is measured and recorded. The paw/tail withdrawal threshold before and after treatments/surgery can then be compared. This testing has been widely used to evaluate the inflammatory pain response (Bujalska and Gumulka 2001, Lee, Li et al. 2001, Anseloni, Ennis et al. 2003) and neuropathic pain response (Santos-Nogueira, Redondo Castro et al. 2012).

Assessment of thermal nociception in rodents

The acute application of thermal stimuli to an animal is another most commonly used assays *in vivo* to evaluate the nociceptive processing for testing physiological manipulation/ effects of drugs. The thermal nociception assays rely on a principle to assess animal's ability to response to noxious thermal stimuli. The nociception responses are measured by behaviours such as withdrawal, licking and vocalisation.

Hargreaves' apparatus

The Hargreave's test measures animals' response to an infrared heat stimulus. An infrared source is focused at the plantar surface of the animal's hind paw, while a timer records the exposure time of the radiant source. When the animal feels pain, it will withdraw the paw. The withdrawal of the paw will cause a sudden reduction in the reflected radiation, which stops the timer and cut off the infrared source. The time latency to the paw withdrawal and the infrared intensity are hence automatically recorded by a fibre optic sensor. The infrared source is calibrated and allows the same power thermal stimulus to be delivered in the same given length of time. The Hargreaves's apparatus allows the thermal stimulus to be given to the animal automatically and also enable the experiment to be conducted without relying on investigator's observation of the animals' behaviours.

The radiant heat from the Hargreaves's apparatus activates the high threshold sensory

fibres that innervate the skin. The infrared source increases the temperature of the skin on the hind paw, and the axons that innervate this area of the skin transduce the temperatures into electric signals, which convey the 'painful signal' to the brain. The mechanism of transduction is believed to involve the temperature-induced opening of thermosensitive channel (TrpV1 channel) (Caterina, Leffler et al. 2000) and ATP receptor (P2Y₂ and P2X₃ receptors) (Malin, Davis et al. 2008). The keratinocytes in the epidermis releases ATP in response to the heat generated from the infrared source. The ATP then subsequently acts as secondary messenger at the cutaneous afferent terminals, which activates ATP receptors and conveys signalling in the sensory neurons. The increase in skin temperature also activates TrpV1 channels on the afferent terminal, and hence modulates the signal transduction.

Hot plate

The hot plate apparatus assesses the acute response of the animal to a strong thermal stimulus by allowing the animal to stand on a heated metal plate. The hot plate is preheated to a selected target temperature (typically between 50 and 55°C), and the animal is placed onto the hot plate while a timer is started immediately. The timer is immediately stopped when the animal displays the criterion behaviours including jumping, restless behaviours, licking, shaking and withdrawal of the hind paws, repeated elevation of hind paw(s), vocalisation and increasing walking activity. The animal is immediately removed from the hot plates when the timer is stopped to prevent tissue damage. The latency to observe criterion behaviour is compared before (baseline) and after physical manipulation/drug treatments. Nevertheless, it has been shown by Wilson and Mogil repeated testing on hot plate within 30 minutes shows decreased in latencies, even in sham animals that are not given any treatment. This phenomenon is described as behavioural tolerance (Wilson and Mogil 2001).

Tail-flick test apparatus

The tail-flick test requires restraining the animal to the apparatus. The animal is placed in a small cylinder with the tail in a tail groove exposing to a beam of light that produces high intensity of heat. A built-in sensor in the tail groove can detect the tail flick of the animal, and a timer will measure the latency and/or temperature threshold when the animal flicked their tail out of the beam. The test is often carried out with a 10 second cut-off time of exposure to the beam to prevent tissue damage.

2.2. Aim

Despite the growing evidences in the literature today suggest that VGF-derived peptides play roles in the pain pathway, the target cells of the VGF-derived peptides at the periphery still remain unknown. The molecular mechanisms of how VGF-derived peptides signal are also incompletely understood. The aim of this chapter is to:

1. Develop a calcium-based functional assay to investigate the effects of VGF-derived peptides on the intracellular calcium levels on different cell types that have been implicated in the pain pathway (DRG neuron and the primary macrophage).
2. Better understand the mechanism of action of the VGF-derived peptide on its target cell by determining the source of calcium and repetitive applications of the peptide in the calcium imaging.
3. Investigate the effect of VGF-derived peptide-stimulated cell *in vivo* by peripheral intraplantar injection and behavioural assessment.

2.3. Material and method

2.3.1. Animal

All the experiments conducted here conformed to the guidelines of the Committee for Research and Ethical Issues of the IASP. The experimental protocols were approved by the United Kingdom Home Office (under project license PPL70/7162). All experiments were carried out using adult Wistar rats (between 150 – 250 g). The rats were housed with standard laboratory conditions, and the food and water were available *ad libitum*. Artificial lighting was given on a fixed 12 hours light-dark cycle.

2.3.2. Primary culture of adult DRG neurons

Female Wistar rats (6 weeks old, ~150 g) were sacrificed by CO₂ asphyxiation. Spinal dorsal root ganglia (~40 per dissection) were removed from the rats and desheathed from the roots using Vannas micro-dissection scissor (Fine Science Tools) under a stereomicroscope. The dissected ganglia were digested in 0.125 % collagenase XI (Sigma Aldrich) and 0.1 mg/ml deoxyribonuclease II (Sigma Aldrich) for 90 minutes at 37 °C. After enzymatic digestion, DRGs were mechanically dissociated by gentle trituration for 10-15 times with a cut 1 ml tip, until a cloudy cell suspension was obtained. Dissociated DRG neurons were subsequently filtered through a 70 µm cell strainer (BD Biosciences). The filtered DRG cell suspension was gently applied to a 10 % BSA cushion (PAA) and centrifuged at 1100 rpm for 7 minutes. The cell debris and non-neuronal cells were removed from the interphase of the cushion after centrifugation, and the DRG neurons were recovered from the pellet. The pellet was resuspended in 1ml pre-warmed Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco Invitrogen). 10 µl of cell suspension was removed and applied to a haemocytometer to perform cell counting. The remaining cell suspension was centrifuged at 1100 rpm for 3 minutes, and the pellet was resuspended to a final volume adjusted.

The harvested DRG neurons were seeded on a 35 mm borosilicate glass bottom plate (10mm glass, MatTek) that had been pre-coated with 100 µg/ml poly-L-lysine (Sigma

Aldrich) and 10 µg/ml laminin (Invitrogen) at a cell density of 8000 cells/plate. 2ml DMEM medium supplemented with 10% Fetal bovine serum, Penicillin-Streptomycin solution (P/S, 1:100, Sigma Aldrich) and NGF (50ng/ml, Peprotech) was added to each plate. Cells were kept in a humidified incubator at 37 °C in a 5 % CO₂-enriched atmosphere. After 5 hours incubation, 10 µM Aphidicolin (Sigma) was added to each plate. The culture was used for calcium imaging 2 days after initial plating.

2.3.3. Bone-marrow derived macrophage culture

Bone marrow-derived macrophage cultures were generated using the protocol derived from Zhang *et al.* (Zhang, Goncalves et al. 2008). Female Wistar rats (6 weeks old, ~150 g) were euthanized, and the femur and tibiae were removed bilaterally using aseptic techniques. Marrow cores were flushed from the bones into a sterile 50 ml Falcon tube using a 50 ml syringe fitted with 25G needle and filled with ice-cold sterile Dulbecco's phosphate buffered saline (PBS, PAA). The bone marrow was triturated 10 times using 10 ml stripettes. The dissociated cells were filtered through a 70 µm cell strainer (BD Biosciences), followed by centrifugation at 1500 rpm for 5 minutes at 4 °C. PBS was carefully removed and discarded, and the pelleted cells were recovered in 100 ml pre-warmed macrophage culture medium (C-IMDM). The C-IMDM comprises the Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen), 10 % fetal bovine serum (FBS, GIBCO Invitrogen), Penicillin-Streptomycin solution (P/S, 1:100, Sigma Aldrich) and 5 ng/ml macrophage colony stimulating factor (M-CSF, Peprotech). The additional supplementation of M-CSF is needed in order to promote the differentiation of bone marrow cells into macrophages.

The cells were plated onto 10 cm tissue culture dish (BD Bioscience) with 10 ml of cell suspension per plate. The plated cells were allowed to differentiate for 3 days in a humidified incubator at 37 °C with 5 % CO₂- enriched atmosphere. The plates were washed once on the 3rd day *in vitro* (DIV), and re-supplemented with 10 ml fresh pre-warmed C-IMDM. The cells were then allowed to further differentiate for 3 more days in the incubator. After 6 DIV, the cells were ready to be re-plated onto the 35mm borosilicate glass bottom plate (10 mm glass, MatTek) for subsequent calcium imaging. After two washes with 5 ml PBS to remove residual media and undifferentiated cells, each plate of cells was incubated with 4ml pre-warmed non-enzymatic cell dissociation

solution (Sigma Aldrich) at 37 °C for 5 minutes. The cells were harvested by gentle pipetting and collected into a 50ml Falcon tube, followed by centrifugation at 1500rpm for 5 minutes at 4°C. The cell pellet was washed twice in 5ml pre-warmed C-IMDM to remove residual dissociation solution and carefully resuspended in 2 ml of pre-warmed C-IMDM. The cells were counted and plated at 40,000 cells per plate on the 35 mm glass bottom dishes. Cells were allowed to settle for 24 hours in 37 °C in a 95 % air / 5 % CO₂ humidified incubator. The media was replaced with 2 ml IMDM with P/S only for 24 hours prior to the calcium imaging experiments. To promote macrophage polarisation into M1, M2a or M2c macrophages, DIV7 cells (24 hours after replating) were treated with LPS (1 µg/ml, Sigma Aldridge), IL-4 (20 ng/ml, Peprotech) or IL-10 (20 ng/ml, Peprotech), respectively, for 24 hours in IMDM + P/S only media.

Macrophage conditioned media (MCM) for *in vitro* experiment was prepared from supernatant of stimulated macrophages. DIV8 cells (2nd day post replating, on the day of experimentation) were treated with 100 nM TLQP-21, 100 nM ScrTLQP-21 or vehicle (same volume of sterile water) in 1 ml of extracellular solution (see calcium imaging session for detail) for 4 hours. Two additional plates were treated with 100 nM TLQP-21 for 1 hour and 2 hours respectively. The supernatants were collected from the plate, centrifuged for 5 minutes at 4 °C at 1200rpm to remove cell debris. The spun supernatants were transferred to a sterile tube, clearly labelled and kept on ice until needed for experiments. The MCMs were removed from ice and leave at the room temperature for 15 minutes before experimentation to prevent activating temperature sensitive channels on DRG neurons.

Stimulated macrophages for *in vivo* experiment were prepared by stimulating DIV6 macrophages (in 10 cm plates) with 10 ml of IMDM+P/S media supplemented with either 500 nM TLQP-21 or 500 nM ScrTLQP-21, for 24 hours. The stimulated cells were washed three times in sterile PBS before harvest. The cells were dislodged from the plate using non-enzymatic cell dissociation solution as described before. The final volume is adjusted so that the harvested cells were resuspended in PBS at density of 35,000 cells/10µl. 10µl of the stimulated cells (i.e. 35000 cells) were injected to the rat intraplantarly using a 30G needle attached to a Hamilton syringe.

2.3.4. Calcium imaging

Fluo-4 AM staining protocol

The calcium fluorescent dye Fluo-4 AM (Molecular probes, Invitrogen) was reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 4 mM (1000X solution). The reconstituted solution was aliquoted at 1 µl per tube and stored at -20 °C. Primary DRG neuronal culture and primary bone marrow derived macrophage culture were prepared prior to calcium imaging experiment as described in session 2.3.2 and 2.3.3. 1 hour prior to the calcium imaging experiment, the media was removed from the cultures and the cells were washed three times in 1ml balanced extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM D-Glucose and 10 mM HEPES buffer, pH 7.4). 1ml of balanced extracellular solution was added to an aliquot of Fluo-4 to dilute it to the working concentration (4 µM). 500 µl of working concentration Fluo-4 was applied to the cells, and allowed to stain for 30 minutes at room temperature protected from light exposure. The cells were subsequently washed three times with 1ml balanced extracellular solution, followed by 30 minutes incubation in fresh extracellular solution to allow de-esterification of the dye. The cells were washed for another three times with 1ml extracellular solution, and left in 1ml fresh extracellular solution ready for imaging. All solutions used in staining protocol were sterile filtered in the tissue culture hood using a 0.2 µm filter, and all the compounds and reagents used were tissue culture grade.

The composition of calcium-free extracellular media used was the same as the balanced extracellular solution except the CaCl₂ was omitted and 2 mM EGTA was supplemented. The cells used to investigate the effect of TLQP-21 in calcium-depleted extracellular solution were still stained in the balanced extracellular solution (with calcium) before experiment to maintain healthiness of the cells. However, the cells were washed three times with 1ml calcium free extracellular solution after de-esterification, and left for 5 minutes in 1ml of fresh calcium free extracellular solution prior to imaging (to allow EGTA to chelate residual calcium). All treatments (i.e. TLQP-21 and ATP) applied in calcium free experiment were diluted in calcium free extracellular solutions.

Imaging acquisition and analysis

The cells were imaged on a Leica SP5 inverted confocal microscope and the acquisition was performed using Leica LAS AF Lite software. The cell images were acquired in both fluorescent and bright field. This allowed the detection in change in fluorescence intensity and possible morphology change throughout the experiment. Figure 2.1 A, 2.1 B and 2.1 C are the representative pictures of macrophage stained with Fluo-4 in fluorescence channel (in pseudocolor), bright field and both channels overlaid, respectively. The Fluo-4 staining protocol used here showed staining of 100% of the cells within the field of view.

To start, the acquisition was set to record the emitted fluorescent every 6 seconds (per frame) using 20x objective, and the maximum pinhole aperture at 600 μm , determining minimal confocality, was used. Two lines average was performed throughout acquisition. The cells were imaged at room temperature in the balanced (or calcium free) extracellular solution. The plate was placed in the recording chamber, and a random field of view was selected by looking down the eyepiece. The Fluo-4 dye was excited with Argon laser (15% laser maximum power) at 488 nm wavelength, and the emission fluorescence was captured between 500-570 nm wavelength. The gain and offset of the acquisition was adjusted so that minimum saturated and black pixels were observed.

The cells were first recorded without any treatment for at least 20 frames. This was essential because such baseline recordings could provide information of cells that were already spontaneously increasing intracellular calcium prior to any treatment. These spontaneous active cells were not therefore included in the data analysis at the end of the experiment. After baseline recording, a VGF derived peptide was applied to the cells, followed by ATP/KCl (1 mM ATP for primary macrophage and 60 mM KCl for primary DRG neurons) treatment as a viability assay to ensure the cells tested were alive. Only the cells that responded to ATP/KCl were selected for subsequent analysis.

The change in fluorescence recorded corresponds to the change in intracellular calcium level after treatment. Thus, the fluorescence recorded was quantified using

the LAS AF Lite software after imaging. To do so, *all* the cells from the field of view were circled and defined as the region of interest (ROI) (as illustrated in figure 2.1D). The changes in the fluorescent intensities in the ROIs were quantified by the software and exported as an excel file as represented in figure 2.1E. These raw data were further analysed by expressing the fluorescence intensity in respect to their baseline. Any cell that showed an increase in fluorescence more than 20 % of the baseline was classified as “responsive” and those below 20 % of the baseline was known as “non-responsive” to the stimulation. The cut off was set to be 20 % of the baseline because a small increase in the background fluorescence could be observed when any solution was applied to the plate during recording, including vehicle control. Therefore, a 20 % baseline cut off was set to reduce false positive selection of non-responsive cells as responsive. Figure 2.1F is an illustrative example of the averaged fluorescence profile of ‘non-responsive’ cells. In this demonstrative picture, the fluorescent intensity was not changed after peptide application. However, the ATP viability control showed a significant increase in the intracellular calcium level, indicating that the cells were viable. On the other hand, figure 2.1G demonstrates the typical averaged fluorescence profile from the “responsive” cells in an experiment. Application of peptide in this experiment induced a robust and immediate increase in the intracellular calcium level that is greater than 20 % of the baseline, which gradually returned to the baseline. ATP stimulation at the end of this experiment also showed that the cells analysed were viable and able to respond to the positive control.

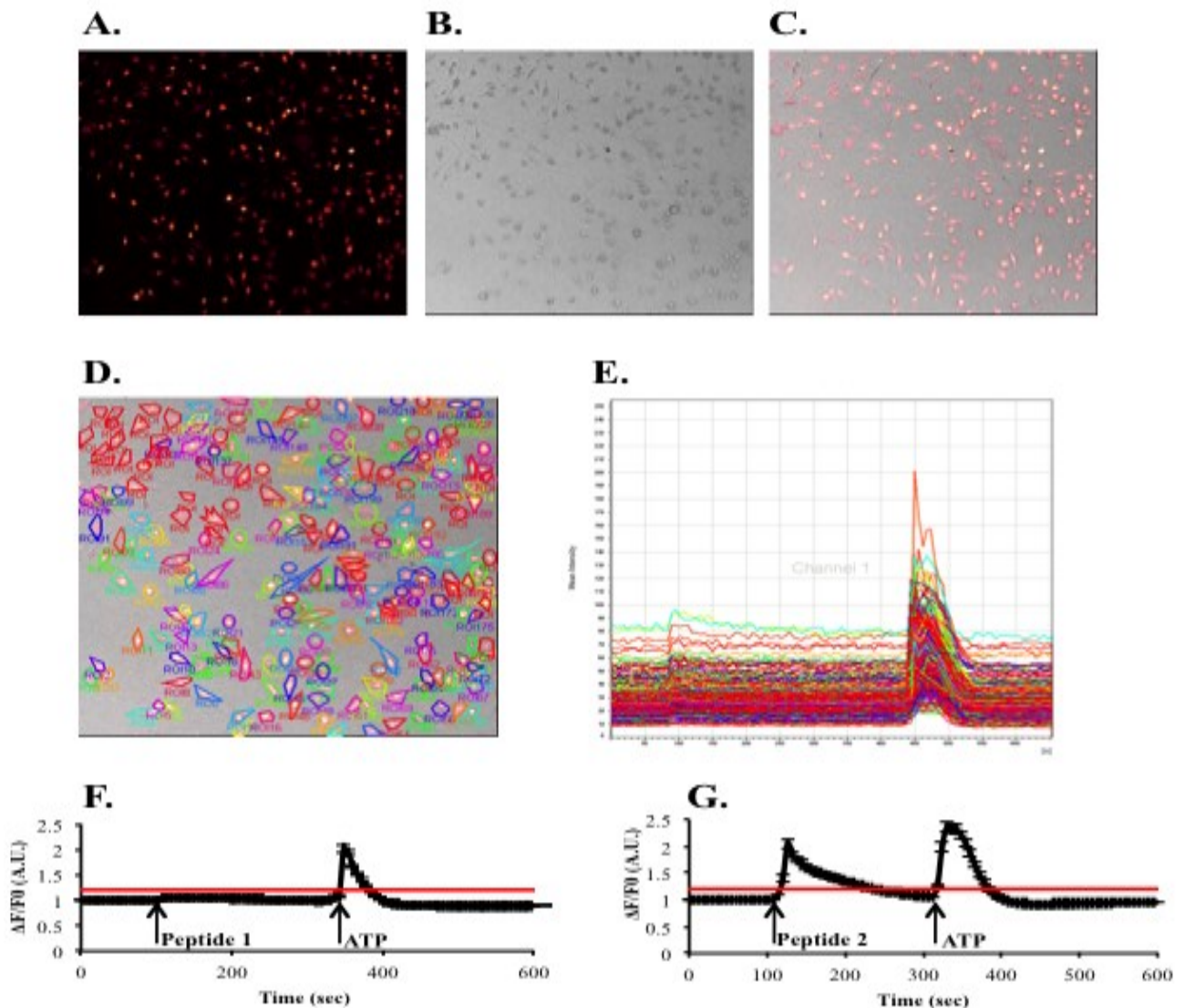


Figure 2.1 Representative field of view, cell selection and fluorescence profiles

(The staining and acquisition protocol for calcium imaging is demonstrated here using primary macrophage as an example.) The stained cells were visualised under confocal microscope and real-time live cell imaging was performed while the peptides and positive controls (ATP) were added. All cells from the field of view were selected and the fluorescence profiles of the cells were exported and analysed. The cells were classified to be “responsive” when the fluorescent intensity is increased by more than 20 % of the baseline after peptide application. Any cell with an increase in fluorescence less than 20 % of the baseline is classified as “non-responsive”. **A.** Representative field of view in fluorescence (shown in red pseudo-colour). **B.** Representative field of view in bright field. **C.** Overlay image of bright field and fluorescence. **D.** Image of region of interest (ROIs) cell selections in the field of view. **E.** Raw data of fluorescence recorded from different ROIs. **F.** A representative averaged fluorescence profile of all selected cells, showing the cells were not responsive. ATP (cell viability control) at the end of the experiment showed the cells were able to elicit an increase in calcium response. **G.** A representative averaged fluorescence, showing an increase in fluorescence intensity 20 % above the baseline after peptide application, indicating the cells were responsive to the peptide.

2.3.5. Quantitative real time polymerase chain reaction

Total RNA from cultured cell was extracted using RNAeasy extraction kit (Qiagen), and cDNA was synthesized from total RNA using the Superscript III first-strand synthesis RT kit (Invitrogen). Real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) in combination with the Fast SYBR Green technology to accurately measure the expression levels of the target genes. The reaction was performed in a total reaction volume of 20 µl, consisting of 10 µl Fast SYBR Green master mix (Applied Biosystems) of 0.5 µM of primer mix of target-interested, and 20ng cDNA. The primers sequences for internal control/reference gene (Cyclophilin A), and gene of interests are shown in the table below. All experiments were repeated three times with independent batch of treated samples. The reaction was run with initial heating step at 95 °C for 20 second, followed by 40 cycles of denature step at 95 °C for 3 seconds, annealing step at 60 °C for 15 seconds, and extension step at 72 °C for 15 seconds. Product composition was verified at the end of each reaction with Melting Curve measurement. Melting Curve stage was performed at initial heating step to 95 °C for 15 seconds, followed by 0.6 °C increment increase steps from 60 °C to 95 °C. The melt curve allows verification of the purity of the PCR products; pure product is characterised by one sharp peak in melt curve graph.

Targets	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Cyclophilin A	GCAAGACCAGCAAGAAGATC	TCAGTGAGAGCAGAGATTACAG
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC
Arg-1	TATCGGAGCGCCTTTCTCTA	ACAGACCGTGGGTTCTTCAC
IL-10	GTTGCCAAGCCTTGTCAGAAA	TTTCTGGGCCATGGTTCTCT

Table2. 1 Primer sequences for Cyclophilin A, iNOS, Arg-1 and IL-10

2.3.6. Behaviour assessment: Electronic von Frey

A custom-built isolation chamber with individual Plexiglas boxes (23 x 18 x 14cm) was used in behavioural study. In order to reduce the confounding affects of stressed animals, the animals received two sessions of 1 hour habituation in the Plexiglas boxes on two consecutive days. During the habituation (and behaviour testing later on), all animals were housed individually in the Plexiglas boxes and received

consistent handling from the experimenter. Two baseline measurements, on two separate consecutive days (at least 24 hours apart), prior to any treatment were recorded after two days of habituation. On the days of baseline measurement, the animals were moved into the testing room in their home cage group at least 20 minutes prior to testing to allow acclimatisation. The animals were transferred to the Plexiglas boxes and allowed to acclimatise for another 15 minutes. The baseline paw withdrawal threshold was measured using a calibrated electronic von Frey device with 0.5mm diameter tip (Electronic von Frey device, Somedic, type 735). The force transducer was applied to the surface of the hind paw at a rate of 8-15 g/sec until a limb withdrawal response was observed. Five applications were made to each of the hind paw and the mean baseline paw withdrawal thresholds were calculated.

10 µl TLQP-21 stimulated macrophage/ScrTLQP-21 stimulated macrophage was injected intraplantarly to the left hindpaw of the animals the day after second baseline recording. In the *in vivo* experiments, the experimenter was blinded to the treatment group of all the animals tested. The male Wistar rats (~200g) were first anaesthetised under 2% isoflurane in 1% O₂ and 1% N₂O. The plantar surface of the left hindpaw was sterilised using isopropanol swab. A 30G needle attached to a Hamilton syringe was used to deliver 10 µl of stimulated macrophage intradermally at the sterilised area. The needle was allowed to rest in place for 1 minute after injection to prevent solution back-flow. The animals were allowed to recover for 24 hours in their home cage groups. The mean paw withdrawal thresholds were determined again on post operative day 1 (POD1), day 2 (POD2) and day 5 (POD5) as described. After POD5 measurement, the experiment was terminated by euthanising the animal with CO₂ asphyxiation. (The described *in vivo* study looking at the effect of TLQP-21-stimulated macrophages was done in collaboration with Dr Alessandro Pristera. The behaviour assessment was kindly performed by Dr Alessandro Pristera).

2.4. Results

Vgf expression has been shown to be upregulated in different neuropathic pain animal models. However the cells that VGF-derived peptides target in the pain pathway still remain as an intriguing question to be answered. In this study, I sought to investigate what cell types do VGF-derived peptides work on, and what are the subsequent consequences upon their binding to the target cell(s), and the mechanism of action of the peptides on its target cells. In order to access what cell types do VGF-derived peptides work on, a functional assay, calcium imaging, was established (see section 2.3.4 for detail protocol). Calcium is well known to be an important signalling ion involved in the majority of the intracellular and extracellular processes. Calcium imaging was exploited in this study as the functional assay to investigate the effect(s) of the peptides on intracellular calcium of different cell types.

2.4.1. Effects of the VGF-derived peptides on the intracellular calcium level of different cell types: Primary DRG neurons.

After nerve injury, the VGF-derived peptides are released from the storing vesicles of the sensory neurons upon depolarization. One of the most obvious cell targets that the VGF derived peptides could act on is the DRG neuron itself. It could be hypothesised that, upon nerve injury, DRG neurons release the VGF-derived peptides, which target the postsynaptic sensory neurons to exert their function, or they could target the neurons that they were released from allowing, for example, more peptides to be produced/released. Based on this hypothesis, we first assessed if different VGF-derived peptides induce a change in the intracellular calcium level in the rat primary DRG sensory neurons. Published data suggested, both *in vitro* and *in vivo* VGF derived peptides work at concentrations lower than 1 μ M concentration (Moss, Ingram et al. 2008, Rizzi, Bartolomucci et al. 2008, Severini, Ciotti et al. 2008, Riedl, Braun et al. 2009), therefore, 1 μ M maximum concentration of each peptide was used in this investigation. Figure 2.2 shows that the primary DRG neurons were not responsive to 1 μ M LQEQ-19 (figure 2.2A), 1 μ M TLQP-21 (figure 2.2B) and negative control peptide ScrTLQP-21 (figure 2.2C), in our calcium-based functional assay. Nevertheless, the 60 mM KCl applied after each peptide application induced a

robust and prolonged increase in the intracellular calcium level in the DRG neurons, indicating the cells were viable to respond to the stimulations.

2.4.2. Effects of the VGF-derived peptides on the intracellular calcium level of different cell types: Primary bone marrow-derived macrophages.

I next evaluated if VGF-derived peptides exert the functional effects by changing the intracellular calcium level of the primary macrophages. Macrophages are known to infiltrate DRG after nerve injury and depletion of macrophages has been shown to relieve neuropathic-pain-like behaviours in animals (Liu, van Rooijen et al. 2000, Mert, Gunay et al. 2009). Based on this evidence, I hypothesized that VGF-derived peptides might be released from the DRG neurons after the nerve injury and subsequently, instead of acting on the neurons itself, acts on the adjacent cells such as the infiltrated macrophages to exert the function(s). To test this hypothesis, primary macrophages were differentiated from bone marrow stem cells using macrophage colony stimulating factor (M-CSF), and cultured for 7 days, followed by live cells imaging. Figure 2.3 shows the Fluo-4 fluorescence in pseudo-color on the left. The clipped pictures are associated with different time points during the recording, pre-application of VGF-derived peptide, application of VGF-derived peptide, post application of VGF-derived peptide and application of ATP, respectively. The graphs on the right in Figure 2.3 show the averaged fluorescence profiles of each experiment and the time point associated with the clipped pictures were numbered. Figure 2.3 A demonstrates that the primary macrophage were not responsive when an equal volume of vehicle control was applied to the plate. In addition, Figure 2.3 B and 2.3 D further demonstrate both LQEQ-19 and TLQP-62 VGF-derived peptides did not induce an increase in the intracellular calcium when applied to the primary macrophage *in vitro*. Nonetheless, when 1mM ATP was applied at the end of the experiments, the cells were able to induce a robust and transient increase in the intracellular calcium, which returned to the baseline within 2 minutes. This demonstrates that the cells were viable and were able to respond to ATP stimulation. Interestingly, when TLQP-21 peptide was applied to the primary macrophage, unlike TLQP-62 and LQEQ-19 that did not induce a response, it induced an immediate increase in the intracellular calcium that gradually returned to the baseline (Figure 2.3 C).

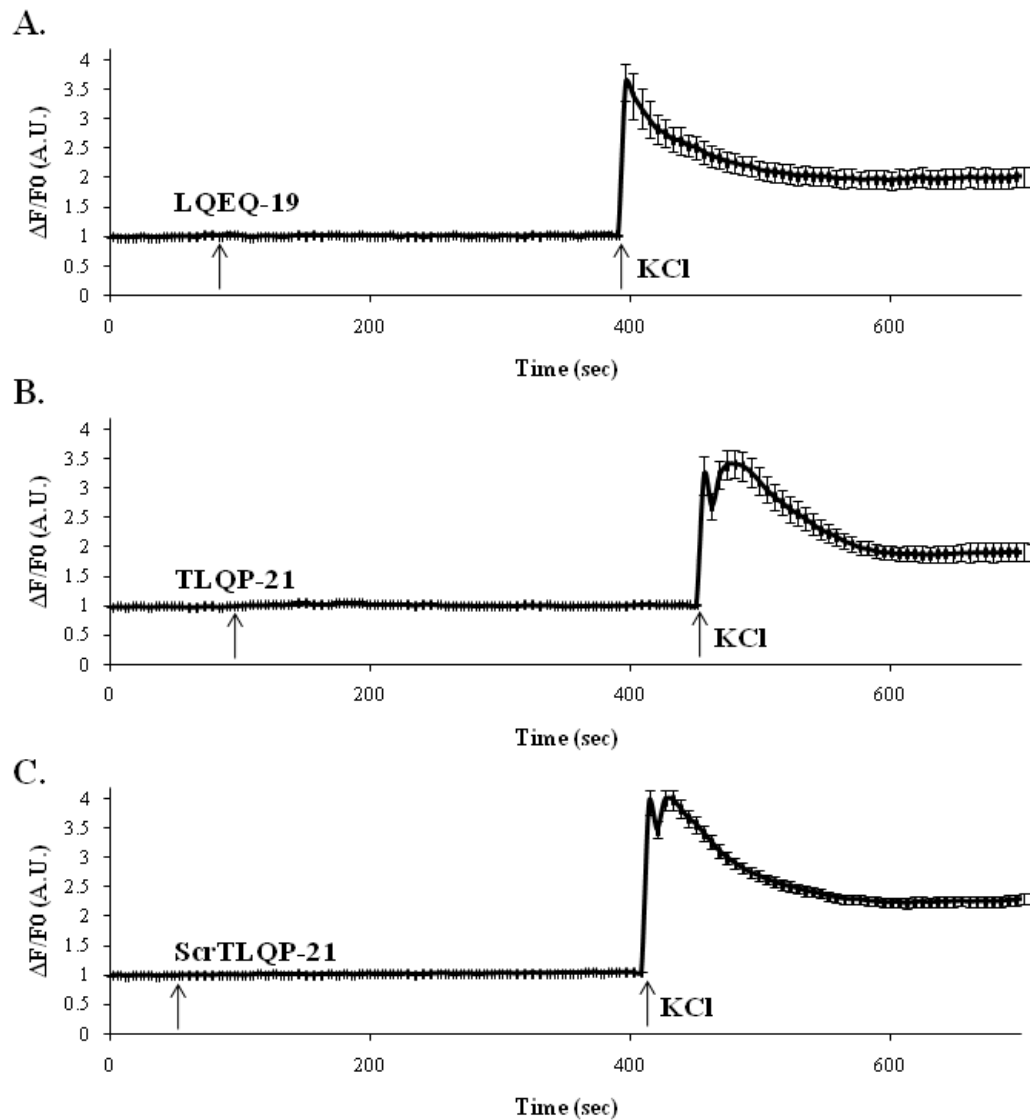


Figure 2.2 Effect of VGF derived peptides, LQEQ-19, TLQP-21 and ScrTLQP-21, on primary DRG neurons.

Average fluorescence profile of DRG neurons. The time points, at which each treatment was applied to the cells, are indicated by arrows. **A.** 1 μ M of LQEQ-19 was applied to cultured DRG neurons, followed by 60 mM KCl positive control. The fluorescence profile shows the cells were not responsive to LQEQ-19 peptide and KCl was able to elicit an increase in intracellular calcium after application. **B.** 1 μ M of TLQP-21 did not induce an increase in calcium response in cultured DRG neurons. **C.** Negative scrambled control peptide did not elicit a calcium response in cultured DRG neurons.

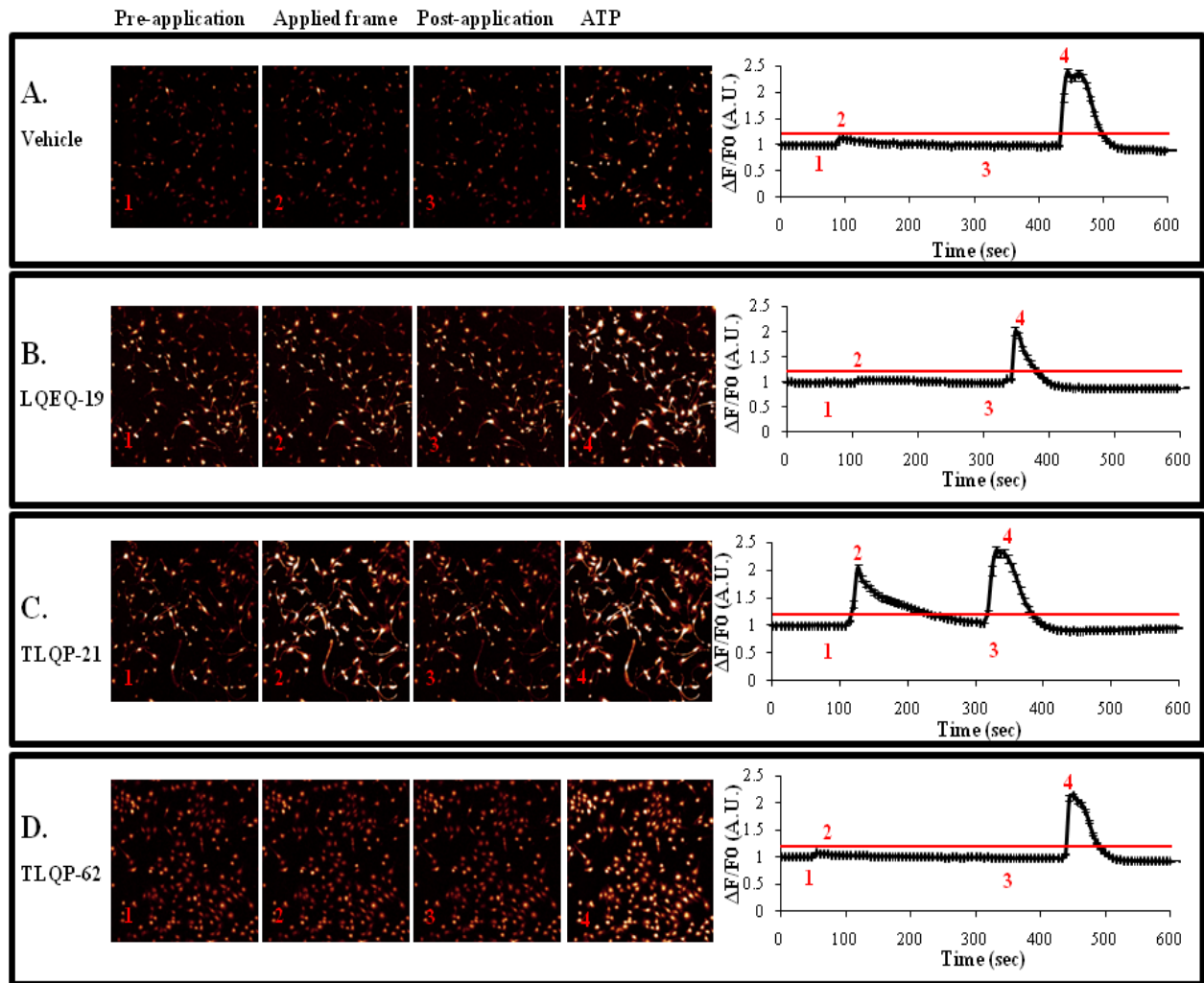


Figure 2.3 Effects of different VGF-derived peptides on primary macrophage.

Pseudo-color images of intracellular calcium levels at different time points are displayed on the left and the averaged fluorescence profile is shown on the right for macrophages stimulated with A. Vehicle B. LQEQ-19 C. TLQP-21 D. TLQP-62

2.4.3. The TLQP-21 peptide specifically induced an increase in the intracellular calcium level in the primary macrophage, in a dose-dependent manner.

To assess the specificity of the TLQP-21-induced calcium response in the primary macrophage, I tested its control peptide at the same molar concentration. The scrambled TLQP-21 peptide (ScrTLQP-21) is a synthesized peptide with the same amino acid residues but scrambled in the sequence. This control peptide, therefore, exhibits the same charge and retains in the same length as the wild type sequence. Figure 2.4 shows that TLQP-21 was able to induce an increase in the intracellular calcium level when applied to the primary macrophages at 100nM concentration. However, when the scrambled control peptide ScrTLQP-21 at the same concentration

was applied, the increase in the intracellular calcium was not observed. This demonstrates the specificity of action of TLQP-21 peptide on the primary macrophage.

These data provide the first evidence, to our knowledge, of a VGF-derived peptide specifically acts on the primary macrophage, at least *in vitro*, in a calcium-dependent manner. To elucidate this further, we tested different concentrations of TLQP-21 and ScrTLQP-21 peptides. This provides information on the minimum and saturating concentrations of TLQP-21 that could elicit responses in the primary macrophages.

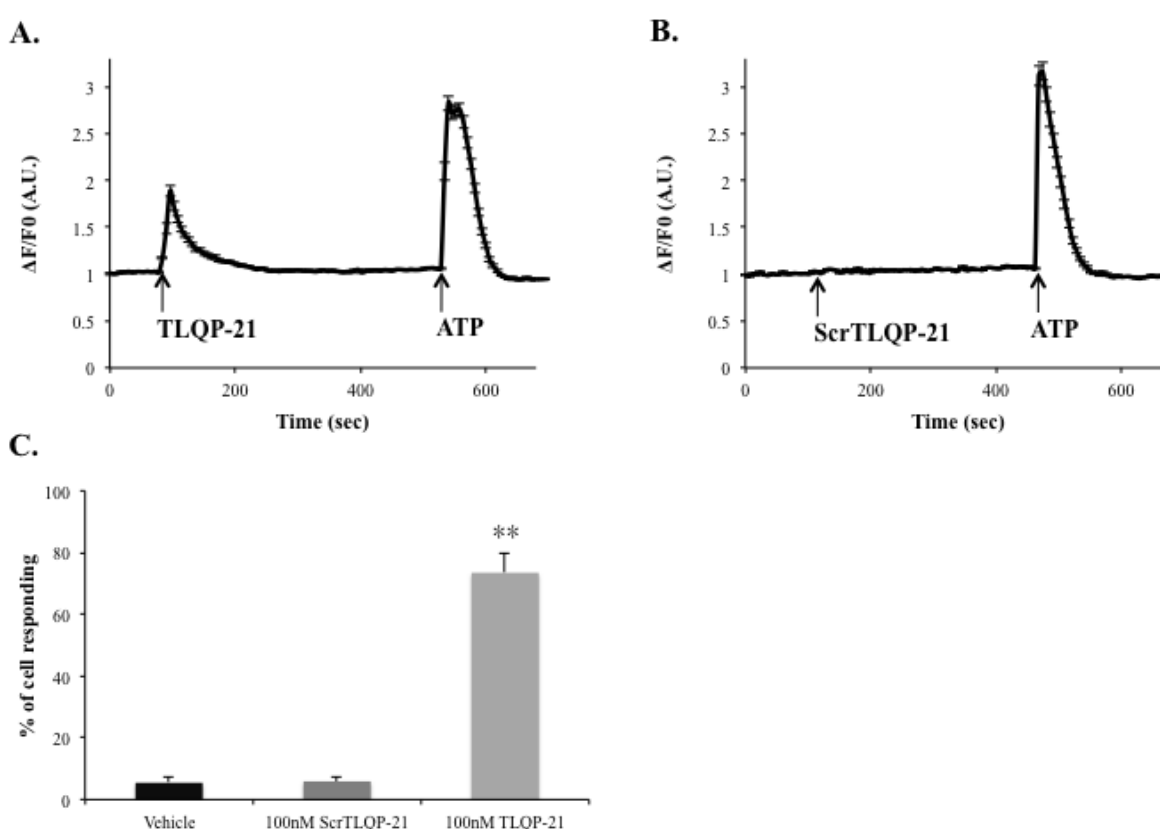


Figure 2.4 Effect of TLQP-21 and its scrambled control peptide, Scr-TLQP-21, on primary macrophage.

A. Fluorescence profile showing 100 nM TLQP-21 specifically induced an increase in intracellular calcium in primary macrophage. **B.** The scrambled control peptide with same amino acid components and charge failed to elicit a calcium response at 100nM concentration in primary macrophage. The ATP viability control at the end of the experiments indicates the cells were viable and able to elicit an increase in intracellular calcium. **C.** Bar chart comparing percentage of cell responding to 100 nM TLQP-21 and 100nM ScrTLQP-21 to vehicle control. All data points were a combination of 4 experiments. The percentage of cell responding to TLQP-21 was significantly different to that responded to vehicle and ScrTLQP-21 (** $P < 0.01$, One way ANOVA, Tucky post hoc test). The percentage of cell responding to ScrTLQP-21 is not statistically significant from that to vehicle control, indicating specific action of TLQP-21 on primary macrophage *in vitro*.

Figure 2.5 A shows the dose response curve for the wild type TLQP-21 peptide on the primary macrophages. For each of the concentrations tested, minimum of 4 independent repeats were performed. The primary macrophages were tested for different concentrations at 10 nM, 100 nM, 1 μ M, 10 μ M and 50 μ M. The fluorescence profile was analysed as described before, and a further analysis was carried out to calculate the percentage of cells responding (with 20% baseline cut off) to the peptide at a given concentration. All data sets of each concentration were combined and the graph was plotted with the percentage of cells responding against the log concentration of the peptide. It must be noted that there were always a small number of cells respond to any stimulation given (including vehicle negative control). This is to be expected, as macrophages are immune surveillance cells that respond to the subtle changes in the environment. To set the “background noise”, the primary macrophage was tested with vehicle negative control (same volume of water in the extracellular solution). A combination of 4 experiments shows that an average of $5.4 \pm 1.7\%$ of the primary macrophages responded to the vehicle control, which is plotted as the red line in figure 2.5 (as the background activity).

As shown in figure 2.5 A, 1 nM of TLQP-21 (at log scale) induced an increase in the intracellular calcium in $9.7 \pm 2.6\%$ of the cells, however, this is not statistically significant from the vehicle control. The lowest concentration that showed a significant increase in the percentage of cells responding to the wild-type TLQP-21, compared to the vehicle control, was at 10nM ($p < 0.01$, One way ANOVA, Tukey post hoc test). 10 nM of TLQP-21 peptide stimulated $56.1 \pm 6.2 \%$ of the primary macrophages in a calcium dependent manner. The percentage of cells responding to TLQP-21 further increased to $73.5 \pm 6.5 \%$ and plateaued at 100 nM concentration. The percentages of cells responding to 1 μ M, 10 μ M and 50 μ M TLQP-21 were $73.9 \pm 8.3 \%$, $74 \pm 8.0 \%$ and $74.1 \pm 4.4 \%$, respectively, which did not significantly differed to the percentage of cells responding to 100nM TLQP-21, indicating no further increase in the percentages of responding cells to TLQP-21.

Figure 2.5 B shows the dose response curve for scrambled control peptide, ScrTLQP-21. 100 nM, 1 μ M, 10 μ M and 50 μ M of ScrTLQP-21 peptides were used to test for the primary macrophages responsiveness. The percentages of cells responding

to ScrTLQP-21 were not significantly different from vehicle control at 100 nM, 1 μ M and 10 μ M concentrations. Even though at 10 μ M concentration, the percentage of cell responding increased to 39.5 ± 17.1 %, the data was not significantly differed when compared to the vehicle control ($p > 0.07$, One way ANOVA, Tukey post hoc test). The percentage of cell responding to 50 μ M of the ScrTLQP-21 was significantly different from the vehicle control ($P < 0.05$, One way ANOVA, Tukey post hoc test), with 57.1 ± 6.1 % of the total analysed cells exhibited an increase in intracellular calcium.

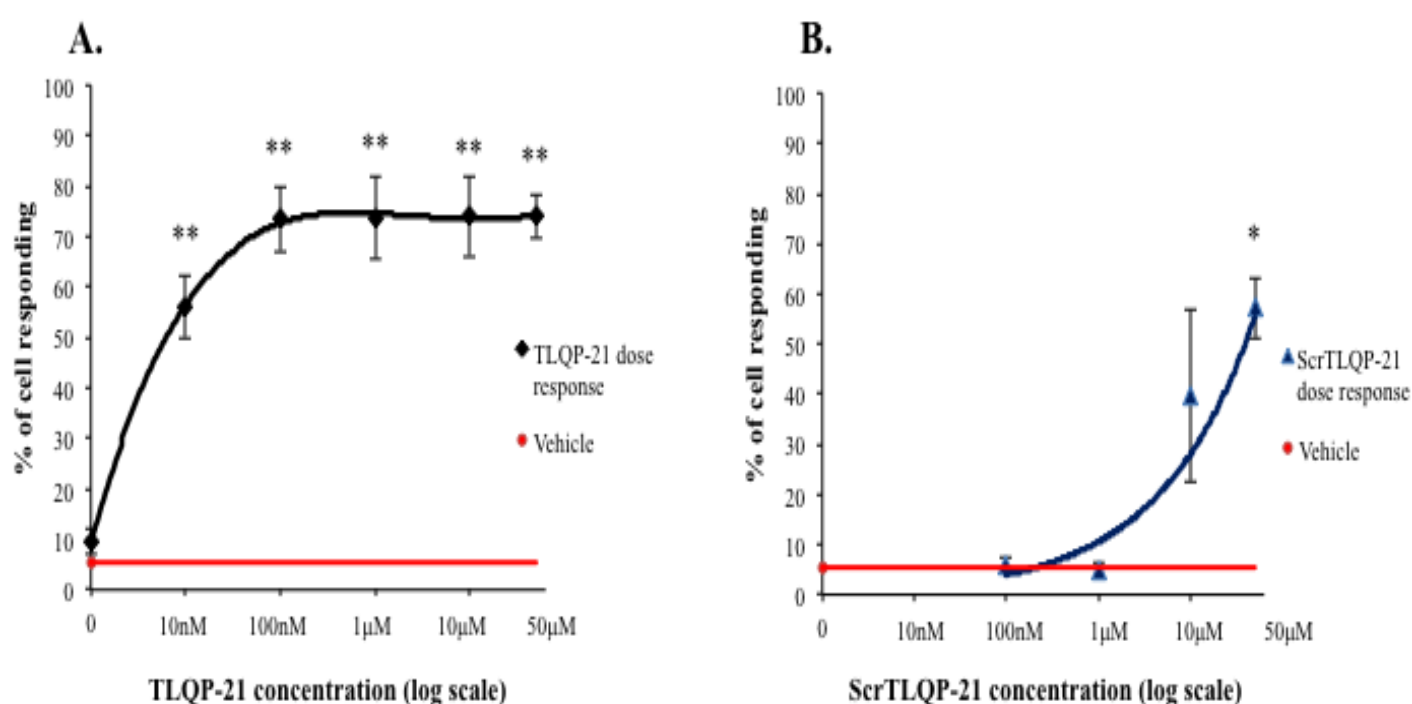


Figure 2.5 Dose response of TLQP-21 and ScrTLQP-21 on primary macrophage.

A. Dose response curve of TLQP-21 on primary macrophage. 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M and 50 μ M concentrations of TLQP-21 peptide were tested on primary macrophages. The responsiveness of cells was expressed in percentage of cell responding to different concentration stimulation. Each data set is a combination of at least 4 experiments. The data point is expressed in average percentage of cell responding $\pm$ S.E.M. (** $P < 0.01$, One way ANOVA, Tukey post hoc test). The percentage of cells responding to vehicle negative control is plot in red line as background noise that observed. **B.** Dose response curve of ScrTLQP-21. 100 nM, 1 μ M, 10 μ M and 50 μ M concentrations of ScrTLQP-21 were tested. (* $P < 0.05$, One way ANOVA, Tukey post hoc test).

2.4.4. TLQP-21 desensitises primary macrophage to further TLQP-21 stimulation

In order to further investigate the effects of TLQP-21 on the primary macrophage, a second application of TLQP-21 was applied to the cells. Macrophage are known to exhibit characteristic properties of desensitisation in the calcium imaging when, for example, ATP is applied repeatedly (Oshimi, Miyazaki et al. 1999). In this experiment, I sought to investigate if repeated application of TLQP-21 causes desensitisation (abolish or diminished in response), sensitisation (increased in response) or the same response as the first application.

In this experiment, 100 nM of TLQP-21 was applied to the primary macrophage after the baseline recording, as this is the lowest dose that elicits the maximum number of cells responding to the wild type peptide. The cells were recorded for further 6 minutes to allow the intracellular calcium level to return to the baseline. A second dose of 100 nM TLQP-21 was then applied to the cells after the fluorescence returned to the baseline. It was observed that the first TLQP-21 elicited a robust and immediate increase in the intracellular calcium as described before. However, when the second dose was applied, the cells were not responsive to the second stimulation of TLQP-21 (Figure 2.6 A). 1 mM ATP was applied to the cells 6 minutes after the second dose of TLQP-21, and an increase in the intracellular calcium level was observed. This indicates that the cells were viable and able to elicit a calcium response. In order to rule out the possibility that the failure to induce an increase in the intracellular calcium by the second TLQP-21 dose was due to the insufficient time allowed for the cells to replenish the intracellular calcium, I allowed a longer time interval in between the first and the second applications. Figure 2.6 B shows the fluorescence profile of the experiment with two applications of 100nM TLQP-21, and a 30 minutes time interval was allowed in between the applications. The first dose of TLQP-21 was applied to the cells after baseline recording, the cells were further recorded for 8 minutes and allowed to rest for 20 more minutes without recording. The recording was turned off during longer “resting time” to prevent laser damage to the cells that arises from long time period recording. The recording was switched back on afterwards and the second dose of the peptide was applied 2 minutes after the second recording started. The total time allowed between two applications was added up to be

30 minutes. The primary macrophage was still observed to be non-responsive to the second dose of TLQP-21 after a 30 minutes recovering time was allowed prior to the second dose application. The longest total time allowed between the two applications was one hour, as the live cells imaging was performed in extracellular solutions without serum. It was to ensure that the cells were in healthy condition prior to the second dose treatment, thus one hour time interval was the longest tested between two TLQP-21 applications. Figure 2.6 C shows that one hour after first TLQP-21 application, the second dose of TLQP-21 still fail to elicit an calcium response in the primary macrophage, indicating a possible desensitisation mechanism in the macrophages for this peptide after repetitive treatments. The percentage of cells responding to the second dose of TLQP-21 after the first stimulation was $5.1 \pm 1.8 \%$, $4.8 \pm 0.75 \%$ and $11.6 \pm 9.6 \%$ for experiments with 8 minutes, 30 minutes and 60 minutes time intervals, respectively (figure 2.6 D). The percentages of cells responding to the second stimulation are not significantly different from each other in all the experiments, indicating desensitisation can be still observed up even one hour time interval was allowed between the two applications.

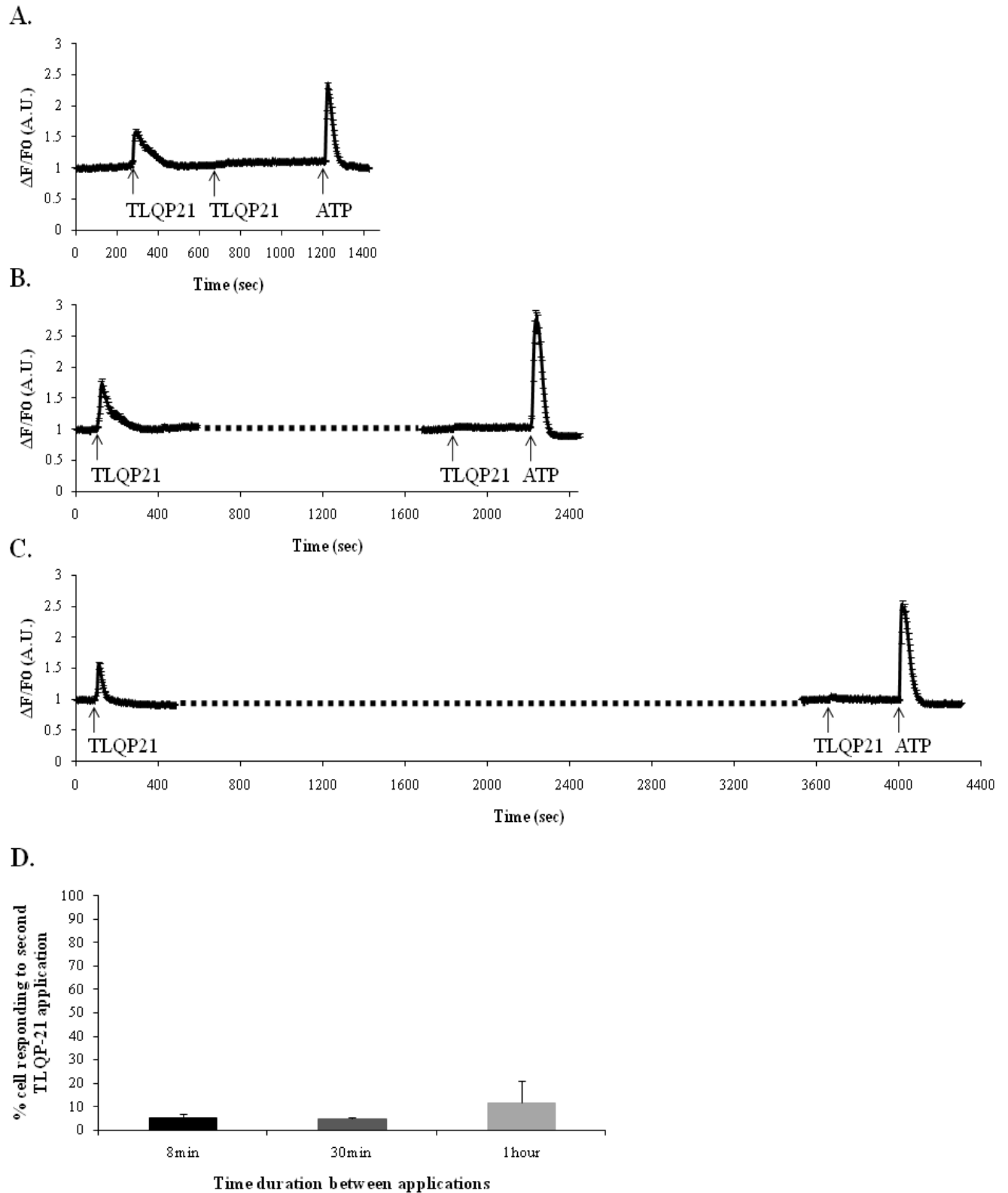


Figure 2.6 Desensitisation effect of TLQP-21 on primary macrophage.

TLQP-21 desensitises the primary macrophages to the second TLQP-21 application. The desensitisation effect is observed with time intervals of, **A.** 6 minutes **B.** 30 minutes **C.** 1 hour, between the two applications. **D.** Quantification of percentage of cells responding to the second stimulation after elicited a response to the first TLQP-21 stimulation.

2.4.5. Pre-application of ScrTLQP-21 at high concentration also desensitised the primary macrophage to further TLQP-21 stimulation, but not with pre-application of TLQP-62.

Having demonstrated TLQP-21 desensitises the primary macrophage to a second TLQP-21 application. Here I sought to further exploit the sensitivity of the primary macrophage to the VGF-derived peptides. TLQP-62 is a longer VGF-derived peptide that harbours TLQP-21 peptide sequence at its N-terminus. I tested if pre-treatment with high concentration of TLQP-62 can cause desensitisation to a subsequent TLQP-21 stimulation in the macrophage. Figure 2.7 A shows when 10 μ M of TLQP-62 was applied to the primary macrophage, the following 100 nM TLQP-21 treatment was able to elicit an immediate increase in the intracellular calcium similar to what was observed before. This possibly implies that pre-treatment of TLQP-62 does not interfere with the TLQP-21 signalling pathway.

It was demonstrated previously in this chapter that high concentration of ScrTLQP-21 could cause a calcium response in the primary macrophage. I, therefore, tested if the TLQP-21 desensitisation can still be observed if high concentration of ScrTLQP-21 was applied as the first stimulation. As shown in figure 2.7B, after 50 μ M of ScrTLQP-21 stimulation, which induced a calcium response, the subsequent 100 nM TLQP-21 application failed to evoke calcium increase in macrophage. This suggests ScrTLQP-21 at extreme high concentration could possibly mimic the TLQP-21-induced response in the primary macrophage.

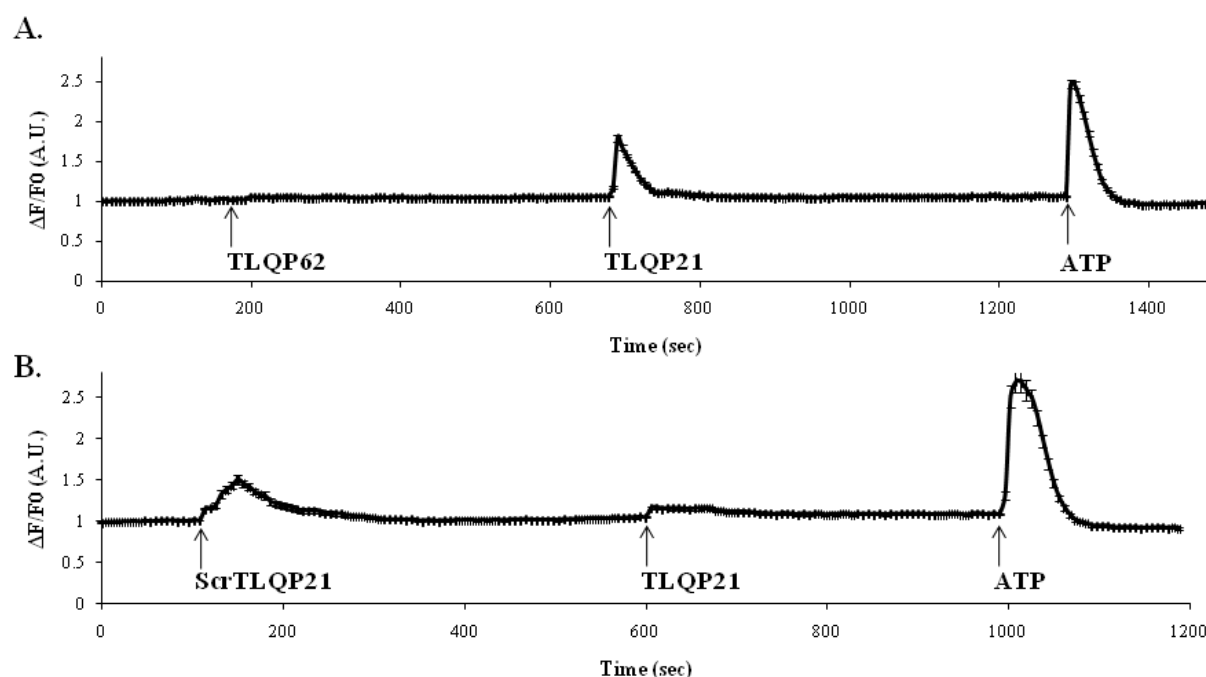


Figure 2.7 Effect of other VGF derived peptides on subsequent TLQP-21 induced calcium response in the primary macrophage.

A. TLQP-62 pre-application does not interfere with the subsequent TLQP-21 response in primary macrophage. **B.** Pre-treatment with high concentration (50 μ M) of ScrTLQP-21 desensitises the primary macrophage to the following TLQP-21 stimulation. ATP response at the end of the experiment indicates the cells were viable, and the time allowed for calcium replenishment was sufficient.

2.4.6. The TLQP-21-induced increase in the intracellular calcium was still observed when the calcium was excluded from the extracellular solution.

I have demonstrated that TLQP-21 peptide specifically acts on the primary macrophage in a dose- and calcium-dependent manner, and it desensitises the target cells to further TLQP-21 stimulation. I was interested to further dissect whether the increase in the calcium level was a result of influx of calcium from the extracellular solution, or a release of calcium from the intracellular store. In this experiment, I investigated the percentage of cell responsiveness upon TLQP-21 application in the medium devoid of calcium and supplemented with 2 mM EGTA. The percentage of cells responding to TLQP-21 in the ‘calcium free’ extracellular solution was then compared to that tested in the medium with calcium. As expected when calcium was present, an immediate increase in the intracellular calcium level was observed, which gradually returned to the baseline as shown in figure 2.8A. Interestingly, when calcium was omitted from the medium, an increase in the intracellular calcium level in the primary macrophage, after 100 nM TLQP-21 stimulation, was still observed

(Figure 2.8B). It is worth noting that the ATP response in the 'calcium free' condition was relatively smaller when compared to that in the presence of calcium. This is expected as ATP works through both P2X and P2Y receptors and allowed both extracellular and intracellular calcium to go into the cytosolic space. Therefore, when the extracellular calcium was omitted, the change in the calcium level after ATP stimulation was expected to be lower, because the only calcium source was from the intracellular store. Figure 2.8 C compared the percentage of cells responding to TLQP-21 with presence or absence of calcium in the extracellular solution. Even though, the average percentage of cell responding to TLQP-21 in the 'calcium free' condition is slightly less than that in the condition with calcium (48.3 ± 8.3 % in calcium free condition compared to 60.9 ± 10.1 % in the presence of calcium), the two groups are not significantly different from each other (student *t* test). This indicates that TLQP-21 peptide, at least at 100 nM concentration tested, was able to elicit a similar response in the primary macrophage in media with or without calcium. The results here showed that the extracellular calcium is not essential for TLQP-21-induced calcium response in the primary macrophage. This suggests the calcium source of the observed TLQP-21-induced response was, at least partly if not all, coming from the intracellular calcium store.

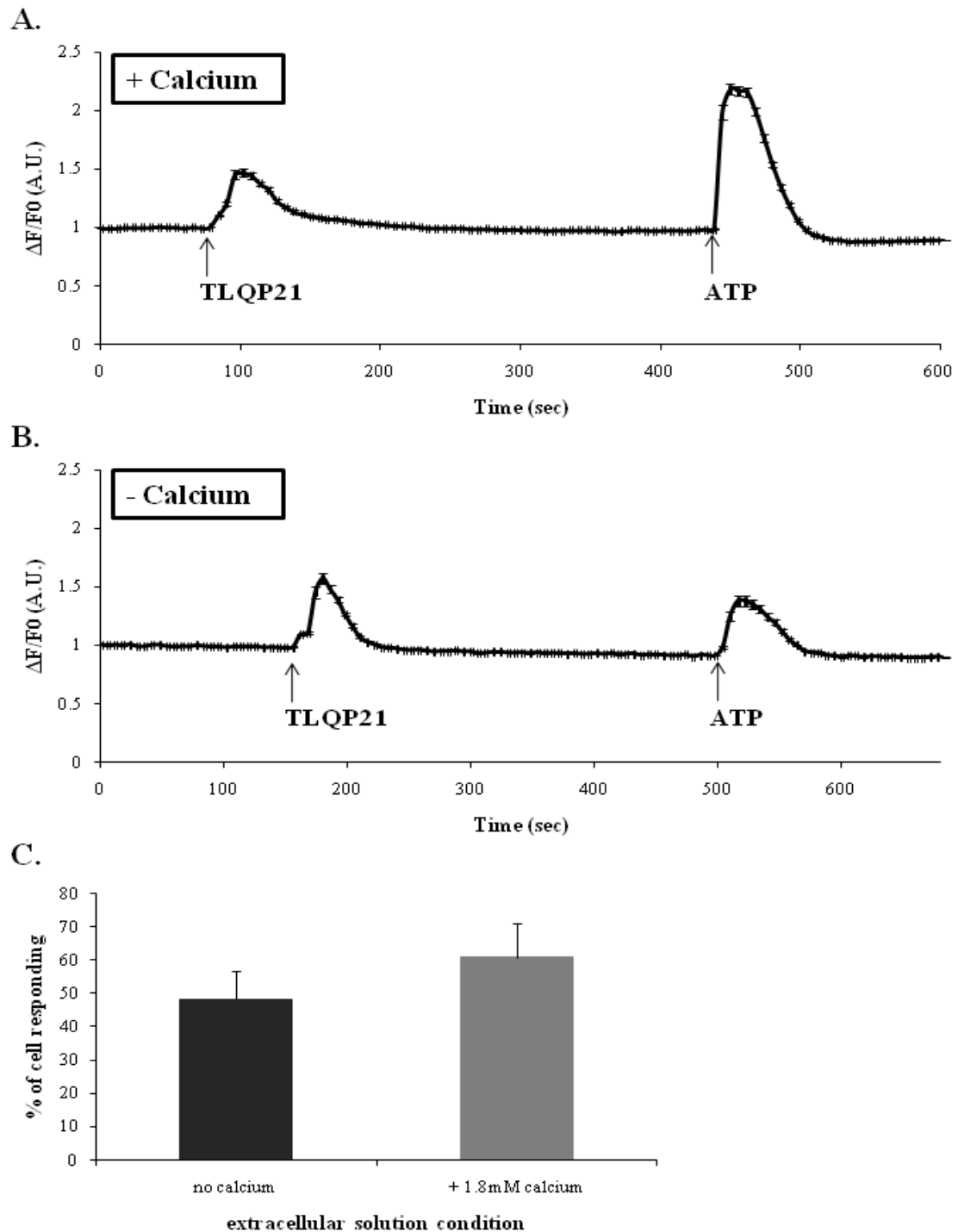


Figure 2.8 Effect of extracellular calcium on TLQP-21 induced calcium response in primary macrophages.
A. 100 nM TLQP-21 induces a calcium response in primary macrophage when calcium is present in extracellular solution. **B.** 100 nM TLQP-21 also elicits a calcium response in primary macrophages bath in extracellular solution devoid with calcium. **C.** Quantification of percentage of cells responding to 100 nM TLQP-21 in the extracellular solution devoid of calcium and with 1.8mM calcium.

2.4.7. The TLQP-21 induced intracellular calcium increase is observed in both M1 and M2 macrophages.

In vitro, different distinctive stimuli endow macrophages with characteristic molecular phenotypes and effector functions (Mantovani, Sica et al. 2004, Mosser and Edwards 2008). Activating the cultured macrophages with lipopolysaccharide (LPS) promotes the “classical activated” M1 state. In these cells, oxidative metabolites (such as nitric oxide and superoxide) and pro-inflammatory cytokines are characteristically produced at very high levels. Therefore, M1 activated macrophage population typically displays high inducible nitric oxide synthase/ Arginase (iNOS/Arg1) ratio in the gene expression (Mills, Kincaid et al. 2000). Conversely, stimulations of the cultured macrophages with cytokines Interleukin-4 (IL-4) and Interleukin-10 (IL-10) encourage “alternatively activated” M2a and “anti-inflammatory” M2c phenotypes, respectively. Unlike M1 macrophage, M2 macrophages (both M2a and M2c subtypes) downregulate oxidative metabolites and facilitate expression of Arginase, and hence a low iNOS/Arg1 ratio (lower than 1) is phenotypically observed. Furthermore, M2c macrophage is known to be anti-inflammatory facilitating high expression of anti-inflammatory cytokine IL-10.

It is evident that polarized macrophage populations are associated with systemic diseases (Gordon 2007) and polarisation of macrophages can contribute to the disease progression (Sica, Larghi et al. 2008, Villalta, Nguyen et al. 2009, Suganami and Ogawa 2010). It is appreciated that distinct macrophage subsets exhibit divergent effects and can react towards the same stimulus differently. It is currently unknown if distinctive macrophage subsets respond to TLQP-21 differently. Therefore, I sought to investigate here if certain macrophage subset responds predominately to the TLQP-21 peptide.

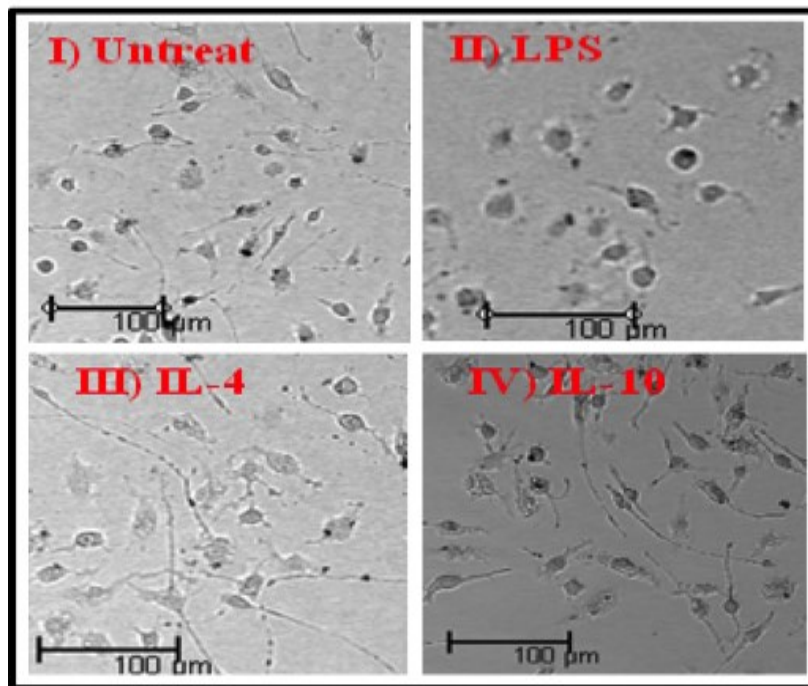
The experiment was set up by first differentiating the cultured macrophage *in vitro* into M1, M2a or M2c subsets by 24 hour stimulation with 1 µg/ml LPS, 20 ng/ml IL-4 and 20 ng/ml IL-10, respectively. Figure 2.9A shows bright field images of untreated, LPS, IL-4 and IL-10 treated macrophage, respectively, after 24 hours of treatment. Remarkably, after 24 hours of treatments, the macrophages displayed

altered morphologies. The LPS-treated M1 macrophages were more flattened and rounded, and appeared as "fried egg" morphology with an enhanced number of lamellipodia compared to the untreated control. On the other hand, IL-4 and IL-10 treated M2 macrophages were more elongated in shape when compared to the control cells. Interestingly, untreated control macrophages appear to possess morphologies of both extremes, with some elongated cells and some rounded cells.

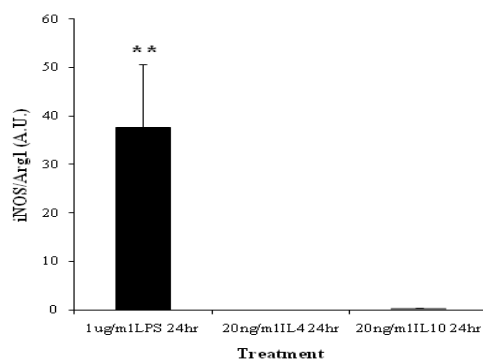
To further confirm that the cells were differentiated into distinctive subsets, qRT-PCR was exploited to check the expression of M1 and M2 phenotypic markers. First, the iNOS and Arg1 expression of each subset was checked, and the gene expression was normalised against the untreated control. The iNOS/Arg1 ratio was then compared between the different subsets. Figure 2.9 B shows iNOS/Arg1 ratio in arbitrary units. As expected, the M1 macrophage subset expressed a high level of iNOS, and hence the iNOS/Arg1 ratio is much bigger than 1 arbitrary unit (37.7 ± 13.0). In contrast, both of the M2 macrophage subsets possess iNOS/Arg1 ratio lower than 1 (0.001 ± 0.0002 , 0.38 ± 0.1 for M2a and M2c, respectively) indicating a much higher expression of Arg1. To further dissect between M2a and M2c subset, the IL-10 expression levels between these two groups were compared. Figure 2.9 C shows a significant IL-10 up-regulation by 43.7 folds in the M2c subset of macrophages when compared to that of M2a ($p < 0.05$, student t test), indicating significantly higher level of anti-inflammatory cytokine production.

Having confirmed that the macrophages had been differentiated into the distinctive subsets, I subsequently tested the different states of macrophages in the calcium imaging for their responsiveness to the TLQP-21 peptide. Figure 2.9 D shows the percentages of cells responding to 100 nM TLQP-21 after each treatment. The average percentages of cells responding to TLQP-21 are $67.6 \pm 7.5\%$, $69.6 \pm 7.28\%$, $64.7 \pm 7.3\%$ and $65.1 \pm 10.6\%$ for control, LPS-treated, IL-4 treated and IL-10 treated macrophages, respectively. The result here demonstrated that all subsets of macrophages responded to the TLQP-21 peptide, and the percentages of cells responding to the peptide were not significantly different from each other.

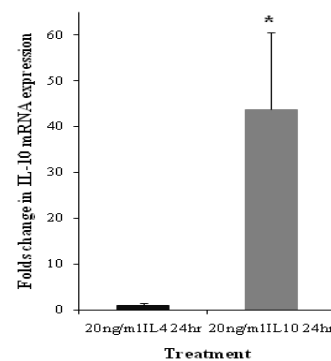
A.



B.



C.



D.

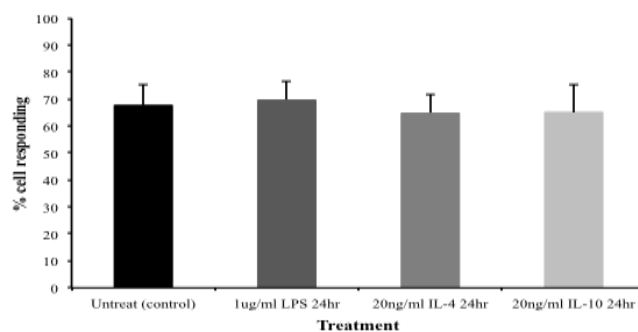


Figure 2.9 Effects of macrophage polarisations on TLQP-21 induced calcium response.

A. Bright field images of macrophages undergo I) no treatment II) 1 µg/ml LPS 24 hours treatment III) 20 ng/ml IL-4 24 hours treatment and IV) 20 ng/ml IL-10 24 hours treatment. **B.** qRT-PCR data of iNOS/Arg1 ratio expression in macrophages treated with LPS, IL-4 and IL10. (** $p < 0.01$ One way ANOVA, Tucky post hoc test) **C.** Fold changes in IL-10 expression of macrophages treated with IL-4 and IL10 (* $p < 0.05$, student t test). **D.** Calcium imaging result of the percentages of cells responding to 100 nM TLQP-21 in control, LPS-primed, IL-4-primed and IL-10-primed macrophages.

2.4.8. Investigating the effects of the conditioned media of the TLQP-21-stimulated macrophages on the primary DRG neurons.

From the above data, it is clear that VGF-derived peptide TLQP-21 specifically targets primary macrophages and elicits the release of intracellular calcium. I hypothesised that some molecular effectors are likely to be released from the macrophage to the extracellular space after TLQP-21 stimulation. The released effectors, in turn, can act on the DRG neurons. If such hypothesis is true in the neuropathic pain condition, the released effectors from the macrophage could exacerbate the secondary damage to the neurons after injury and may contribute to the initiation and/or maintenance of neuropathic pain.

To test whether the TLQP-21-stimulated macrophages confer effects on activating the sensory neurons, the primary DRG neurons were cultured for 2 days (DIV2), loaded with Fluo-4 and the macrophage conditioned medium (MCM) or control was applied to the neurons during imaging recording. The MCM was prepared from the supernatant of TLQP-21-stimulated macrophages, so if any effector was released that could activate the primary DRG neurons, fluorescence increases would be detected in the neurons. The primary macrophages were conditioned with 100 nM TLQP-21 for 1 hour, 2 hours or 4 hours. The MCM was collected and spun to remove any cell debris. Two controls were used in this experiment; 100 nM ScrTLQP-21 and the same volume of vehicle conditioning for 4 hours.

Results in figure 2.10 shows that the vehicle control has little effect on the primary DRG neurons, 17.9 % of the total analysed neurons responded to the vehicle control. Four hours ScrTLQP-21 MCM induced an increase in the intracellular calcium level in 15.0 % of the total analysed neurons. Conversely, the condition media of four hours TLQP-21 treatment elicits a calcium response in 29.8 % of the total neurons. The four-hour time point appears to stimulate the highest number of cells to respond, as one-hour and two-hour TLQP-21 MCM treatments only induce calcium response in 8.33% and 18.75%, respectively, of the total analysed cells. The number of neurons responding to four-hour TLQP-21 MCM is approximately 2 folds of that treated with ScrTLQP-21 MCM. This indicates that the effectors in the conditioned media of TLQP-21-stimulated macrophages can potentially excite the primary DRG neurons, at

least at the four-hour time point tested. Nevertheless, the experiment still awaits to be repeated to draw a more reliable conclusion.

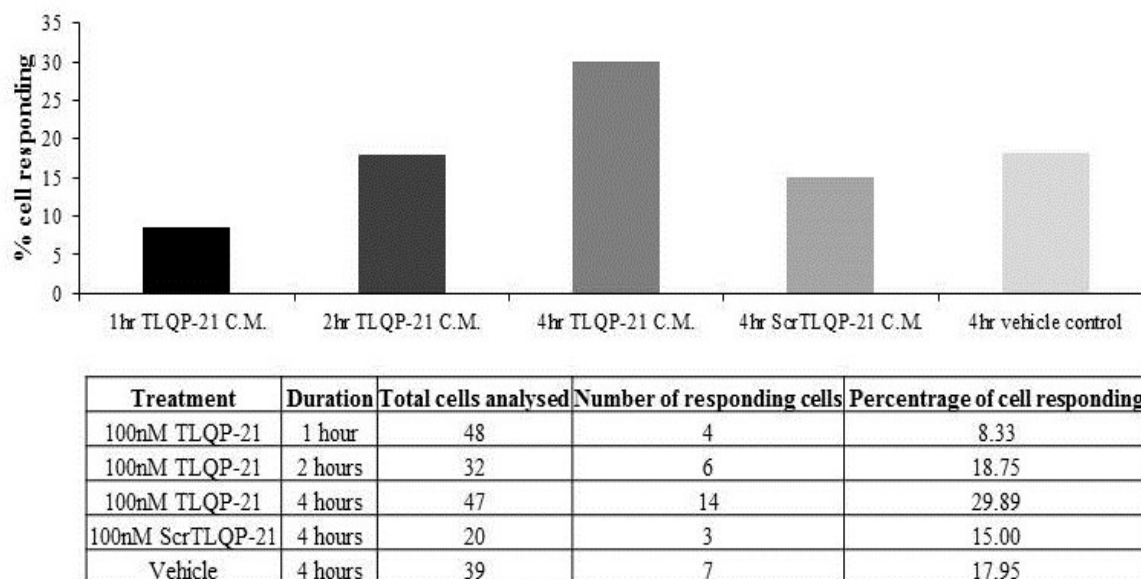


Figure 2.10 Effect of TLQP-21 conditioned media from macrophage on DRG neurons. The primary macrophages were stimulated with vehicle control, 100 nM ScrTLQP-21 or 100 nM TLQP-21, and the conditioned media were collected and applied to the DRG neurons during calcium imaging. The percentages of cell responding to different conditioned media (1 hour, 2 hours, 4 hours 100 nM TLQP-21 treated MCMs, 4 hours 100 nM ScrTLQP-21 treated MCM and 4 hours vehicle treated MCM) were compared.

2.4.9. Intraplantar injection of TLQP-21-stimulated macrophages induced the pain-like behaviour *in vivo*.

To further investigate the hypothesis that TLQP-21 acts on the macrophages and stimulates the release of the macrophage effectors, which subsequently convey the signals in the pain pathway, I next tested this hypothesis in an *in vivo* study.

The overview of the experimental set up is shown in figure 2.11. Briefly, 200 g male Wister rats were habituated in the individual Plexiglas testing chambers for 1 hour for two consecutive days prior to the behavioural testing. This was necessary for the animals to get used to handling. The baseline paw withdrawal threshold (PWT) to the mechanical stimulation of each individual animal was measured by automate von Frey filament for the next two days before any treatment was given. The two baseline PWT measurements were taken at least 24 hours apart, in order to prevent viability caused by too frequent exposures to the testing technique. Primary macrophage was

cultured a week before the experiment and treated with either 500 nM TLQP-21 or ScrTLQP-21 for 24 hours before being used for the intraplantar injection. The conditioned macrophages were washed and harvested after treatment, and 35,000 harvested cells in 10 μ l of PBS were injected to the rats' hind paw using a 30G needle attached to a Hamilton syringe. The rats' PWTs to the mechanical stimulation, of both treated paw (ipsilateral) and untreated paw (contralateral), were measured on the post operative day 1 (POD1), day 2 (POD2) and day 5 (POD5). The ipsilateral PWTs were then compared between the two groups injected with either TLQP-21-treated macrophages or ScrTLQP-21-treated macrophages.

Figure 2.12 shows the animals tested in this study displayed consistent baseline to the mechanical stimulation. The raw data of the baseline measurements of each individual animal used were summarised in the tables of figure 2.12 A and 2.12 B (prior to injections with TLQP-21-treated macrophages and ScrTLQP-21-treated macrophages, respectively, $n = 9$ for both groups). Figure 2.12 C shows the averaged baseline measurements of the nine animals that were used the next day for injection with TLQP-21-treated macrophage. The averaged baseline measurements of the nine animals on day 1 and day 2 were not significantly different from each other, indicating a consistent response to the mechanical stimulation given. The baselines were also steady for the group of animals that were used the next day for injection with ScrTLQP-21-treated macrophages (figure 2.12 D).

If TLQP-21, as hypothesised, specifically targets the primary macrophages and activates them for the subsequent cellular events in the pain signalling, it would be expected to observe a reduction in the PWTs (Hypersensitive to the mechanical stimulation) in the animals injected with TLQP-21-treated macrophages. Indeed, when compared to the control group injected with ScrTLQP-21-treated macrophages, the PWTs after TLQP-21-treated macrophages injection were significantly reduced on POD 1, POD 2 and POD5 (Figure 2.13 A, $p < 0.01$, two way ANOVA, Tukey post hoc test). This implies that the TLQP-21-treated macrophages elicit some downstream events that subsequently produce a pain-like behaviour in the animals. Moreover, when compared to its own baseline, the PWTs of the animals injected with TLQP-21-treated macrophages were significantly reduced on the POD1 and POD2 ($P < 0.05$,

two way ANOVA, Tukey post hoc test), but not on the POD5 (Data of each individual animal is tabulated in figure 2.13B). This shows the effect induced by the TLQP-21-treated macrophage injection is likely to be temporary. Nevertheless, the PWTs of the animals injected with ScrTLQP-21 treated macrophage, on any given testing day, were not significantly differed from its own baseline (Tabulated in figure 2.13 C). This indicates that the effect induced by the injected macrophages is TLQP-21 treatment-specific, as the reduced in PWTs were not observed in the control group.

Injection of TLQP-21-treated macrophages to the hind paw resulted in a marked reduction in the ipsilateral PWT to punctuate mechanical stimulus up to 5 days post-surgery when compared to the ScrTLQP-21-treated macrophages injection group. To test if the injected macrophages exert an effect on the contralateral paw of each group, the contralateral paw withdrawal threshold to the von Frey filament was also measured. Figure 2.14 A shows there was no difference between baseline and post-injection withdrawal threshold in the contralateral limb of the animals injected with TLQP-21-treated macrophages. No alternations between baseline and post-injection contralateral PWTs were observed in the animals injected with ScrTLQP-21-treated macrophages. The tabulated data of all animals tested were shown in figure 2.14 B and figure 2.14 C for the groups injected with TLQP-21-treated macrophages and ScrTLQP-21-treated macrophages, respectively.

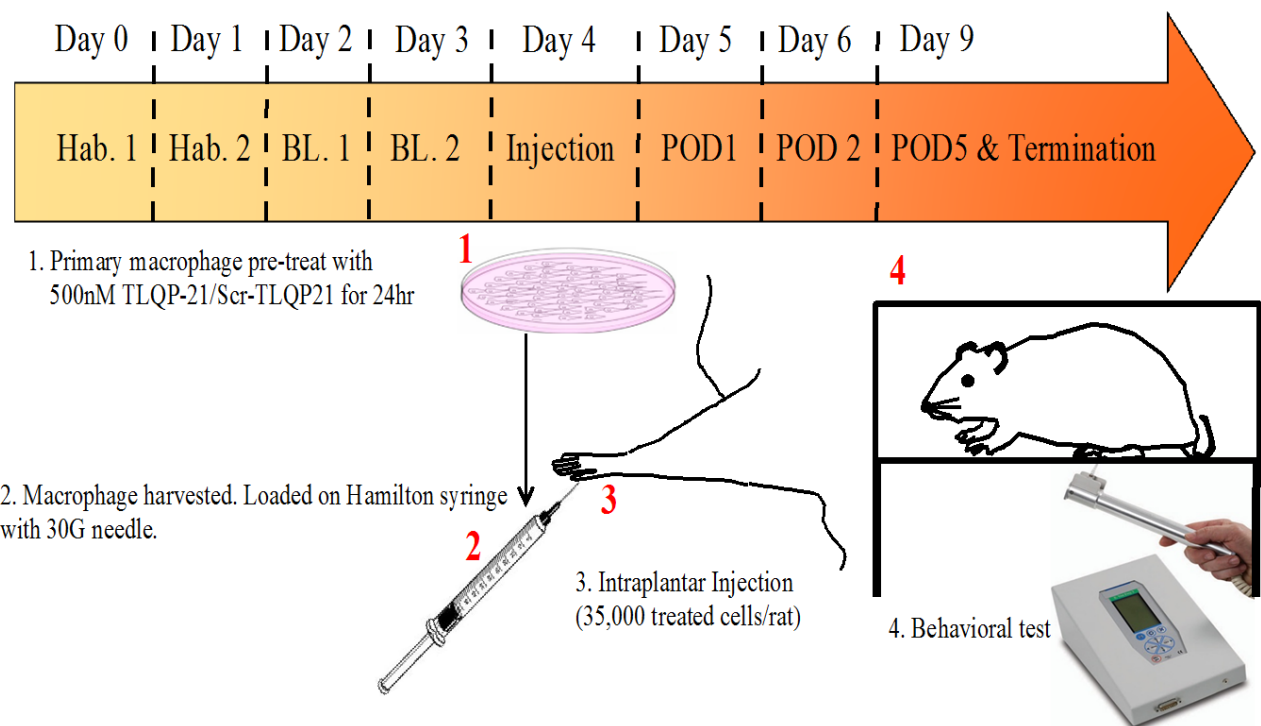


Figure 2.11 Overview of experimental set up for *in vivo* TLQP-21 treated macrophage intraplantar injection behavioural study.

The primary macrophage was treated with 500 nM TLQP-21/ScrTLQP-21 for 24 hours prior to be used for *in vivo* experiment. On the day of injection, the dishes were washed three times to remove residual peptides and the cells were collected in PBS. 35,000 TLQP-21/Scr-TLQP-21 stimulated cells were injected to the plantar skin of the rat. The behaviours to mechanical stimulation were assessed by electronic von Frey on post operative day 1, day 2 and day 5.

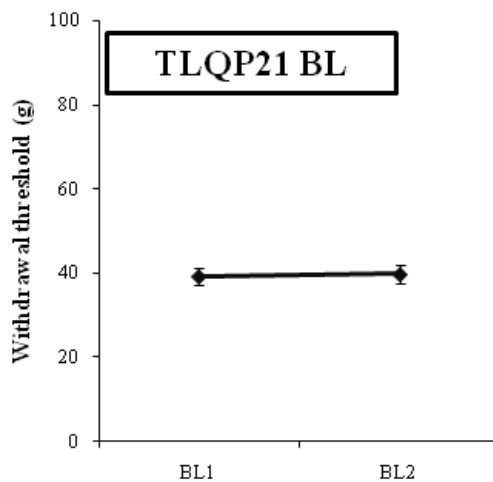
A.

Intraplantar injection with TLQP-21 treated macrophage		
	Baseline Day 1	Baseline Day 2
	Threshold (grams)	
Animal 1	51.03	45.40
Animal 2	34.84	30.79
Animal 3	42.39	36.15
Animal 4	40.09	48.95
Animal 5	38.99	47.33
Animal 6	27.38	33.35
Animal 7	37.50	38.16
Animal 8	42.93	39.60
Animal 9	38.49	38.46

B.

Intraplantar injection with ScrTLQP-21 treated macrophage		
	Baseline Day 1	Baseline Day 2
	Threshold (grams)	
Animal 1	51.71	44.56
Animal 2	37.27	44.53
Animal 3	55.40	43.38
Animal 4	49.31	44.06
Animal 5	51.82	50.72
Animal 6	41.99	39.01
Animal 7	33.34	37.70
Animal 8	36.37	43.76
Animal 9	33.46	38.45

C.



D.

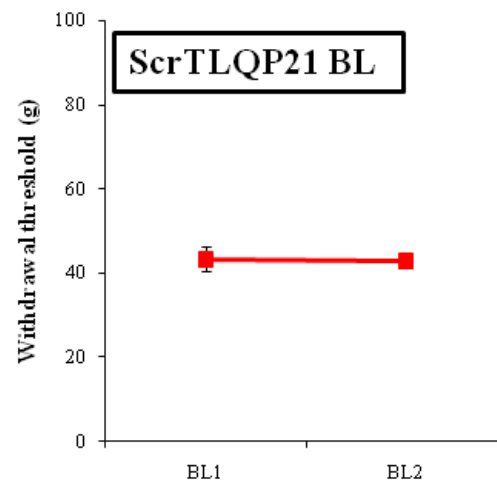
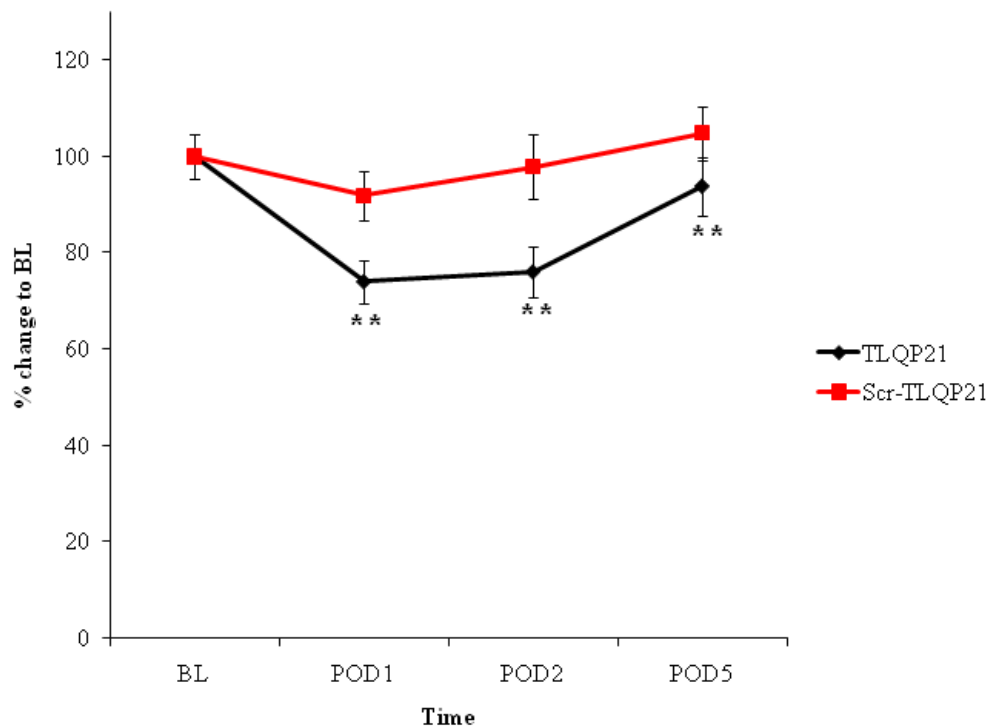


Figure 2.12 Baseline hind paw withdrawal thresholds to punctuate mechanical stimuli in the naïve rats before injections.

A. Tabulated data of two consecutive days baseline PWTs in all the animals that were later assigned to inject with TLQP-21-treated macrophages. **B.** Tabulated data of two consecutive days baseline PWTs in all the animals that were later assigned to inject with ScrTLQP-21-treated macrophages **C.** Averaged baseline 1 and baseline 2 of all nine animals assigned to be injected with TLQP-21-treated macrophages. **D.** Averaged baseline 1 and baseline 2 of all nine animals assigned to be injected with ScrTLQP-21-treated macrophages.

A.



B.

Ipsilateral paw: TLQP-21 treated macrophage				
	Mean Baseline	POD1	POD2	POD5
	Threshold (grams)			
Animal 1	121.9	84.9	75.3	80.3
Animal 2	83.0	86.3	87.1	121.4
Animal 3	99.3	76.6	49.1	97.0
Animal 4	112.6	87.3	76.8	102.1
Animal 5	109.1	50.2	74.0	100.3
Animal 6	76.8	80.9	99.0	92.6
Animal 7	95.7	66.0	64.9	54.0
Animal 8	104.3	77.1	64.8	99.3
Animal 9	97.3	56.8	93.7	97.2

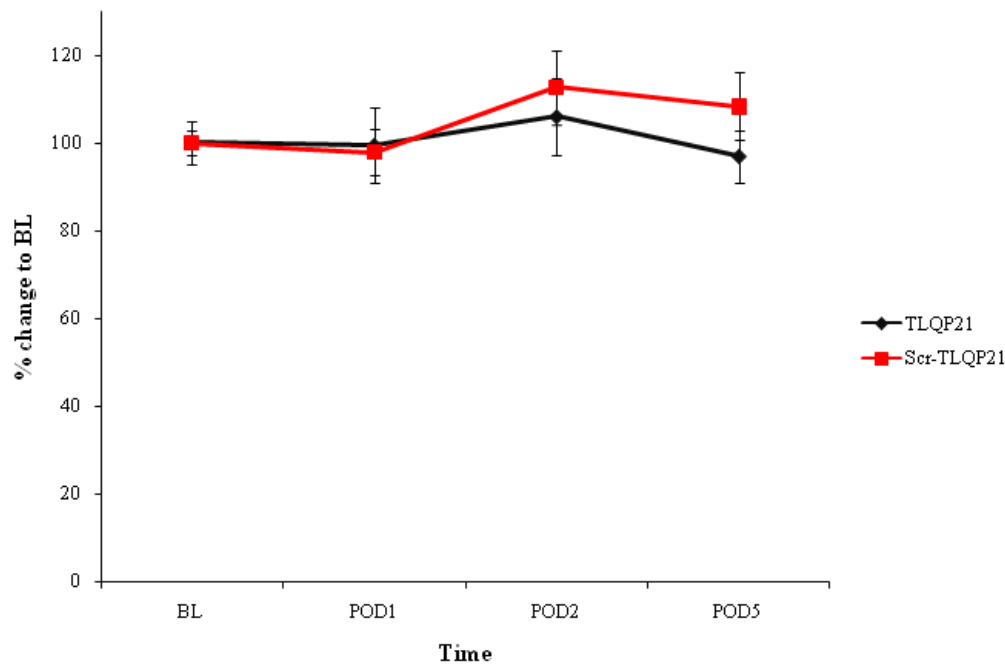
C.

Ipsilateral paw: ScrTLQP-21 treated macrophage				
	Mean Baseline	POD1	POD2	POD5
	Threshold (grams)			
Animal 1	111.5	75.8	76.2	107.0
Animal 2	94.8	108.3	120.9	138.0
Animal 3	114.4	70.1	72.8	80.2
Animal 4	108.2	107.6	112.5	115.1
Animal 5	118.8	103.8	88.0	115.0
Animal 6	93.8	91.7	90.7	95.1
Animal 7	82.3	98.6	121.6	90.7
Animal 8	92.8	71.9	115.9	103.3
Animal 9	83.3	99.7	82.1	98.3

Figure 2.13 Effects of TLQP-21/ScrTLQP-21-treated macrophages intraplantar injections on the ipsilateral paw withdrawal thresholds to punctate mechanical stimulation.

A. Intraplantar injection of TLQP-21 treated-macrophage significantly reduced the ipsilateral hind paw withdrawal threshold to the punctate mechanical stimulation when compared to the group injected with ScrTLQP-21-treated macrophages. (**P<0.01, Two way ANOVA vs scramble, Tuckey post hoc test). **B.** Tabulated data of ipsilateral PWTs on averaged baseline, POD1, POD2 and POD3 of all the animals injected with TLQP-21-treated macrophages. **C.** Tabulated data of ipsilateral PWTs on averaged baseline, POD1, POD2 and POD3 of all the animals injected with ScrTLQP-21-treated macrophages.

A.



B.

Contralateral paw: TLQP-21 treated macrophage				
	Mean Baseline	POD1	POD2	POD5
	Threshold (grams)			
Animal 1	112.4	88.9	61.5	77.5
Animal 2	130.7	76.4	75.1	79.1
Animal 3	101.3	63.6	110.3	84.9
Animal 4	96.6	102.8	114.8	120.9
Animal 5	76.7	148.5	131.4	113.8
Animal 6	101.6	124.3	91.2	81.2
Animal 7	96.9	115.3	126.2	91.4
Animal 8	94.2	86.5	103.0	106.6
Animal 9	89.5	89.0	141.0	117.1

C.

Contralateral paw: ScrTLQP-21 treated macrophage				
	Mean Baseline	POD1	POD2	POD5
	Threshold (grams)			
Animal 1	99.1	86.8	156.3	115.5
Animal 2	99.7	124.0	120.2	145.1
Animal 3	109.1	103.1	95.5	90.7
Animal 4	104.8	99.1	76.3	107.2
Animal 5	97.2	83.6	134.2	139.3
Animal 6	91.6	72.2	108.9	96.8
Animal 7	90.2	99.5	127.9	95.8
Animal 8	92.3	117.2	111.7	112.7
Animal 9	116.0	95.8	83.9	72.3

Figure 2.14 Effects of TLQP-21/ScrTLQP-21 -treated macrophages intraplantar injection on the contralateral paw withdrawal thresholds to mechanical stimulation.

A. Intraplantar injection of TLQP-21-treated macrophages/ScrTLQP-21-treated macrophages does not affect contralateral hind paw withdrawal threshold to the punctate mechanical stimulation when compared to its own baseline. No difference in the contralateral PWT is observed when compared between the two groups. **B.** Tabulated data of contralateral PWTs on averaged baseline, POD1, POD2 and POD3 of all the animals injected with TLQP-21-treated macrophages. **C.** Tabulated data of contralateral PWTs on averaged baseline, POD1, POD2 and POD3 of all the animals injected with ScrTLQP-21-treated macrophages.

2.5. Discussion

2.5.1. Effects of VGF-derived peptides on the intracellular calcium level in the primary DRG neurons.

VGF has been demonstrated, both *in vitro* and *in vivo*, to be upregulated in the DRG sensory neurons after peripheral neuronal injuries (Moss, Ingram et al. 2008, Rizzi, Bartolomucci et al. 2008, Riedl, Braun et al. 2009). However, the effects of VGF peptides on the DRG neurons have not been investigated in the literature. The present study looked at the effects of different peptides on the cultured primary DRG neurons by calcium imaging. I demonstrated that both LQEQ-19 and TLQP-21 (and its control peptide ScrTLQP-21) did not induce a calcium response in the DRG neurons in the setting used in this study. It is worth noting that the concentration tested, 1 μ M, might not be the “physiological functioning concentration” on the DRG neurons, even though evidence suggested 1 μ M of LQEQ-19 can activate BV-2 microglial cells and increase p38 phosphorylation in these cells (Riedl, Braun et al. 2009). Moreover, 1 μ M of TLQP-21 has been shown to prevent cerebellar granule cells (CGCs) apoptosis induced by serum and potassium deprivation and is able to induce a significant increase in the intracellular calcium level in a subpopulation of CGCs (Severini, Ciotti et al. 2008). Although these evidences suggested 1 μ M of VGF-derived peptides could activate other cell types including neurons in the central nervous system, it is still possible that DRG neurons are activated by lower/higher concentration at the periphery. Further work using different concentrations of VGF-derived peptides could be carried out to provide more information to this question. Moreover, there is a possibility that VGF-derived peptides only act on a subset of DRG neurons. It is possible that the effects of the peptides on the small population of the neurons are masked by the background noise, as cultured DRG neurons are highly heterogeneous. Future work to investigate the effect of VGF-derived peptides on longer DRG neuronal culture supplemented with different neurotrophins can be done. This isolates different populations of neurons (such as supplementing NGF to isolate small diameter sensory neurons) and hence allows a more reliable conclusion of the effects of the VGF-derived peptides on the primary DRG neurons to be drawn. Furthermore, as reported by Riedl *et al*, VGF is upregulated in the cultured DRG neurons within 24

hours, it is possible that the cultured DRG neurons were already desensitised by the VGF-derived peptides released in the culture prior to the experiment, and hence a further response could not be elicited during the calcium imaging. As demonstrated in our study, repetitive applications of VGF-derived peptide on the macrophages cause cell desensitisation to further stimulation. Unlike macrophage that does not synthesise VGF and hence is able to respond to the peptide applied during experiment, DRG neurons in the culture could potentially be desensitised by the endogenous VGF-derived peptides before being used for the experiment.

The functional assay used in this study, calcium imaging, has been widely employed as the assay to investigate the neuronal excitability *in vitro* and *in vivo* (MacLean and Yuste 2009, Grienberger and Konnerth 2012). However, the acquisition time *in vitro* is often at millisecond scale, rather than seconds. Due to technical limitations, the measurements of the assay were made every 6 seconds in our investigation. It is possible that the VGF-derived peptides-induced calcium responses are rapid and transient within seconds, and hence acquiring measurements every 6 seconds may miss the detail of such subtle changes. Although, Severini's observation of TLQP-21-induced increase in the CGCs intracellular calcium was performed in 6 seconds interval, it is still possible that the mechanism involved in the DRG neurons are more rapid and different from that of CGCs. A more thorough study using higher resolution cameras to increase temporal resolution may further increase the sensitivity of the assay and hence further dissect the role of the peptides on the primary DRG neurons.

In this study, calcium imaging was used as a functional read out to investigate the role of VGF-derived peptides on the DRG neurons. This assumed that VGF-derived peptides-induced neuronal activation is a calcium-dependent process, and the calcium response is an indicative of the neuronal action potential activity. Although depolarization of neurons are often accompanied by a change in the intracellular calcium, it is possible that VGF-derived peptides can induce action potential via calcium-independent pathway as it has been shown action potential can be modulated by calcium-independent process in the sensory neurons (Zhang and Zhou 2002, Zheng, Fan et al. 2009). This question can be answered by utilizing the voltage sensitive dye, such as DiBAC, in combination with the high temporal resolution

imaging or employing the high resolution simultaneous voltage and calcium imaging to correlate electrical and chemical activities in the neurons (Vogt, Gerharz et al. 2011) upon VGF-derived peptides stimulations.

It is also possible that the VGF-derived peptides tested in this study (i.e. LQEQ-19 and TLQP-21) are not the biologically active peptides on the sensory neurons. To date, there are at least 13 VGF-derived peptides been reported, other peptides could still potentially act on the sensory neurons. There is also a possibility that VGF-derived peptides do not, at all, signal through DRG neuron itself in an autocrine manner. It is likely that VGF derived-peptides are synthesised and released by DRG neurons upon depolarisation, and subsequently exert its function on the adjacent cell types such as dorsal horn neurons and microglia as suggested by Moss *et al* and Riedl *et al*, respectively. Nevertheless, the conclusion could not been drawn until the questions mentioned are answered.

2.5.2. Effects of VGF-derived peptides on the intracellular calcium level in the bone-marrow derived macrophages.

Macrophages play a pivotal role in the immune response and have been implicated in the development and/or maintenance of neuropathic pain including injury-induced neuropathic pain (Shi, Zekki et al. 2011). It is well established that a change in the intracellular calcium concentration is the initiation step of many ordinary macrophage cellular processes, including pro-inflammatory mediators productions (Park, Jun et al. 1996) (Park, Jun et al. 1996, Watanabe, Suzuki et al. 1996), phagocytosis (Gronski, Kinchen et al. 2009) (Kruskal and Maxfield 1987) and chemotaxis (Smith, Kessler et al. 1982). In the cultured macrophage, the cytosolic calcium concentration of the resting cells is kept at low level, ranged from ~50 to 150 nM (Malik, Denning et al. 2000), which can be evoked to a dramatically high level in response to various stimuli and subsequently carries out the ultimate cellular functions. As mentioned, several evidences in the literature suggested that VGF-derived peptides act on cells adjacent to the sensory neuron terminals in the central nervous system, including dorsal horn neurons and microglia. I was interested to elucidate if VGF-derived peptides also signal in such paracrine manner at the periphery. Having demonstrated that VGF-derived peptides at 1 μ M did not elicit a calcium response in the DRG neuron, I

further examined if the VGF-derived peptides, which are increased in the gene expression and released from the DRG neurons after nerve injuries, signal through the primary macrophage in a calcium-dependent manner.

Here I demonstrated, for the first time, a VGF-derived peptide (TLQP-21) acts on the bone-marrow derived primary macrophage. Other VGF-derived peptides (LQEQ-19 and TLQP-62), at the concentration tested, did not induce a change in the intracellular calcium, indicating unique calcium-dependent function of TLQP-21 on the primary macrophage. Even though LQEQ-19 and TLQP-62 did not elicit a calcium response in the macrophages, it cannot be ruled out that these peptides could still act on the macrophages by other means such as inducing epigenetic changes. The TLQP-21 is functional at concentration as low as 10 nM in the primary macrophage, and the percentage of cell responding to this peptide plateaued at 100 nM. Although the percentage of cell responding to the peptide did not further increase at concentration higher than 100 nM, it does not mean that 100 nM is the saturating concentration as it is possible that the amount of calcium flux was different at the higher peptide concentrations while the percentages of cell responding remained similar. Future experiment can be carried out to quantify the amount of calcium flux in the macrophages after high concentrations of TLQP-21 applications using ratiometric calcium dye such as Fura-2. This will provide more detailed information on how TLQP-21 induces changes in the intracellular calcium level in the macrophages at higher peptide concentrations.

As demonstrated in this chapter that TLQP-21 induces a dose-dependent, rapid but transient, calcium response in the primary macrophages, this could possibly indicate that TLQP-21 acts on the primary macrophage by binding to its target receptor on the membrane. Another line of evidence suggesting a receptor is involved is that not all cultured macrophage responded to the TLQP-21 peptide. Cultured bone-marrow derived primary macrophage is shown to be a mixture of different subtypes of macrophages. It is possible that TLQP-21 only acts on the receptor-expressing subtypes of macrophages, and hence a small number of cells per experiment did not respond to the peptide. It is also possible that the receptor is only expressed on cell surface at specific cellular phase, for example the receptor can be internalized during cell division and hence no response can be observed. Low concentration of TLQP-21

(1 nM) is unable to elicit a calcium-dependent response in the primary macrophage, indicating dose-dependent specificity of this peptide on the macrophages and possible ligand kinetic on the target receptor.

One issue must be raised here is that the primary macrophages used in this study were derived from the bone-marrow pluripotent stem cells, which yield a relatively more homogenous cells. Although this is the widely accepted protocol in studying macrophage function, it is important to note that they are not like resident macrophages *in vivo* as the resident macrophages themselves are not homogeneous where they differentiate according to the environmental cues to suit the microenvironment of the tissue and possible pathogens they encountered. The major reason bone-marrow derived macrophage was used in this study was that isolation of resident macrophages from the animal tissues results significant lower number of cells available for the study and the procedure is relatively more complex. Future work could be carried out *in vitro* to further confirm TLQP-21 activity using the resident macrophages isolated from animal tissues. Moreover, *in vivo* study using transgenic mice systematically expressing fluorescent calcium sensor can be carried out to investigate the TLQP-21 mechanism *ex vivo* as demonstrated recently (Sasaki, Takahashi et al. 2010).

The scrambled control peptide ScrTLQP-21 possesses the same charge, same amino acid components (but scrambled in sequence) as TLQP-21 peptide. Here, I demonstrated in the study that vehicle control and scrambled peptide control (ScrTLQP-21) at physiological concentration did not elicit a calcium response in the primary macrophage, this evidence further strengthens the postulation of TLQP-21 specific action on the primary macrophage. Nevertheless, the scrambled peptide was shown to be functional to the primary macrophage at extreme high concentration (50 μ M). The possible explanation for the functionality of ScrTLQP-21 at high concentrations could be that the sequence of the scrambled peptide was not randomised enough. The TLQP-21 harbours four arginine residues possessing positive charges, showing in bold in the sequences below. The four arginine residues in the scrambled peptide after randomisation were still in close proximity at the C-terminus of the peptide, this could possibly account for the non-specific effect observed at extreme high concentration.

TLQP-21 sequence TLQPPASSRRRHFFHHALPPAR

ScrTLQP-21 sequence PSFLLPPHHSRAQHRTPEAAR

It is also possible that the secondary structure of the scrambled peptide remains similar though the sequence is not identical from the wild type. Therefore, the 'active domain' of the peptide was not completely disrupted, which resulted in partial functionality of the peptide at extreme high concentration. Furthermore, it is also possible that TLQP-21-induced response in macrophage is partially charge-dependent, and hence the scrambled peptide possessing the same charge and same amino acid component can partially elicit a non-specific effect at extreme high concentration.

2.5.3. The molecular mechanism of actions of the TLQP-21 peptide on the bone-marrow derived primary macrophages.

Change in the intracellular calcium level is important in initiating various macrophage functions such as recognition of foreign antigens challenges including bacterial substances (Hotchkiss, Bowling et al. 1997) (Dewamitta, Nomura et al. 2010) yeast component (Kelly, Wang et al. 2010) and viral protein (Hegg, Hu et al. 2000, Lee, Liu et al. 2003). Not surprisingly, numerous disease pathogeneses are associated with dysregulation in macrophage calcium signalling. For example, *mycobacterium tuberculosis* interferes with macrophage antimicrobial response to achieve immune evasion by inhibiting the increase in the intracellular calcium that is required for the macrophage phagocytosis (Malik, Denning et al. 2000). Macrophage intracellular calcium dynamic is also altered in the pathological conditions such as Sepsis. Deviant of calcium signals in the macrophage induced by bacterial lipopolysaccharide contributes to the aberrant cytokines productions that underlies the consequences of organ failure during sepsis (West, Clair et al. 1996, Zhang, Guo et al. 2011). Regulated calcium signalling is pivotal for macrophage to ultimately function correctly. Thus, understanding the molecular mechanism by which calcium signals after TLQP-21 stimulation is important for understating how the cell functions upon VGF-derived peptide challenge.

I have demonstrated that TLQP-21 application to the primary macrophage induced an

immediate but transient increase in the intracellular calcium. I was interested to investigate further the effect of multiple applications of TLQP-21 on the primary macrophages to further dissect the mechanism involved in TLQP-21 signalling. It was observed that TLQP-21 desensitised the primary macrophage to further TLQP-21 stimulation without affecting ATP-induced calcium response. In order to eliminate the possibility that the lack of response was not due to insufficient time for replenishing the intracellular calcium, the cytosolic calcium was allowed to return to the basal level before subjecting the cells to another stimulation after first application. In addition, I also tested longer time intervals between the two applications up to an hour, and the primary macrophages were still desensitised to the second dose of TLQP-21 treatment one hour after the first stimulation. Moreover, the ATP induced calcium response at the end of the experiment was not affected, implying the calcium depletion from intracellular store is invalid.

The rapid TLQP-21-induced desensitisation of calcium response to the repetitive peptide stimulation in the primary macrophage could be explained by several mechanisms. As seen in the current study, TLQP-21-induced calcium response in the macrophage is rapid but transient, like many responses (including chemokine- and complement-induced responses) observed mediating through G protein-coupled receptors (GPCRs) that quickly uncouples from G protein-mediated signalling by kinase phosphorylation of the receptors (Oppermann, Mack et al. 1999, Rebres, Moon et al. 2010). It is possible that TLQP-21 induces an increase in intracellular calcium via the GPCRs, and the termination of the signalling is due to the receptor internalization or desensitisation. There are two documented desensitisation forms of GPCRs, the homologous- and heterologous-desensitisation (Murakami, Imamichi et al. 1993, Capra, Accomazzo et al. 2010). The homologous desensitisation is the process of downregulating activated GPCR itself, whereas heterologous desensitisation is the phenomenon of activated receptor downregulating a different GPCR. In the current study, calcium response was observed after first TLQP-21 application, the same macrophages were stimulated again with the same amount of TLQP-21 peptide. No calcium response was observed to the second dose of TLQP-21, but the macrophages still showed the calcium response to ATP stimulation at the end of the experiments. This suggests that the desensitisation observed is likely a homologous one and that the signal transduction pathway for TLQP-21 is different

from that of ATP. The key reaction of GPCR desensitisation is initiated by phosphorylation of intracellular domain of the activated receptors by GPCR kinases (GRKs), which consequently results in binding of arrestin proteins to the phosphorylated receptor. Upon binding of the arrestin adaptor proteins, the phosphorylated receptor is prevented from binding to the G proteins, and hence disrupting its activation. This mechanism effectively turns off the receptor for a short period of time (Lattin, Zidar et al. 2007). The phosphorylation of the GPCRs could also results in internalisation of the receptor (Krueger, Daaka et al. 1997, Zimmermann, Conkright et al. 1999), although this process is less likely to account for the results observed here. As demonstrated by Zimmerman *et al.* the internalisation of GPCR CC chemokine receptor-3 (CCR-3) was merely 76 % and 24 % after 3 hours of RANTES (reduced on activation T cell expressed and secreted) and eotaxin stimulation, respectively. Moreover, the desensitisation described in the current study was observed in more than 90% of the cells within minutes of time scale. Therefore, the internalisation of receptor is probably not fast enough to account entirely for our results. Nevertheless, there is still a possibility that the receptor that TLQP-21 targets exhibits a fast kinetic in receptor internalisation and hence account for the desensitisation effect seen here.

Another possible mechanism that could account for the absence of the calcium response after successive applications of TLQP-21 could be a negative feedback mechanism of the macrophage. The first TLQP-21 stimulation on macrophage could results in the secretion of certain factor from the cells that, in response to further exposure of TLQP-21, acts in an autocrine manner and negatively regulates the cells from further activation. In fact, it has been demonstrated such negative feedback mechanism exists in primary macrophage. It has been shown that upon LPS challenge, activated macrophage secretes nitric oxide that exerts a negative feedback effect on production of the downstream inflammatory mediator TNF- α in response to further activation by LPS (Fahmi, Charon et al. 1995).

I have also showed in the study, pre-treatment of TLQP-62 that possesses TLQP-21 at its N-terminus, did not induce heterozygous desensitisation in the primary macrophages. This implies TLQP-62 signal transduction pathway is probably different from that of TLQP-21. It is also possible that TLQP-62 is not a functional

peptide on the primary macrophage at all. More intriguingly, when a high dose of ScrTLQP-21 was applied to elicit a non-specific response in the primary macrophage, the successive wild type TLQP-21-induced response was not observed. This suggests that the scrambled peptide may be acting non-specifically in a similar manner and sharing a common calcium flux pathway as wild type TLQP-21. To further elucidate the desensitisation mechanism involved in the TLQP-21 calcium flux pathway, the experiments could be carried out in the future to test the phosphorylation state of the membrane proteins, find the receptor and track the receptor for internalisation.

Having demonstrated that TLQP-21 elicits a calcium response in the primary macrophage, I was interested to further investigate what was the source of the calcium in the calcium response observed. Is it a result of influx of extracellular calcium or a release of calcium from internal store? This was investigated by using a calcium free extracellular solution, containing 2 mM calcium specific chelator EDTA, during calcium imaging. If the source of calcium is from the extracellular solution, the calcium response induced by TLQP-21 will be abolished when extracellular calcium is depleted. As demonstrated in the study, the cells still responded to the TLQP-21 when the extracellular calcium was excluded, implying the TLQP-21-induced calcium response is, at least partly, from the internal stores. The extracellular calcium is not essential for initiating the TLQP-21 induced calcium response, as the percentage of responding cells in extracellular calcium free condition was not significantly different from that in the normal condition. Nevertheless, it is still possible that a small influx of extracellular calcium is subsequent to TLQP-21 induced calcium response. This may not be observed in our experiment because the magnitude of ATP response was altered in calcium free condition. Therefore, the ratio between TLQP-21/ATP induced responses may not be directly compared, and hence unable to conclude if the magnitude of TLQP-21 induced calcium response was changed in calcium free condition. This question can be investigated by future experiment to quantify the absolute calcium concentration induced by TLQP-21 in both calcium present and calcium free conditions using dyes such as Fura-2 (Invitrogen). The ratiometric calcium dye as such allows conversion of fluorescent calcium signals into calcium concentrations, which allows direct comparison of calcium response induced by TLQP-21 in different bathing solutions. This experiment will allow us to further conclude if the calcium observed in TLQP-21 induced calcium response was solely a release of calcium from intracellular

store to the cytoplasm, and independent to extracellular calcium.

The data presented here suggested that the calcium source of TLQP-21-induced calcium response is, at least partly, from the internal stores of the macrophage. To further test if the calcium released from the internal stores is the only calcium source, pharmacological agents such as thapsigargin can be used in calcium imaging. Thapsigargin depletes the internal stores of calcium by inhibiting the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) family of calcium pumps (Rogers, Inesi et al. 1995), which has been shown to be present in a variety of organelles including nuclear envelope (Xu, Morita et al. 2001), sarcoplasmic reticulum (Arruda, Nigro et al. 2007), endoplasmic reticulum (Papp, Dziak et al. 2003) and Golgi body (Vanoevelen, Raeymaekers et al. 2004). Pretreatment primary macrophages with thapsigargin can block the restoration of calcium that causes the internal stores to become depleted. If calcium from internal store is the only core source of calcium, pretreatment of thapsigargin would expect to abolish the subsequent TLQP-21-induced calcium response. It is established in literature that the major pathways that regulate internal store calcium homeostasis involve calcium channels such as inositol 1,4,5-trisphosphate (IP₃) receptors and ryanodine receptors (RyR), and calcium pumps such as SERCAs and PMCAs (plasma membrane calcium ATPases). Future experiments can be carried out to dissect which pathway and which calcium channel/pump is downstream to TLQP-21 stimulation in macrophage. For example, PLC inhibitor, U73122, can be used to check if the TLQP-21 induced calcium response involves G-protein activation of phospholipase C and IP₃ production (Mogami, Lloyd Mills et al. 1997). Moreover, inhibitors of RyR such as ruthenium red and high concentration of ryanodine can be used to determine the involvement of RyR downstream to TLQP-21 activation (Gerasimenko, Maruyama et al. 2003). The use of pharmacological agents as such in calcium imaging would not only further confirm the source of calcium, but also reveals the nature of the pathway that TLQP-21 mediated through. The major intracellular organelles involve in calcium dynamic in the cells are the endoplasmic reticulum (ER) and the mitochondria, although it must be noted that other organelles, such as Golgi body, nucleus, lysosomes and secretory vehicles have also been implicated in modulating intracellular calcium level (Michelangeli, Ogunbayo et al. 2005). To further elucidate if the internal store of calcium was released from specific organelle(s), studies using

calcium sensitive photoprotein, such as aequorin, targeting specific organelle (such as mitochondria) can be carried out. Chimeric proteins of aequorin with organelle targeting sequences can be generated (Rizzuto, Simpson et al. 1992), and the calcium change in these organelles can be specifically monitored upon TLQP-21 application. All together, these experiments would provide more evidences to draw a more reliable and convincing conclusion on how TLQP-21 acts on the primary macrophage through calcium signalling.

After determining that the calcium released from the internal stores plays an imperative role in TLQP-21 signalling in the primary macrophage, I was interested to next investigate if different subtypes of macrophage response to the peptide differently *in vitro*. Macrophage is established to adapt multiple subtypes and can be highly polarised in response to their microenvironment. The polarisation of macrophages has been implicated in many disease progressions. For example, macrophage is shown to shift from M1 subtype to M2 subtype during tumor progression (Sica, Larghi et al. 2008). A shift from an M1 phenotype to an M2 phenotype is also observed during the course of the disease in animal model of muscular dystrophy (Villalta, Nguyen et al. 2009). On the other hand, a change in macrophage phenotype from M2 to M1 has been evident in obesity (Suganami and Ogawa 2010). The high plasticity of macrophages plays a role in the disease development and polarised macrophages may respond to the same stimulus differently. To examine whether TLQP-21 acts on different subtypes of macrophage differently, I attempted to convert the cultured macrophages into M1 population by LPS treatment, M2a population by stimulating with IL-4 and M2c population with IL-10. After 24 hours stimulations, the polarised populations were able to produce phenotypical markers (high iNOS/Arg1 expression for M1 population and low iNOS/Arg1 expression for M2 populations. M2c also produces high levels of IL-10). In addition, the morphologies of M1 macrophages were observed to be more rounded in shape and M2 macrophages were more elongated, which are consistence with the literature finding (Mills, Kincaid et al. 2000). The data presented in this investigation surprisingly suggests TLQP-21-induced calcium response was not affected by the polarisation of the macrophages. The percentages of cells responding to TLQP-21 in different macrophage subtypes were not significantly different from each other, suggesting all subtypes of macrophages responded to TLQP-21 peptide in a calcium

dependent manner. However, it is still possible that the pathways downstream to calcium increase are different in different macrophage subtypes, and hence result in different functional outcomes after TLQP-21 stimulation. Although the percentages of responding cell to TLQP-21 were not significantly differing between groups, the calcium concentrations increased in different subtypes could still be different.

It has been demonstrated that media isolated from activated macrophages show effect on DRG neuron axon outgrowth and neurotoxicity *in vitro* (Gensel, Nakamura et al. 2009). For this reason and because macrophages are shown to be infiltrated to the DRG after peripheral injury (Kim, You et al. 2011), the effect of TLQP-21-stimulated macrophage conditioned media (MCM) on cultured DRG neurons were assessed. The preliminary data in this study suggests that the 4 hours TLQP-21 treated MCM induces about 2 times more DRG neurons to respond when compared to that stimulated with 4 hours ScrTLQP-21 treated MCM. This suggests TLQP-21 stimulated macrophage could possibly release certain functional effectors, which in turn activates DRG neurons. Nevertheless, the experiment needs to be repeated in order to confirm the hypothesis. In addition, MCM from longer treatment with TLQP-21 can be tested to examine if the percentage of responding neurons further increases.

2.5.4. Effect of TLQP-21 treated primary macrophage on nociception at the periphery *in vivo*.

Having demonstrated VGF-derived peptide, TLQP-21, specifically acts on the primary macrophage in a dose-dependent and calcium-dependent manner *in vitro*. I next sought to investigate the effect of TLQP-21-stimulated macrophage *in vivo*. This is the first study that investigates the involvement of VGF-derived peptide-stimulated macrophage in the pain modulation. The data presented here suggests that TLQP-21-stimulated macrophages exhibited a functional role in eliciting mechanical hypersensitivity in rat when injected peripherally. This effect is specific, as the hypersensitivity was not observed when ScrTLQP-21-stimulated macrophages were injected. The TLQP-21-stimulated macrophages-induced hypersensitivity behaviour was demonstrated to be temporary in our study, which was observed in the animals up to 3 days post-injection. The paw withdrawal threshold of the animal to the mechanical

stimulation on day 5 post-injection of TLQP-21-treated macrophages was not significantly different from the baseline before injection. The observed TLQP-21-stimulated macrophage effect *in vivo* is transient could be due to a couple of possible reasons. Firstly, it is possible that the injected macrophages may require constant exposure to TLQP-21 for a persistent effect. In fact, prolonged upregulations of VGF mRNA and protein were observed in the DRG neurons after peripheral nerve injury in the Spared nerve injury (SNI) model of neuropathic pain. Such increase in the expression is maintained for at least 21 days (Moss, Ingram et al. 2008). Moreover, the macrophage is highly polarised, and the polarisation of macrophage can be reversed when the polarising stimulus is removed or repolarised when challenged with other stimuli (Mosser and Edwards 2008, Ambarus, Santegoets et al. 2012). The animals used in this study were naïve, so the VGF derived peptides were most likely not presented in the microenvironment surrounding the injected macrophages *in vivo*. Therefore, the injected TLQP-21-stimulated macrophages were removed from its stimulus upon delivering to the naïve rats, and hence likely to be reversed to the resting state after several days without exposure to TLQP-21. Secondly, it is also possible that the turnover rate of TLQP-21-stimulated macrophage is quick, and hence the majority of the stimulated macrophages died out before day 5 behavioural assessment. Indeed, differentiated and activated macrophage has been evident to have a limited lifespan. It is believed that they enter apoptotic death at the site of injury after activation by nature ligands/foreign pathogens (Marriott, Bingle et al. 2005).

Another important point derived from this *in vivo* experiment that needs to be noted is that the hypersensitivity effect observed here could be due to the residual TLQP-21, as it has been shown to induce hyperalgesic response in mice when inject peripherally (Rizzi, Bartolomucci et al. 2008). Although the cultured macrophages were washed thoroughly before being harvested for the intraplantar injection, it is still possible that some peptide remained in the residual solution, and hence was carried over and injected to the animals. Though, the possibility of the carried over peptide inducing the nociceptive behaviour is very low, as the concentration of TLQP-21 used to treat the culture macrophage was 500 nM and washes were performed before harvesting. Moreover, the literature suggested that concentration to elicit a hyperalgesic response was 4 mM, and lower doses at 1 mM and 2 mM do not induce a hyperalgesic response when injected. Therefore, it is almost unlikely that the residual TLQP-21 carried over

could elicit the robust mechanical hypersensitivity as observed.

Summing all the results presented in this chapter, I have demonstrated for the first time that a VGF-derived peptide, TLQP-21, acts on the primary macrophage *in vitro*. The mechanism of action of TLQP-21 on the macrophages is dose-dependent. It elicits a robust but transient increase in the intracellular calcium, which is resulted from, at least partly, the release of calcium from the internal stores. I also demonstrated that TLQP-21 desensitises the primary macrophage to further TLQP-21 stimulation and the TLQP-21-induced response is observed in both M1 and M2 subtype of macrophages. Macrophage conditioned media after TLQP-21 treatment may be able to cause excitation of the DRG neurons *in vitro*, although more experiments need to be carried out to confirm the crosstalk in the neuro-macrophage signalling. Lastly, the intraplantar injection of TLQP-21-stimulated macrophages induces the mechanical hypersensitivity *in vivo* on POD1 and POD2, suggesting a role of TLQP-21 in the pain pathway both *in vitro* and *in vivo*.

CHAPTER 3:
GC1QR AS THE POTENTIAL RECEPTOR FOR TLQP-21 ON
PRIMARY MACROPHAGES

Synopsis

The data in the previous chapters suggested that TLQP-21 acts on the primary macrophages possibly through a receptor-mediated pathway, as desensitisation was observed in the TLQP-21-induced response in the macrophages stimulated repeatedly. In this chapter, the identification of the potential receptor, GC1qR, for TLQP-21 is described. In addition, the verification of GC1qR as the receptor for TLQP-21 on the primary macrophages by neutralising antibodies and siRNA approaches has been outlined here. I also present and discuss, in the context of neuropathic pain, the effects of neutralising GC1qR *in vivo* in the partial sciatic nerve ligation animal model.

3.1. Introduction

3.1.1. GC1qR

The receptor for the globular head of complement C1q (GC1qR) is a conserved eukaryotic protein. The protein homologues have been identified in a wide range of eukaryotic species spanning from fungi to mammal. The protein is expressed in multiple compartments within a cell and is reported to be expressed in a variety of cell types including endothelial cells (Peerschke, Smyth et al. 1996), fibroblast (Deb and Datta 1996), immune cells (Ghebrehiwet and Peerschke 2004) and various cell lines at high level, such as the Raji and Daudi cell lines (Ghebrehiwet, Lim et al. 1994). The molecular weight of GC1qR is 33kDa and is also known as p33, p32, C1qBP, HABP1 and TAP (Deb and Datta 1996, Yao, Eisen-Vandervelde et al. 2004). In addition to its multi-compartmental expression, the protein has also been shown to bind multiple ligands and exerts multiple functions (see later ‘function of GC1qR and its interactive proteins’).

Cellular Expression of GC1qR

The cellular distribution of GC1qR is detected in several of cellular compartments, including the plasma membrane (Ghebrehiwet, Lim et al. 1994, Ghebrehiwet, Lim et al. 2001, Mahdi, Madar et al. 2002, Fogal, Zhang et al. 2008), cytoplasm (Laine, Thouard et al. 2003), endoplasmic reticulum (ER), mitochondria (van Leeuwen and

O'Hare 2001) and the nucleus (Krainer, Mayeda et al. 1991, Laine, Thouard et al. 2003). Although the cellular expression of GC1qR is still controversial, it is suggested that the protein is produced as a full-length pre-pro-protein of 282 amino acids, which appears to be directed mainly to the mitochondria by its N-terminus targeting sequence (Dedio, Jahnen-Dechent et al. 1998). The 73-residue-long pre-sequence is predicted to harbour the mitochondrial targeting sequence (MTS), which leads the pre-pro-protein for mitochondrial accumulation. It has been demonstrated that fusing of such pre-sequence alone to a green fluorescent protein directs the protein to the mitochondria matrix (Dedio, Jahnen-Dechent et al. 1998). Cleavage or modification of the first 73-residue-long targeting sequence results in re-targeting of the pro-protein to other cellular compartments. Indeed, it has been demonstrated that the addition of a short flag tag to the N-terminus of GC1qR results in disrupting the targeting sequence and re-directing the protein to the ER and cell surface (van Leeuwen and O'Hare 2001). GC1qR on the cell surface is proposed to serve as a multifunctional receptor for many extra- and intracellular proteins (Lim, Reid et al. 1996, Nguyen, Ghebrehiwet et al. 2000, Pupo and Minneman 2003, Yao, Eisen-Vandervelde et al. 2004).

Although it is expressed on the cell surface, the translated amino acid sequence of GC1qR surprisingly lacks transmembrane domains and GPI anchor. Some suggested that the protein possibly induces a transmembrane signal via association with other transmembrane proteins, such as β 1-integrin and DC-SIGN, to channel the downstream signalling events inside the cell (Ghebrehiwet, Feng et al. 2003, Hosszu, Valentino et al. 2012). Though there is neither predicted transmembrane segments nor GPI anchors on GC1qR, strong evidences in the literature demonstrated its expression on the cell surface. First, labelling peripheral blood lymphocytes with sulfo-NHS-LC-biotin that is impermeable to the cell membrane, showed specific incorporation of biotin in the surface expressed GC1qR but not the intracellular counterpart (van Leeuwen and O'Hare 2001). Second, western blot analysis on membrane proteins purified from Raji cells showed corresponding bands for GC1qR protein, implying it membrane localisation. Moreover, confocal laser scanning microscopic analysis using GC1qR specific monoclonal antibodies revealed specific surface staining on 90-95% of the Raji cells (Ghebrehiwet, Lim et al. 1994). Furthermore, the surface expression of GC1qR has also been demonstrated by

Fluorescence-activated cell sorting (FACS) analysis on various cell types (Ghebrehiwet, Lim et al. 2001, Vegh, Goyarts et al. 2003). Lastly, functionally, the membrane expression of gC1q-R was shown to be increased by inflammatory cytokines (INF- γ , TNF- α and LPS) stimulations on endothelial cells (Guo, Ghebrehiwet et al. 1999).

Structure of GC1qR

The structure of GC1qR was first reported by Jiang *et al* in 1999 at 2.25 Å resolution (Jiang, Zhang et al. 1999). Except for mutating the Leucine to Methionine on the residue at position 74 for expression purpose, the molecule expressed was identical to the mature form of GC1qR without the first 73 targeting sequence.

The primary structure of GC1qR contains a protein kinase C (PKC) phosphorylation site at residue 207, a tyrosine kinase recognition site at position 268, and three consensus N-glycosylation sites at position 114(Asparagine-Glycine-Threonine), 136(Asparagine-Asparagine-Serine) and 223(Asparagine-Tyrosine-Threonine). As mentioned previously, the primary structure of GC1qR does not predict consensus amino acid sequence for transmembrane segments and GPI anchors.

The crystal structure revealed the secondary structure of GC1qR monomer, which consists seven consecutive β -strands forming an antiparallel β -sheet flanked by α -helices (one at N terminus and two at C terminus of the protein). The region spanning the second (α B) and the third (α B) α -helice is suggested to be pivotal for oligomerisation of the monomers, and is shown to be conserved in homologous proteins among different species. Despite the simplicity of GC1qR monomer structure, it does not relate to any known structure folding of proteins.

Three tightly bound GC1qR monomers constitute to a doughnut-shaped tertiary trimer structure, forming a channel of $\sim 20\text{\AA}$ in diameter in between. The channel wall is formed by the β -sheets of all three subunits, where each antiparallel β -strands is highly twisted, thus arranging into an up-and-down β -barrel structure. The inner and outer diameters of the trimer are measured approximately $20\text{\AA}$ and $75\text{\AA}$, respectively, and the resultant thickness of the trimeric structure is about $30\text{\AA}$. The trimeric structure

of GC1qR is suggested to increase the structural integrity of the protein and has been postulated to be the physiological structure. Indeed, the postulation is consistent with the literature evidences where GC1qR and its homologue in *Saccharomyces cerevisiae* are shown to behave as trimer in gel filtration chromatography (Ghebrehiwet, Lim et al. 1994, Seytter, Lottspeich et al. 1998). Moreover, oligomeric nature of GC1qR has been suggested to play a pivotal role for its affinity to multivalent ligands such as high molecular weight kininogen (HK) and C1q (Herwald, Dedio et al. 1996).

The doughnut-shaped structure of GC1qR exhibits characteristic of highly negative charged channel and asymmetric surface charge distribution on the sides of the doughnut (one side is highly negatively charged and the other is less charged). The high net negative charge of the molecule makes the protein to be highly acidic with a calculated pI of 4. The distinctive characteristic of different charges on the two sides of the doughnut may suggest its asymmetric functional role.

Function of GC1qR and its interactive proteins

GC1qR was first characterised as C1q binding protein with K_d of 50-100 nM. It has been demonstrated that GC1qR binds to both non-denatured and denatured C1q on its A-chain and, to a less extend, the C-chain. Although GC1qR was originally identified as the binding protein to the globular head of C1q, there is growing literature suggesting that it is in fact a multi-ligand binding protein that binds to a variety of extra- and intracellular proteins, thus exerting multiple cellular functions.

The first group of interactive proteins that GC1qR binds are the plasma/extracellular proteins, which includes C1q (Ghebrehiwet, Lim et al. 1994), thrombin (Ghebrehiwet and Peerschke 1998), vitronectin (Lim, Reid et al. 1996), high molecular weight kininogen (HK), factor XII (Joseph, Ghebrehiwet et al. 1996) and fibrinogen (Lu, Galanakis et al. 1999). GC1qR was first isolated from Raji cell and demonstrated to bind C1q by solid phase binding assay. 250 ng of full length C1q, globular region of C1q, Collagen-like domain of C1q or BSA were coated on a microtiter plate and the binding of ^{125} I-labelled GC1qR to each of these ligands was accessed. It was clearly shown that GC1qR binds to C1q and the globular head of C1q with high affinity but

not to the collagen-like stalk of C1q or BSA. This suggested the specific binding of GC1qR to the globular head of C1q for the first time and lead to the further investigation of the role of GC1qR in complement pathway. Indeed, it is demonstrated then that GC1qR functions to inhibit complement haemolytic activity by binding to C1q, which often triggers the pathway by binding to the immune complexes (Peterson, Zhang et al. 1997). It is also demonstrated that GC1qR plays a role in activating C1q dependent Classical pathway of the complement system in human blood platelets, and hence contributing to the process of inflammation (Peerschke, Yin et al. 2006). In addition to activating the classical pathway of the complement system, GC1qR also exacerbates inflammatory process by interacting with HK and factor XII in the bradykinin-generating cascade, which later exerts proinflammatory effect (Kaplan, Joseph et al. 2002). GC1qR has also been evident to interact vitronectin alone and the vitronectin-thrombin-antithrombin complex to initiate clearance of vitronectin-containing complexes, thus inhibiting complement-mediated cell lysis (Lim, Reid et al. 1996).

Some intracellular proteins have also been shown to interact with GC1qR. This includes cC1q-R, which is another receptor to C1q. GC1qR has been suggested to form complex with CC1qR and possibly other proteins, and initiates cross talk between the associated proteins (Ghebrehiwet, Lu et al. 1997). $\alpha 1\beta$ -adrenergic receptor has also been evident to interact with GC1qR via its cytoplasmic domain of the Carboxyl-terminus by yeast-two hybrid screening and co-immunoprecipitation experiments (Xu, Hirasawa et al. 1999). Recently, the $\alpha 1$ -adrenergic receptor has been demonstrated to positively regulate cytokine production in human monocytes and macrophages (Grisanti, Woster et al. 2011) and GC1qR has been suggested to regulate cellular expression of $\alpha 1\beta$ subunit of $\alpha 1$ -adrenergic receptor where co-expression of GC1qR with $\alpha 1\beta$ reduced the receptor membrane expression (Xu, Hirasawa et al. 1999). It is therefore plausible that GC1qR controls human monocyte and macrophages cellular functions by coupling with $\alpha 1\beta$ and controlling its expression and downstream signalling cascades. Furthermore, GC1qR has been identified to interact with intracellular PKC- μ . It is demonstrated that GC1qR binds to PKC- μ protein via its kinase domain, possibly function to regulate PKC- μ kinase activity, yet does not serve as a substrate. Indeed, PKC- μ aldolase phosphorylation, but not autophosphorylation, is shown to be inhibited in a dose-dependent manner by

presence of GC1qR in the kinase assays (Storz, Hausser et al. 2000).

GC1qR has also been demonstrated to bind to proteins from pathogenic microorganisms including viral and bacterial proteins. The strong interactions of GC1qR to the viral and bacterial proteins have been implicated in the pathogenicity and oncogenicity of these pathogenic microorganisms directly or indirectly. GC1qR is shown to bind to the core protein of Hepatitis C virus (HCV) and function to promote viral infection and persistence (Kittlesen, Chianese-Bullock et al. 2000, Waggoner, Hall et al. 2007). HCV utilised GC1qR to evade the immune response, in which HCV core protein interacts with GC1qR and subsequently arrests the proliferation of T cells, which is reversible by anti-GC1qR antibody (Yao, Eisen-Vandervelde et al. 2003). GC1qR has also been implicated in viral gene expression regulation of HIV-1 and herpes simplex virus by interacting with their accessory proteins HIV-1 Tat (Yu, Loewenstein et al. 1995), HIV-1 Rev (Luo, Yu et al. 1994) and open reading frame P of herpes simplex virus (Bruni and Roizman 1996). It is also evident that GC1qR interacts with bacterial proteins such as protein A of *Staphylococcus aureus* (Peerschke, Bayer et al. 2006) and Internalin B of *Listeria monocytogenes* (Pizarro-Cerda, Sousa et al. 2004). In both of the cases, GC1qR functions as the initiating step of invasion mechanism of the pathogen and enable subsequent intracellular growth.

All together, the cellular expression, structure, examples of functions and interactive proteins of GC1qR demonstrates its regulatory roles in intracellular and extracellular signalling. The localisation of the protein and which ligand it interacts dictate its ultimate cellular functions. Thus, GC1qR, as agreed in the literatures, is shown to be a multi-ligand binding protein exerting multiple cellular functions.

3.1.2. Animal models of neuropathic pain

Human studies of pain patients and control volunteers provides invaluable information towards our understanding of the mechanism of neuropathic pain, which serves as strong stepping stone for future research and treatment therapeutic drug developments. One example of the importance of human research is the identification of SCN9A as one of the ‘pain genes’. SCN9A gene has been mapped to be the gene causing

phenotype from patients with complete inability to sense pain in 2006 (Cox, Reimann et al. 2006). It encodes the alpha subunit of the voltage-gated sodium channel, Nav1.7, which is now one of the major targets for pain drug developments in the pharmaceuticals and remained to be the major focus of research by a number of international laboratories. Although human studies are essential in the pain research, the evaluation of neuropathic pain in human is complex and more than often, the stimuli/procedures required to induce neuropathic pain result in permanent damage and are all subject to limitation. Moreover, it is difficult to recruit a large number of patients with similar symptoms for testing. Therefore, there is a need for reproducible surrogate animal models to validate, and in some case guide, human research and broaden the knowledge of the mechanism involved in the neuropathic pain development, thus shedding the light for better pharmacotherapies development.

The major advantages of the animal models of neuropathic pain include that they allow pathophysiological assessment in the nervous system, both centrally and peripherally, in the neuropathic state. They also enable neurophysiological, morphological and biochemical changes to be evaluated during disease state and allow molecular and cellular mechanism underlying the development of such dis-regulated pain to be investigated. Moreover, they are invaluable for analgesic pharmacology where they could enable drug efficacy to be examined and allow identification of rational targets for future analgesic drugs developments.

In this session, some of the most commonly employed animal models of neuropathic pain in the pain research will be introduced. The existing animal models of neuropathic pain could be divided into two broad categories, nerve injury models of neuropathic pain and disease-associated models of neuropathic pain. Common models from both of the categories will be presented here.

Nerve injury model of neuropathic pain

Partial sciatic nerve ligation (PSNL or Seltzer) model

In my studies I have used the partial sciatic nerve ligation (PSNL) model of peripheral injury neuropathic pain model. Seltzer, Dubner and Shir (Seltzer, Dubner et al. 1990) developed this model by partially ligating the sciatic nerve, which is one of the most

commonly employed animal model of neuropathic pain. In this model, the sciatic nerve at upper thigh level of the left hind leg is exposed under anaesthesia. About one-third to half width of the sciatic nerve is tightly ligated with non-absorbable suture (table 3.1). This procedure results in partial denervation of one of the hind paws while keeps some of the sensory input intact. The damage to the peripheral nerves results in infiltration of mast cells, macrophages and T lymphocytes that produce inflammatory mediators such as proinflammatory cytokines and chemokines, which play pivotal roles to the initiation and/or maintenance of neuropathic pain. The behavioural alterations of the animal after the surgery includes spontaneous pain in forms of wound guarding, excessive licking of the ipsilateral hind paw and avoidance of putting body weight on the injured paw. The behavioural changes to the hind paw like cold allodynia, hyperalgesia to mechanical, thermal and chemical stimuli have been shown to develop within 1 week after the surgery and could persist up to several months post-surgery. The PSNL model produces unilateral peripheral mononeuropathy, and symptoms developed after surgery has been suggested to manifest those observed in causalgiform pain syndrome in patients. Furthermore, the model has been extended to other species like mice (Malmberg and Basbaum 1998, Harvey and Dickenson 2009), and it has been demonstrated there is changes in the electrophysiological properties of the neuronal activity. It has been noted that increases in C-fibre evoked response, spontaneous activity and wind-up of wide dynamic range (WDR) neurons have been observed after PSNL surgery. Wind-up is a phenomenon where repetitive electrical stimulation of the peripheral C-fibres results in progressively increasing response in the corresponding spinal cord WDR neurons. Increase in wind up of WDR neurons is correlated to the central sensitisation, implying development of central sensitisation in the PSNL model of neuropathic pain.

Spinal nerve ligation (SNL or Chung) model

The other most commonly employed nerve injury model of neuropathic pain is the Spinal nerve ligation (SNL) model. Kim and Chung developed this model with tight ligation at the L5 and L6 spinal nerves distal to the dorsal root ganglion (Kim and Chung 1992). Briefly, under anaesthesia, a small incision is made at the lower back to expose the left posterior iliac crest. The left posterior iliac crest is crushed to expose the lumbar spinal nerves. The L5 and L6 spinal nerves are identified, carefully separated from L4 and ligated with non-degradable suture (table 3.1). L4 spinal nerve is often

kept intact in this model because it consists large amount of motor fibres, ligation to this spinal nerve will result in disrupting motor function and hence complicate interpretation of the behavioural outcome. Within 48 hours, the operation results in long-lasting behaviour change including cold and mechanical allodynia, thermal hyperalgesia and persistent pain, which are maintained for up to 16 weeks. This is a highly reproducible model and the symptom mimics that of chronic neuropathic pain state in human. The SNL model offers a major advantage that the L5 and L6 spinal nerves are designed to be denervated, thus the specific spinal segments that are damaged/intact are known, allowing subsequent investigation of the spinal cord (central) mechanism of neuropathic pain.

Chronic constriction injury (CCI) model

The chronic constriction injury (CCI) model, developed by Bennett and Xie, is a model of mononeuropathy induced by self-strangulation of the sciatic nerve. Unlike PSNL in that a single partial ligation is performed on the sciatic nerve, four ligatures are loosely tied around the sciatic nerve and result in slight constriction to the nerve (Bennett and Xie 1988)(Table 3.1). The constriction of the sciatic nerve is associated with intraneural oedema, focal ischemia and Wallerian degeneration. Thus, the actual cause of the nerve damage in the CCI model is not resultant from the ligatures, but the ‘strangulation’ of the nerve caused by oedema-induced nerve swelling. In addition, it is suggested the myelinated sensory afferents are affected more severely in this model than the unmyelinated fibres (Bennett, Chung et al. 2003). The post surgery behaviours of the animals includes decreased threshold in paw withdrawal to thermal (both hot and cold), mechanical and chemical stimuli. Spontaneous pain behaviours are also observed. These phenotypes can persist for 8-12 weeks post-surgery. Moreover, the affected hind paw are shown to be unusually warm/cool in one third of the animals and more than half of the operated animals developed overgrown claws on the injured side. The CCI model was originally developed in the rats, but it has been successfully established in the mice as well (Sommer, Schmidt et al. 1997).

Spared nerve injury (SNI) model

Spared nerve injury (SNI) model is the latest peripheral nerve injury model of neuropathic pain developed by Decosterd and Woolf in 2000. In this model, the rats are anaesthetised and the sciatic nerve of the left hind leg and its three terminal branches are

exposed (sural, tibial and common peroneal nerves). Two of these sciatic nerve terminal branches are tightly ligated with non-degradable sutures followed by 2mm axotomy (typically the tibial and common peroneal nerves) and the sural nerve is left intact (table 3.1), and hence the model was named the ‘spared nerve’ injury model. The spared nerve injury model results in mechanical and thermal hyperalgesia. The behavioural changes are observed within 24 hours, and could persist up to 6 months. The reproducibility of this model is considered to be higher when compared to PSNL and CCI models, and it is claimed that all animals are responders after the surgery (Decosterd and Woolf 2000). Unlike PSNL, SNL and CCI models result in a mixture of intact and degenerating axons and the behavioural testing outcome is a summation output of all these nerves, SNI model permits testing of the non-injured skin surface where the sural nerve innervates and the denervated areas of the axotomised common peroneal and tibial nerves. This allows direct comparison and simultaneous investigation of the thermal and mechanical sensitivities between the injured skin territories and the neighbouring intact sensory afferents.

Disease-associated model of neuropathic pain

Diabetes-induced neuropathy model

Diabetic neuropathy is resulted from the nerve damage caused by chronically high blood sugar and diabetes. It is estimated about one in five people with diabetes develops painful diabetic neuropathy, which is a devastating condition and often leads to foot amputation. The clinical conditions of diabetes-induced neuropathy include paraesthesia, allodynia, hyperalgesia, spontaneous pain and sensory loss (Calcutt 2002). The most common animal model for painful diabetes-induced neuropathy is the streptozotocin (STZ) model. In this model, STZ, which is toxic to the insulin-producing β cell of the pancreas, is administered intra-peritoneally/subcutaneously/ intravenously to the animals (normally between 50-200mg/kg concentration) (Courteix, Eschalier et al. 1993, Aley and Levine 2001, Anjaneyulu and Chopra 2003). The behavioural symptoms post-injection include thermal and mechanical hyperalgesia and allodynia to thermal and mechanical stimuli that often do not elicit pain response. These symptoms occur gradually and are observed most prominently 2 weeks after diabetes induction. Although the model can be easily induced by solely STZ injection, it presents drawbacks such as that the animals develop other symptoms complicating the

interpretation of nociception, these include reduced growth and motor activity, fall in skin temperature, alterations in lipid metabolism, ketoacidosis, polyuria and diarrhea. These complications thus have to be considered when choosing the suitable models for neuropathic pain studies.

Cancer treatment-induced neuropathy model

Chemotherapeutic agents used to treat cancers have been reported to induce sensory neuropathy and resulting in neuropathic pain in patients. These chemotherapy drugs include Vincristine and Paclitaxel. Cancer treatment-induced neuropathy animal models of neuropathic pain have hence been developed in hope to understand the pathogenic mechanisms underlying the development of drug-induced neurotoxicity and better understand the pathophysiological, morphological and electrophysiological changes due to chemotherapeutic agents. The two most commonly utilised chemotherapy drug-induced neuropathy models are described here -the vincristine-induced and paclitaxel-induced neuropathy model.

Vincristine has been used in current clinic routinely to treat breast cancer, leukemia, lymphomas and primary brain tumours. Several toxic effects have been attributed to vincristine, including peripheral neurotoxicity and the subsequent resultant sensory-motor neuropathy (Hilken and van den Bent 1997). Different animal models of vincristine-induced neuropathy have been developed in which they differ from the dose regimen and the numbers of dosages. The most frequently employed protocol is developed by Aley et al (Aley, Reichling et al. 1996) where 100 µg/kg of Vincristine is delivered daily intravenously to the tail vein of rats over a period of two weeks. Thermal and mechanical hyperalgesia has been reported to develop on the second day after vincristine treatment, and persist up to 24 days. However, this model has been demonstrated that higher dose (200 µg/kg) of vincristine treatment leads to loss of ~12.5% body weight during the course of treatment and significant mortality.

Paclitaxel is one of the other chemotherapeutic agents currently used in the clinic, which has been extensively used for the treatment of numerous malignancies including breast, ovarian, head, neck and non-small cell lung cancers (Jordan and Wilson 2004). Like Vincristine, Paclitaxel has been reported to cause adverse side effects including peripheral axon neuropathy, cardiotoxicities, gastrointestinal

toxicities and mucositis, which may ultimately lead to disabling symptoms and resultant in reduced quality of life for the patients. Paclitaxel-induced neuropathy model of neuropathic pain has hence been developed to understand not only the mechanism underlying neuropathic pain, but also the pathogenesis of paclitaxel-induced neuropathy in hope to prevent or delay neurotoxicity. If the neurotoxicity side effect could be greatly improved by the better understanding of the pathogenesis from the model, it may leads to more effective therapeutic regimens. In this model, typically 1-2 mg/kg of paclitaxel is administered intra-peritoneally to the animals on four alternate days (day 0, 2, 4 and 6). This dose regimen of paclitaxel results in cold and mechanical allodynia and endoneural oedema of the sciatic nerve. In addition cold and mechanical hyperalgesia has also been observed on day 5 post- first dose injection and persist for almost 3 weeks after the last dose. Intriguingly, this model results in little or no alteration to the sensitivity to noxious pain as heat hyperalgesia is often not observed (Polomano, Mannes et al. 2001).

Viral infection-induced neuropathy model

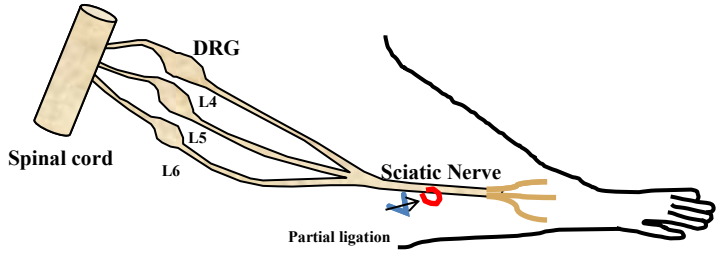
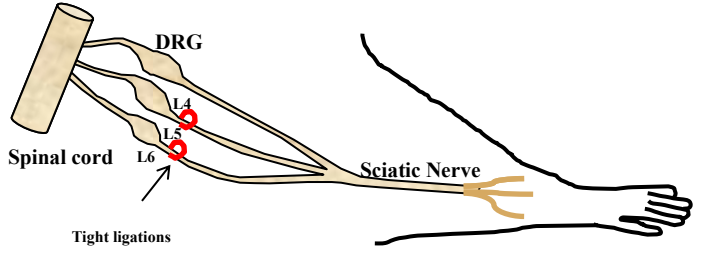
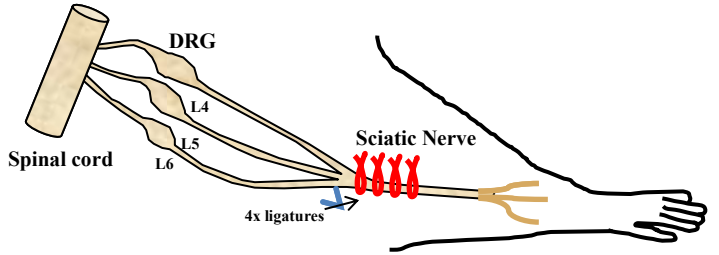
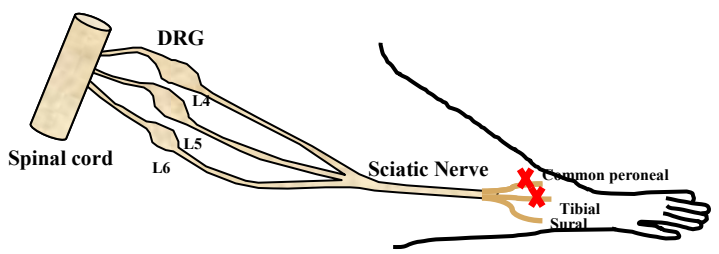
Sensory peripheral neuropathy associated neuropathic pain is frequently observed in patients with viral infections, which include the Human Immunodeficiency virus Type 1 (HIV-1) and Varicella zoster virus (VZV) infection. HIV-induced sensory neuropathy is common among infected patients, which has been shown to affect about 35% of the hospitalised patients (So, Holtzman et al. 1988). The HIV-induced sensory neuropathy, which often associates with devastating neuropathic pain, is reported at high rate globally and remains to be a major global health concern. The animal model hence was developed to better understand the pathogenesis of HIV-induced sensory neuropathy in hope to improve symptom, identify risk factors and develop effective therapeutic treatments for affected sufferers. In the HIV infection-induced neuropathy model, the rat's left sciatic nerve is exposed and wrapped with oxidised cellulose (oxycellulose) soaked with 200ng HIV-1 envelope protein gp120. The oxycellulose serves as a carrier matrix, which slowly release viral protein gp120 ectopically, mimicking physiological viral infection (Wallace, Blackbeard et al. 2007). Such perineural administration of gp120 results in mechanical, but not thermal or cold, hyperalgesia. This hypersensitivity is observed from day 9 post-gp120 treatment, peaked at day 12, and persists until day 21 post-surgery. Moreover, the rats also exhibit anxiety-like behaviour in the open field behavioural experiment. In addition, the mechanical

hypersensitivity observed is shown to be reversible by treatment with gabapentin, morphin and cannabinoid. This model not only allows investigation of the mechanism underlying HIV-1 induced sensory neuropathy, but also provides invaluable system for preclinical testing of drugs available for treatments.

Varicella zoster virus (VZV) is a virulent neurotropic herpesvirus that causes approximately 4 million cases of chickenpox annually. After primary infection, the virus retrogradely transports along the sensory axon and quiescent in the sensory ganglia. The quiescent virus can be reactivated when the immunity is weaken and causing acute herpes zoster, also known as shingles. Neuropathic pain is more than often associated with relapse of viral infection. Thus, the VZV infection-induced neuropathy has been replicated in rats, in which animals are anesthetised and injected with VZV infected cells ($6-8 \times 10^6$ cells in 50 μ l) to the left mid-plantar glabrous foot pad. 3 days after the viral infected cells injection, the rats develop mechanical allodynia and hyperalgesia, which reach the maximal level before day 21 (Hasnie, Breuer et al. 2007).

Although growing numbers of animal models of neuropathic pain have been developed in the recent years, each animal model still presents its drawbacks. For example, in the PSNL model, because 30-50% of the sciatic nerve axons are ligated, it is impossible to ensure the same amount of axons, and the exact location of sciatic nerve, is ligated in different animal. Therefore, resulting in different amount of nerve damage in different animals, which may contribute to the variability observed in the behaviours among animals underwent surgery. Moreover, over tightening/loose tightening of the ligatures in the CCI models could lead to complete axotomy/no nerve constriction. Hence, the model required researchers with extensive experience to perform minimal but appropriate constriction to the sciatic nerve. Furthermore, most of the disease-associated models of neuropathic pain are limited with dosage cytotoxicity. Apart from these technical drawbacks, it is impossible for a single animal model to manifests all the symptoms from human patients and exhibits the whole spectrum of neuropathic pain mechanisms. Therefore, comparisons between animals models and combined used of the models are essential in the pain research and would benefits the future research for better understanding of the mechanism of neuropathic pain.

Nerve Injury models of Neuropathic pain

Name	Procedure	Schematic diagram	Reference
Partial sciatic nerve ligation (PSNL or 'Seltzer' Model)	Tightly ligate 30-50% of the sciatic nerve of the left hind at upper thigh level	 A schematic diagram of a rat's left hind limb. The spinal cord is shown with dorsal root ganglia (DRG) at levels L4, L5, and L6. The sciatic nerve is shown exiting the spinal cord and running down the leg. A blue arrow points to a red circle on the sciatic nerve, labeled 'Partial ligation'.	(Seltzer, Dubner et al. 1990)
Spinal nerve ligation (SNL or 'Chung model')	Tight ligation of the L5 and L6 spinal nerves distal to the dorsal root ganglia	 A schematic diagram of a rat's left hind limb. The spinal cord is shown with DRG at L4, L5, and L6. Red circles with arrows pointing to them are labeled 'Tight ligations'.	(Kim and Chung 1992)
Chronic constriction injury model (CCI)	Four ligatures are loosely tied around the sciatic nerve of the left hind leg resulting in chronic constriction to the nerve	 A schematic diagram of a rat's left hind limb. The spinal cord is shown with DRG at L4, L5, and L6. The sciatic nerve is shown with four red 'X' marks labeled '4x ligatures'.	(Bennett and Xie 1988)
Spared nerve injury model (SNI)	The common peroneal and Tibial nerves are ligated followed by axotomy transection while the sural nerve is left intact (Spared nerve)	 A schematic diagram of a rat's left hind limb. The spinal cord is shown with DRG at L4, L5, and L6. The sciatic nerve is shown with red 'X' marks labeled 'Common peroneal' and 'Tibial Sural'.	(Decosterd and Woolf 2000)

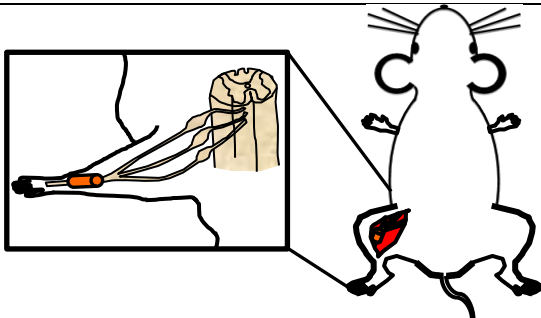
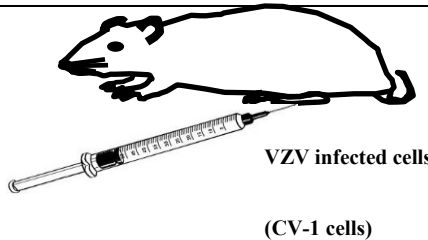
Diseases-associated models of neuropathic pain			
Name	Procedure	Schematic diagram	Reference
Diabetes induced neuropathy model	Subcutaneous injection of streptozocin (STZ)	 Streptozocin	(Courteix, Eschaliere et al. 1993)
Cancer treatment-induced neuropathy model	Paclitaxel and vincristine injection	 Paclitaxel/Vincristine	(Aley, Reichling et al. 1996, Polomano, Mannes et al. 2001)
HIV-1 induced neuropathy model	Perineural administration of gp120 glycoprotein of HIV-1 by wrapping oxidised cellulose soaked with gp120 around the left sciatic nerve.		(Wallace, Blackbeard et al. 2007)
Varicella zoster virus (VZV)-induced neuropathy model	Injecting VZV infected cells ($6-8 \times 10^6$ cells in $50 \mu\text{l}$) to the left mid-plantar glabrous foot pad.	 VZV infected cells (CV-1 cells)	(Hasnie, Breuer et al. 2007)

Table 3.1. Summary of the procedures, schematic diagrams and original references for different animal models of neuropathic pain.

3.1.3. Identification of VGF receptor – GC1qR

I have demonstrated in the previous chapter that macrophage is one of the target cells, if not the only one, for VGF-derived peptide TLQP-21. Despite the results in the previous chapter suggesting TLQP-21 acts on primary macrophage in a dose-dependent and intracellular calcium-dependent manner, the receptor for TLQP-21 and other VGF derived peptides still remain unknown in the current literature. Recently in our laboratory, an attempt was made to identify the receptor(s) for TLQP-21. To identify the receptor for TLQP-21, two approaches were employed.

The first method exploited was the chemical cross-linker in combination with mass spectrometry techniques. Sulfo-EMCS amine-to-sulfhydryl heterobifunctional cross-linker was used in this experiment, which contains N-hydroxysuccinimide (NHS)-ester and maleimide reactive groups at each end as shown in figure 3.1. The NHS-ester functional group and the maleimide functional groups react with primary amine and sulfhydryl group, respectively, and are separated by a medium-length spacer arm (9.4 angstroms). For receptor identification, a synthetic TLQP-21 peptide (>95 % purity, Peptide Synthetics, PPR Ltd) was generated with an extra cysteine added to the C-terminus and a biotin covalently attached to the N-terminus via the amine bond (see figure 3.1). TLQP-21 naturally consists no cysteine in the protein sequence, and the extra cysteine added to the C-terminus allows Sulfo-EMCS cross-linker to be utilized in the receptor identification experiment. In addition, the extra biotin at the N-terminus allows the ease for detecting the receptor/ligand complex (e.g. using streptavidin-HRP). Moreover, this modification disrupted the primary amine structure on the TLQP-21 peptide, which prevents the TLQP-21-conjugated cross-linkers reacting with the peptide itself or the adjacent peptides. This eliminates the concern of the multiple cross-linkers joining TLQP-21 peptides together, and the NHS-ester reactive arms are left to only react to the primary amines groups on the potential receptor(s).

The synthetic peptide was conjugated to the sulfo-EMCS cross-linker via the maleimide reactive arm, which reacts with the sulfhydryl group on the cysteine at the C-terminus. To confirm the peptide was successfully conjugated to the cross-linker, the mixture was analysed by MALDI-TOF mass spectrometry. Figure 3.2 A shows the

result from the MALDI-TOF analysis. The predicted monoisotopic mass of the synthesised TLQP-21 conjugated to Sulfo-EMCS is 3147.46 Da. The most abundant peak observed in the spectrum was the conjugated form at m/z 3148.4, which corresponds to the (TLQP-21+cross-linker+H)⁺ form of the conjugate. Although the unconjugated peptide was also observed in the spectrum at m/z 2764.6, the intensity of such peak is much weaker than the conjugated peptide+cross-linker peak. To identify the receptor, the peptide-conjugated cross-linker was mixed with membrane proteins prepared from adult rat brains and spinal cord. The free NHS-ester reactive group covalently reacted with the primary amine of receptor protein(s) that binds to TLQP-21+cross-linker conjugate. Western blotting using streptavidin-HRP was employed to detect the receptor/peptide complex. Figure 3.2 B reveals a ~30 kDa band was detected when the conjugated peptide was applied to the membrane proteins. This band was barely visible in the control lane loaded with same amount of membrane proteins, suggesting the specificity of the reaction and the presence of a receptor at ~30 kDa for TLQP-21.

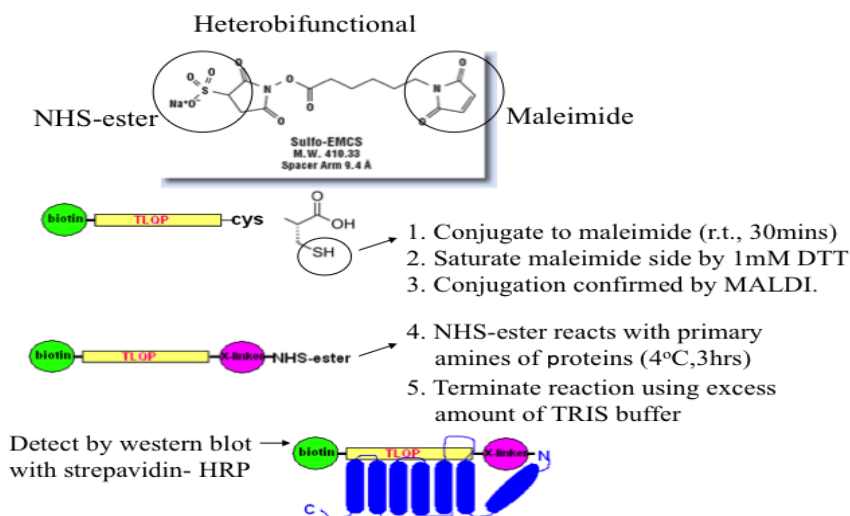


Figure 3.1 Schematic diagram illustrating the experiment procedure exploited for the identification of TLQP-21 receptor by chemical cross-linker.

Sulfo-EMCS crosslinker with a medium-length spacer arm was used. The crosslinker was conjugated to the synthesised biotin-TLQP-21 peptide via the sulfhydryl group on the cysteine at the C terminus of the peptide. The reaction was carried out in MES buffer at pH 6.5 for 30 minutes at room temperature to allow stable formation of thioether bonds between the crosslinkers and sulfhydryl group of the cysteine residue. The reaction was stopped by adding excess amount of DTT to react with the free crosslinkers. The conjugations were confirmed by MALDI TOF mass spectrometry. The conjugated peptides were then incubated with membrane proteins homogenates prepared from CNS tissue (whole brain and spinal cord) in boric acid buffer at pH 8 with 150mM NaCl for 3 hours on ice to allow identification of the potential receptor. The reaction was terminated by adding 50mM Tris buffer, and the samples were resolved on a Tricine-PAGE and analysed by Western immunoblotting.

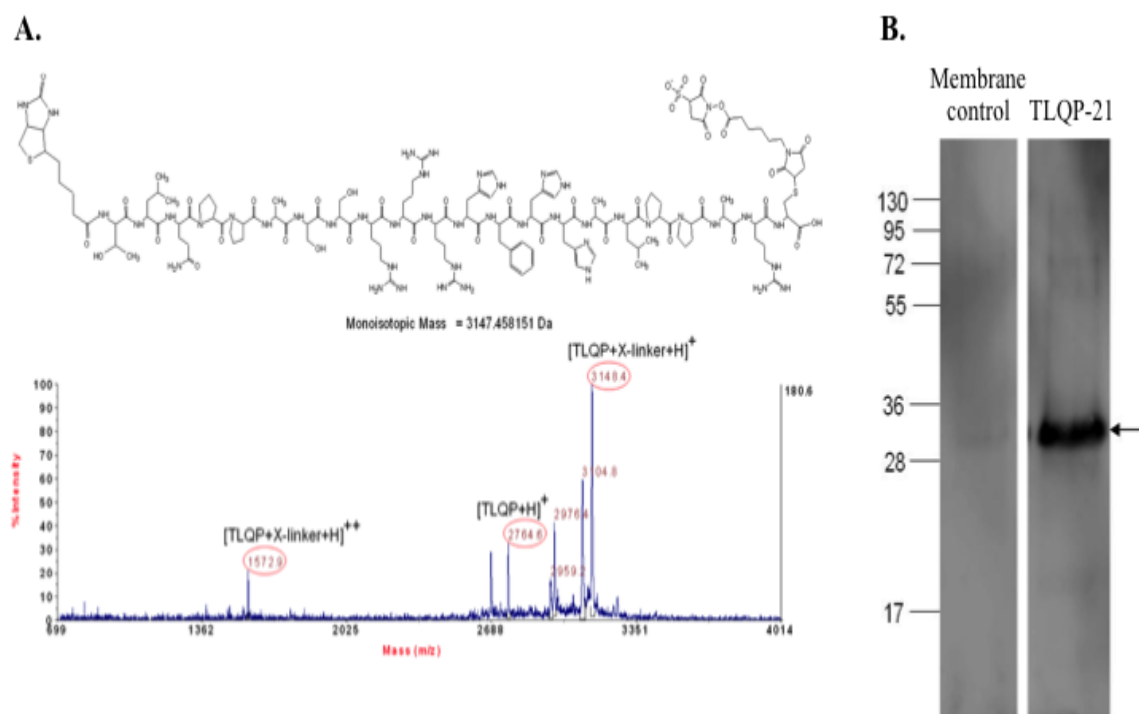


Figure 3.2 Receptor identification by chemical crosslinker method (Data generated by Dr Mahmood Ayub, see thesis for more methodology detail).

A. The chemical structure of the conjugated TLQP-21 with sulfo-EMCS is illustrated, and the predicted monoisotopic mass is calculated to be 3147.458151 Da. The TLQP-21 conjugates were cleaned-up and eluted from a C₁₈ resin Zip Tip, followed by MALDI-TOF mass spectrometry analysis to confirm conjugation. MALDI-TOF mass spectrometry result confirmed successful in conjugation and the major peak observed was the Sulfo-EMCS-conjugated TLQP-21 species. The major peak with m/z of 3148.4 represents the species of TLQP-21 conjugated to sulfo-EMCS, and that with m/z of 1572.9 represents the double protonated form of such conjugate. Unconjugated TLQP-21 in protonated form is shown at the peak of m/z 2764.6 **B.** Western blotting revealed a distinct band at ~30kDa (indicated by an arrow) in the TLQP-21 crosslinking reaction compared to its corresponding control.

This is the first time, to our knowledge, a receptor was identified for TLQP-21 peptide. To elucidate the identity of this receptor and confirm the binding of the complex without covalent chemical reaction, a second methodology was employed: affinity chromatography combined with mass spectrometry (figure 3.3). The synthetic TLQP-21 (10 μ M) was attached to a monomeric avidin resin via the N-terminus biotin, and membrane proteins prepared from the rat brains was applied through the column. The potential receptor that bound to the TLQP-21 peptide would be retained in the column after several washes, while others were washed away. The potential receptor bound to TLQP-21 was subsequently eluted and resolved by SDS-PAGE. A control experiment was carried out in parallel, where the same chromatography was performed without attaching TLQP-21 peptides to the monomeric avidin column. Any protein eluted after washes in the control chromatography experiment would be those interacted with the resin non-specifically, such as the endogenous biotinylated proteins. Figure 3.4A shows the result of a silver-stained SDS-PAGE gel from the affinity chromatography experiment. A $\sim$ 30 kDa band was observed in the elution from TLQP-21 attached avidin column (indicated by arrow), but not in the elution from control. To reveal the identity of this $\sim$ 30 kDa proteins, which appeared in both chemical cross-linking based and affinity chromatography based experiment, the band was excised from the silver-stained gel, followed by de-staining and in-gel digestion with trypsin. The resulting peptide was analysed by nanoLC-MS/MS Orbitrap. Figure 3.4 B shows the result from nanoLC-MS/MS Orbitrap experiment. The identity of the band was revealed to be a multimeric, multi-functional protein – GC1qR, with three unique peptide coverage shown in the ion-assignment spectra and highlighted in red in the protein sequence. To further verified the identity of the $\sim$ 30 kDa band is GC1qR (as revealed in the mass spectrometry analysis), Western blot analysis was performed on the elutions from affinity-chromatography experiment using GC1qR specific antibody. A monoclonal antibody from Abcam that targets amino acids 76-93 at GC1qR N-terminus was used in the analysis. It was found that a visible band at $\sim$ 30 kDa was apparent in the lane corresponds to the elution from TLQP-21 packed column (Figure 3.4C). The band is barely visible in the adjacent lane corresponding to the elution from negative control. The data here validates the LC-MS/MS data that indicates TLQP-21 binds to GC1qR and suggests the identity of the $\sim$ 30 kDa band we observed in the cross-linker experiment was most likely to be GC1qR. All together, these experiments suggest GC1qR could be the potential receptor for TLQP-21

peptide and the function of TLQP-21 could possibly be mediated via pathways downstream to this receptor.

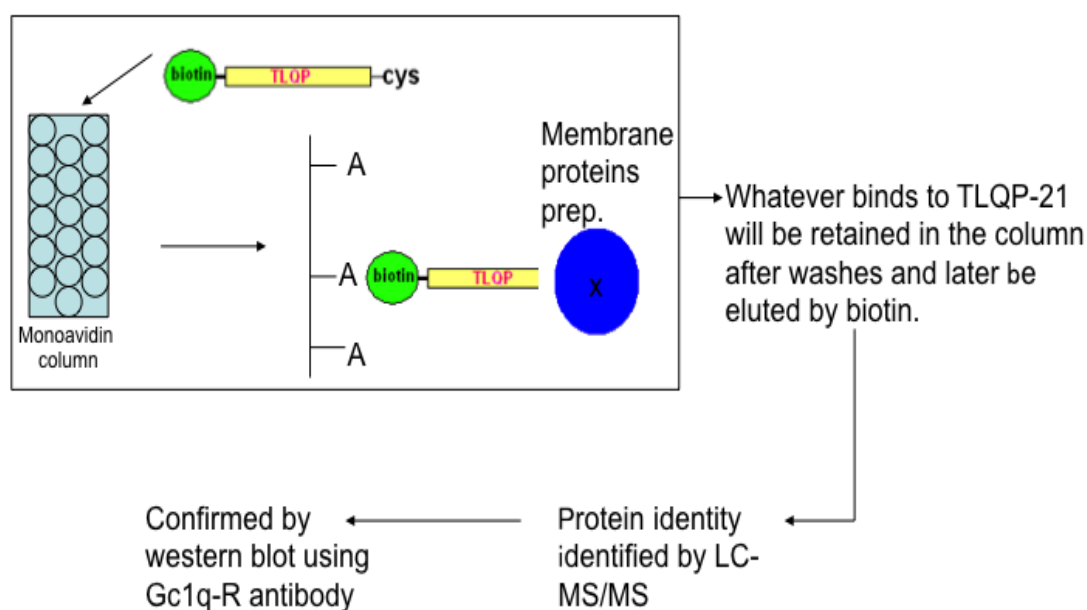


Figure 3.3 Schematic diagram illustrating the experiment procedure exploited for the identification of TLQP-21 receptor by monoavidin affinity chromatography.

10 μ m of synthesised TLQP-21 was attached to the monomeric avidin (monoavidin) column, via their biotin tag, and the membrane proteins were applied to the column. The membrane proteins were prepared from cerebral hemispheres of post natal day 4 rats prior to the experiment. Any membrane protein that binds to TLQP-21 peptide would be retained in the column after a number of washes, while others would be washed away. The potential TLQP-21-bound receptor is then eluted from the column, resolved by SDS-PAGE, followed by silver staining. A negative control chromatography without TLQP-21 peptide attached to the column would also be performed. The proteins eluted in the control would be non-specific interactive proteins to the resin/column itself. Any protein that is eluted specifically in TLQP-21 attached column would be TLQP-21 specific interactive protein, and hence, the potential receptor for TLQP-21. The identity of such potential receptor would then be revealed by LC-MS/MS, followed by confirmation using western blot analysis with the target specific antibody.

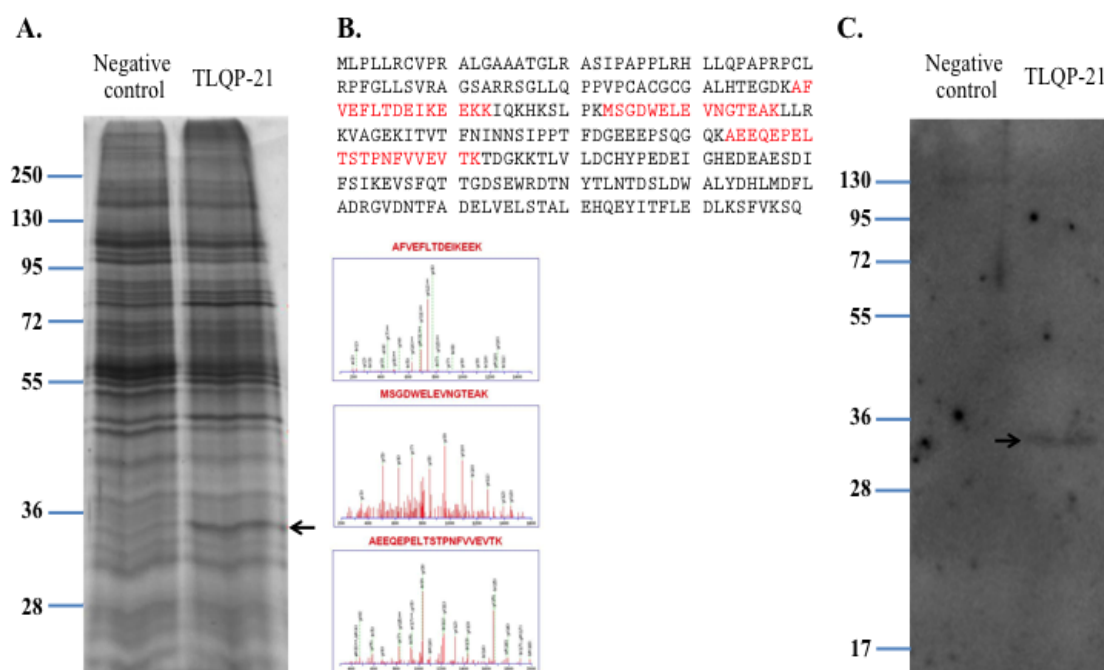


Figure 3.4 Receptor identification by monoavidin affinity chromatography method (Data generated by Dr Mahmood Ayub, see thesis for more methodology detail).

A. The sample eluted from TLQP-21 packed monoavidin column and monoavidin only column were analysis by SDS-PAGE, which were subsequently silverstained by SilverQuest Silver staining kit (Invitrogen, mass spectrometry analysis compatible). A band at ~30kDa was observed in the eluents from the TLQP-21 attached monoavidin column (as indicated by an arrow), indicating TLQP-21 interacts with a ~30kDa protein. The band was not observed in the eluent from the monoavidin only column (negative control), suggesting the specificity of interaction. **B.** LC-MS/MS analysis revealed three unique peptide coverages (as shown in red) for GC1qR. The LC-MS/MS analysis from a second independent affinity chromatography experiment was repeated and GC1qR was identified in both analysis. The fragmentation spectra indicates the three fragment ions assignments for the GC1qR peptides identified. **C.** Western blot analysis using GC1qR specific antibody confirmed the identity of the ~30 kDa protein that interacted with TLQP-21 was GC1qR (as indicated by an arrow).

3.2. Aim

Despite the accumulative evidences shown in the previous chapter and the literatures suggesting the involvement of VGF-derived peptide in the pain pathway, the receptors for all VGF-derived peptides still remain unknown. Recently in our laboratory, a potential receptor for TLQP-21 was identified. The aim of this chapter is, therefore, to:

1. Verify, functionally, if GC1qR is the potential receptor for TLQP-21 on macrophage by using neutralising antibody in combination of calcium imaging.
2. Establish siRNA transfection into the primary macrophage to aid to verify involvement of GC1qR in the molecular mechanism of TLQP-21 signalling in macrophage.
3. Clone and overexpress murine GC1qR in cells that don't respond to TLQP-21, and to investigate such overexpression could aid TLQP-21 signalling in these cells.
4. To better understand the involvement of GC1qR in the development of neuropathic pain *in vivo* by localised treatment of neutralising antibody against GC1qR after partial sciatic nerve ligation.

3.3. Material and method

3.3.1. Transfection of siRNA oligonucleotides into rat primary macrophages

RNA interference was carried out using double-stranded short interference RNA oligonucleotides purchased from Qiagen (listed in table below). The differentiated macrophages were transfected using either the Neon capillary electroporation system (Invitrogen) or the GenePulser cuvette electroporation (BioRad). The bone marrow derived primary macrophages were cultured and differentiated for 6 days as previously described (section 2.2.3). On the day of transfection, the cells were washed three times with PBS and harvested using 5ml of non-enzymatic dissociation buffer (Sigma). The conditions used for Neon Capillary electroporation system is listed in table 3.2 in the result section. The pulse condition for GenePulser cuvette electroporation were performed at 50V, 100V, 200V or 400V with constant resistance (100 Ω) and capacitance (150 μ F). The electroporation was performed in an electroporation cuvette (gap width 0.2 cm). For each electroporation, 1,000,000 cells in 50 μ l of optiMEM medium (Invitrogen) and 3 μ g of double-stranded siRNA were used. Immediately after electroporation, 2 ml of warmed 10 % FBS in DMEM (no antibiotics) was added to the cells. The mixture was plated onto a 6 well plate and cultured for up to 48 hours. The transfected cells were lysed at 24 or 48 hours post-transfection for total RNA and protein lysates preparation (see later for detail).

Short interference RNA	Sequence (5'-3')
siRNA1	UAGGUGGUCAUACAAGGCCCA
siRNA2	UUCUCCGGCAACUUUGCGCAA
siRNA3	UAAUUUAGCCUCCGUGCCGTT
siRNA4	UAAUUGGAGGUGUAAACGGCGA

siRNA oligonucleotide sequences used for transiently knock-down GC1qR

3.3.2. Trypan blue viability assay

The cell survival after electroporation was evaluated by dye exclusion assay using trypan blue. Non-viable cells will be stained blue and the viable cells will exclude the dye and remain unstained. The electroporated cell suspension (or untreated cells) was topped up to 1 ml by complete media. 10 μ l of the cell samples were diluted 1:1 with a sterile filtered 0.4 % Trypan Blue solution. The dilution was carefully pipetted into a hemocytometer, which was allowed to stand for 2 minutes at room temperature. The cells were counted under the microscope in four 1 x 1 mm squares and the average number of cells per square was determined. The number of trypan blue positive cells were also counted and the cell viability was access by the percentage of trypan blue positive cells (Number of trypan blue positive counted $\div$ total number of cells counted x 100%).

3.3.3 Quantitative real time polymerase chain reaction

Total RNA extraction from cultured cells, cDNA synthesis and Quantitative RT-PCR was performed as described in the previous chapter (section 2.2.5). To obtain the total RNA for the *ex vivo* investigation of *vgf* expression in the PSNL model study, the L4 and L5 DRGs were removed from both ipsilateral and contralateral sides of injured rats on post-operative day 1, 3 and 5. The total RNAs were extracted from the harvested DRGs using Qiashedders (Qiagen) and RNAeasy extraction kit (Qiagen) according to the protocols provided by the manufacturer. The primers used in the qRT-PCR studies of this chapter are listed below:

Targets	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Beta-actin	TCCTGTGGCATCCATGAAACT	AACGTCACACTTCATGATGGAATT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Cyclophilin A	GCAAGACCAGCAAGAAGATC	TCAGTGAGAGCAGAGATTACAG
Vgf	ACTTCCTGGTCCCAAACGG	GGCTGGGAGACAGACACTTCA
GC1qR 1	GTATTGGGCGCCTGGTCACC	CGCTCCTGGAAGATGGTGATGG

3.3.4. Western blot analysis

48 hours after siRNA electroporation, the transfected macrophages were harvested for western blot analysis to check the GC1qR protein expression level.

Sample lysis and preparation

The cell culture dish were placed on ice and washed with ice-cold PBS three times to remove residual serum proteins in the culture. The PBS was aspirated and cells were lysed in 100 µl ice-cold Radio Immuno Precipitation Assay (RIPA) lysis buffer with protease inhibitors. The RIPA buffer comprised 150 mM NaCl, 0.1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS) and 50 mM Tris-HCl, which is adjusted to pH 8.0. The lysed cells were scraped and transferred to a pre-chilled 1.5 ml eppendorf tube, where the cell suspensions were incubated with agitation at 4 °C for 2 hours, followed by centrifugation at 16,000 x g for 20 minutes at 4 °C in a refrigerated centrifuge. The lysate supernatants were carefully removed and transferred to a pre-chilled eppendorf tube and the pellets were discarded. The protein concentrations were determined by BCA protein assay (Pierce). 10 µg of proteins from each sample were mixed with 5X Laemmli buffer (10 % SDS, 50 % glycerol, 25 mM DTT, 0.01 % bromphenol blue, 0.312 M Tris-HCl, pH 6.8) and incubated for 1 hour at room temperature.

SDS poly-acrylamide gel electrophoresis (SDS-PAGE), transfer and blotting

Equal amounts of protein were subjected to SDS-PAGE. Samples along with protein marker were loaded into the wells of the 12.5 % poly-acrylamide gel. The proteins were separated at constant voltage at 80 V until the marker was well separated. After SDS-PAGE, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Amersham) using the iBlot™ dry transfer system (Invitrogen). The blot was then incubated with 5 % blocking solution, consisted of 5 % dried fat-free milk in phosphate buffered saline supplemented with 0.1 % Tween-20 (Sigma) (PBS-T), at 4 °C overnight to block the non-specific sites. The membrane was subsequently incubated with primary antibody anti-GC1qR 60.11 (ab24733, Abcam) diluted 1:1000 for 2 hours at room temperature. Following three 15 minutes washes with PBS-T, the membrane was incubated with anti-mouse secondary antibody (DAKO) conjugated to Horseradish Peroxidase (HRP) at 1:1000 dilution for 1 hour at room temperature. The membrane was washed for three times, 15 minutes each, with PBS-T, followed by signal development. The enhanced chemiluminescence western blotting detection system kit (Applichem) was used for the signal development and signal detection was performed using Fujifilm LAS-3000 Imaging System.

The membrane was stripped with stripping buffer (2 % SDS, 100 mM β -mercaptoethanol, 50 mM Tris-HCl, adjusted to pH 6.8) at 50 °C for 30 minutes with constant agitation. Following three washes in PBS-T, the membrane was blocked in 5 % blocking solution overnight at 4°C and subsequent re-probing with second primary antibody (beta-actin) at 1:1000 dilution for 1 hour at room temperature. The anti-rabbit HRP antibody was used at 1:1000 dilution and the signal was detected as previously described. All signal quantification and analysis was performed using

ImageJ image analysis software (<http://rsbweb.nih.gov/ij/>).

3.3.5. Molecular cloning of recombinant GC1qR

Polymerase Chain Reaction

The primer sequences used in the PCR reaction are shown in the below table. The final concentrations of the following components were mixed: 1x PCR Master Mix (Promega) consisting Taq DNA polymerase/dNTP/MgCl₂, 1.5 µM of each primer and 100 ng of rat macrophage cDNA templates. The polymerase reaction was activated 95°C for 2 minutes, followed by 30 cycles of reaction at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute.

Primer	Sequence (5'-3')
Forward (Bam HI site)	AT GGATCC ACCATGGTCCCTCTGCTGCG
Reverse (Xho I site)	GG CTCGAG CCCTGACTCTTGACAAAGC

Primer sequences used for GC1qR gene amplification by PCR

DNA gel electrophoresis, purification and TA cloning

The PCR reaction products were separated by gel electrophoresis according to the size of the amplified fragments. The PCR products were run on a 1.5 % w/v agarose gel along with 10 µl of TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen, UK) at 100 volts for 45 minutes. The expected size of the product is 840 bp. The correct size bands were excised and the DNA was purified from the gels using gel purification kit (Qiagen). The concentration of recovered DNA was then determined using nanodrop; purified PCR products were subsequently ligated with 50 ng pGEM-T easy vector at 3:1 ratio overnight at 16 °C. The ligated clones were then transformed into XL1Blue E. Coli cells, and plated on LB plates supplemented with 100 µg/ml Ampicillin (Amp),

1mg of Xgal in DMF and 1mg of IPTG. The plates were incubated overnight at 37 °C. To check if cloning was successful, white colonies were selected and inoculated the next day, and incubated at 37 °C with agitation overnight. Restriction enzyme digestion with BamHI and XhoI was then carried out on the plasmids purified from the overnight culture using mini-prep kit (Qiagen), followed by DNA gel electrophoresis analysis of the digestion reaction. The clones with the correct insert sizes were then sent to be sequenced using Eurofins MWG operon's DNA sequencing service, followed by checking the sequence using BLAST software (<http://blast.ncbi.nlm.nih.gov/>).

Subcloning into expression vector

The insert was cut out from the pGEM-T vector and the expression vector pDG1-MN1 was linearised by BamHI and XhoI restriction enzyme digestion at 37 °C for 2 hours. The digestion reactions were run on 1.5% agarose gel to separate the digested insert from vector backbone and separate the digested pDG1-MN1 from the undigested. The 840 bp insert and 4.7 kb vector bands were excised from the gel and purified using Gel purification kit (Qiagen). The insert was ligated to pDG1-MN1 vector at 3:1 ratio at 16 °C overnight. The ligation reaction was then transformed to XL1blue competent cells and the colonies were picked the next day to check the success in subcloning (as described previously). The molecular cloning of GC1qR construct was done with help of a undergraduate student, Mr Yosuke Hamada, during his final year project.

3.3.6 HEK293T cell transfection

HEK293T cells were cultured in DMEM supplemented with 10 % FBS, penicillin

and streptomycin. The cells were re-plated one day before cell transfection. 1×10^5 cells were plated on glass-bottom plate and allowed to divide overnight. On the day of transfection, 1 μ l of lipofectamine was added to 25 μ l of OptiMEM and 250 ng of dronpa-GC1qR DNA construct was mixed with 25 μ l. Both mixtures were allowed to stand for 5 minutes at room temperature, followed by mixing both mixtures together and incubate for further 20 minutes to allow DNA-lipofectamine complexes to form. Meanwhile, the cell cultures were washed 3 times with OptiMEM. The DNA-lipofectamine complexes (50 μ l total) was subsequently added to the plates of cells. The cells were transfected overnight with 10% FBS supplemented to the cells 6 hours after transfection. The cells were washed and replaced with fresh media (DMEM + 10% FBS and penicillin and streptomycin) the next day.

3.3.7 Rhodmine 3 calcium imaging

The Rhod-3AM imaging kit (Invitrogen) was used to allow investigation of calcium level changes. Briefly, the cells were washed 3 times in balanced extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM D-Glucose and 10 mM HEPES buffer, pH 7.4) and incubated with 2 ml of loading buffer (consisting 20 μ l of 100x PowerLoad concentrate, 2 μ l of 10mM Rhod-3AM and 20 μ l of 250 mM probenecid, topped up to 2 ml with balanced extracellular solution) for 30 minutes. The cells were washed twice in balanced extracellular solution, followed by incubation with 2 ml of incubation buffer (balanced extracellular solution containing 2.5 mM probenecid) in the dark for 30 minutes. The cells were washed twice and live-cell imaging was performed subsequently using confocal microscopy with HeNe543 laser. The emission wavelength was captured at 570-630 nm. This protocol was used because of the emission wavelength of Dronpa protein overlapped

with Fluo-4 calcium dye. Calcium imaging of dronpa-GC1qR transfected cells was hence carried out using Rhodamine 3 calcium dye instead. The calcium imaging experiment of Dronpa-GC1qR transfected HEK293T cells were carried out 1 day and 3 days post transfection.

3.3.8. *In vivo* surgery and behavioural assessment

Three weeks old male Wistar rats were used for the *in vivo* experiment. The partial sciatic nerve ligation was performed under general anaesthesia. About 50 % of the sciatic nerve on the left hind leg was tightly ligated using 7.0 non-absorbable silk suture (Mersilk, Ethicon). Immediately after ligation, the injured sciatic nerve was wrapped loosely with oxidized regenerated cellulose soaked with 25 µg of GC1qR neutralising antibody, 25 µg control IgG antibody or PBS only. The mechanical sensitivity of the ipsilateral and contralateral hind paws were examined using electronic Von Frey filaments as previously described in section 2.2.6. The experimenter was blinded to the treatment each animal group received and the experimental groups of the animals were randomised. The behavioural assessment was carried out on the post-operative day 1,3 and 5. The described *in vivo* study was done in collaboration with Dr Alessandro Pristera. The behaviour assessment was kindly performed by Dr Alessandro Pristera

3.4. Results

3.4.1. Verification of GC1qR as the receptor for TLQP-21 on primary macrophage by neutralising antibodies

In the previous chapter, it was observed that TLQP-21 induced an increase in intracellular calcium, in a dose-dependent manner, in the primary macrophage. It is also suggested in our laboratory that GC1qR is the potential receptor for TLQP-21. Here I sought to understand if TLQP-21 induced increase in calcium level in macrophage was mediated via this potential receptor. Two experimental approaches were employed here, blocking the receptor by neutralising antibodies and knock down the receptor by siRNA, in combination with calcium imaging to examine if interfering GC1qR signalling affects TLQP-21 induced calcium response in the primary macrophage. This enables us to further verify if GC1qR is the receptor for TLQP-21 on primary macrophage.

Effect of GC1qR neutralising antibodies on TLQP-21 induced calcium response in primary macrophages

Two monoclonal antibodies neutralise GC1qR were used in conjunction with calcium imaging. One of which targets the N terminus of GC1qR at amino acid 76-93 (MAb60.11) and the other targets the C terminus at amino acid 204-218 (MAb74.5.2). The MAb60.11 antibody recognises the mature form (74-282) of GC1qR on the putative C1q binding site. The MAb74.5.2 antibody, on the other hand, reacts with both the mature and truncated forms (lack residues 74-95) of GC1qR. The interactive side of this antibody on GC1qR is the portion where

high-molecular-weight kininogen (HK) and Factor XII (FXII) bind.

The neutralising antibodies were incubated with cultured primary macrophages, either singly or combined, at 3 $\mu\text{g/ml}$ for 15 minutes prior to calcium imaging. Figure 3.5 A shows the averaged fluorescence profile of all cells recorded during calcium imaging after treatment with both antibodies. The previous observed TLQP-21 induced increase in intracellular calcium was abolished in the majority of the cells after neutralising antibodies treatment, whereas, the 1 mM ATP was able to elicit a robust increase in calcium level, indicating the cells were viable after antibodies treatment. To further elucidate the effect of neutralising GC1qR on the cell responsiveness to TLQP-21 in the calcium imaging, I further quantified the percentages of cells responding to the peptide after treatment of MAb60.11 only, MAb74.5.2 only, or both antibodies together. Figure 3.5B shows that after treatment of both antibodies for 15 minutes, the percentage of cells responding to 100 nM TLQP-21 peptide significantly decreased from $67.04 \pm 2.24 \%$ to $10.56 \pm 3.97 \%$ ($p < 0.01$, $n = 3$, One way ANOVA, Tukey post hoc test), indicating there was a decrease in responsiveness to TLQP-21, at 100 nM, by 84.25 %. Moreover, when the cells were pre-treated with MAb60.11 or Mab74.2.5 antibody only, the percentages of cells responding to the peptide also significantly decreased to $18.46 \pm 7.84 \%$ and $32.45 \pm 6.67 \%$, respectively ($p < 0.05$, $n = 3$ for both treatments), indicating a drop of 72.46 %, and 51.60 % in responsiveness to the peptide. Together these data suggest neutralising, or blocking, GC1qR interferes the responsiveness of primary macrophages to TLQP-21, at least at the concentration tested (100 nM) here. This implies the GC1qR is likely to be the potential receptor for TLQP-21, and the function of TLQP-21 was signaled, at least partly, via GC1qR.

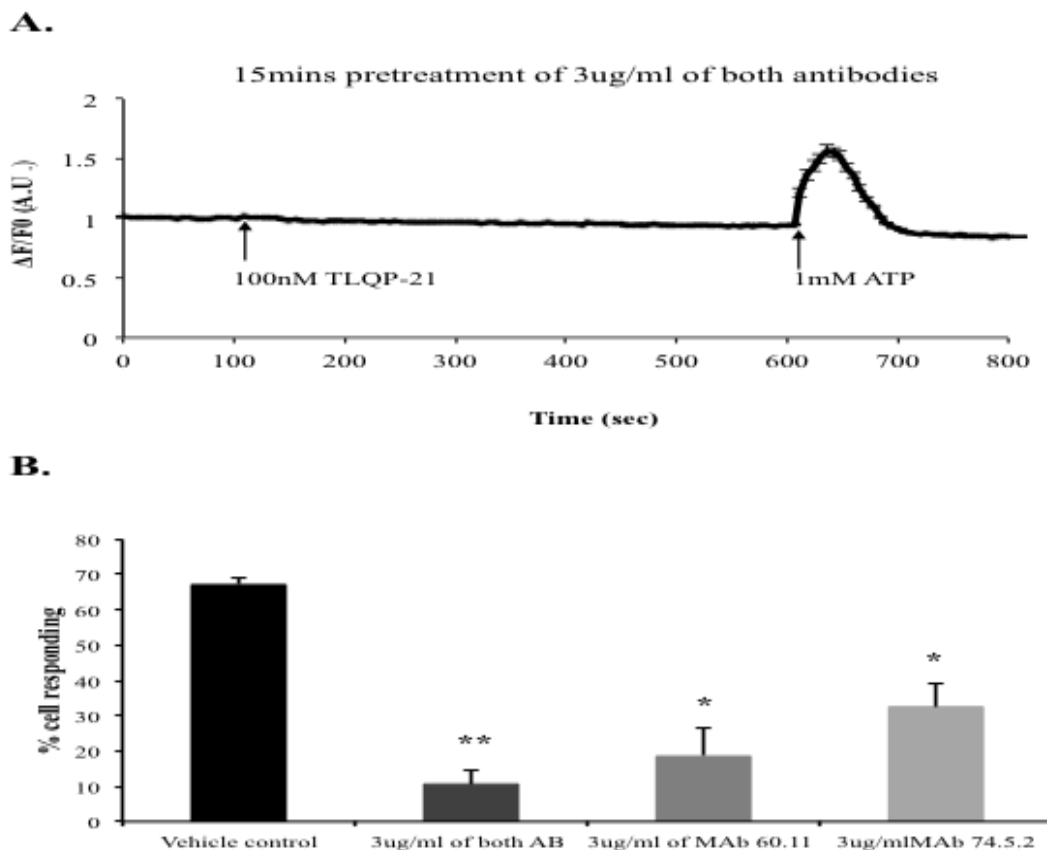


Figure 3.5. 15 mins pre-incubation of anti-GC1qR antibodies attenuates the TLQP-21 induced calcium response and reduces the percentage of cells responding to the peptide.

A. Represented averaged fluorescence trace from calcium imaging of macrophage pre-treated with both GC1qR neutralising antibodies (3 μ g/ml). The averaged fluorescence demonstrated the calcium levels were not increased after 100 nM TLQP-21 application. Yet, the positive control at the end of experiment, 1 mM ATP, induced a robust increased in intracellular calcium, indicating the cells were viable and able to respond. **B.** Pre-incubation with GC1qR specific antibodies MAb60.11, MAb74.5.2 or both antibodies together (3 μ g/ml) significantly reduced the percentage of cells responding to 100 nM TLQP-21 peptides (* p <0.05, ** p <0.01, One way ANOVA, Tukey post hoc test, n = 3)

Experiment number	Percentage of cells responding (%)	Number of cells responded/total cells for analysis
Vehicle control		
1	70.8	92/130
2	63.0	75/119
3	67.3	136/202
3ug/ml of both antibodies		
1	12.5	16/128
2	2.9	5/171
3	16.3	13/80
3ug/ml of MAb 60.11		
1	17.1	25/146
2	5.6	8/143
3	32.7	49/150
3ug/ml MAb 74.5.2		
1	45.1	78/173
2	22.4	33/147
3	29.8	45/151

Table 3.1 Table listed the number of cells analysed and responded to 100nM TLQP-21 in each experiment (A minimum of 80 cells analysed for each experiment).

3.4.2. Verification of GC1qR as the receptor for TLQP-21 on primary macrophage by siRNA approach

To further confirm that TLQP-21 signals through GC1qR to exert its function on the primary macrophage, a second method was employed, small interfering RNA (siRNA) gene manipulation, to interfere with this pathway. siRNA is a method of ‘silencing’ gene expression *in vitro* and could be used to down-regulate endogenous GC1qR expression. Although the silencing effect will only be transient and the level of knock-down in gene expression may be incomplete, it may still be enough to deliver a functional change in response to TLQP-21. Here we sought to silence GC1qR expression in the primary macrophage by siRNAs, and examine the subsequent cell responsiveness to TLQP-21. To achieve this, a protocol to introduce siRNAs into the primary macrophage needed to be first established.

Primary macrophage siRNA transfection optimisation

Delivering siRNAs to primary macrophage using lipid-based transfection method has been evident to alter macrophage physiology and often yield low transfection rate (Zhang, Edwards et al. 2009). To attempt delivering siRNA into the primary macrophage, I employed two electroporation systems, the Neon capillary electroporation system (Invitrogen) and the GenePulser cuvette electroporation (BioRad). The GenePulser cuvette electroporation allows a higher electric field to be delivered, which could be advantageous for cells hard to be transfected. On the other hand, the capillary electroporation system is suggested to generate a more uniform electric field that results in better cell survival and allows better maintenance.

Alexa Fluor 546- tagged control siRNA, that does not show any homology to any mammalian gene, was introduced to the cells to aid visual determination of successful transfection. The electroporation conditions tried using the Neon system failed to deliver siRNA into the primary macrophage (table 3.2), except for one cell which was transfected using 2200V voltage, 20ms Pulse width with 2 electroporation pulses condition. Nevertheless, cuvette electroporation using GenePulser system successfully showed positive transfection in three of the conditions tested, 100V:150 μ F:100 Ω , 200V:150 μ F:100 Ω and 400V:150 μ F:100 Ω (voltage: resistance: capacitance, figure 3.6). Keeping the siRNA concentration constant (3 μ g), 400V:150 μ F:100 Ω appeared to produce the greatest number of transiently transfected cells. The number of cells transfected with siRNA in the 200V:150 μ F:100 Ω condition was high as well. However, 100V:150 μ F:100 Ω condition showed relatively lower number of transfected cells. The untreated control showed no background fluorescence, indicating the fluorescence observed in the transfected cells were not derived from cell autofluorescence. Moreover, the same amount of siRNA was used for electroporation for 50V:150 μ F:100 Ω condition, and the result shows little fluorescence, indicating the success transfection using other three conditions were not due to residual siRNAs in the media.

Furthermore, I tested the cell viability in the three conditions that shows positive transfections by using trypan blue. As shown in figure 3.7, the percentages of trypan blue positive cells after electroporation were 4.47%, 10.71%, 11.21% and 28.73%, for untreated control, 100V, 200V and 400V conditions, respectively. It is worth noting that there was a low percentage of cells showed trypan blue positive staining even in the untreated control, which may possibly indicates some cells became unhealthy during the harvesting procedure. Nevertheless, electroporation of the cells

caused further increase in numbers of trypan blue positive cells with a particular high percentage when using 400V voltage. Together with these data, in terms of both cell survival and transfection efficiency, 200V:150 μ F:100 Ω was shown to be the optimal condition for high transfected cell number and cell survival. It was hence chosen to be the condition used for all the subsequent work involved siRNA transfection into primary macrophage.

Pulse voltage (V)	Pulse width (ms)	Number of pulses	Cell density (cell/ml)	siRNA concentration used (μ g)	Number of cells transfected
1680	30	1	5 x 10 ⁵	0.3	0
1680	20	1	5 x 10 ⁵	0.3	0
1680	10	2	5 x 10 ⁵	0.3	0
1900	30	1	5 x 10 ⁵	0.3	0
1900	20	1	5 x 10 ⁵	0.3	0
1900	10	2	5 x 10 ⁵	0.3	0
2200	20	1	5 x 10 ⁵	0.3	0
2200	10	2	5 x 10 ⁵	0.3	0
2200	20	2	5 x 10 ⁵	0.3	1

Table 3.2 Table listed all the conditions tested using the Neon capillary electroporation system.

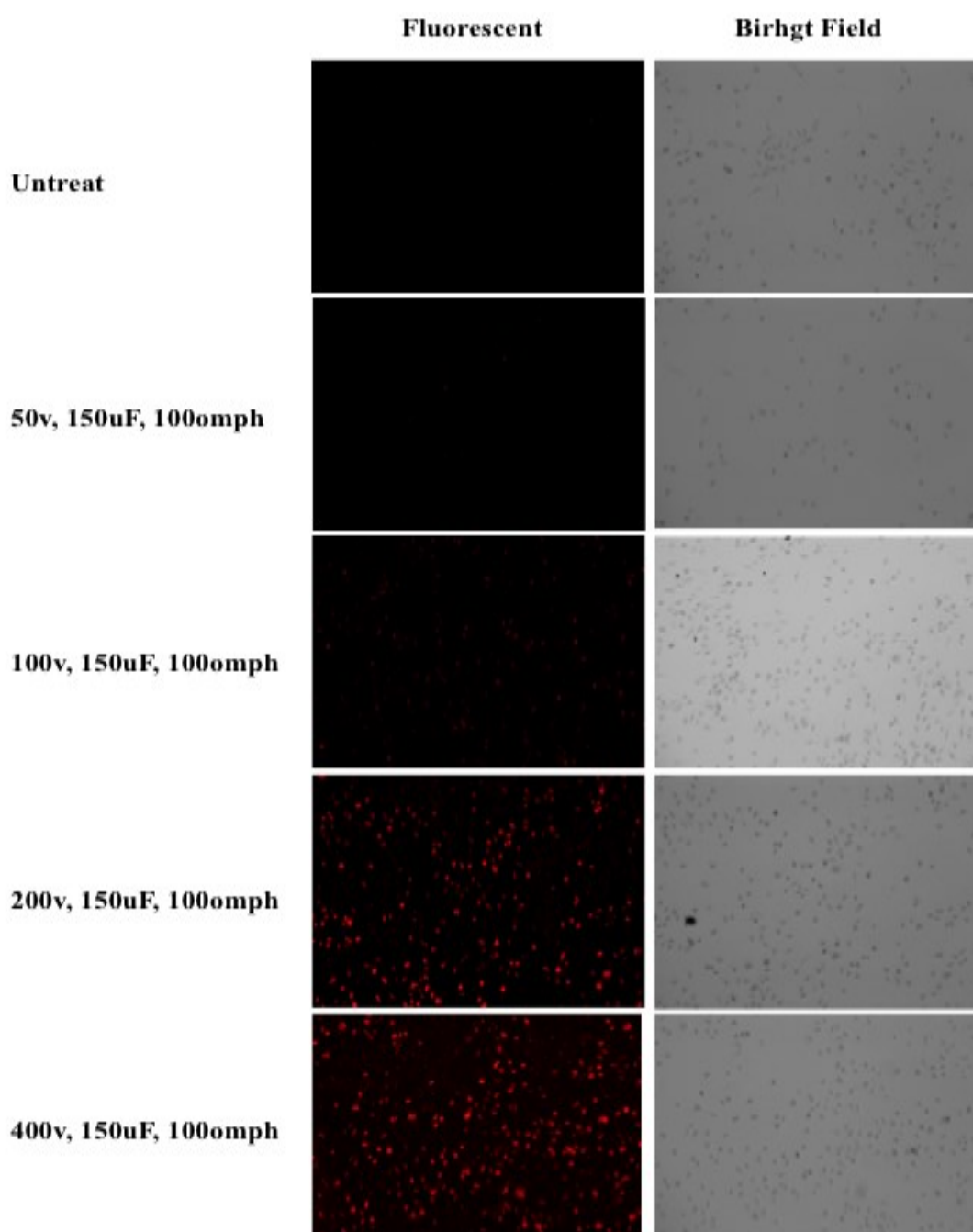


Figure 3.6 GenePulser electroporation system successfully introduced siRNA into the primary macrophages.

Alexa Fluor 546 tagged non-targeting siRNA was used to optimise the electroporation conditions for primary macrophage siRNA transfection. The fluorescent images and its corresponding bright field images were taken, and the success in transfection was monitored by the red fluorescence observed inside the cells. Condition 100V:150 μ F:100 Ω , 200V:150 μ F:100 Ω and 400V:150 μ F:100 Ω (voltage: resistance: capacitance) showed successful transfection of siRNA as indicated by the red florescence. The untreated control showed no fluorescent, indicating the fluorescence observed in the mentioned conditions were derived from the Alexa Fluor 546 tagged, not from the auto-fluorescence of the cells.

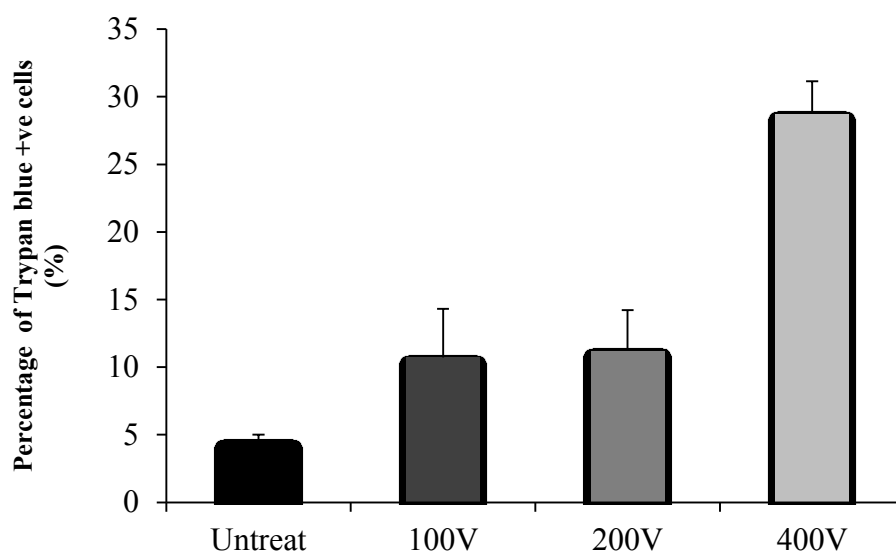


Figure 3.7 Cell viability assay by Trypan blue after electroporation.

Primary macrophages were transfected with non-targeting siRNA by electroporation (1,000,000 cells per electroporation) using the following conditions: 100V:150 μ F:100 Ω , 200V:150 μ F:100 Ω and 400V:150 μ F:100 Ω (voltage: resistance: capacitance). The untreated cells were harvested under the same, but did not underwent electroporation. The cell suspension was topped up to 1ml by complete media after electroporation. 10 μ l of the cell samples were diluted 1:1 with a sterile 0.4% Trypan Blue solution. The dilution was filled into a hemocytometer and allowed to incubate for 2 minutes at room temperature. The non-viable cells would be blue and the viable ones would be unstained. The cells were counted under a microscope and the percentage of trypan blue positive cells was calculated. In the untreated cells that underwent the harvesting procedure, about 4.47% of the cells were non-viable, and the percentage of non-viable cells increased to 10.71%, 11.21% and 28.73% after electroporation using 100V:150 μ F:100 Ω , 200V:150 μ F:100 Ω and 400V:150 μ F:100 Ω settings, respectively. The error bar was expressed as standard deviation between the two independent experiments carried out.

Percentage of Trypan blue +ve cells %(no. of +ve cells/total cells)				
Condition	Untreated	100V	200V	400V
	4.08 (4/98)	8.16 (8/98)	9.09 (8/88)	30.43 (21/69)
	4.85 (5/103)	13.25 (11/83)	13.33 (12/90)	27.03 (30/111)
Average	4.47	10.71	11.21	28.73

Table 3.3 Table listed the number of blue cells (non-viable cells), total number of cells counted and the percentage of trypan blue positive cells in the hemocytometer for each experiment.

Validation of downregulation by qRT-PCR

Having established the optimal electroporation condition for siRNA transfection into the primary macrophages, the cells were transfected with different siRNAs targeting GC1qR. To explore the changes in GC1qR expression levels, real-time quantitative PCR was employed as the readout assay. The two most commonly used analyses for qRT-PCR are the absolute quantification (standard curve method) and relative quantification ($2^{-\Delta\Delta C_T}$ method). The absolute quantification allows the input copy number to be determined and a standard curve for each gene target is essential to relate the PCR signal. On the other hand, instead of measuring the absolute amount of gene expression, the relative quantification compares the PCR signal of the target gene in a control group to that of the treatment groups. The relative quantification, i.e. $2^{-\Delta\Delta C_T}$ method, is a convenient analysis to determine the relative changes in gene expression after siRNA knock down experiment, where the absolute expression of the gene is not as important, and hence was used here as the analysis method.

In order to perform the relative quantification from the qRT-PCR experiment, and for the $2^{-\Delta\Delta C_T}$ calculations to be valid, the amplification efficiencies for the target gene primer and house keeping gene primer must be approximately equal. Therefore, the primers efficiencies were first verified by performing qRT-PCR on a range of dilutions of the macrophage cDNA. The amplification curves and the melt curves for the target genes (GC1qR 1 and GC1qR 2) and house keeping genes (CyclophilinA, Beta actin, GAPDH) were monitored (figure 3.8). The amplification curves show all the primers tested, at all template concentrations, increased in the fluorescent detected, implying the success in PCR amplification. Moreover, all the amplification curves showed good C_T values, suggesting detectable copy numbers of gene at the

dilation made. The melt curve for GC1qR 1 and Cyclophilin A show perfect single peak curve, indicating only one product was generated and the primer specificities. On the other hand, beta actin (at high concentration only), GC1qR 2 and GAPDH all show additional product peak at higher melting temperature, implying multiple products were produced. The additional peaks in the melt curve for beta actin and GC1qR 2 were minimal when compared to the main product peak, which could possibly be optimised by reducing primer concentration used, and hence were still used for the subsequent test of the primer efficiencies. However, the GAPDH primer shows strong amplifications of more than one product, which may make the interpretation of primer efficiency difficult.

The differences in efficiencies between the target and house keeping genes primers were calculated (ΔC_T), and the log cDNA concentrations were plotted against the resultant ΔC_T . The slope of the linear trendline was the indicator of the variability between the efficiencies of the primers. If there were no differences in the efficiencies of the two primers, it will result a slope value of 0. If the slope values were good (<0.1), this indicates there were minimal variations between the amplification efficiencies using the two primers compared, and hence are suitable for the use in $2^{-\Delta\Delta C_T}$ analyses. However, if the slope value is >0.1 , the primers should not be used together for the $2^{-\Delta\Delta C_T}$ analysis to prevent false interpretation of the qPCR results.

The validation experiment for the GC1qR primers and the house-keeping gene, beta actin was performed, which resulted a slope of 0.0019 (GC1qR 1) and 0.0546 (GC1qR 2), confirming comparable primer efficiencies between the two GC1qR primers to beta actin primer (figure 3.9A). Figure 3.9B also shows minimal

variations in efficiencies between the two GC1qR primers and the GAPDH primer, as the resultant slope were 0.0022 and 0.0549 for GC1qR 1 and GC1qR 2, respectively, when compared to GAPDH. Comparable efficiencies were also observed between the two GC1qR primers and Cyclophilin A primer (figure 3.9C), as indicated by the slope of 0.0448 (GC1qR 1) and 0.0879 (GC1qR 2). These data confirmed the primer efficiencies between the primers for target gene (GC1qR 1 and GC1qR 2) and that for house-keeping genes (Beta actin, GAPDH and Cyclophilin A) were similar with minimal variations. However, when the primers were tested in a no-template reaction, GC1qR 2 primer showed an increase in amplification curve with C_T value around 27. This indicates the primer could amplify in a qPCR reaction without template, which could lead to wrong interpretation of gene expression, and hence was not suitable for subsequent studies.

From the results obtained here, GAPDH primer resulted in multiple products generated (as indicated in melt curve) and GC1qR 2 primer exhibited self-amplification property in no-template reaction, and hence both primers were discarded from use in the later studies. Nevertheless, GC1qR 1 primer shows good amplification efficiency and the efficiency showed minimal variation to beta actin and cyclophilin A primers, thus were utilised in the subsequent experiments.

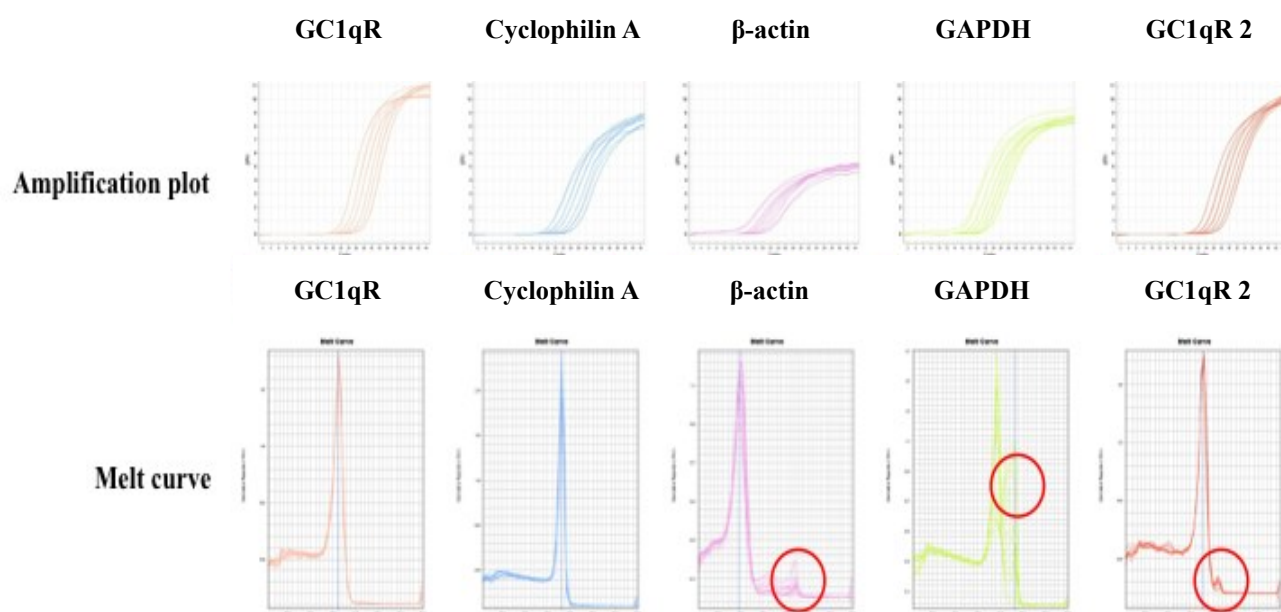


Figure 3.8 Amplification plot and Melt curve of the primers tested using qPCR revealed successful amplifications after serial dilutions.

The GC1qR, Cyclophilin A, Beta actin, GAPDH and GC1qR2 primers sets were tested for suitability to be used in qPCR experiment. The primers tested by qPCR experiments on a range of dilutions of the macrophage cDNA. The melt curves of all primer tested showed desirable C_T values, and the melt curves showed a major single peak for majority of primers tested, except for GAPDH. Beta actin and GC1qR2 primers also showed a small additional peak at higher melting temperature (additional peaks indicates by red circles.)

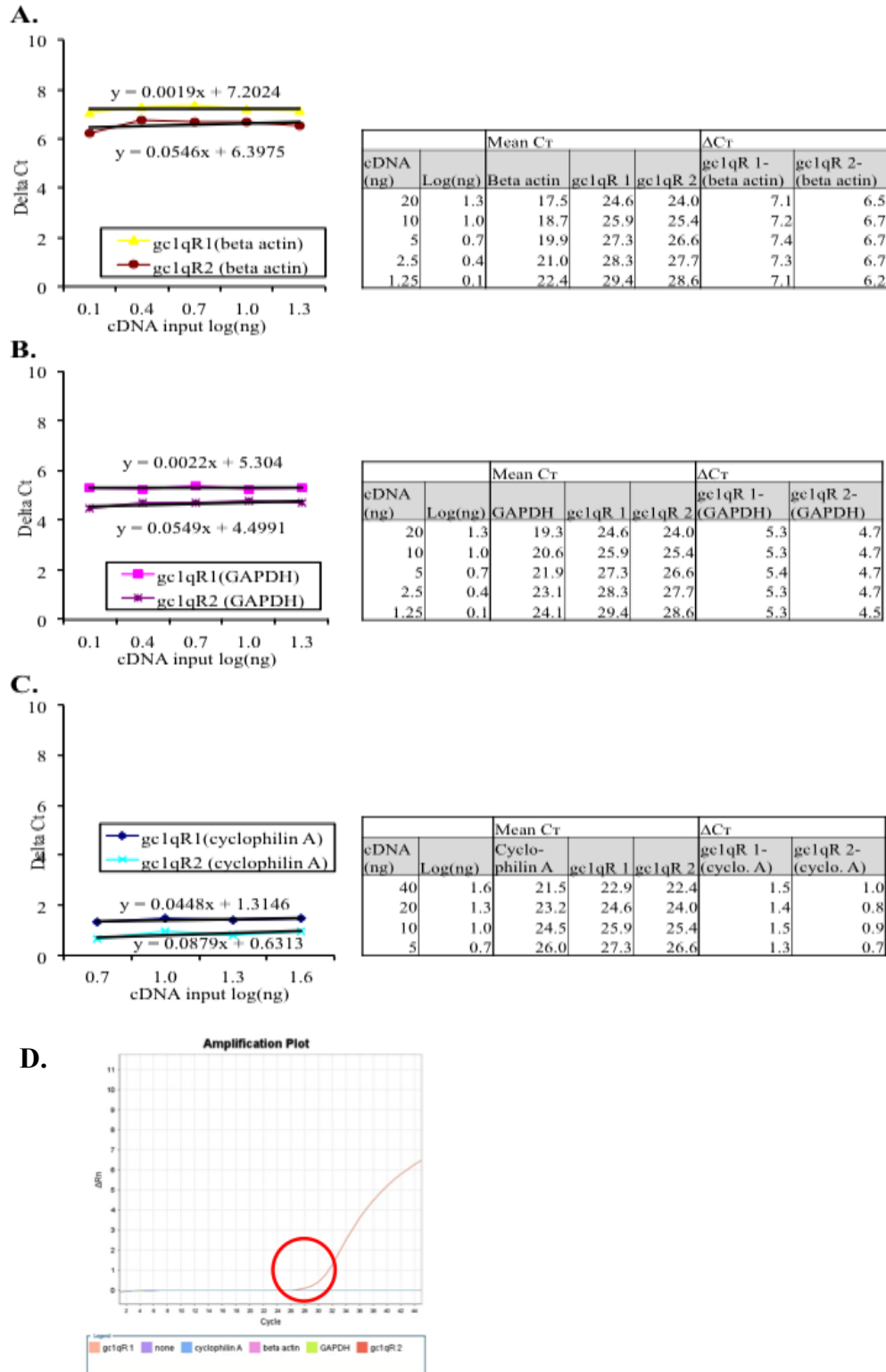


Figure 3.9 The slopes of the primers efficiencies tests revealed there were only minimal variations between the primers for target gene and that for reference genes.

Primer efficiencies test between **A.** GC1qR1/GC1qR2 and beta actin. **B.** GC1qR1/GC1qR2 and GAPDH **C.** GC1qR1/GC1qR2 and Cyclophilin A. **D.** Primer sets tested in the absence of cDNA template revealed self annealing property of GC1qR2.

Having determined the suitability of the primers being used, the siRNA-mediated down-regulation of GC1qR was validated by qPCR. The GC1qR expressions were examined in untreated cells, non-targeting siRNA transfected cells and GC1qR-siRNAs transfected cells. Figure 3.10 shows the amplification plot and melting curve for GC1qR, beta-actin and cyclophilin A in the 24 hrs-post transfection samples. The amplification curve of the house-keeping genes (beta actin and cyclophilin A) showed similar C_T values in all samples, indicating reliability in pipetting and cDNA synthesis. A shift in amplification curve of GC1qR was observed, implying there was a difference in GC1qR expression between samples. The melt curves showed single peaks for all products generated, confirming good PCR quality and amplification specificity. To quantify the changes in GC1qR expression between samples, $2^{-\Delta\Delta C_T}$ calculations were performed. Figure 3.11A and 3.11B show the percentage of GC1qR expression in all samples at 24 hrs- and 48 hrs-post transfection, respectively (the raw data are listed in table 3.4). 24 hrs- post transfection, GC1qR mRNA expressions decreased 73.5%, 22.6%, 77.6%, 81.8%, 36.1%, and 47.6% by siRNA1, siRNA2, siRNA3, siRNA4, siRNA mix and modified siRNA, respectively, when compared to the non-targeting siRNA transfected (nt-siRNA) control. It is worth noting that untreated control (without electroporation) showed similar expression to the nt-siRNA control 24hrs after transfection (101.7% and 100% for untreated and nt-siRNA, respectively), indicating the electroporation treatment itself did not alter GC1qR expression. 48hrs post transfection, all samples showed at least 60% decreased in GC1qR expression at mRNA levels when compared to nt-siRNA control (reduced in GC1qR expression by 64.1%, 85.9%, 75.6%, 79.3%, 78%, 63.2% for siRNA1, siRNA2, siRNA3, siRNA4, siRNA mix and modified siRNA transfected samples, respectively). Still, the GC1qR expression in the untreated control was similar to nt-siRNA control 48

hours after transfection (84.7% and 100% for untreated and nt-siRNA, respectively). Together, these data show robust down-regulation of GC1qR at mRNA level by all the siRNA tested.

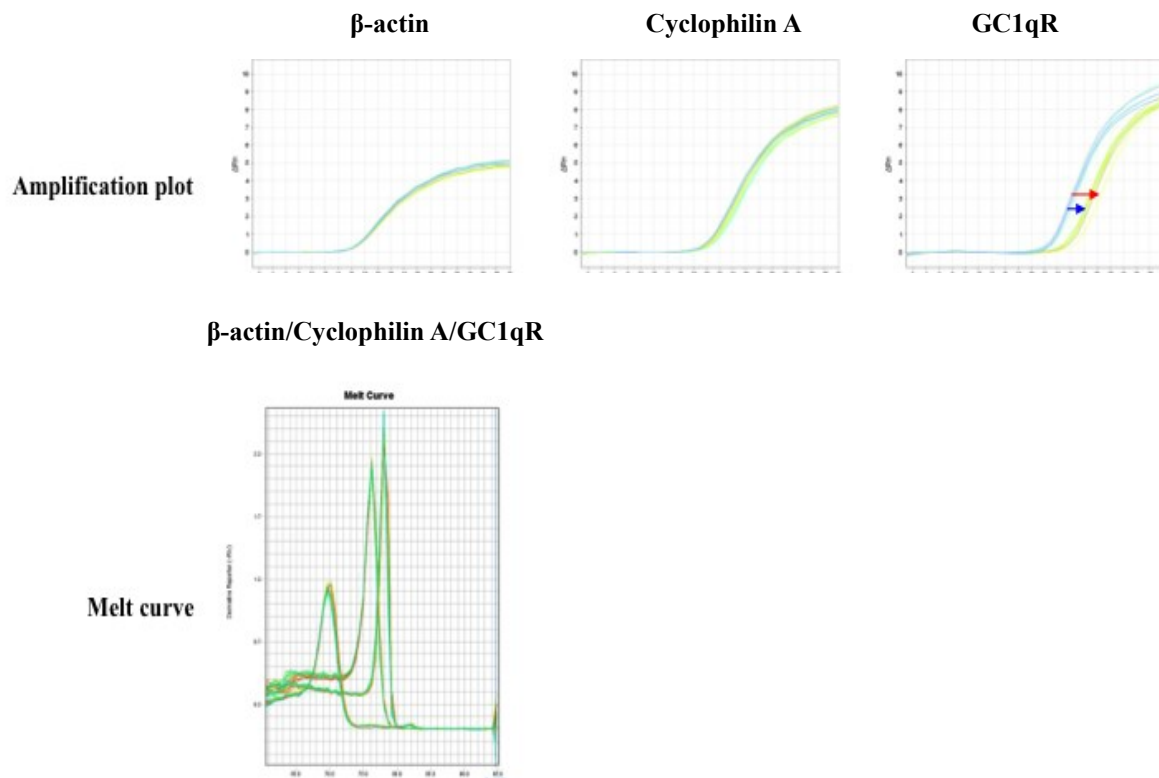
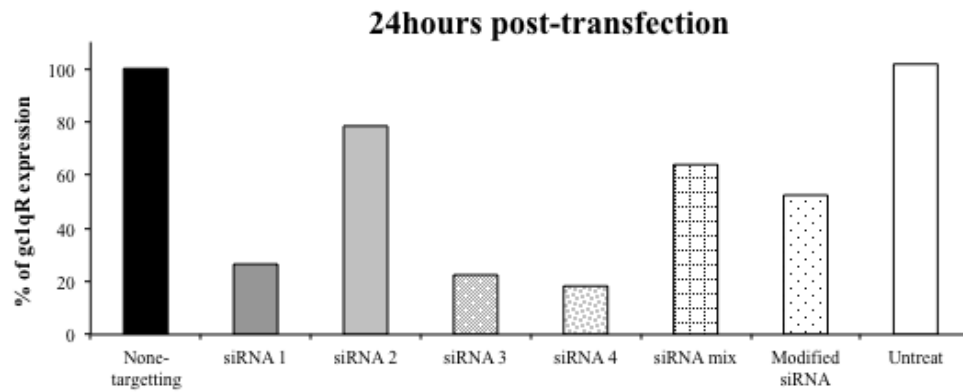


Figure 3.10 qPCR results from the siRNA transfected cells versus untreated control. Amplification plot showed consistency of C_T values on reference genes amplifications across all samples. Whereas, changes in C_T values were observed in GC1qR amplifications (as indicated by red and blue arrows). The melting curves demonstrated all amplifications were resulted from a single amplified product, as indicated by a single peak produced.

A.



B.

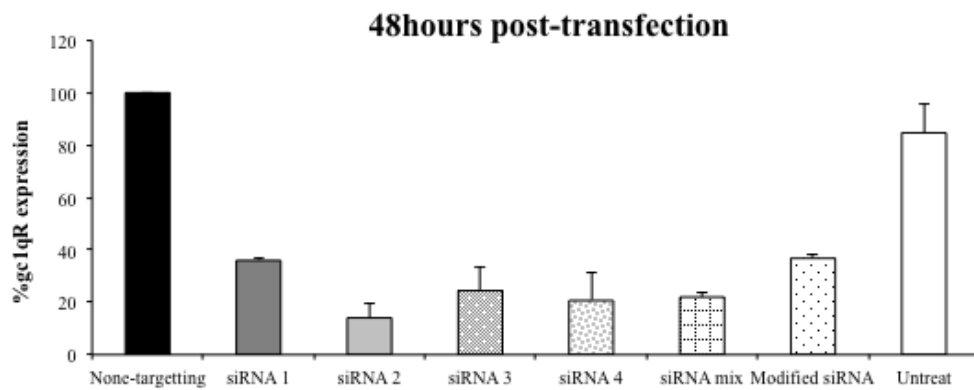


Figure 3.11 qPCR revealed downregulation of GC1qR mRNA in primary macrophage 24 hours- and 48 hours- post-transfection.

Transfection of siRNA against GC1qR (siRNA 1-4, mixed siRNA and modified siRNA with Alexa Fluor 546) into primary macrophage successfully reduced GC1qR mRNA expression **A.** 24 hours and **B.** 48 hours after transfection when compared to both untreated and non-targeting siRNA transfected controls. The data presented were normalised to Cyclophilin A house-keeping gene, and were expressed as percentage of non-targeting siRNA control.

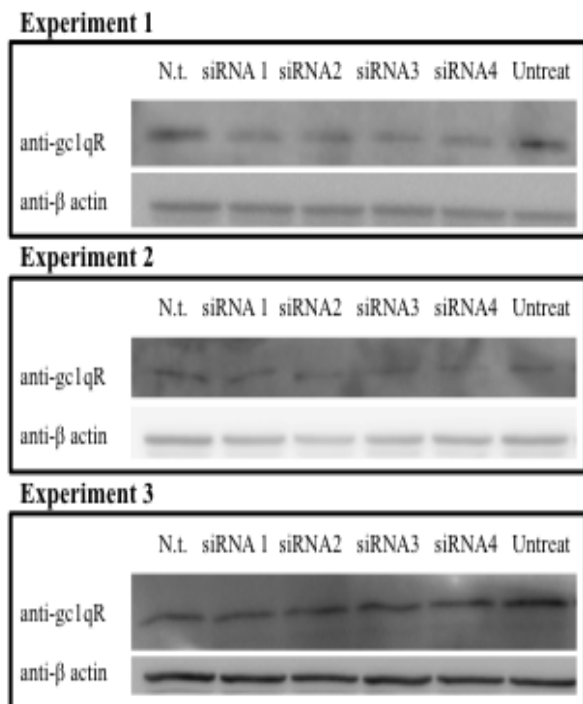
% gc1qR mRNA expression 24hours after transfection								
	None-targetting	siRNA 1	siRNA 2	siRNA 3	siRNA 4	siRNA mix	Modified siRNA	Untreat
Experiment 1	100.0	26.5	78.4	22.4	18.2	63.9	52.4	101.7
% gc1qR mRNA expression 48hours after transfection								
	None-targetting	siRNA 1	siRNA 2	siRNA 3	siRNA 4	siRNA mix	Modified siRNA	Untreat
Experiment 1	100.0	35.2	17.8	30.7	28.3	20.7	76.7	35.7
Experiment 2	100.0	36.7	10.3	18.1	13.1	23.2	92.7	37.9
Average	100.0	35.9	14.1	24.4	20.7	22.0	36.8	84.7
S.D.	0.0	1.0	5.3	8.9	10.8	1.7	1.5	11.3

Table 3. 4 Table listed the raw data of all qPCR experiments carried out for figure 3.11.

Validation of downregulation by Western blotting

Although the GC1qR expressions were shown to be downregulated at mRNA level by siRNA tested, the changes in GC1qR protein expressions still need to be examined as mRNA expression level does not always correlate to its protein expression level. It was observed after electroporation, there were much debris in the culture, and such debris possibly made the cells more restless as spontaneous increase in calcium were observed 24 hours after electroporation in the majority of the cells in the later calcium imaging experiment. The spontaneous activations of the cells made the calcium imaging analysis difficult, as it would be hard to interpret if the increase in intracellular calcium was due to peptide stimulation or spontaneous activity of the cells. For this reason, the cultures were washed twice and the media were changed 24 hours after electroporation, and the cells were allowed to settle for another 24 hours before being used for calcium imaging. GC1qR protein expressions were hence quantified and compared, by western blotting, 48 hours after transfection. Figure 3.12A shows the GC1qR protein expressions in the nt-siRNA control, siRNAs-transfected samples and the untreated control in all western blotting experiments. The percentages of GC1qR protein expressions to nt-siRNA control significantly decreased to $50.6 \pm 10.2\%$, $40.5 \pm 13.1\%$, $58.7 \pm 9.9\%$ and $55.5 \pm 14.5\%$ by siRNA1, siRNA2, siRNA3 and siRNA4, respectively (figure 3.12B * $p < 0.05$, ** $p < 0.01$, raw data listed in table 3.5). The GC1qR protein expressions in nt-siRNA controls did not significantly differ from that of untreated controls ($98.3 \pm 4.0\%$). The data here suggests all four siRNAs tested showed successful downregulation of GC1qR at protein level 48 hours post transfection.

A.



B.

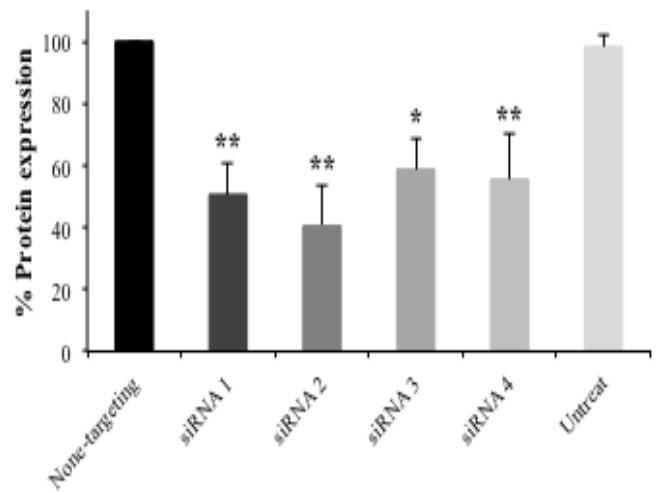


Figure 3.12 Western blot analysis demonstrated the downregulation of GC1qR protein in primary macrophages 48 hours after transfection.

Transfection of siRNA against GC1qR (siRNA 1-4) into primary macrophages significantly reduced GC1qR protein expression after 48 hours. Non-targeting siRNA (negative control) and untreated control did not show alteration in GC1qR expression. **A.** Western immunoblots from three independent experiments **B.** Quantification of the bands intensity from part A by ImageJ analysis. Error bar expressed as standard error means. * $p < 0.05$, ** $p < 0.01$, One way ANOVA Fisher's post hoc test, $n = 3$

	None-targeting	siRNA 1	siRNA 2	siRNA 3	siRNA 4	Untreat
experiment 1	100	60.8	32.5	38.9	52.4	103.3
experiment 2	100	30.1	22.7	69.5	32.1	90.5
experiment 3	100	60.8	66.1	67.6	82.1	101.1
Average% protein expression	100	50.6	40.5	58.7	55.5	98.3

Table 3.5 Raw data for the quantification of the band intensities from immunoblots. Protein expression is normalised to the non-targeting control for comparison.

Effect of GC1qR knock-down by siRNAs on TLQP-21 induced calcium response in primary macrophage

To elucidate if knocking down GC1qR by siRNA alters the TLQP-21 induced calcium response in the primary macrophage, calcium imaging was performed on siRNA-transfected cells, nt-siRNA transfected control cells and untreated cells. The percentages of cells responding to 100nM were determined for each condition, and the averages of the percentages of cells responding for each condition was normalised to the nt-siRNA control. As shown in figure 3.13, the percentage of cells responding to 100nM TLQP-21 was significantly reduced by 27.7%, 43.5%, 33.8% and 29.3% 48 hours after transfection of siRNA1, siRNA2, siRNA3 and siRNA4, respectively, when compared to nt-siRNA control (*p<0.05, **p<0.01, n=4). The percentage of cells responding to the peptide in nt-siRNA transfected cells did not significantly differ from that in untreated control. The raw data of each experiment was summarised in table 3.6.

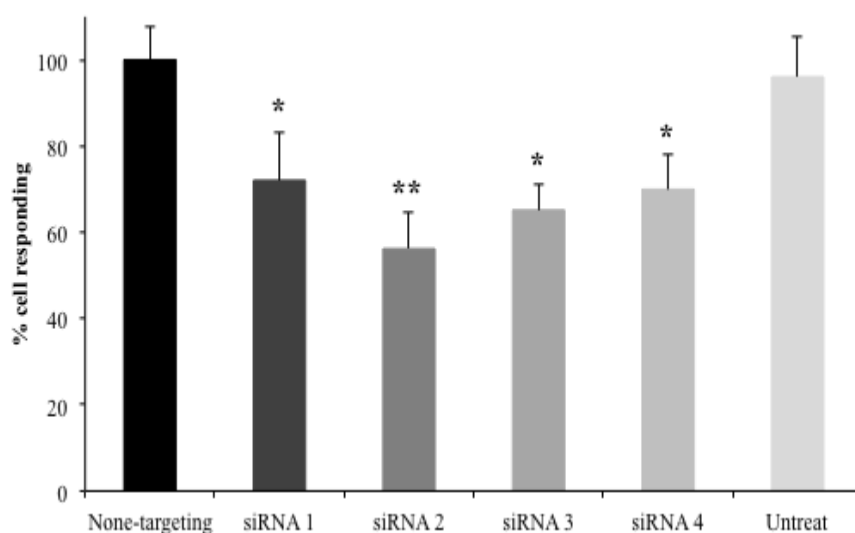


Figure 3.13 GC1qR knock-down by siRNA significantly reduced the percentage of cells responding to 100nM TLQP-21 48hours post-siRNA transfection.

Error bars expressed as standard error means. *p<0.05, **p<0.01, One way ANOVA Fisher's post hoc test (n=4). A minimum of 183 cells were analysed per experiment.

	None-targeting	siRNA 1	siRNA 2	siRNA 3	siRNA 4	Untreat
Experiment 1	84.2% (154/183)	40.1% (99/247)	54.8% (120/219)	64.5% (158/245)	44.8% (137/306)	91% (110/120)
Experiment 2	81.8% (202/248)	54.7% (110/201)	26.0% (81/312)	46.3% (184/397)	72.4% (142/196)	91.8% (201/219)
Experiment 3	60% (117/195)	42.9% (79/184)	34.1% (63/185)	31.7% (98/309)	35.5% (110/310)	42.8% (89/208)
Experiment 4	86.4% (228/264)	88.1% (193/219)	61.4% (129/210)	64.3% (151/235)	68.1% (154/226)	79.9% (191/239)
% responding cells (%R.C.): Average	78.1	56.5	44.1	51.7	55.2	76.4
%R.C. normalised to none-targeting control	100.0	72.0	56.2	65.1	70.0	96.0

Table 3.6 Table summarises the results the percentage of cells responding to 100nM TLQP-21 after GC1qR knockdown by siRNA.

3.4.3. Overexpression of GC1qR-dronpa fusion protein in HEK-293T cells

Whilst having demonstrated that TLQP-21 induced response on primary macrophage was reduced when GC1qR was ‘blocked’ or ‘downregulated’, over-expressing recombinant GC1qR in cells that are naturally unresponsive to TLQP-21 and examine its effect would provide us with further evidence on the involvement of GC1qR in the TLQP-21 pathway. HEK-293T cells, as had been demonstrated by Dr Mahmood Ayub previously, are unresponsive to TLQP-21 peptide by using the calcium imaging. Here, I aimed to clone and overexpress recombinant GC1qR with Dronpa fluorescent protein at its C terminus in HEK-293T cells, followed by examining the effect of TLQP-21 peptide on the GC1qR overexpressed HEK-293T cells.

Cloning and overexpressing GC1qR in the HEK-293T cells

For obtaining the GC1qR clone, T/A cloning was first employed. The rat macrophage cDNA was used as template for PCR amplification using Taq polymerase, which allowed the resultant PCR product to harbor 3’ A-overhangs. The PCR product was ran on a DNA gel to confirm the size. As shown in figure 3.14A,

the resultant PCR products had an expected size of 840bp, which were subsequently purified from the gel and ligated with pGEM®-T Easy (pGEM-T) vectors. Positive clones were selected by blue-white colony screening in the presence of IPTG and Xgal. The plasmid DNA was purified from the selected positive clones, and subsequently treated with restriction enzymatic digestion to confirm the insert sizes. The two positive clones selected showed expected sizes of 840bp and 3015bp for GC1qR insert and pGEM-T vector respectively, after enzymatic digestion (Figure 3.14B). The insert was excised from the pGEM-T cloning vector using BamHI and XhoI restriction enzyme and subcloned into the CoralHue® Dronpa-Green expression vector. The success in subcloning was confirmed by restriction enzymatic digestion followed by resolving fragments on a DNA gel. As indicated in figure 3.14C, the two fragments resulting from the enzymatic digestion showed correct sizes of 840bp and 4696bp for GC1qR insert and pDG1-MN1 vector, respectively. The sequence of GC1qR in this clone was confirmed by DNA sequencing.

Having obtained the desire construct, the HEK-293T cells were transfected with the either the Dronpa-GC1qR construct or Dronpa (vector only) control using lipofetamine based method. The Dronpa protein is a photochromic version of the Green fluorescent protein (GFP). The transfected cells were hence observed under a fluorescence microscope 1 day- and 3 days- post transfection. The cells transfected with Dronpa control showed homogeneous green fluorescence in the whole cell without particular compartment accumulation of fluorescence (figure 3.15).

Interestingly, most of the Dronpa-GC1qR transfected cells exhibited ‘ring-shape’ green fluorescence in the cell 1 day after transfection. Such expression was observed in the majority of the transfected cells 3 days post-transfection, suggesting specific compartmentalisation of GC1qR expressed in the HEK-293T cells.

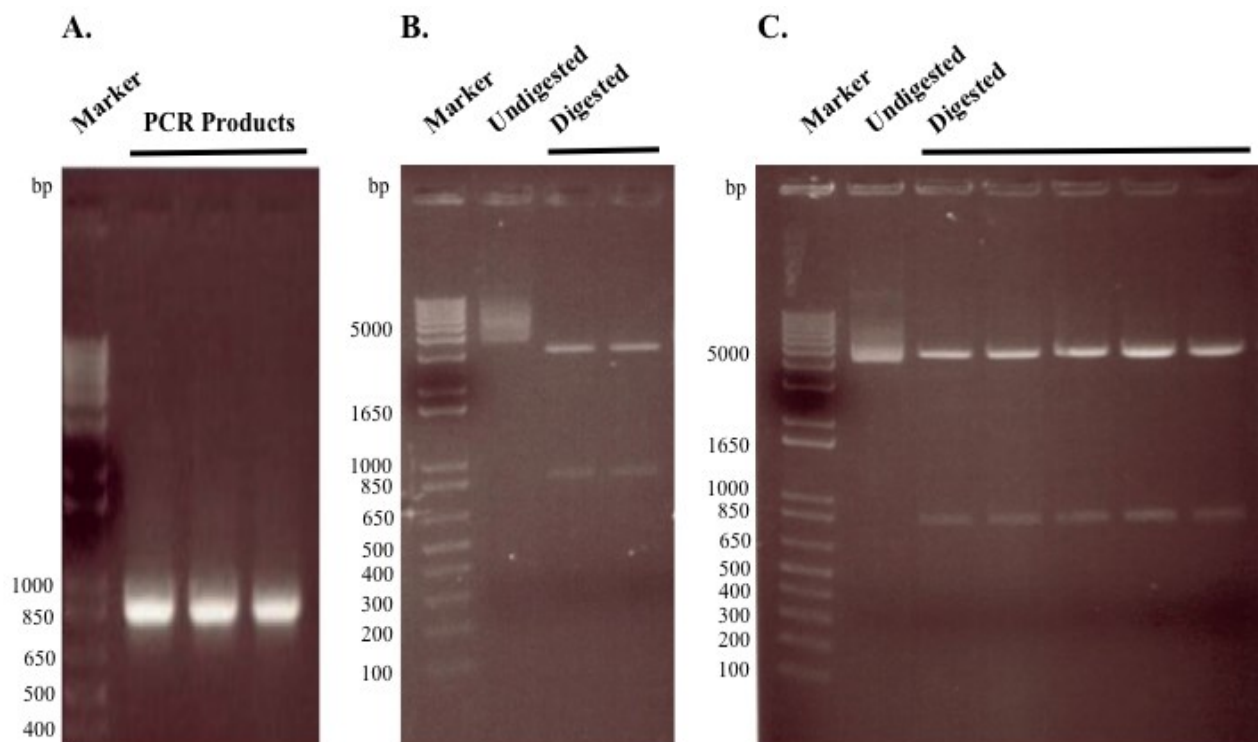


Figure 3.14 Successful recombinant DNA construct was generated for GC1qR expression with Dronpa fluorescent protein tag at the C-terminus.

DNA gel revealing **A.** Successful amplification of GC1qR sequence from macrophage cDNA

B. Successful cloning of GC1qR sequence into pGEM-T vector and **C.** Successful inserted

GC1qR coding sequence into Dronpa vector.

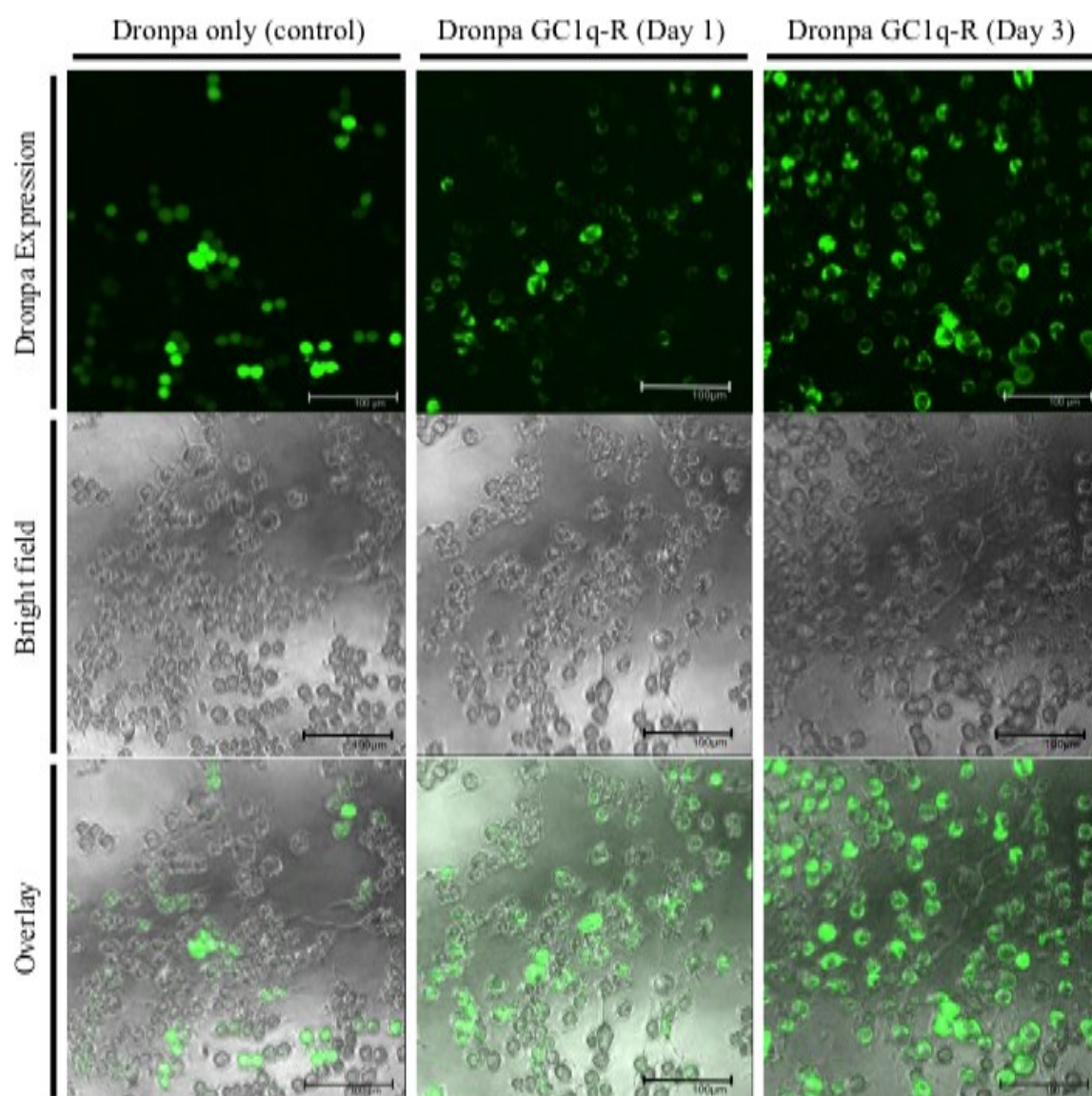


Figure 3.15 Microscopic images revealed successful transfection of HEK-293T cells with Dronpa-GC1qR and Dronpa only constructs.

Representative fluorescence pictures of HEK-293T cells transfected with Dronpa only (control construct) or Dronpa-GC1qR construct are shown. The green fluorescence picture, bright field and the overlay of the two channels are displayed. Scale bars represent 100µm.

Effect of GC1qR overexpression in HEK-293T on its responsiveness to TLQP-21

In order to examine the functionality of TLQP-21 peptide on Dronpa-GC1qR overexpressed HEK-293T cells, calcium imaging using Rhod-3 calcium indicator was employed. In these experiments, Rhod-3 was used instead of Fluo-4 calcium dye because the emission wavelength of Fluo-4 dye overlapped with the green fluorescence emitted from Dronpa. Rhod-3 with longer excitation/emission maxima of 556/579 nm allowed simultaneous investigation on calcium level change in Dronpa transfected cells emitting green fluorescence. Prior to the HEK-293T experiment, the experiment protocol was first established using the primary macrophage. Primary macrophage was stained with Rhod-3 calcium dye and tested to see if TLQP-21 induced increase in calcium could be observed in the modified calcium imaging protocol (a positive control experiment to test the experimental set up). Figure 3.16 shows all the primary macrophages within the field of view were stained with Rhod-3 using the described staining protocol, and 100nM TLQP-21 stimulation induced an expected immediate increase in intracellular calcium. The subsequent application of 1mM ATP also elicited an instant increase in intracellular calcium, indicating cell viability. The result here demonstrated that the Rhod-3 staining protocol and the experimental set up in calcium imaging were good for the investigation of TLQP-21 induced calcium response.

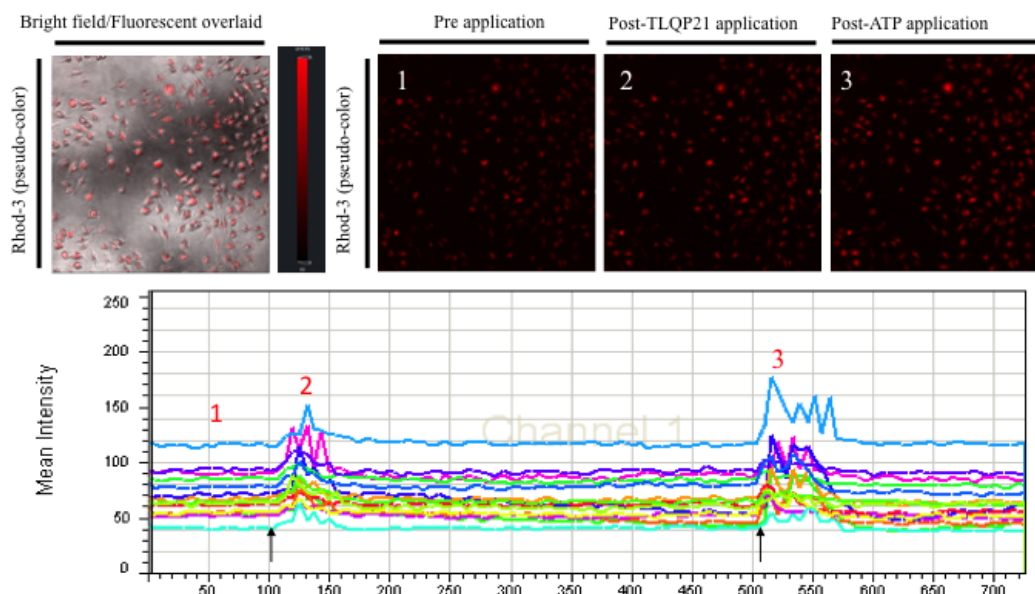


Figure 3.16 Calcium imaging with Rhod3 calcium indicator revealed that primary macrophages were able to respond to 100nM TLQP-21 peptide and 1mM ATP under the RhoD3 staining protocol and the modified experiment procedure.

Having established the experiment protocol and staining condition, I next examined the effect of TLQP-21 on Dronpa-GC1qR transfected HEK-293T cells 1 day after transfection. Figure 3.17 shows the majority of cells were transfected with Dronpa-GC1qR, and the cells expressed notable green fluorescence indicating successful expression. 100nM TLQP-21 was applied to the transfected cells, no significant increase in fluorescent intensity was detected, indicating no change in intracellular calcium level after peptide application. Since HEK-293T cells do not endogenously express P2X subtype receptors for ATP (Morelli, Chiozzi et al. 2003), Ionomycin was used as a positive control at the end of the experiment instead. Ionomycin induced the increase in intracellular calcium by stimulating the release of calcium from intracellular stores and enhancing calcium influx by activation of endogenous entry pathways (Morgan and Jacob 1994). Positive control with 2 μ M of ionomycin applied at the end of the imaging experiment elicited a robust increase in intracellular calcium in Dronpa-GC1qR expressing HEK-293T cells immediately

after stimulation, indicating the capability of the cells to elicit a calcium response.

The effect of TLQP-21 on Dronpa-GC1qR/Dronpa only transfected HEK-293T cells was also tested on post-transfection day 3. 100nM TLQP-21 application was shown to elicit no increase in intracellular calcium in both Dronpa-GC1qR transfected and Dronpa only transfected HEK-293T cells on post-transfection day 3 (as shown in figure 3.18A and 3.18B, respectively). Nevertheless, 2 μ M ionomycin at the end of the experiment demonstrated that the cells were viable and were able to respond to the positive control stimulation. Together, these data failed to demonstrate overexpression of GC1qR in HEK-293T cells enable a functional response of TLQP-21 peptide on these cells on post transfection day 1 and 3.

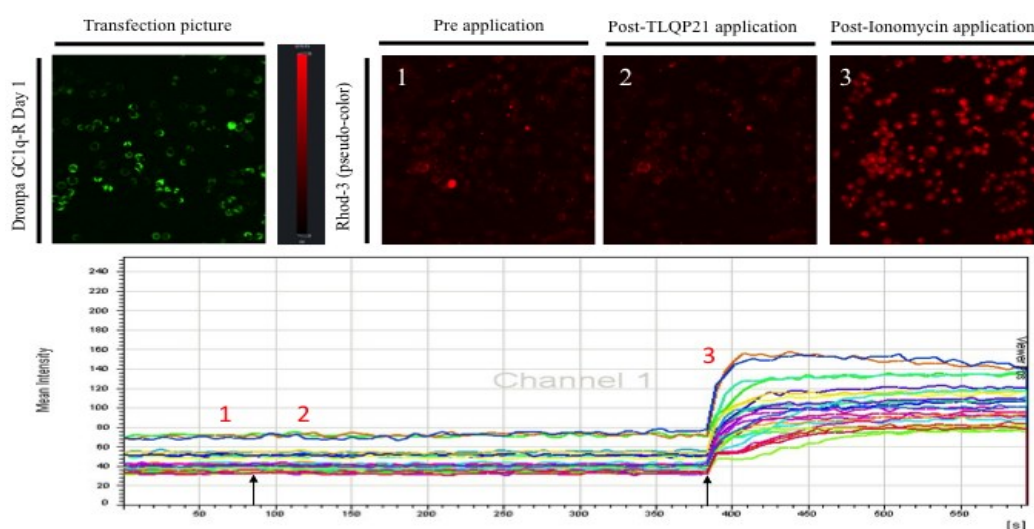


Figure 3.17 Calcium imaging using RhoD3 demonstrated HEK-293T cells transfected with Dronpa-GC1qR construct did not respond to 100nM TLQP-21 1 day after transfection. Ionomycin positive control, however, induced a robust response.

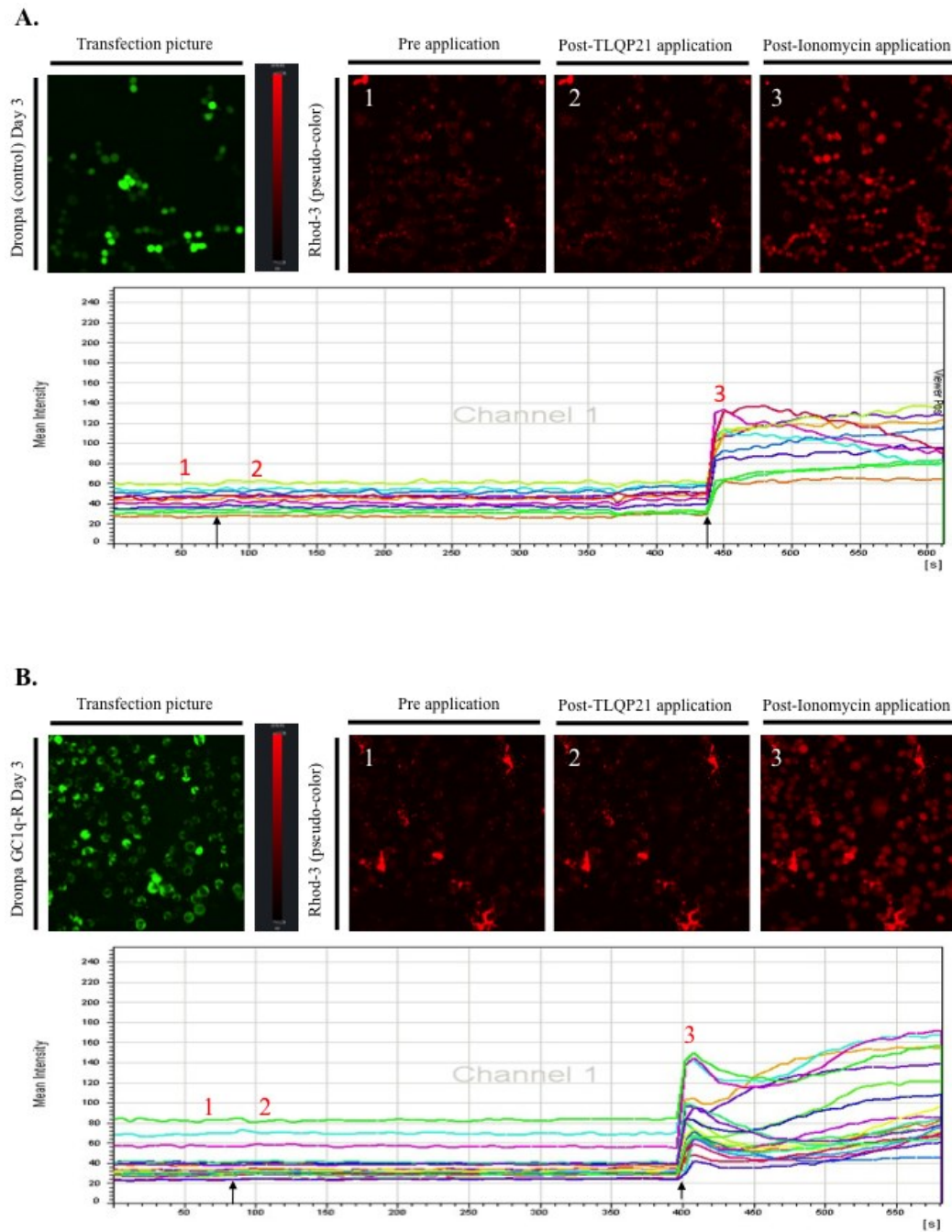


Figure 3.18 Calcium imaging using Rhod3 calcium dye illustrated the cells were not responsive to TLQP-21 peptide 3 days after transfection.

HEK293-T cells transfected with **A.** Dronpa only control construct and **B.** Dronpa GC1qR construct, failed to demonstrate a TLQP-21 stimulated calcium response 3 days after transfection. Nevertheless, the positive control with ionomycin elicited a sustained increase in intracellular calcium level after application.

3.4.4. VGF expression in Partial Sciatic Nerve Ligation (PSNL) model

The presented *in vitro* data together suggested that GC1qR is the potential receptor for TLQP-21 on primary macrophages. Macrophages were shown to infiltrate to the site of injury and dorsal root ganglion (DRG) after peripheral nerve injury (Hu and McLachlan 2003, Dubovy, Tuckova et al. 2007, Komori, Morikawa et al. 2011), where *vgf* has been shown to be released and upregulated in expression (Riedl, Braun et al. 2009). It was hence hypothesised that the recruited macrophages after nerve injury were stimulated by TLQP-21 via GC1qR at the periphery, which affects the excitability of sensory neurons and resulting in a change in the nociception. To validate this hypothesis, I first sought to demonstrate if *vgf* expression is upregulated in the partial sciatic nerve ligation (PSNL) model of neuropathic pain, followed by elucidating if interfering TLQP-21/GC1qR pathway *in vivo* alters the hypersensitivity to the mechanical stimulation seen in this rat animal model of neuropathic pain (see next section).

Vgf has been demonstrated to be upregulated in a variety of animal models of neuropathic pain, including spinal nerve ligation (SNL) model, chronic constriction injury (CCI) model, spare nerver ligation (SNI) model, HIV-1 gp120 model, varicella zoster virus (VZV) model (Maratou, Wallace et al. 2009, Riedl, Braun et al. 2009). However, its expression has not been monitored in the rat PSNL model of neuropathic pain, and hence was investigated in this study. The experimental animals were divided into two groups; one group underwent PSNL surgery and the other received sham surgery. The sham surgery was necessary to isolates the specific effects of PSNL surgery as opposed to that caused by anaesthesia, incisional trauma, or pre- and postoperative care. Figure 3.19A shows the immunohistochemistry image of the

sciatic nerve in Sham animal and that of PSNL animal. The nerve sections were stained with neuronal tubulin and Hoechst to reveal neuronal fibres and nuclei, respectively. As observed in the PSNL picture, a great number of cells were infiltrated to the side of injury where the suture was made, presumably including macrophages as evident in literature. The immunohistochemistry confirmed the successful ligation and revealed cell infiltration in PSNL models.

Both the ipsilateral and contralateral dorsal root ganglions were collected from the two groups of animals on post operative day 1 (POD1), post operative day 3 (POD3) and post operative day 5 (POD5). The *vgf* mRNA expressions were measured by quantitative RT-PCR and the data were expressed as percentage of *vgf* expression in ipsilateral DRG over the contralateral DRG. As shown in figure 3.19B, the *vgf* expressions were at approximately the same level in the ipsilateral and contralateral DRGs of the sham animal on POD1, POD3 and POD5 (shown as blue bars), as the percentages of expression (ipsi/contra) were close to 100% ($119.3 \pm 7.8\%$, $144.2 \pm 15.7\%$ and $121.0 \pm 19.5\%$ for POD1, POD3 and POD5, respectively). However, in the animals underwent PSNL surgery, the *vgf* expressions were robustly increased by 3.2 ± 0.2 , 3.4 ± 0.4 , 2.8 ± 0.5 folds on the ipsilateral DRG on POD1, POD3 and POD5, respectively, when compared to the contralateral DRG. Moreover, the percentages of *vgf* expression (ipsi/contra) on POD1, POD3 and POD5 were significantly different in the PSNL animals when compared to Sham ($n=3$, $p<0.01$ for POD1 and $p<0.05$ for POD3 and POD5). The presented data here confirmed the *vgf* expression was upregulated in the ipsilateral DRG, up to post-operative day 5, after PSNL surgery and the expression was not altered in the sham-operated animals.

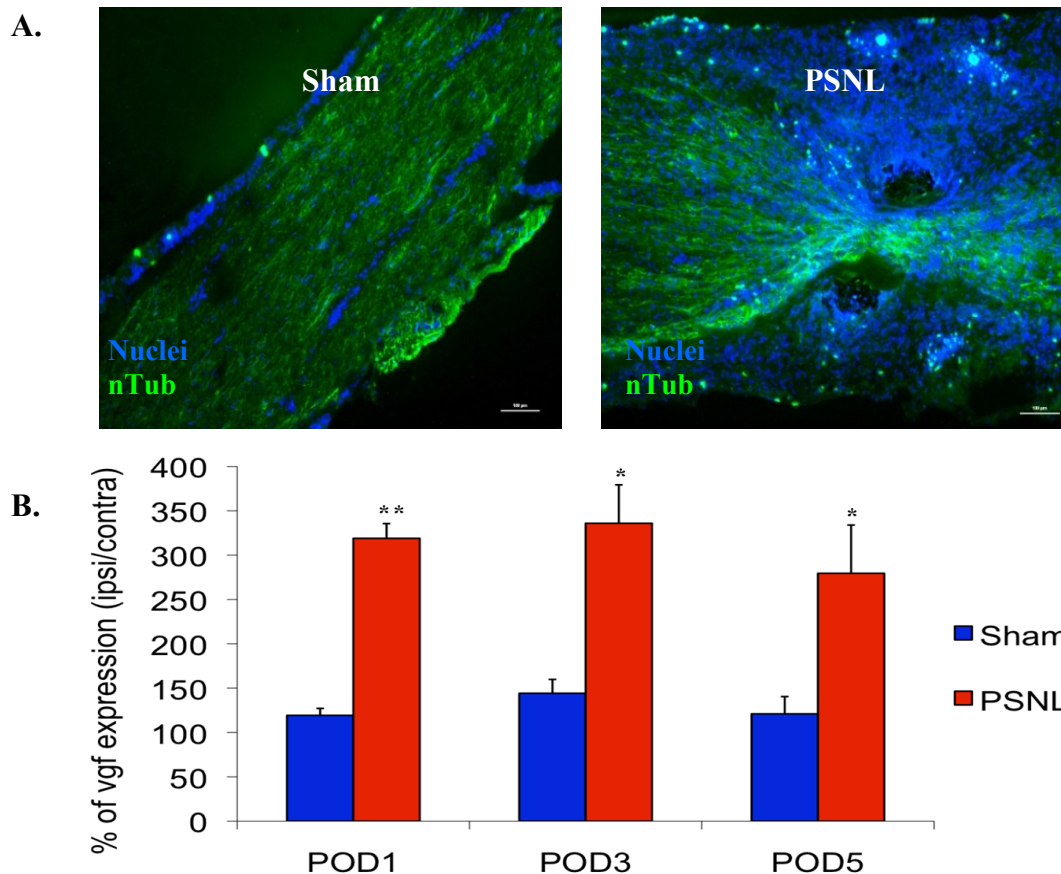


Figure 3.19 *Vgf* expression was significantly upregulated at mRNA level in PSNL animal models of neuropathic pain on post operative day 1, 3 and 5.

A. Immunohistochemistry illustrating the staining of neuronal tubulin (green) and cell nuclei (blue) at the ipsilateral side of sciatic nerve in Sham and PSNL operated animal. **B.** qPCR data showed significant upregulation of *vgf* mRNA in DRG of PSNL rats (red bars) 1 day (POD1, ** $p < 0.01$), 3 days (POD3, * $p < 0.05$), and 5 days (POD5, * $p < 0.05$) post operation when compared to sham operated rats (blue bars) ($n = 3$, Student's t-test vs sham).

3.4.5. Effect of blocking GC1qR on mechanical hyperalgeia of the PSNL neuropathic pain animal model

The data so far has demonstrated that TLQP-21 can robustly activate bone-marrow derived macrophages and result in the release of the intracellular calcium from the internal stores. *In vitro* antibodies neutralisation and siRNA knock-down of GC1qR on the primary macrophage interfered the TLQP-21 signalling and reduced the percentage of cells responding to the peptide. In addition, *vgf* expression has also been demonstrated to be upregulated *in vivo* at the periphery of the PSNL animal model of

neuropathic pain. To validate the results presented *in vitro* and that the culture models tested were biologically relevant and to implicate the GC1qR/TLQP-21 pathway in macrophage to neuropathic pain, the effect of neutralising GC1qR *in vivo* was examined, in the PSNL animal model of neuropathic pain.

The *in vivo* experimental set up is shown in figure 3.20. Here I sought to investigate if there is a change in the mechanical hypersensitivity that is often observed in animal model of neuropathic pain, after topical application of GC1qR neutralising antibody at the ligation site. The rats were divided into three groups receiving equal volume of PBS vehicle, control IgG, or anti-GC1qR neutralising antibody, after partial sciatic nerve ligation surgery. The treatments were soaked in oxidised regenerated celluloses, which were wrapped around the suturing ligature after ligation to allow slow release of treatments. The mechanical sensitivity to electronic Von Frey was measured on the hind paws before (baselines) and after the surgery (POD1, POD3 and POD5), and the resulting paw withdrawal threshold data were expressed as percentage to baseline.

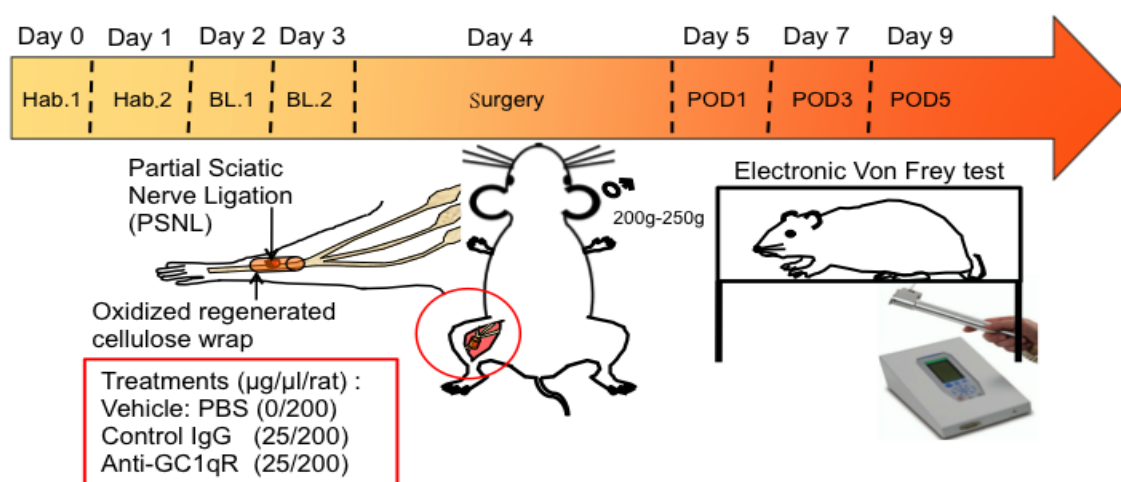


Figure 3.20 Schematic diagram illustrating the experimental set up for *in vivo* study.

The treatments (vehicle, control IgG or anti-GC1qR) were soaked in an oxidised regenerated cellulose, which were wrapped around the ligation sutures. The paw withdrawal thresholds to mechanical stimulation were assessed by electronic von Frey on post operative day 1, 3 and 5.

Figure 3.21A shows that the animals received PBS vehicle control after PSNL developed typical hypersensitivity to mechanical stimulus as seen in the animal model of neuropathic pain. The paw withdrawal threshold to the mechanical stimulus significantly dropped by $33.5 \pm 9.1\%$ ($n=8$, $p<0.05$) on POD1, which is further reduced to $48.2 \pm 7.4\%$ and $42.2 \pm 10.3\%$ of the baseline on POD3 and POD5, respectively, indicating a development of mechanical hypersensitivity in animals receiving vehicle control after surgery. Animals receiving control IgG treatment was also demonstrated to develop hypersensitivity to the mechanical stimulus (figure 3.12B), as the paw withdrawal threshold was significantly reduced by $36.9 \pm 6.1\%$, $49.4 \pm 3.6\%$, $47.8 \pm 7.6\%$ ($n \geq 7$, $p<0.001$) on POD1, POD3 and POD5, respectively, compared to the baseline. This indicates that the animals received control IgG after PSNL surgery still developed mechanical hypersensitivity as observed in the vehicle control, suggesting the treatment of control IgG did not interfere with the development of hyperalgesia in the animal model of neuropathic pain. Interestingly, the animals received anti-GC1qR treatment after surgery showed a delayed in development of mechanical hypersensitivity. As shown in figure 3.21, the paw withdrawal threshold on POD1 was not significantly differed from that of the baseline ($74.2 \pm 6.8\%$ for POD1 compared to $100 \pm 8.9\%$ of baseline), even though there was a trend of reduction. Nevertheless, the paw withdrawal thresholds on POD3 and POD5 were significantly reduced by $40.7 \pm 6.9\%$ and $37.8 \pm 13.3\%$ ($n \geq 5$, $p<0.001$), indicating a development of mechanical hypersensitivity on POD3 and POD5. It is worth noting though when the percentages reduction in withdrawal threshold were compared for day 1 between PBS, control IgG and anti-GC1qR groups, the results were statistically insignificant ($P=0.535$, One Way ANOVA). This indicates the possibility that the lack of significance in the day 1 of the GC1qR antibody treated group could likely be driven by the big variations in response between animals or the higher than normal

variability in the baseline of the animals.

The data presented here may suggest that topical treatment of GC1qR neutralising antibody after PSNL surgery could potentially delay the development of mechanical hypersensitivity in the rat PSNL model of neuropathic pain. Although future experiments with larger n numbers should be carried out to draw a more reliable conclusion, the data presented here may implicate a role of GC1qR in the development of neuropathic pain *in vivo*.

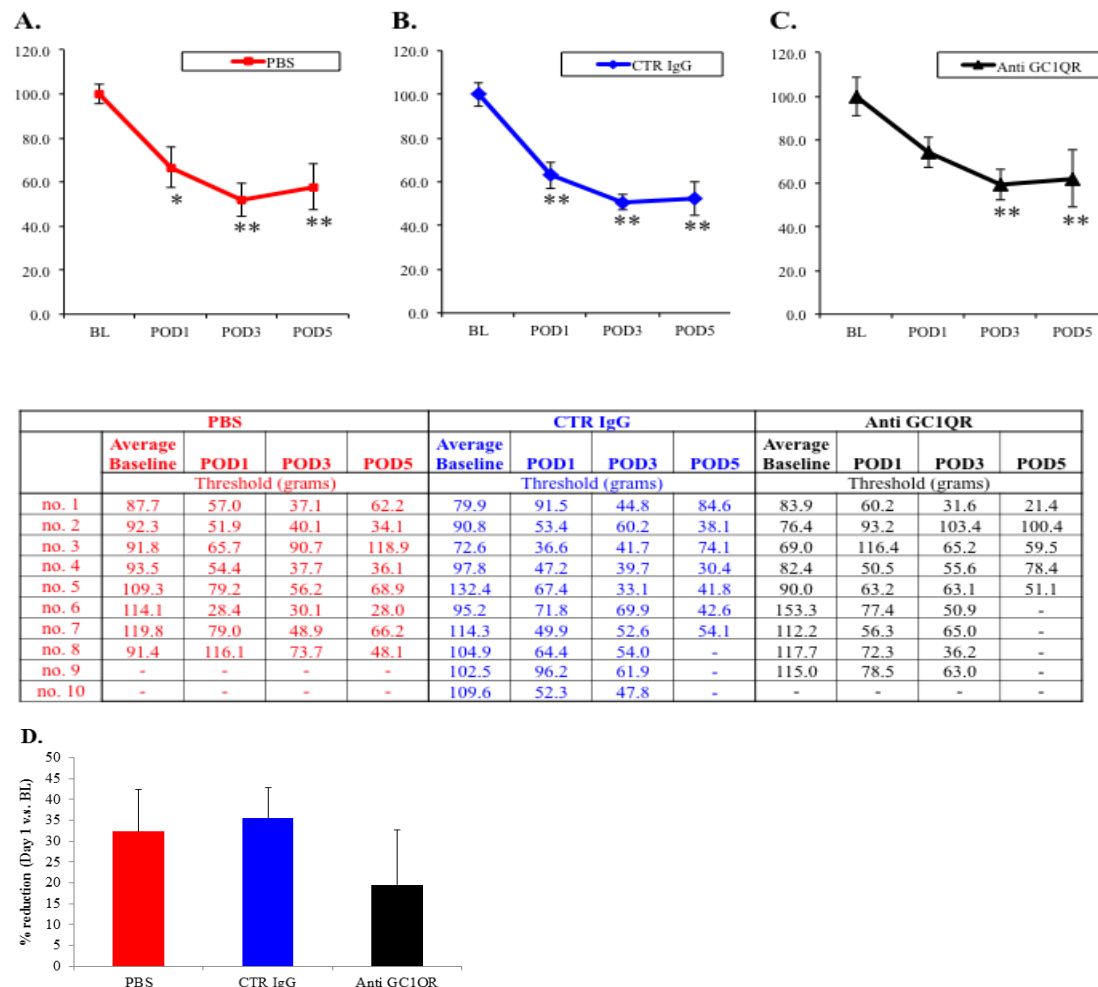


Figure 3.21 Anti-GC1qR neutralising antibody, but not the vehicle nor control IgG antibody, attenuated mechanical hypersensitivity on post-operative day 1 in PSNL animal model of neuropathic pain.

Ipsilateral paw withdrawal threshold to electron von Frey were examined on post operative day 1, 3 and 5 on PSNL rats treated with **A.** Vehicle, PBS (n=8) **B.** 25 µg Control IgG (n≥7) **C.** 25 µg Anti-GC1qR neutralising antibody (n≥5). The ipsilateral paw withdrawal threshold for each n number in all experiments is listed in the table. The animals treated with vehicle and control antibody showed reductions in mechanical thresholds one day after PSNL surgery (*p<0.05 and **p<0.01 vs. BL for POD1 vehicle and POD1 control IgG, respectively.). In contrast, the PSNL rats treated with GC1qR antibody exhibited higher mechanical threshold to the electronic von Frey when compared to the control antibody treated rats and the vehicle treated rats. Moreover, the paw withdrawal threshold POD1 GC1qR treated PSNL rats were not statistically differing from the baseline (not significant, p = 0.12, for POD1 anti-GC1qR). *p<0.05, **p<0.01, Two way ANOVA Tukey's post hoc test. **D.** The percentages reduction in withdrawal threshold of PBS, control IgG and anti-GC1qR treated groups. The percentage reduction between groups were statistically insignificant (p = 0.53515, One way ANOVA). Error bar expressed as standard error means.

3.5 Discussion

In the previous chapter, it was demonstrated that macrophage is one of the target cells, if not the only one, for TLQP-21 in the periphery. TLQP-21, at the concentration as low as 10nM, acts on the primary macrophage and induce a pronounced increase in the intracellular calcium level. Intriguingly, TLQP-21 stimulation on the primary macrophage desensitised the cells from responding to the second dose of TLQP-21, implying the presence of a receptor for this peptide on the primary macrophage. This potential receptor for TLQP-21 was identified as GC1qR in our laboratory by two independent experiments, chemical crosslinking and affinity purification in combination with mass spectrometry. In this chapter, I aimed to verify, functionally, if GC1qR is the receptor for TLQP-21 on the primary macrophage *in vitro*, and examine its involvement in the neuropathic pain development *in vivo*.

3.5.1. Effect of GC1qR neutralisation and downregulation on TLQP-21 induced calcium response in the primary macrophage *in vitro*

I first demonstrated in this chapter that neutralisation of the receptor using two monoclonal antibodies specifically targets GC1qR (MAb60.11 and MAb74.5.2) and results in a reduction of calcium response induced by TLQP-21 in the primary macrophages. The effect is seen most pronounced when the antibodies were used together at the concentration of 3µg/ml (of each antibody). Though there is no statistical significance between the percentages of cells responding to TLQP-21 after MAb60.11 and MAb74.5.2 pre-incubation, there is a trend of more cells being able to respond after MAb74.5.2 treatment. The possible reason for this could be that

MAb60.11 only recognises the mature form of GC1qR, whereas MAb74.5.2 interacts with both mature and truncated forms of GC1qR. It is possible that TLQP-21 interacts with the mature forms of GC1qR with higher affinity and subsequently exerts its function upon binding, and hence the effect of MAb74.5.2 treatment was diluted as it also recognised the truncated form. Moreover, MAb 60.11 and MAb74.5.2 target specific GC1qR epitopes at amino acids 76-93 and 204-218, respectively, where they block distinctive functional domains of GC1qR. It is also plausible that blocking of the functional domain that MAb74.5.2 targets is less effective in disrupting the interactions between GC1qR and TLQP-21 stoichiometrically. Nonetheless, blocking of GC1qR using either neutralising antibodies or combined, showed significant reduction of percentage of cells responding to TLQP-21 in the primary macrophage. This indicates that GC1qR is, at least partly, involved in TLQP-21 induced calcium signalling in the primary macrophage.

This finding is in line with the GC1qR downregulation experiment by siRNA oligonucleotides, where the GC1qR downregulation in the primary macrophage also showed to reduce the percentage of cells responding to TLQP-21. It is worth noting though, the abolishment in calcium response effect caused by GC1qR downregulation was not as pronounced as that of GC1qR neutralisation by antibodies. This could be explained by that the siRNA oligonucleotides used in the experiment not completely eliminating all the endogenous GC1qR, and the residual proteins expressed in some of the cells could still exert functions. Indeed, the Western blot analysis 48 hours after siRNA transfection showed the downregulation efficiency of GC1qR was about 50% at protein level on average. Although, the percentage of knockdown at the mRNA level was achieved between 70-80%, it is possible that

GC1qR has a long protein half-life. As a result of this, though the mRNA was knocked down to a significant level, the protein remained stable inside cells and hence partially masking the siRNA effect in some of the cells. Future experiments could be carried out using different siRNA sequences or carrying out extensively optimisation on concentration used and time point for the current siRNAs to achieve better knock down at protein level. This achievement would be relevant to achieve a more pronounced functionality change in macrophage upon TLQP-21 stimulation.

3.5.2. Effect of GC1qR overexpression on TLQP-21 stimulation of the HEK293T cells *in vitro*

Having demonstrated GC1qR receptor neutralisation and knockdown both affect the ability of primary macrophage responding to TLQP-21, I intended to proof the functionality and involvement of GC1qR in the TLQP-21 induced calcium signalling on primary macrophage by a third experimental approach. Other than knocking down of the target gene by RNAi technology, one of the most common ways to dissect the function of a gene in a pathway is to overexpress the gene. One of the main concerns associated with RNAi experiment is the off-target effect on other genes. However, if result obtained from the overexpression experiment shows the opposite functional read out to siRNA, it is less likely the results of siRNA-mediated reduced in percentage of cell responding were due to non-specific off target effects. For this reason, the GC1qR construct was cloned and transfected into a cell line which did not respond to TLQP-21. It was thus hypothesised that overexpression of GC1qR into a cell line that does not normally respond to TLQP-21 could potentially allow them to become responsive, at least in terms of calcium response, to the peptide. The human embryonic kidney cell line 293 (HEK293T) has been demonstrated previously in our

laboratory by Dr Mahmood Ayub to be unresponsive to TLQP-21 stimulation in the calcium imaging and was hence chosen to be the in vitro system for overexpression experiment. Unexpectedly, it was demonstrated in this chapter that HEK293T cells overexpressed GC1qR failed to induce an intracellular calcium response when TLQP-21 was applied. The possibility that HEK293T cells were unable to elicit a calcium response was eliminated as ionomycin positive control induced a robust transient calcium response after its application to the cells. These cells failure to respond could be for a number of reasons. It is possible that HEK293T cells may not have the correct downstream molecular components to process the interaction of GC1qR and TLQP-21, as HEK293T cells are human embryonic kidney cells, the endogenous molecular machinery is likely to be very different from macrophage. It is also plausible that HEK293T cells may have the correct machinery, but the GC1qR construct transfected was a rodent clone, which failed to interact or weaken the interaction with the endogenous downstream signalling molecule so that the subsequent functional outcome cannot proceed. Although this possibility exists, it is very unlikely to be the case as the rodent sequences of GC1qR are 89.9% identical to the human sequence and the essential amino acids at functional domains are often conserved among different species. Furthermore, it is worth noting that GC1qR lacks consensus sequence for a predicted transmembrane segment, it is hence hypothesised to initiate downstream signalling by coupling with transmembrane proteins. Here, I cannot formally rule out other factors such as cell type specific accessory/adaptor/transmembrane proteins might be needed and are involved to dictate the cell type specific response to be induced following TLQP-21 stimulation. Although, the complete signalling mechanism has yet to be determined, the finding that TLQP-21 and GC1qR interact and interfering with this interaction, either by siRNA genetic manipulation or antibody neutralisation, results in reduced

TLQP-21-induced calcium response, suggests a role of GC1qR in the TLQP-21-mediated cellular response in the primary macrophage. Future experiments could be carried out to consolidate the finding of the involvement of GC1qR in TLQP-21 signalling in primary macrophages, where siRNA targeting of the untranslated region of GC1qR mRNA could be designed and used to downregulate the endogenous GC1qR in the primary macrophage. The percentage of cells responding to TLQP-21 after GC1qR knock down by such siRNA will be expected to reduce. An investigation could be carried out subsequently to examine if overexpressing exogenous GC1qR could rescue the siRNA-mediated effect (i.e. rescue the percentage of cell responding to TLQP-21).

3.5.3. Effect of GC1qR neutralisation *in vivo* on pain behaviour in the PSNL animal model of neuropathic pain

Despite that the evidences demonstrated in this chapter linking GC1qR in the TLQP-21 induced calcium-dependent signalling pathway in the macrophage *in vitro*, its involvement *in vivo* in neuropathic pain development had not yet been examined, and hence was investigated. As demonstrated in figure 3.21, a single dose of local anti-GC1qR antibody treatment, but not control IgG or vehicle treatment, after PSNL surgery delayed the neuropathic pain-like behaviour in the animal model, suggesting neutralisation of GC1qR at the site of injury may result in delay in the development of neuropathic pain. This indicates that GC1qR may play an important role in initiating and/or maintenance of neuropathic pain after nerve injury.

If GC1qR is pivotal to neuropathic pain development, why is the effect observed transient? There could be a number of reasons for this. Firstly, in the experimental set

up, the antibody was only delivered to the animal as a single dose of 25µg. It is possible that the concentration of the antibody was not high enough to neutralise all the GC1qR readily available at the site of injury, as mentioned previously that large number of macrophages infiltrate to the injured nerve after periphery nerve injury. The concentration of the antibody delivered in the experiment was already the highest possible from the commercial available purchase. For future experiment to optimise the antibody dose in the *in vivo* experiment, a higher-titer MAbs could be produced through commercial source, and be used at higher concentration to increase neutralisation effect *in vivo*. The second possible reason for the transient effect observed in the *in vivo* study could be that the antibody was delivered as a single dose. Antibodies are proteins, which exhibit distinctive half-life. It is possible that the antibody has a short half –life and the stability of the antibody *in vivo* was one of the reasons, if not the only one, contributing to the transient effect. Although it has been demonstrated by Morecell et al. (Morell, Terry et al. 1970) that the average biological half-life of IgG antibodies were 21 days for G1, G2 and G4 subtypes, while IgG3 was only 7.1 days. The monoclonal antibody used in the *in vivo* experiment was IgG1 subtype, and hence the possibility that the antibody exhibit a short half-live *in vivo* is less likely to the reason. Although this possibility cannot be firmly ruled out as it has been demonstrated binding of endogenous antibody to the ‘therapeutic’ antibody, as an immune response to eliminate the foreign antibody, forms immune complexes that could shorten the half-life of the therapeutic antibody and compromise its effectiveness (Chan and Carter 2010). To overcome this problem, future experiment could be design to deliver antibody at multiple dosage using technology such as osmotic minipump, which allows constant and long-term delivery of the antibody at the site of injury, which has been shown successful in the literature (Krum, Mani et al. 2008, Clausen, Hanell et al. 2009). Moreover,

production of a rat monoclonal neutralising antibody against GC1qR may improve the possibility that cross species immune response resulting in shortening of antibody stability *in vivo*.

Moreover, it is worth noting that macrophages infiltrate into the injured nerve 1 day after nerve injury, and GC1qR on these macrophages proximal to the treatment wrap could possibly be neutralised. However, 2 days after PSNL surgery, another wave of M2 macrophage infiltrate the dorsal root ganglia (DRG), where the sensory neurons cell bodies lied (Komori, Morikawa et al. 2011). The ectopic application of the anti-GC1qR antibody at the site of injury distal to the DRG ganglia may be insufficient to neutralise the function of the receptors on this second wave infiltrated macrophages, and hence the development of neuropathic pain was delayed but not abolished. There is also a complication where the endogenous macrophages are constantly being replenished and replacing the ‘neutralised’ macrophages. Therefore, the existence of endogenous circulating macrophage may mask the effect of ectopic application of the GC1qR antibody. To overcome these problems, future experiments could be carried out to deliver the antibody systemically. Though the required antibody for systemic injection would be much higher and it could possibly cause a secondary effect, it could allow a more distinctive phenotype to be observed as the neutralisation condition is more stringent and multiple dosages by systematic injection would be experimentally possible. To help dissecting further if neutralising GC1qR on macrophage could delay/abolish the development of neuropathic pain *in vivo*, an experimental global macrophage depletion could be carried out in the future experiment. This could be done by either total body irradiation (Matute-Bello, Lee et al. 2004) or clodronate liposomes technique (Van Rooijen and Sanders 1994).

Clodronate liposomes techniques rely on the nature that macrophages are phagocytic and ingestion of clodronate (toxin) packed liposome could kill the host cells. Both of these methods selectively deplete circulating monocytes and macrophages, as microglia in the CNS are not phagocytic and proliferate very slowly, thus making them more resistant to these treatments. After systemic depletion of endogenous macrophages, *in vitro* cultured macrophages treated with either anti-GC1qR or IgG control could be transplanted to the animal after PSNL surgery. If the anti-GC1qR treated macrophage-transplanted animals fail/delay to develop neuropathic pain and that of the IgG group develop neuropathic pain normally, this data will draw a more convincing result to support the involvement of GC1qR in the development of neuropathic pain.

Another weakness of the *in vivo* experiment that should be noted is that neuropathic pain animal models often exhibit characteristic thermal and mechanical hyperalgesia and in the *in vivo* experiment set up, only the mechanical hypersensitivity was monitored after neutralising antibody treatment. There is a possibility that a more dramatic effect, in terms of delaying/abolishing neuropathic pain development, could be observed in the noxious heat read out assay using Hargreaves's apparatus.

Summing all the results presented in this chapter, I have demonstrated for the first time that GC1qR could be the potential receptor on primary macrophage for VGF derived peptide, TLQP-21. The interaction between GC1qR and TLQP-21 is essential for TLQP-21 induced calcium response in macrophage, as shown in this chapter that disrupting this interaction by GC1qR downregulation/neutralisation results in reduced percentage of cells responding to TLQP-21 in calcium imaging. I also demonstrated that *vgf* was significantly upregulated in the DRG of PSNL animal model of

neuropathic pain, and this upregulation was sustained at least until POD5. Lastly, I demonstrated in this animal model, interfering the TLQP-21/GC1qR pathway by neutralisation of GC1qR *in vivo* results in a delay of neuropathic pain development. Together, this chapter presents the data examining and verifying the involvement of GC1qR in the TLQP-21 signalling pathway and suggests TLQP-21 provokes the intracellular calcium response in the primary macrophage, at least partly, via GC1qR. Though further optimization is still required, GC1qR is suggested here to play a role in the development of neuropathic pain *in vivo*, and this involvement is likely to be downstream of TLQP-21 stimulation.

CHAPTER 4:
INVESTIGATION OF TLQP-21 DOWNSTREAM SIGNALLING
PATHWAY IN THE PRIMARY MACROPHAGES BY GENETIC
APPROACHES

Synopsis

The work presented in this chapter aims to investigate the downstream mechanisms underlying the TLQP-21 signalling in the primary macrophages. Individual inflammatory mediators that are common produced from the macrophages and the GC1qR receptor has been tested for their changes in gene expression by qRT-PCR following TLQP-21 treatment. In addition, PCR array is utilised to investigate if the common transcription factors involved in various biological processes are dis-regulated in the primary macrophages after TLQP-21 stimulation. Genome-based approach has been also been employed, such that the microarray analysis of the transcriptomes of primary macrophage treated with TLQP-21/scrambled peptide are compared at different time points.

4.1. Introduction

4.1.1. Methods of differential gene expression detection for investigating gene function.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Polymerase Chain Reaction (PCR) is a revolutionary technology developed by Saiki *et al.* in 1985 (Saiki, Scharf et al. 1985). Ever since then, this technique has been widely employed in the field of molecular biology for cloning, genetic engineering and sequencing. This technology allows exponential amplification of small DNA fragments, which requires a pair of primers (usually ~ 20 nucleotides) complementary to, and flanking, the sequence of interest. In addition, thermo-stable DNA polymerase is essential to perform the sequence extension from the primers by utilising the deoxynucleotide triphosphates supplemented in the reaction. The amplification can be

repeated for multiple cycles, which results in an exponential amplification of the target sequence. Quantitative Real Time PCR (qRT-PCR) is an advance development from such PCR technology, in which the products generated during each cycle of amplification can be precisely detected and measured (Freeman, Walker et al. 1999). Because the reliable quantification of the product could be measured, qRT-PCR has been widely employed in the quantification of gene expression, differential gene expression analysis, quantifying pathogen loads such as bacterial, fungal and viral infections (Deepak, Kottapalli et al. 2007). The principle of qRT-PCR derived from that of PCR, in which sophisticated detection and quantification methods differentiate them from the conventional PCR and hence enable reliable “real-time” measurement of gene expression.

The most common detection methods in qRT-PCR are the fluorescent double-stranded DNA(dsDNA) dye and the fluorescent reporter probes consisting of a reporter and a quencher. In the first method, a fluorescent DNA-binding dye (such as SYBR green) non-selectively and preferentially binds to any double-stranded DNA in the PCR, resulting in fluorescent emission. The binding ability of the dye to the single-stranded DNA (ssRNA) is often much lower than that to the dsDNA (at least 11 folds less for SYBR green dye)(Zipper, Brunner et al. 2004). As the dsDNA is amplified during the qRT-PCR cycles, the fluorescent intensity is increased along the reaction cycles. The quantification of the PCR product can be made by exciting the fluorophore at certain wavelength ($\lambda_{\text{max}} = 488 \text{ nm}$ for SYBR green) and subsequently measures the fluorescent emission ($\lambda_{\text{max}} = 522\text{nm}$ for SYBR green) after each cycle of the PCR, thus monitoring the gene amplification in “real-time”. A major drawback of this detection method is that fluorescent dsDNA dye could bind to all dsDNA, including the primer-dimers and the off-target amplified products in the reaction.

Such non-specific products could contribute to the output fluorescent intensity and prevent accurate quantification of the gene of interest. In order to overcome this problem, a melt curve analysis is often performed after the qRT-PCR reaction. During the analysis, all dsDNA PCR products are denatured with increasing temperature, which results in a reduction in fluorescent intensity. The temperature where 50% of the dsDNA is dissociated is known as the melting temperature. Different dsDNA PCR products will have different melting temperatures as the sequences are not identical. This helps to assess if undesirable multiple products are generated during the qRT-PCR reaction and if repeated qRT-PCR reaction could generate the same melting temperature for the gene of interest (Ririe, Rasmussen et al. 1997). The latter quantification method involves oligonucleotide probes consisting DNA labelled with fluorescent reporter at the 5' end and a quencher at the 3' end (Holland, Abramson et al. 1991). The most widely used fluorescent reporter probes are the TaqMan probes. During qRT-PCR cycles, the probe hybridises to the complementary target sequence and the fluorescent reporter is cleaved during PCR due to the 5' nuclease activity of Taq polymerase. The cleavage releases the fluorophore from the quencher and hence permitting fluorescent emission to allow subsequent quantification of the PCR product. The detected fluorescent in the qRT-PCR using TaqMan probes is, therefore, directly proportional to the fluorophore released from the sequence specific probe and the amount of target DNA generated. Non-specific amplification signal is hence reduced as no signal will be generated from any background amplification that may be formed. In addition, the probes can be labelled with different fluorescent dyes to permit detection of different target sequences in a single reaction. However, the synthesis of the reporter-and-quencher-labelled probes is required for all the targets of interest, which results to a higher experiment cost of this detection method compared to others.

The quantification methods performed in a qRT-PCR can be either an absolute or a relative quantification. The absolute quantification of qRT-PCR data involves calibrating the experiment with a standard curve generated from serial dilution of a sample with known concentration (Bustin 2000), while the relative quantification measures the relative changes in gene expression level of the target gene to a reference gene. Relative quantification is generally easier to perform than absolute quantification because the generation of a standard curve is not needed. However, a good reference/housekeeping/control gene with stable expression is essential for the relative quantification to be carried out, as the quantification is heavily relied on comparing the expression levels of the target gene to the housekeeping gene and it is often necessary to normalise the expression of the gene of interest over the consistently expressed reference gene (Radonic, Thulke et al. 2004). The data from relative quantification method are often analysed by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). In this method, it is assumed that the primers for the target gene and the reference gene exhibit the same efficiency and the amplification of the genes results in similar productivities. The $2^{-\Delta\Delta C_T}$ algorithm is implemented in the $\Delta\Delta C_{Tq}$ quantification method. The data analysed are expressed as a fold change to the corresponding control and the absolute amount of gene expression in the control remains unknown.

Microarray as tools in genetic research to investigate gene function

Microarray is a chip-based technique that allows simultaneous measurement of a large number of genes. The microarray chip is made by attaching small fragment DNA probes to a solid surface. An array can contain thousands of DNA probes, and hence enabling the survey for the expression of thousands of genes at the same time. The principle of the microarray relies on the phenomenon that complementary nucleic

acid sequences are able to bind to each other via hydrogen bonds formations between Watson-Crick nucleotide base pairing. The total mRNA is often extracted from the sample of interest, followed by labelling with fluorescent detector. The common fluorescent dyes used for cDNA labelling are Cy3 and Cy5, which emit fluorescence at 570nm (green fluorescence) and 670 nm (red fluorescence), respectively. The labelled target sequences are then hybridised to the probes on the array. The perfect complementary sequences exhibit tight associations via strong non-covalent bindings, which remain hybridised while non-specific annealing is washed. The gene expression level is detected from the fluorescent emission, where the total amount of fluorescent signals corresponds to the amount of dye-tagged sequence hybridised to the probes on the chip (Gibson 2003). The level of expression can hence be quantified by comparing the intensities of all the genes of a sample to those of another sample.

Because of its advantage to investigate the changes in expression of large numbers of genes in one experiment, microarray analysis has become popular choice for identifying differential gene expression in different disease states. Once identified, the varying expression levels of specific gene can be confirmed and further investigated for its functional roles in the disease. Indeed, such an approach was found to be useful in the field of pain research for investigating changes in gene expression of the neuropathic pain animal model compared to naïve animal (Costigan, Befort et al. 2002), examining common genes that are dis-regulated among different animal models of neuropathic pain (Maratou, Wallace et al. 2009), identifying inflammatory mediators produced in the sensory ganglia during neuropathic pain development (Strong, Xie et al. 2012), and comparing gene profile between different type of pain (neuropathic versus inflammatory pain(Rodriguez Parkitna, Korostynski et al. 2006)) .

4.2. Aim

Having demonstrated that VGF-derived peptide, TLQP-21, acts on the primary macrophage in a dose- and calcium-dependent manner, and the calcium response evoked by TLQP-21 is, at least partly, via GC1qR. *In vivo* studies in the previous chapters also showed that TLQP-21-activated macrophages significantly induced mechanical hyperalgesia in the naïve animal and interfering with the GC1qR pathway delayed the neuropathic pain development in the PSNL animal model of neuropathic pain. Despite these novel findings allowed better understanding of the TLQP-21 signalling and its involvement, together with GC1qR receptor, in the neuropathic pain development, the molecular mechanism downstream to TLQP-21-evoked calcium response in the primary macrophage is largely unknown. Thus, the aim of this chapter is to investigate *in vitro*, the downstream signalling event following the TLQP-21-induced calcium response in the primary macrophages by:

1. Quantitative RT-PCR to investigate if the characteristic cytokines, inflammatory mediators and receptor GC1qR are dis-regulated after 3 hours of 100nM TLQP-21 treatment.
2. PCR array to investigate if any differential gene expression of the 84 common transcription factors can be detected after 30 mins/3 hours of 100nM TLQP-21 exposure.
3. Microarray analysis to investigate, in the genome wide level, the differential gene expression in the macrophages after 30 mins/3 hours/24 hours of TLQP-21 stimulation.

4.3. Material and method

4.3.1. Sample preparation

Primary macrophage cultures were established as outlined previously in section 2.2.3. The differentiated cells were isolated from the undifferentiated cells 48 hours prior to the experiment by re-plating ~1,000,000 cells/90mm culture dish. For quantitative real-time PCR, PCR array and microarray experiment, the media of the culture were changed to serum free IMDM 24 hours prior to the treatment. The cells were then treated with either 100nM wild type TLQP-21, 100nM scrambled TLQP-21 or vehicle (same volume of sterile water) for 30 minutes 3 hours or 24 hours. Total RNA was extracted from the cells after corresponding peptide/vehicle treatment, followed by subsequent procedures to perform qRT-PCR, PCR array and microarray (see later).

4.3.2. Quantitative Real-time PCR

Total RNA extraction from cultured cells treated with either scrambled TLQP-21/wild type TLQP-21, cDNA synthesis and quantitative RT-PCR was performed as described in the previous chapter (section 2.2.5). The primers used in the qRT-PCR studies of this chapter are listed below:

Targets	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Beta-actin	TCCTGTGGCATCCATGAAACT	AACGTCACACTTCATGATGGAATT
Cyclophilin A	GCAAGACCAGCAAGAAGATC	TCAGTGAGAGCAGAGATTACAG
TNF- α	ATCCGAGATGTGGAAGTGGCA	CCGGACTCCGTGATGTCTAAGT
IL-1 β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
IL-6	TCCTACCCCAACTTCCAATGCTC	TGGATGGTCTTGGTCCTTAGCC

iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC
BDNF	AAGGCACTGGAACCTCGCAAT	AACCGCCAGCCAATTCTCTT
Arg1	TATCGGAGCGCCTTTCTCTA	ACAGACCGTGGGTTCTTCAC
Cox2	TGTATGCTACCATCTGGCTTCGG	GTTTGGAACAGTCGCTCGTCATC
GC1qR	GTATTGGGCGCCTGGTCACC	CGCTCCTGGAAGATGGTGATGG

4.3.3. NanoDrop™ spectrophotometer

For checking the RNA quantity and quality, the NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific) was used following the manufacturer's instructions. Briefly, the instrument was initialised by 2µl of RNase free water. A blanking cycle was performed using 2µl of pure RNase free water before any measurements were performed. 1.5µl of RNA from the samples was loaded onto the sample loading platform and the concentration of the RNA was obtained in ng/µl. The 260/280 absorbance ratio as well as the 260/230 ratio were also obtained from the measurement. For 260/280 ratio, a value greater than 2.0 generally indicate sufficient quality in the RNA purity. If the value is lower than 2.0, it is likely the RNA was contaminated with proteins. 260/230 ratio with value greater than 1.7 indicates minimal contamination with solvent (such as ethanol).

4.3.4. Assessment of RNA integrity

The RNA integrity assessment was performed using the Agilent Total RNA Nano 6000 Labchip™ kit and Agilent Bioanalyser according to the manufacturer's manual. Briefly, all reagents from the Nano 6000 Labchip kit were allowed to equilibrate at room temperature for 30 minutes prior to be used. 550µl of the Agilent RNA 6000 Nano gel matrix was placed into the top of the spin filter provided, which is then

centrifuge at 1500g for 10 minutes. The filtered gel was then divide into 65µl aliquots in 0.5ml RNase-free tubes, and stored at 4°C. 1µl of well-mixed RNA 6000 Nano dye concentrate from the kit was added to a 65µl aliquot of the filtered gel, followed by thorough vortex and centrifugation at 13000g for 10 minutes at room temperature. The mixture of filtered gel and dye was allowed to equilibrate at room temperature for 30 minutes in the dark. The gel/dye mixture was loaded to the chip via a plunger system on the chip priming station. The RNA samples used were at concentrations between 158-492ng/µl. Prior to the RNA samples loading, 5 µl of the RNA 6000 Nano marker was loaded into all the wells. 2µl of the RNA samples were denatured at 70°C for 2 minutes to minimise secondary structure. 1µl of the heated samples/RNA ladder were loaded into the corresponding wells. The chip was placed into the IKA vertex mixer and vortex at 2400rpm for 1 minute. The chip was then placed into the Agilent 2100 Bioanalyzer and analysed by the Eukaryote Total RNA Nano series II programme.

4.3.5. Transcription factor PCR array

cDNA sample for PCR array was synthesized from 3µg of the total RNA using RT² First strand reverse transcription kit (SABiosciences, Qiagen) according to the manufacturer's instruction. Briefly, 8µl of total RNA was mixed with 2µl of 5x genomic DNA elimination buffer. This Genomic DNA elimination mixture was incubated at 42°C for 5 minutes, followed by chilling on ice for another 5 minutes. While incubation, reverse transcription cocktail was prepared by mixing 4µl of 5x RT buffer 3, 1µl of Primer & External control mix, 2µl of RT enzyme mix 3 and 3µl of RNase/DNase free water. The 10µl of Genomic DNA elimination mixture was then mixed with the 10µl reverse transcription cocktail. The mixture were mixed well and

incubated at 42°C for 15 minutes exactly and terminating the reaction immediately after by heating at 95°C for 5 minutes. 91µl of RNase/DNase free water was added to the 20µl cDNA synthesis reaction, which is chilled on ice for 5 minutes. To run the RT² Profiler transcription factor PCR Array (PARN-075ZC, SABiosciences, Qiagen), 102 µl of the diluted first strand cDNA synthesis reaction was mixed with 1350µl of 2x SABioscience RT2 qPCR master mix and 1248µl of RNase/DNase free water. 25µl of the experimental cocktail was loaded into each well of the array by using a eight channels pipettor. After a brief spin at 1000g, the array was run on Applied Biosystems 7500 fast Real-Time PCR machine using the following cycling programme: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes. The raw Ct values were analysed by the comparative expression $2^{-\Delta\Delta C_t}$ method, and the relative fold changes were normalised to five endogenous house-keeping genes; β -actin, β -2 microglobulin, Hypoxanthine phosphoribosyltransferase 1, Lactate dehydrogenase A, Ribosomal protein large P1, and assessed relative to either the scrambled peptide treated control or the vehicle control. Where expression was undetermined in the real-time PCR reaction, the sample was given a default Ct of 35.

4.3.6. Microarray

The RNA samples for microarray were prepared as described in the section 4.3.1. The preliminary microarray work investigating the downstream signalling upon TLQP-21 stimulation in the primary macrophages used the whole genome expression profiling Affymetrix RG230.2 arrays. The quality control of the RNA samples, hybridisation and the microarray analysis were performed by the commercial company AROS. The differential gene expression analysis were performed by Pfizer Neusentis, in which the

statistical analysis was performed using the Linear Models for microarray analysis from the Limma package (Smyth, G.K., et al., Limma: linear models for microarray data, in Bioinformatics and Computational Biology Solutions using R and Bioconductor. 2005, Springer, New York. p. 397-420). The differential expressions was compared between the TLQP-21 treated and scrambled TLQP-21 treated groups at all time points. The p-values were uncorrelated and p values below 0.01 were considered to be significant. The significant probe lists were generated.

4.4. Results

Despite macrophage has been known to upregulate expression of many pro-inflammatory mediators upon activation in the neuropathic pain state, the molecular event downstream to TLQP-21 stimulation in the macrophage still remains unknown. Having demonstrated in the previous chapters that primary macrophage is the target cell, if not the only one, for VGF-derived peptide TLQP-21 and TLQP-21 acts on the primary macrophage, at least partly, via GC1qR, eliciting a dose-dependent increase in intracellular calcium level, here I sought to investigate the TLQP-21 downstream signalling pathway in the primary macrophage. The investigation here will help to address the following questions: are the gene expressions of the characteristic cytokines, chemokines and metabolites produced by macrophages in the pain state altered upon TLQP-21 stimulation? Does TLQP-21 induce changes in gene expressions of other genes after eliciting the calcium response in the primary macrophages?

To examine the TLQP-21 downstream signalling event, three experimental approaches were employed. The first method employed was the quantitative real-time PCR to investigate if the common mediators secreted by macrophages are changed in expression levels after TLQP-21 treatment. Secondly, I employed PCR array to assess if the expression levels of the common transcription factors are altered following TLQP-21 exposure. Lastly, microarray was employed for a high throughput assessment to the genome-wide gene expression changes.

4.4.1. Examining *in vitro* gene expression of the characteristic mediators produced from macrophages after TLQP-21 stimulation by Quantitative

Real-Time PCR.

Macrophages secrete many cytokines, chemokines and metabolites in the pain state, which are important mediators of hyperalgesia (Sommer and Kress 2004). These mediators include tumour necrosis factor α (TNF- α)(Miller, Jung et al. 2009), interleukin 1 β (IL-1 β)(Kiguchi, Kobayashi et al. 2012), interleukin 6 (IL-6)(Kiguchi, Maeda et al. 2009), inducible nitric oxide synthase (iNOS)(Levy, Kubes et al. 2001, Purwata 2011), brain-derived neurotrophic factors (BDNF)(Wong, Liao et al. 2010, Salegio, Pollard et al. 2011), arginase 1 (Arg1)(Kigerl, Gensel et al. 2009), and cyclooxygenase 2 (Cox2)(Ma, Chabot et al. 2010). Targeting some of these mediators has been proposed to be the potential treatment for neuropathic pain (Ma and Quirion 2006).

TLQP-21 has been demonstrated in the literature to play a role in modulating inflammatory pain *in vivo* (Rizzi, Bartolomucci et al. 2008). However, it was demonstrated in chapter 2 that TLQP-21 is likely not acting directly on the sensory neuron, at least not in the functional assay tested. In addition, TLQP-21 was shown in the same chapter to act on primary macrophage *in vitro* and intraplantar injection of TLQP-21-stimulated macrophages elicited pain-like behaviour *in vivo*. These accumulating data enables us to hypothesise that TLQP-21 could potentially exert its function by modulating activity of the primary macrophages at the periphery upon nerve injuries. It could possibly indirectly modulate the neuronal excitability/activity by inducing the release of effectors from the stimulated macrophages. Nevertheless, the possibility that the TLQP-21 peptide acts directly on neurons could not be firmly eliminated, as it is possible that the process is a calcium independent process, and hence was not picked up in our functional assay.

The mentioned mediators play important roles in the initiation and/or maintenance of neuropathic pain, it is hence our interest to investigate if the expression of these mediators are altered in the macrophage upon TLQP-21 stimulation, and if any of these mediators are the downstream effectors of TLQP-21 in the macrophages, which transduces the signal to specific functions and ultimately alters sensory neuronal activity.

To investigate if any of these mediators are involved in the downstream event of TLQP-21 in the primary macrophages, I examined the gene expressions of these common macrophage mediators following 100nM TLQP-21 stimulation by quantitative real-time PCR. In addition, the expression level of the TLQP-21 receptor, GC1qR, was also examined in parallel to investigate if the TLQP-21 positively/negatively regulate the expression of its receptor in the macrophage. Specific primers for TNF- α , IL-1 β , IL-6, iNOS, BDNF, Arg1, Cox2 and GC1qR were designed. The expression level of these mediators, along with housekeeping genes β -actin and cyclophilin A, were accessed by qRT-PCR. Three hours treatment was selected as the time point for the experiment because it has been demonstrated in the literature that the maximal expressions for most of the inflammatory mediators peak at about 6 hours (Wollenberg, DeForge et al. 1993, Chacon-Salinas, Serafin-Lopez et al. 2005). The cultured macrophages were re-plated to 90mm plates at 1,000,000 cells per plate one day prior to treatment. On the day of treatment, the cells were treated with either 100nM TLQP-21 or 100nM scrambled TLQP-21 as control for 3 hours, followed by total RNA extraction, reverse transcription and qRT-PCR analysis. The result was analysed by comparative $2^{-\Delta\Delta C_t}$ method. The data for each gene after TLQP-21/scrambled TLQP-21 was normalised against to the house keeping gene (β -actin), and the gene expression after TLQP-21 treatment was normalised to that

after scrambled TLQP-21 treatment (expressed as percentage of expression to scrambled TLQP-21 treated control). Figure 4.1 shows the expression of house keeping gene cyclophilin A is not significantly altered in expression, indicating treatment of TLQP-21 and scrambled TLQP-21 did not interfere with normal expression of the house keeping genes. Moreover, it was found, rather unexpectedly, none of the common inflammatory mediators implicated in the pain pathway were significantly altered in expression level. This was found to be consistent with either β -actin or cyclophilin A as control.

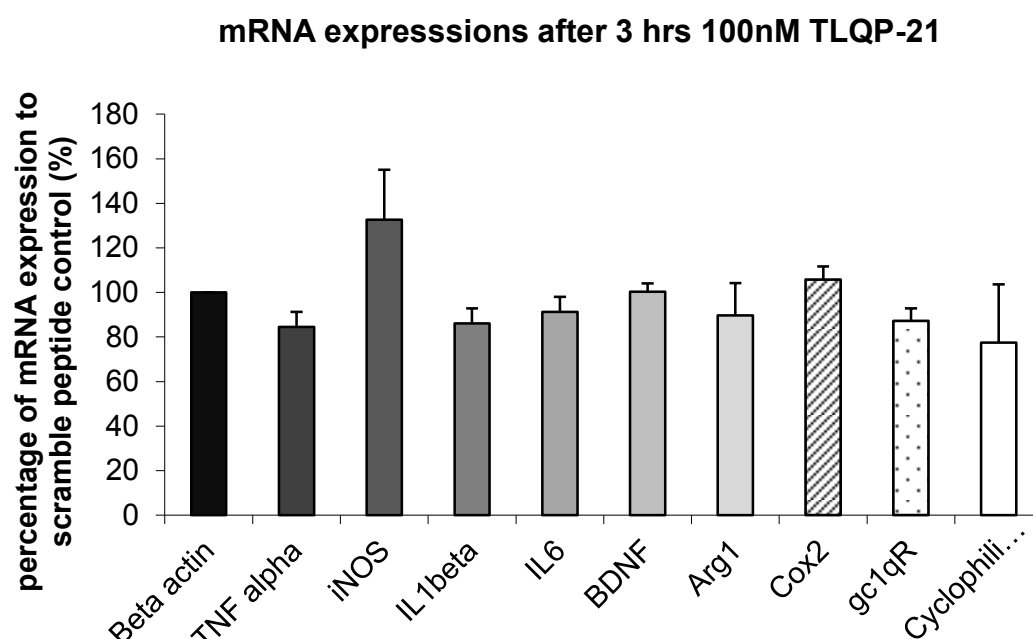


Figure 4.1 Result of quantitative Real-Time PCR shows 100nM TLQP-21 did not induce a significant change in gene expression of common macrophage inflammatory mediators or the GC1qR receptor.

The expression levels of the characteristic macrophage inflammatory mediators were not significantly altered after treating the cells with 100nM TLQP-12 for three hours. Error bars presents as standard deviation.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Average	Standard Deviation
TNF alpha	87.5	87.9	88.2	74.4	84.5	6.7
iNOS	120.0	156.2	107.9	146.2	132.6	22.4
IL1beta	93.7	85.9	77.3	87.4	86.1	6.8
IL6	92.1	90.8	82.8	99.3	91.3	6.8
BDNF	103.5	100.8	96.1	undetermined	100.1	3.8
Arg1	103.8	69.6	91.4	94.1	89.7	14.5
Cox2	112.1	108.3	98.2	104.3	105.7	5.9
GC1qR	81.3	92.5	83.3	91.6	87.2	5.7
Cyclophilin A	104.6	54.4	95.0	56.1	77.5	26.0
Beta actin	100.0	100.0	100.0	100.0	100.0	0.0

Table 4.1 Data of the percentage of gene expression after TLQP-21 treatment from the four quantitative real-time PCR experiments (expressed as percentage to the scrambled TLQP-21 treated control).

4.4.2. Investigation of transcription factors expressions following TLQP-21 treatment by Transcription factor PCR array.

Macrophages are cells with diverse function. To fulfill these functions, macrophages possess the ability to express, produce and release of more than one hundred of different soluble substances that subsequently influence diverse functional pathways (Pollard 2009). The expressions of these effector substances, or change in the expression levels of these substances upon stimulation, are largely dictated by the transcription factors control of macrophages. Here, I ask the question if the expression levels of the common transcription factors in the primary macrophages are altered upon TLQP-21 stimulation. If any of the common transcription factors is shown to be dis-regulated in the expression level after TLQP-21 treatment, it will enable us to narrow down, and further dissect, the TLQP-21 downstream signalling pathway in the macrophages.

To achieve this, I utilized the RT² Profiler transcription factor PCR Array (PARN-075ZC) to simultaneously perform gene expression analysis of 84 common transcription factors, along with 5 house keeping genes (full gene list please refer to Table 4.2). The primary macrophages were cultured in serum-free media for 24 hours prior to the treatment with either vehicle control (same value of water), 100nM TLQP-21, or 100nM Scrambled TLQP-21. The cells were exposed to the treatments for either 30 minutes or 3 hours and the total RNA was immediately extracted, followed by reverse transcription for cDNA synthesis. The cDNA was used to run the real-time PCR cycling program and the analysis of the array was performed (see figure 4.2 for experimental outline).

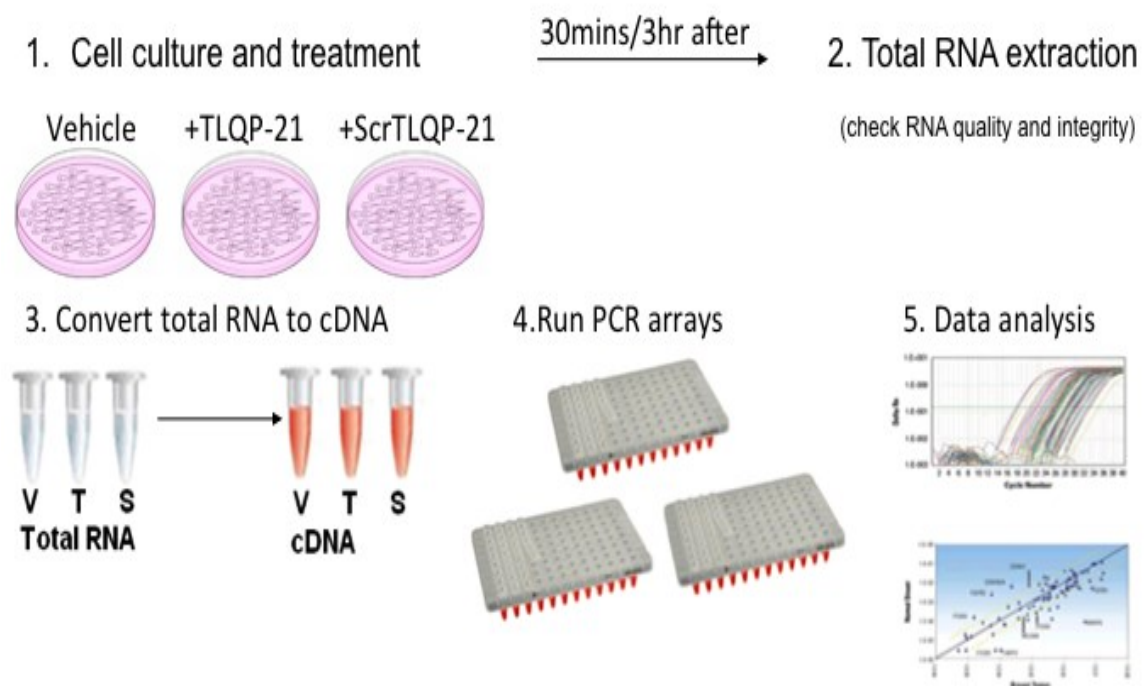


Figure 4. 2 Schematic diagram showing experimental outline of transcription factor PCR array experiment.

GeneBank	Symbol	Gene Name	Also known as
NM_012502	Ar	Androgen receptor	Andr, Tfm
NM_012780	Arnt	Aryl hydrocarbon receptor nuclear translocator	Arnt1
NM_001100895	Atf1	Activating transcription factor 1	Atf-1
NM_031018	Atf2	Activating transcription factor 2	-
NM_012912	Atf3	Activating transcription factor 3	LRF-1, LRF1
NM_024403	Atf4	Activating transcription factor 4	-
NM_012524	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	DBPCEP
NM_024125	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	Il6dbp, LAP, NF-IL6, TCF5
NM_012831	Cebpg	CCAAT/enhancer binding protein (C/EBP), gamma	C, EBP, CEBPRNA
NM_001024294	Clasrp	Clk4-associating serine/arginine rich protein	Sfrs16, Srsf16
NM_031017	Creb1	CAMP responsive element binding protein 1	Creb
NM_133381	Crebbp	CREB binding protein	CBP, RSTS, RTS
NM_053357	Ctnnb1	Catenin (cadherin associated protein), beta 1	Catnb
NM_001011914	Dr1	Down-regulator of transcription 1	-
NM_001100778	E2f1	E2F transcription factor 1	-
XM_233986	E2f6	E2F transcription factor 6	-
NM_012551	Egr1	Early growth response 1	Krox-24, NGFI-A, Ngf1, Ngfi
NM_012689	Esr1	Estrogen receptor 1	ER-alpha, Esr, RNESTROR
NM_012555	Ets1	V-ets erythroblastosis virus E26 oncogene homolog 1	Ets-1, Etsoncb, Tpl1
NM_001107107	Ets2	V-ets erythroblastosis virus E26 oncogene homolog 2	-
NM_022197	Fos	FBJ osteosarcoma oncogene	c-fos
NM_012743	Foxa2	Forkhead box A2	Hnf3b
NM_012560	Foxg1	Forkhead box G1	BF1A, Fkh11, RATBF1A
NM_012764	Gata1	GATA binding protein 1	Eryf1
NM_033442	Gata2	GATA binding protein 2	-
NM_133293	Gata3	GATA binding protein 3	-
NM_031041	Gtf2b	General transcription factor IIB	-
NM_001007711	Gtf2f1	General transcription factor IIF, polypeptide 1	MGC94148
NM_021592	Hand1	Heart and neural crest derivatives expressed 1	MGC114332, eHand
NM_022696	Hand2	Heart and neural crest derivatives expressed 2	-
NM_001025409	Hdac1	Histone deacetylase 1	-
NM_024359	Hif1a	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	MOP1
NM_012669	Hnf1a	HNF1 homeobox A	HNF1, LF-B1, Lfb1, Tcf1
NM_022180	Hnf4a	Hepatocyte nuclear factor 4, alpha	Hnf4, Hnf4alpha
XM_001059031	Hoxa5	Homeo box A5	-

NM_024393	Hsf1	Heat shock transcription factor 1	-
NM_012797	Id1	Inhibitor of DNA binding 1	ID125A, Idb1, MGC156482
NM_012591	Irf1	Interferon regulatory factor 1	-
NM_021835	Jun	Jun oncogene	-
NM_021836	Junb	Jun B proto-oncogene	-
NM_138875	Jund	Jun D proto-oncogene	MGC72300
NM_145095	Kcnh8	Potassium voltage-gated channel, subfamily H (eag-related), member 8	ELK3, Elk1, Ets-1
NM_022210	Max	MYC associated factor X	MGC124611
NM_001014035	Mef2a	Myocyte enhancer factor 2a	-
NM_001017507	Mef2b	Myocyte enhancer factor 2B	-
XM_574821	Mef2c	Myocyte enhancer factor 2C	RGD1563119
NM_012603	Myc	Myelocytomatosis oncogene	MGC105490, c-myc, mMyc
NM_001106783	Myf5	Myogenic factor 5	-
NM_176079	Myod1	Myogenic differentiation 1	MGC156574
NM_001108908	Nanos2	Nanos homolog 2 (Drosophila)	RGD1562436
NM_001107425	Nfat5	Nuclear factor of activated T-cells 5	Nfat, TonEBP
NM_001107805	Nfatc2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-
NM_001108447	Nfatc3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NFAT4
NM_001107264	Nfatc4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	-
XM_342346	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NF-kB
NM_031553	Nfyb	Nuclear transcription factor-Y beta	CBF-B, CBFB, Nfya
NM_012576	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	GR, Gcr, Grl
NM_013001	Pax6	Paired box 6	-
NM_001109599	Pou2af1	POU class 2 associating factor 1	-
NM_013196	Ppara	Peroxisome proliferator activated receptor alpha	PPAR
NM_013124	Pparg	Peroxisome proliferator-activated receptor gamma	-
NM_017045	Rb1	Retinoblastoma 1	-
XM_223688	Rel	V-rel reticuloendotheliosis viral oncogene homolog	-
NM_199267	Rela	V-rel reticuloendotheliosis viral oncogene homolog A	NFkB
XM_001058445	RGD1560225	Similar to nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	-
NM_013130	Smad1	SMAD family member 1	Madh1
NM_019275	Smad4	SMAD family member 4	Madh4

NM_021692	Smad5	SMAD family member 5	Madh5
NM_138872	Smad9	SMAD family member 9	Madh9, Smad8
NM_012655	Sp1	Sp1 transcription factor	-
XM_002726189	Sp3	Sp3 transcription factor	-
NM_032612	Stat1	Signal transducer and activator of transcription 1	-
NM_001011905	Stat2	Signal transducer and activator of transcription 2	-
NM_012747	Stat3	Signal transducer and activator of transcription 3	MGC93551
NM_001012226	Stat4	Signal transducer and activator of transcription 4	-
NM_017064	Stat5a	Signal transducer and activator of transcription 5A	Stat5
NM_022380	Stat5b	Signal transducer and activator of transcription 5B	-
NM_001044250	Stat6	Signal transducer and activator of transcription 6	-
NM_001004198	Tbp	TATA box binding protein	MGC93994, TFIID
XM_001054844	Tcf7l2	Transcription factor 7-like 2 (T-cell specific)	-
NM_001107345	Tcfap2a	Transcription factor AP-2, alpha	Tfap2a
NM_001015020	Tgif1	TGFB-induced factor homeobox 1	MGC109104, MGC156525, Tfig, Tgif
NM_030989	Tp53	Tumor protein p53	MGC112612, Trp53, p53
NM_173290	Yy1	YY1 transcription factor	NF-E1, UCRBP
NM_031144	Actb	Actin, beta	Actx
NM_012512	B2m	Beta-2 microglobulin	-
NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase, Hprt, MGC112554
NM_017025	Ldha	Lactate dehydrogenase A	Ldh1
NM_001007604	Rplp1	Ribosomal protein, large, P1	MGC72935
U26919	RGDC	Rat Genomic DNA Contamination	RGDC
SA_00104	RTC	Reverse Transcription Control	RTC
SA_00104	RTC	Reverse Transcription Control	RTC
SA_00104	RTC	Reverse Transcription Control	RTC
SA_00103	PPC	Positive PCR Control	PPC
SA_00103	PPC	Positive PCR Control	PPC
SA_00103	PPC	Positive PCR Control	PPC

Table 4. 2 Full gene list for the RT² Profiler transcription factor PCR Array (PARN-075ZC).

A total of 84 common transcription factors were analysed along with 5 house-keeping genes (highlighted in orange). Quality controls were monitored at the same time to check for the genomic DNA contamination (highlighted in green) and efficiency of the reverse transcription (highlighted in blue). Moreover, positive controls were performed (highlighted in yellow) to ensure the real-time PCR reaction was at its optimal.

Total RNA quality, integrity assessment and quantification.

Although the quantitative real-time PCR array allows easy and reliable analysis of the expression of a focused group of genes, the technique is highly sensitive and a minute change during the experiment, such as the RNA quality, efficiency of reverse transcription and efficiency of the PCR cycling, could greatly affect the final result. Thus, the initial total RNA quantification and its quality and integrity were carefully assessed prior to the cDNA synthesis. For RNA quantification, the Thermo Scientific NanoDrop™ ND-1000 spectrophotometer was used. The concentration of the RNA (in ng/μl) was measured as well as the 260/280 absorption ratio. The 260/280 ratio absorbance indicates the purity of the RNA, a ratio of >2.0 is generally regarded as sufficient 'pure' for RNA. If such ratio is shown to be lower than 2, it possibly indicates that there is protein contamination in the RNA, which absorbs strongly near 280nm wavelength. As shown in figure 4.3B, all samples prepared for PCR array are at good concentration and the 260/280 absorption ratio are all greater than 2.0, indicating the total RNA extracted are with good purity.

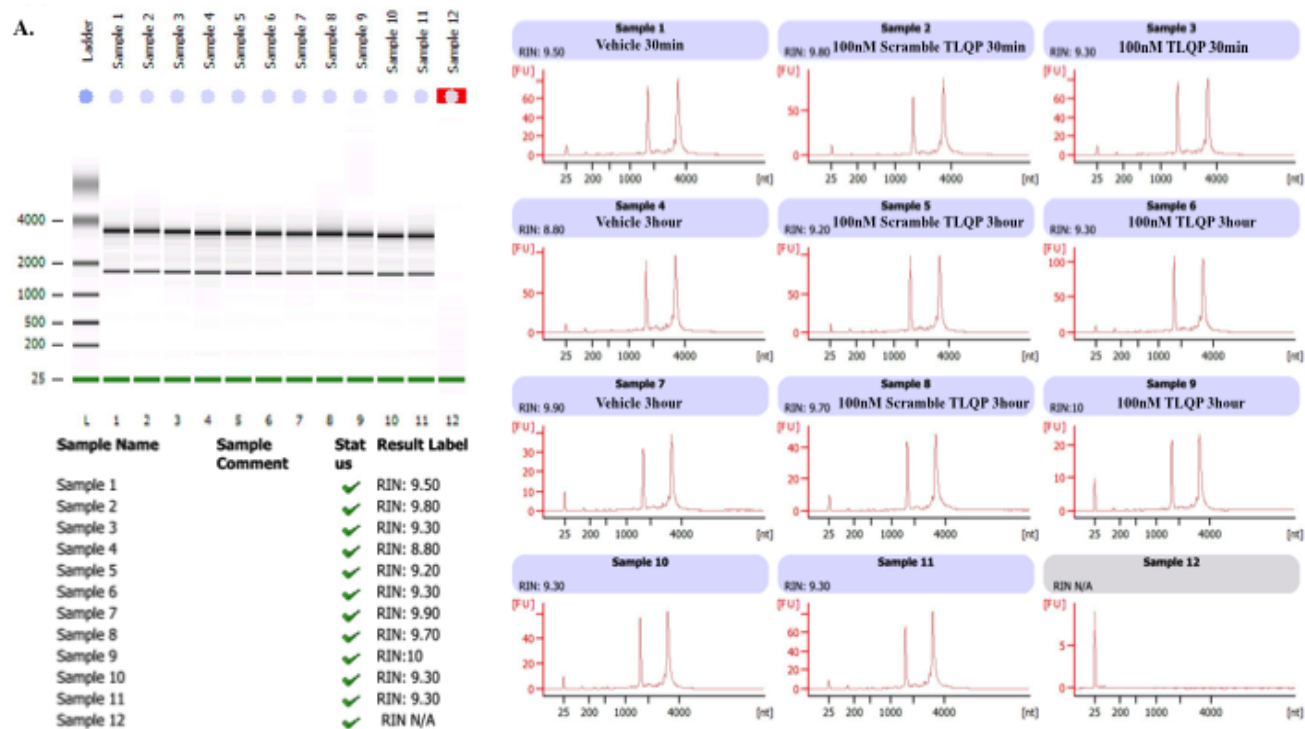
All samples were then assessed for the RNA integrity using an Agilent RNA Nano 6000 electrophoresis Labchip™ and Agilent bioanalyser. The ribosomal RNAs (rRNAs) comprise more than 90% of the mass of total RNA, and the most abundant rRNAs are the 18S rRNA and 28S rRNA. The chip-based nucleic acid analysis system performs capillary electrophoresis of the samples along with a reference marker in the Agilent 2100 bioanalyser. The two abundant peaks shown in electrophoresis result in figure 4.3A (left) are the 18S and 28S rRNAs. The electropherograms of the electrophoresis result of each sample was generated (figure 4.3A right), where sharp single peaks for rRNAs indicate good RNA integrity. The

RIN software algorithm of the bioanalyser then analyse the entire electrophoretic trace and generates a RNA integrity number (RIN) scoring from 1 to 10. The RIN values allows non-subjective interpretation of the RNA integrity from the electropherogram, where value 10 indicates the RNA is at good purity and 1 suggests the RNA is totally degraded. The total RNAs use for sensitive technique such as PCR array and microarray generally require RIN values great than 7. As shown in the figure 4.3A, the RIN values of all samples prepared are greater than 8.8, indicating minimal RNA degradation in these samples and the purity and integrity of the total RNAs are at good quality.

3µg of the total RNAs were hence used to synthesise the cDNA using RT² First strand reverse transcription kit (SABiosciences, Qiagen). The RT² First strand kit enables elimination of genomic DNA, the efficiency of the reverse transcription to be assessed and examines if there is enzyme inhibitors contamination in the RNA samples. These steps are essential for PCR array quality controls as they could alter the final result greatly. The reverse transcription efficiency and enzyme inhibitors contamination are tested with a built-in external RNA control. A last quality control was monitored at the PCR cycling step where a built-in external cDNA was tested for positive control to inspect the PCR efficiency. The Positive PCR Control (PPC) and Reverse Transcription Control (RTC) were repeated three times in the array, and the average Ct and standard deviation between the three repeats were calculated for both RTC and PPC. The PCR reaction reproducibility was assessed by the standard deviation of the three repeats. The smaller the standard deviation between three RTC and PPC indicates better reproducibility of the reaction. To ensure the PCR array is comparable, the standard deviation between RTC and PPC has to fall below 0.1 (0.1 cycle indicates about 7% deviation in gene expression between two repeats). As indicated in figure

4.4 (panel 1), the standard deviations between the three replica of RTC and PPC are lower than 0.8, indicating less than 5% deviation in gene expression between the repeats, and hence suggesting good PCR array reproducibility.

The change in Ct values was calculated between the averaged RTC and PPC, which is an arbitrary number to assess the reverse transcription efficiency. The reverse transcription efficiency is regarded as good efficiency when the ΔC_t (average RTC - average PPC) is less or equal to 5. As shown in figure 4.4 (panel 2), the ΔC_t (average RTC - average PPC) from all samples are less than 3.87, indicating a good efficiency at the first strand reverse transcription step. Lastly, the genomic DNA contamination was assessed by using genomic DNA specific primers in the PCR array, the Ct value of these wells should be undetermined or greater than 35 cycles. In all the samples prepared, the genomic DNA contamination controls are greater than 35, indicating minimal contamination of genomic DNA in the samples. Thus, all the signal amplified and interpreted in the experiment are derived from the first strand cDNA amplified from the total RNA.



B.

Sample ID	Condition	Concentration	A260	A280	260/280	260/230
Sample 1	Vehicle 30 min	395.87	9.897	4.703	2.1	1.79
Sample 2	100nM ScrTLQP-21 30 min	354.35	8.859	4.191	2.11	1.73
Sample 3	100nM TLQP-21 30 min	300.24	7.506	3.548	2.12	2.08
Sample 4	Vehicle 3 hrs	443.43	11.086	5.317	2.08	1.95
Sample 5	100nM ScrTLQP-21 3 hrs	451.4	11.285	5.41	2.09	1.99
Sample 6	100nM TLQP-21 3 hrs	491.89	12.297	5.748	2.14	2.01
Sample 7	Vehicle 3 hrs	232.52	5.813	2.788	2.08	2.03
Sample 8	100nM ScrTLQP-21 3 hrs	245.05	6.126	2.89	2.12	2.04
Sample 9	100nM TLQP-21 3 hrs	264.71	6.618	3.134	2.11	2.14

Figure 4.3 RNA integrity and quality validation using Agilent Bioanalyzer and NanoDrop spectrophotometer.

A) The RNA integrity was validated using Agilent RNA nano 6000 chip and Agilent Bioanalyzer. The micropipillary electrophoresis was run on the chip (top left) and the two 28S and 28S ribosomal RNA bands are shown to be visible in all samples. The electropherogram traces (right) of all the samples were generated from laser-induced fluorescence (LIF) detection of the micropipillary electrophoresis gel. The two peaks for 18S and 28S ribosomal RNAs are detected as distinct sharp peaks in all the samples, indicating good RNA integrity. The RNA integrity number (RIN) scorings of all samples were calculated from RIN software algorithm (bottom left), Samples with RIN value greater than 7 are considered to possess good RNA integrity. **B)** The RNA quantification and quality were accessed by Thermo Scientific NanoDrop™ ND-1000 spectrophotometer. The concentration was highlighted in red column (ng/μl) and the 260/280 ratio of all samples are shown to be greater than 2.0, indicating good RNA quality with minimal protein contamination in all the samples.

1. PCR Array Reproducibility:							
Array	Vehicle 30 minutes	100nM TLQP 30 minutes	100nM Scramble TLQP 30 minutes	Array	Vehicle 3 hours	100nM TLQP 3 hour	100nM Scramble TLQP 3 hours
Average Ct (PPC)	19.66	19.71	19.68	Average Ct (PPC)	19.65	19.71	19.62
ST DEV C _t (PPC)	0.08	0.03	0.04	ST DEV C _t (PPC)	0.03	0.05	0.03
Average C _t (RTC)	23.23	23.39	23.11	Average C _t (RTC)	23.45	23.42	23.49
ST DEV C _t (RTC)	0.05	0.06	0.02	ST DEV C _t (RTC)	0.04	0.04	0.04
2. Reverse Transcription Control (RTC):							
Array	Vehicle 30 minutes	100nM TLQP 30 minutes	100nM Scramble TLQP 30 minutes	Array	Vehicle 3 hours	100nM TLQP 3 hour	100nM Scramble TLQP 3 hours
Delta C _t (AVG RTC - AVG PPC)	3.57	3.68	3.43	Delta C _t (AVG RTC - AVG PPC)	3.8	3.71	3.87
RT Efficiency	Pass	Pass	Pass	RT Efficiency	Pass	Pass	Pass
Criteria: If Delta C _t (AVG RTC - AVG PPC) <= 5, RT Efficiency reports 'Pass'							
3. Genomic DNA Contamination (GDC):							
Array	Vehicle 30 minutes	100nM TLQP 30 minutes	100nM Scramble TLQP 30 minutes	Array	Vehicle 3 hours	100nM TLQP 3 hour	100nM Scramble TLQP 3 hours
C _t (GDC)	35	35	35	C _t (GDC)	35	35	35
Genomic DNA	Pass	Pass	Pass	Genomic DNA	Pass	Pass	Pass
Criteria: If C _t (GDC) >= 35, then the GDC QC reports 'Pass', if C _t (GDC) < 35							

Figure 4.4 Quality controls for the PCR arrays.

1. PCR array reproducibility control 2. Reverse transcription efficiency control 3. Genomic DNA contamination control.

PCR array scatter plot: fold regulation comparison between the treatment and control

Following validating quality controls for reverse transcription efficiency, genomic DNA contamination and PCR array reproducibility, the gene expression of the 84 focused panel of transcription factors were analysed. A 2-fold cut off threshold was set for the PCR array data analysis. The changes in the gene in the primary macrophages after 30 minutes/3 hours peptide incubation were assessed by comparing the gene expression levels between TLQP-21 treatment group and scrambled TLQP-21 treated group. As shown in Figure 4.5, none of the 84 genes tested were shown to be differentially expressed after 30 minutes of 100nM TLQP-21

and 100nM Scrambled TLQP-21 treatment. However, it is shown that the transcription factor Nuclear factor of activated T-cells calcineurin-dependent 2 (Nfatc2) was upregulated by 2.56 folds after TLQP-21 stimulation when compared to its expression in the cells treated with scrambled TLQP-21. The raw Ct data of Nfatc2 in all three groups were listed in Table 4.3.

Although it is shown Nfatc2 transcription factor is upregulated by 2.56 folds after TLQP-21 treatment when compared to that treated with scrambled TLQP-21, there remains a possibility that the Nfatc2 expression was downregulated by 2.56 folds instead after scrambled TLQP-21 treatment, and hence a seemingly increased expression was observed when compared TLQP-21 treated cell to scrambled TLQP-21 treatment. For this reason, the PCR array data was also analysed against the expression in the vehicle control, where equal volume of water was added to the culture medium (without peptide).

As indicated in figure 4.6, it was found that the scrambled peptide showed no differential expression after 30 minutes treatment when compared to the vehicle control (figure 4.6A), and Nfatc2 was, in fact, down-regulated after 3 hours of scrambled peptide treatment (figure 4.6B). The Nfatc2 expression after 3 hours of wild type TLQP-21 peptide treatment was, on the other hand, not differentially expressed when compared to the vehicle control (figure 4.6B). In addition, GATA binding protein 2 (GATA2) was demonstrated to be downregulated by 2.43 and 2.69 folds after 30 minutes and 3 hours stimulations with TLQP-21, respectively. The raw data of the Ct value of GATA2 expressions in all three conditions were listed in the table 4.3.

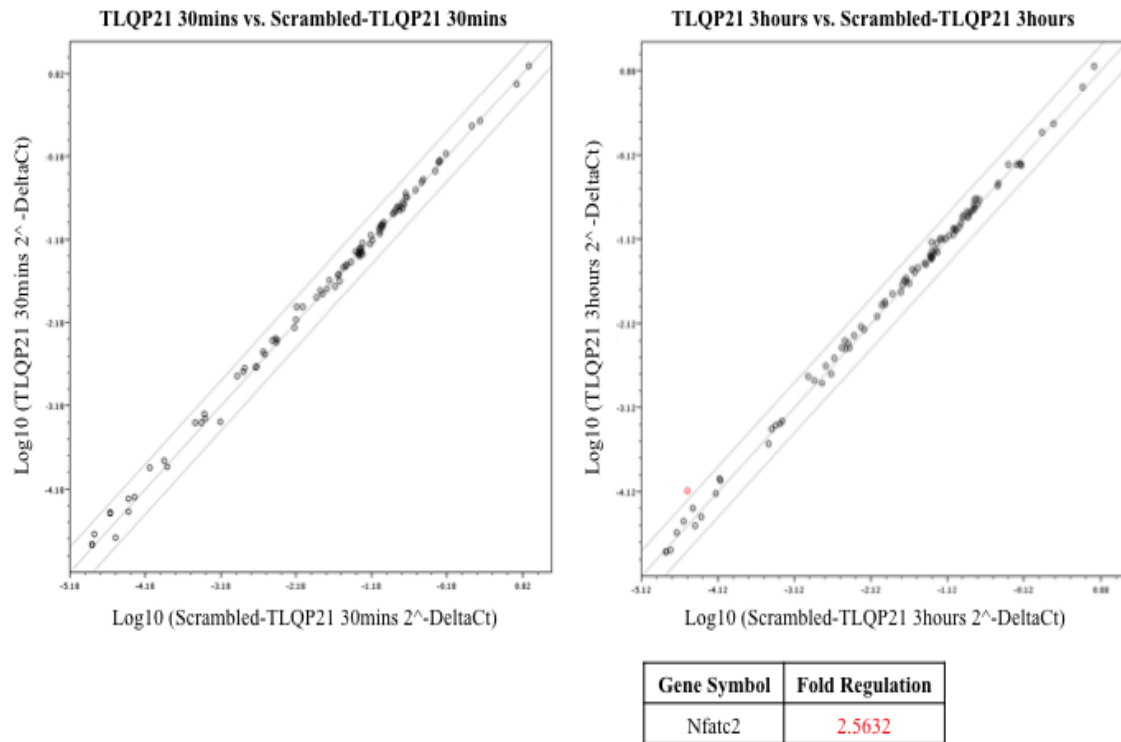
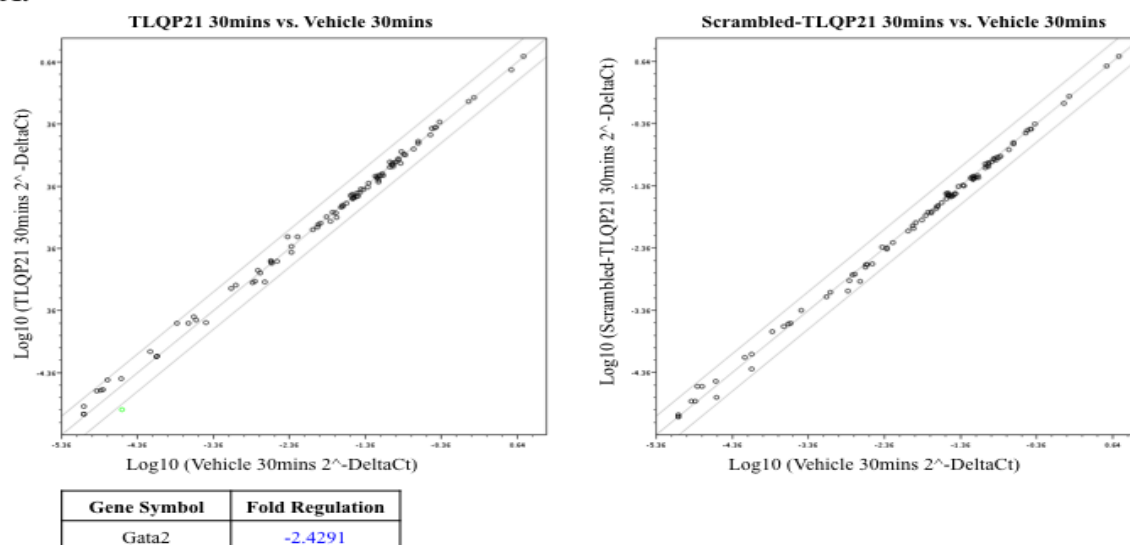


Figure 4.5 Transcription factors PCR array data shows only one gene expression level is altered after 3 hours TLQP-21 treatment when compared to that of scrambled TLQP-21 group.

The raw Ct data from the PCR array experiments were analysed using a 2-fold threshold with $2^{\Delta\Delta Ct}$ comparative expression analysis method. The analysed data is represented in scatter plots showing the boundary line of 2-fold cut off from the scrambled TLQP-21 treated group (control). Genes with more than 2 folds increases in the expression relative to the control fall into the up-left area of the scatter plot. On the other hand, genes with more than 2 folds decreases in the expression relative to the control fall into the bottom right area of the scatter plot. For the genes that are not differentially expressed between the treatment and control (i.e. expression differences are less than 2 folds), they are represented within the two boundary lines along the diagonal line. The genes expressions of all 84 genes tested are not differentially expressed after 30 minutes TLQP-21 treatment and scrambled TLQP-21 treatment (left scatter plot) and Nfatc2 is upregulated by 2.56 folds after 3 hours of TLQP-21 treatment relative to the control.

A.



B.

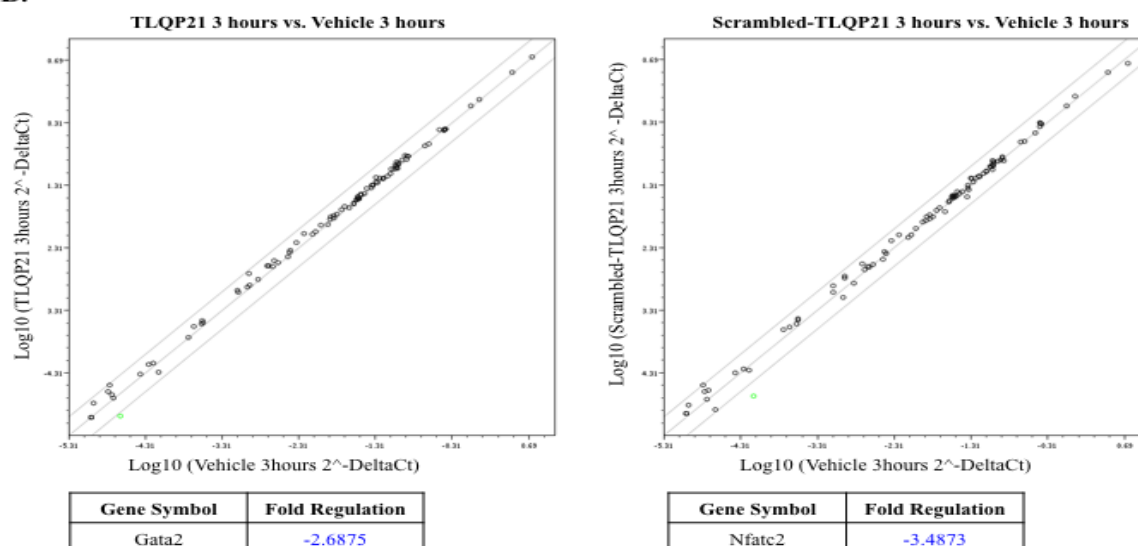


Figure 4.6 Transcription factors PCR array data of changes in gene expressions after TLQP-21/Scrambled TLQP-21 treatment when compared to the expression in the vehicle control.

The scatter plots show only one gene, Gata2, expression level is down-regulated after 30 minute TLQP-21 treatment relative to the vehicle control, which remains at lower expression in the 3 hours TLQP-21 treatment condition. Nfatc2 is shown to be down-regulated after 3 hours treatment with scrambled TLQP-21, and no gene is shown to be differentially expressed relative to vehicle control after 30 minutes of scrambled peptide treatment.

Gene symbol	Vehicle 30mins	TLQP-21 30mins	Scrambled-TLQP-21 30mins	Vehicle 3hours	TLQP-21 3hours	Scrambled TLQP-21 3hours
Nfatc2	32.10	31.66	31.83	32.07	32.61	34.09
Gata2	33.32	34.76	33.97	33.73	34.93	34.80

Table 4.3 Raw Ct data for Nfatc2 and Gata2 in all 6 arrays.

4.4.3. Microarray: high-throughput assessment of gene expression changes in the primary macrophages upon TLQP-21 stimulation.

In order to expand our investigation of TLQP-21 downstream signalling pathway and examine if TLQP-21 stimulation affected the expression of specific genes in the primary macrophages, whole-genome expression analysis was carried out in triplicate for primary macrophages treated with scrambled TLQP-21 and TLQP-21. In order to catch the changes of immediate early genes and late response genes, three time points were selected for the treatments, 30 minutes, 3 hours and 24 hours, to cover a wider range of treatment time. It was observed during the incubation period, the macrophages treated with wild type TLQP-21 peptide drastically change the morphology after 12 hours of incubation. The morphology of the cultured primary macrophage changed from irregular ramified 'resting' shape into a more elongated morphology with long processes (figure 4.7). This indicates that the downstream signalling of TLQP-21 induces a morphological change to the primary macrophage, possibly to adapt for its functional outcome.

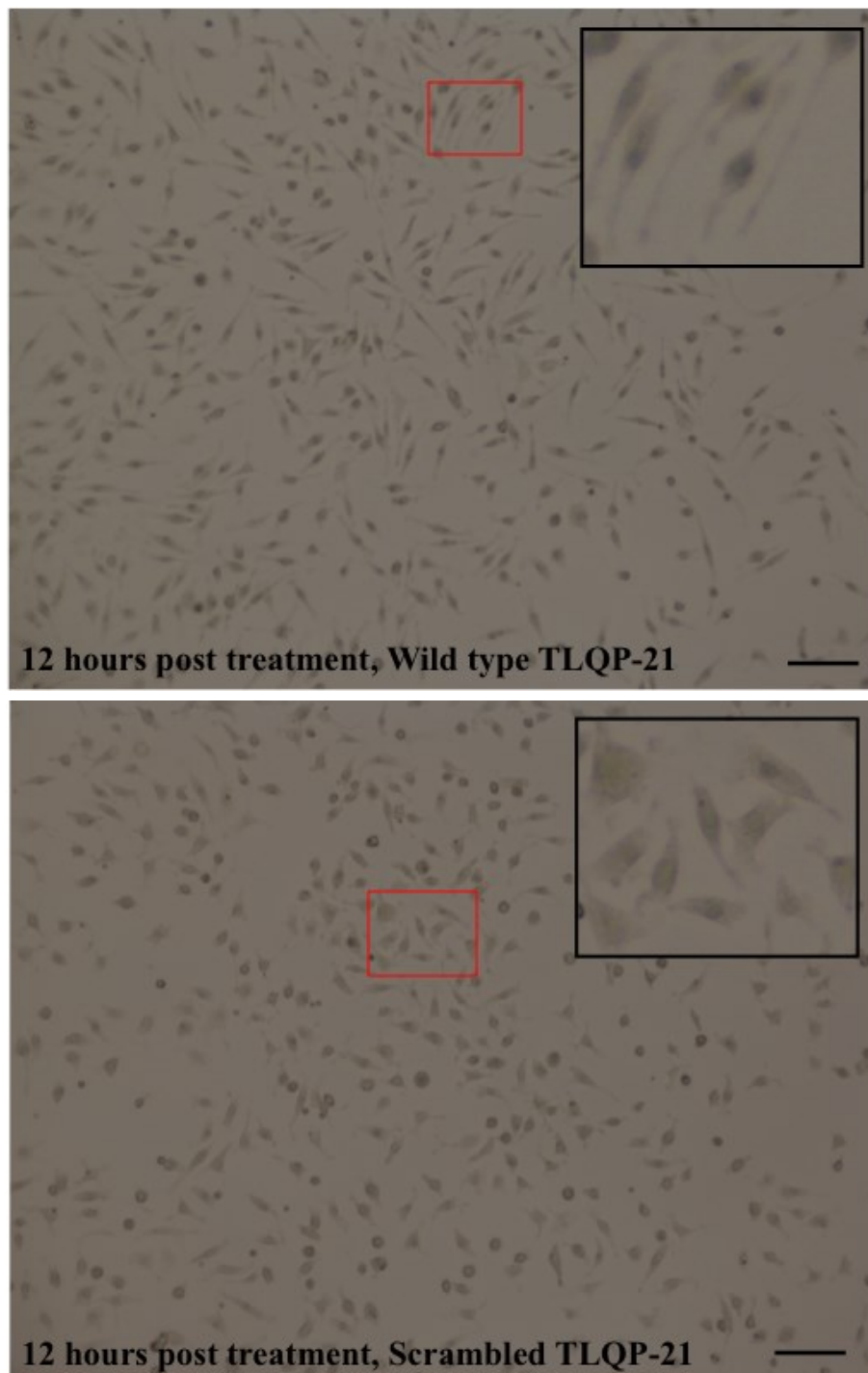


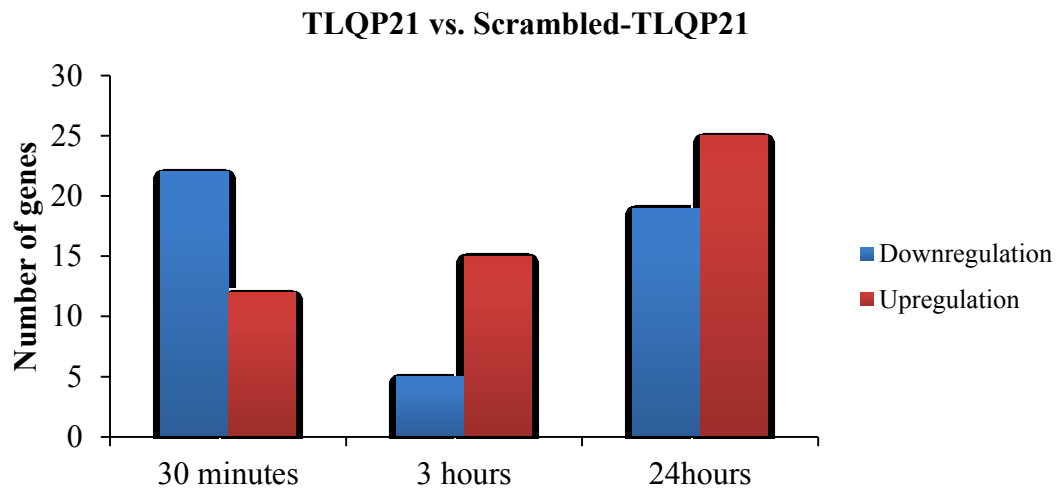
Figure 4.7 Representative pictures in bright field of primary macrophages treated with either Scrambled TLQP-21 or TLQP-21 for 12 hours.

The primary macrophages were treated with 100nM of either scrambled TLQP-21 or wild type TLQP-21. It is observed after long incubation with the wild type peptide, the morphology of the primary macrophages changed to a more elongated morphology (top) from the usual ramified shape in the resting state. The cells treated with scrambled peptide, however, retained the ramified cell morphology (bottom).

After treating the cells with TLQP-21/Scrambled TLQP-21 for corresponding incubation time, the total RNA was extracted from the cells using Qiagen RNAeasy extraction kit. The quality control of RNA was performed by commercial company AROS, using Bioconductor QualityMetrics array, where no significant outliers were detected. The Affymetrix RG230.2 arrays were used for microarray analysis, and the differential gene expression analysis of the raw microarray data was performed by AROS. Briefly, the raw data was first normalised to the corresponding control by robust multi-array analysis (RMA) package (bioconductor), and the data were pre-filtered to remove probes that had little variation across all arrays. This decreases the number of probes taken forward into the differential analysis step and increases the sensitivity for identifying probe sets that significantly altered in expression.

To perform the differential analysis, a less stringent direct modeling of the batch structure with removeBatchEffect function was performed using the linear model in Limma package from Bioconductor. The contrast of interest was formulated to compare TLQP-21 treated sample versus its corresponding Scrambled TLQP-21 treated controls. Figure 4.8 shows the numbers of differential expressed genes in the primary macrophages after 30 minutes, 3 hours and 24 hours TLQP-21 treatments compared to their corresponding Scrambled TLQP-21 treated controls. It was found that 22, 5 and 19 genes were significantly down-regulated ($p < 0.01$) after 30 minutes, 3 hours and 24 hours TLQP-21 treatments, respectively, compared to the corresponding scrambled peptide treated controls. In addition, 12, 15 and 25 genes were significantly up-regulated ($p < 0.01$) in the 30 minutes, 3 hours and 24 hours TLQP-21 treatment group, respectively, compared to the corresponding controls treated with same concentration of scrambled TLQP-21. Table A.1, Table A.2 and

Table A.3 (see appendix) show the summary of all the differentially expressed genes with an uncorrected p-value cutoff of <0.01 for 39 minutes, 3 hours and 24 hours treatments, respectively.



TLQP-21 vs. Scrambled-TLQP-21			
	30 minutes	3 hours	24hours
Number of genes down-regulated	22	5	19
Number of genes with no change in expression	9825	9839	9815
Number of genes Up-regulated	12	15	25

Figure 4.8 Number of genes differentially/constantly expressed after 30 minutes, 3 hours and 24 hours of TLQP-21 stimulation relative to that treated with same concentration of Scrambled TLQP-21.

4.5. Discussion

4.5.1. TLQP-21 peptide treatment and the differential gene expression analysis by qRT-PCR, PCR array and Microarray.

Having demonstrated in the previous chapters that TLQP-21 acts on the primary macrophage in a dose-dependent and calcium-dependent manner, macrophages are desensitised to multiple TLQP-21 treatments, and the signalling of TLQP-21 on the primary macrophages are, at least partly, via the potential receptor GC1qR, several attempts were made in this chapters to elucidate the subsequent downstream signalling event in the primary macrophages upon TLQP-21 stimulation.

The first strategy to investigate the downstream signalling event after TLQP-21-induced calcium response employed the qRT-PCR to investigate the change in expression of the common pro-inflammatory mediators and the receptor. It is evident in the literature that the change in the intracellular calcium level is associated with the macrophage activation (Petricevich, Reynaud et al. 2008, Kelly, Wang et al. 2010). In addition, the activation of another lineage of macrophage in the brain, microglia, has also been coupled downstream to the calcium signalling (Hoffmann, Kann et al. 2003). The subsequent signalling event after the initial calcium response often results in the release of the pro-inflammatory mediators and ultimately leading to the changes in the corresponding gene expressions, which have been evident to peak between 2-6 hours (Wollenberg, DeForge et al. 1993, Brown, Donaldson et al. 2004, Chacon-Salinas, Serafin-Lopez et al. 2005). Using these as guidance, the downstream signalling event of TLQP-21 was first investigated by qRT-PCR as a preliminary investigation. Based on the assumption that the maximal

pro-inflammatory mediators gene expression levels could be reached after 3 hours exposure to TLQP-21 and the scrambled TLQP-21 control peptide would not non-specifically elicit the same response as the wild type, the primary macrophage cultures were stimulated for 3 hours with either 100nM scrambled TLQP-21 or wild type TLQP-21. The gene expression changes of the common pro-inflammatory mediators produced from the macrophages were assessed subsequently using qRT-PCR.

For all the primer sets designed for the qRT-PCR experiment (TNF- α , IL-1 β , IL-6, iNOS, BDNF, Arg1, Cox2 and GC1qR), none of the gene was shown to be significantly dis-regulated after 3 hours treatment of 100nM TLQP-21 versus 100nM scrambled TLQP-21 treatment. There could be a number of reasons for the absence in gene expression alteration. The first reasons could simply be that the target genes investigated here were indeed not involved in the downstream signalling event of TLQP-21 in the macrophages, and hence the changes in gene expressions were not observed after TLQP-21 stimulation. The targets that investigated here are mainly the pro-inflammatory mediators secreted by M1 subtype of macrophages. While there are other different subtype classifications, it is possible that the TLQP-21 downstream signalling event is different from the classical M1 signalling pathway. Indeed, it has been demonstrated that different subtype of macrophages often gave rise to a subtype-specific increase in expression of genes and different stimuli could elicit different subtype macrophage responses (Shalhoub, Falck-Hansen et al. 2011, Engler, Robinson et al. 2012). It is also possible that TLQP-21-induced response is not as robust as other stimuli, such as LPS, which leads to immense up-regulations of various pro-inflammatory effectors. The possibility that TLQP-21, as an endogenous neuropeptide, may induce a more subtle response, which requires a longer stimulation

time, still remains to be further evaluated and cannot be exclusively eliminated. Future experiments could be carried out to optimise for longer time points of TLQP-21 stimulation. Furthermore, there is also a possibility that macrophages may adapt to some immediate physiological responses, such as chemotactic response and increase/inhibit in proliferation, instead of being pro-inflammatory activated. Thus, the changes in expression of the pro-inflammatory mediators were absent. Future experiments could be carried out to investigate the chemotactic effect of TLQP-21 on the primary macrophage using techniques such as Boyden chamber assay, and the proliferative/anti-proliferative effect of TLQP-21 can also be further assessed.

To understanding the downstream signalling pathway following the TLQP-21 stimulated calcium response in the macrophage better, a high throughput PCR array technique was employed to investigate the changes in transcription factor expression. Only one out of the 84 transcription factors tested in the array was dis-regulated after 3-hours TLQP-21 treatment when compared to the corresponding scrambled peptide treated control. It was shown that the expression of *Nfatc2* in the sample treated with 100nM TLQP-21 for 3 hours was 2.56 folds higher than that treated with 100nM scrambled peptide control. 2.56 folds higher expression could equally mean there was a 2.56 folds up-regulation in *Nfatc2* after 3 hours of TLQP-21 treatment, or a 2.56 folds down-regulation in *Nfatc2* in the 3 hours scrambled peptide treated group. Indeed, the raw Ct value indicates that the *Nfatc2* expression was similar in the vehicle control (32.07) and TLQP-21 treated sample (32.61), whereas the Ct value altered after scrambled TLQP-21 treatment to 34.09, indicating a down-regulation of gene expression after scrambled peptide 3 hours treatment. This suggests that the scrambled peptide could potentially elicit non-specific effects, therefore, comparisons of the differential expression between treatment groups to the vehicle control was hence important. When comparing to the vehicle control, *Gata2* was the gene shown

to be down-regulated by 2.43 and 2.69 folds after 30 minutes and 3 hours of TLQP-21 treatments, respectively. However, the Ct value for this gene is very high (between 33 - 34), which is very close to the default value (35) set for undetermined gene, and it indicates the gene was expressed at very low level even before the treatment, and was not further investigated. Here, the transcription PCR array was not particularly successful in identifying potential transcription factors downstream to the TLQP-21 signalling in the primary macrophages. The first possible reason for this could be that TLQP-21 may not change in an alternation in the transcription factors gene expressions, instead, it could alter the transcription factor activity at the epigenetic levels. Post translational modifications of the transcription factors has been shown to modulate the transcription factor activity significantly (Gill 2003, Saxe, Tomilin et al. 2009), and hence it is plausible that though the transcription factor expression level was not changed, the activity of the transcription factors was in fact altered after TLQP-21 stimulation. Moreover, the PCR array monitored the changes in expression at the mRNA level. The mRNA expression level in the cell may not always correspond to the level of protein expression. Therefore, it is still possible that the protein expression was altered while the mRNA expression remains at similar level, and hence the change in protein expression, which ultimately affects function, was not picked up in the functional assay used in this experiment.

Since the first microarray that has been employed (Schena, Shalon et al. 1995), a lot of experiments started to utilise this technique for investigating the dysregulations of genes in a genome wide level. This technique was also employed in this chapter in hope to better understand the downstream signalling event of TLQP-21 in the primary macrophages. The primary macrophages cultures were treated with wild type TLQP-21/Scrambled TLQP-21 for 0 minutes, 30 minutes, 3 hours or 24 hours and the

differential gene expression was assessed by Affymetrix RG230.2 arrays. Small changes in the microarray experiments could easily be interpreted as expression changes. Due to the sensitivity of the microarray technique and eliminating the chance for negative-positive findings, the biological replicates are essential for reliable and reproducible result to be obtained. Each treatment at all time points were hence repeated three times in order to enable a more reliable and conclusive result to be drawn. The differential expressions of genes were first analysed using Limma's empirical Bayes moderated t-test with an FDR corrected cutoff <0.05 (performed by Neusentis). It was shown the down-regulation of E2f8 after 24 hours of TLQP-21 treatment when compared to time 0 control was the only significant result. The reason why the batch correlated analysis showed such low number of gene was differentially expressed could be due to that the macrophages were differentiated from the bone marrow stem cells. M-CSF that used to drive macrophage differentiation could differ from batch to batch when purchased. Therefore, the differentiated macrophages in different experimental replicates were not identical, and hence the endogenous expression of genes could differ from batch to batch. This varying in the background expression before stimulation makes the induced gene expressional changes inconsistent and the result interpretation difficult. Nevertheless, the result derived using a relaxed stringency with an uncorrected p-value cutoff of <0.01 showed that there were 46 and 52 genes up-regulated and down-regulated in gene expression (all time points combined), respectively, after TLQP-21 treatment when compared to scrambled peptide treatment group.

A significant morphological change was noticed after long incubation with TLQP-21 compared to that treated with scrambled peptide and time 0 control. As observed

under the inverted microscope, ramified resting morphology with occasional short process was the typical feature of the cultured primary macrophages without treatment. The short processes were exhibited in the macrophages mainly to allow dynamic surveying their microenvironment and allow movements. This morphology was retained during the course of scrambled TLQP-21 peptide treatment (up to 24 hours). After treatment with TLQP-21 (at least at the 12 hours time point observed), the macrophages changed morphology to an elongated shape with bipolar processes. This result is intriguing, as change in the macrophage morphology has been shown to associate with its activation (Vereyken, Heijnen et al. 2011). The classical activated macrophages are often associated with a flattened and rounded amoeboid shape, which is distinctive from the stretched and elongated rod-shape of alternative activated macrophages (Vereyken, Heijnen et al. 2011). The observed macrophage morphology after TLQP-21 treatment was related closer to the alternative activated macrophage shape, in terms of the cell body was elongated and not amoeboid in shape. However, the morphology was not completely identical to the alternative activated macrophage because of the almost neurite-like long, thin bipolar processes. Though thin processes were frequently observed in the alternative macrophages, they are often not bipolar (i.e. multiple processes could often be observed in a single cell). The distinctive neurite-like, long thin bipolar processes in the macrophage treated with TLQP-21 could be closely related to its functional outcome, just as the amoeboid shape of classical activated macrophage has been associated to the increase in phagocytosis activity and the elongated shape of alternative activated macrophage to an increase in chemotactic activity (Vereyken, Heijnen et al. 2011). The morphology observed after TLQP-21 treatment seems to resemble the morphology of the macrophage lacking GTPase-activating proteins, Abr and Bcr (Cho, Cunnick et al. 2007). It was demonstrated by Cho *et al.* that macrophages

lacking both Abr and Bcr exhibited an elongated morphology with distinctive neurite-like long ends. This morphology can be reproduced by overexpression of Abr and Bcr mutants lacking the GTPase-activating protein domain and continuously activating Rac in the primary macrophages (Cho, Cunnick et al. 2007). Due to the large extent in the morphological change similarity, it could be possible that TLQP-21 stimulation in primary macrophages leads to a sustained Rac activation, which subsequently signal downstream to exert ultimate functions. However, this hypothesis still awaits to be tested.

In the next session, I briefly review the functional relevance of a few interesting genes that have shown to be significantly differentially expressed following TLQP-21 treatment in the macrophages from the microarray experiment. Some of these genes are highlighted here due to its previous implication in the pain pathway and others are examined here to offer novel perspective of TLQP-21 signalling in the macrophage and suggestion for their potential roles underlying TLQP-21 signalling mechanism.

4.5.2. Candidate genes for TLQP-21 downstream signalling events

Interleukin-18 (IL-18, upregulated by 2.16 folds at 3 hours time point)

Interleukin-18 (IL-18), was first known as interferon- γ inducing factor, is a small cytokine belongs to the Interleukin-1 (IL-1) superfamily, which is closely related to IL-1 β (Okamura, Tsutsi et al. 1995). The nucleotide sequence of murine IL-18 is 582 bp in length, encodes for a 192 amino acids precursor protein, which is processed to form a mature protein of 157 amino acids. Murine and human homologues of the IL-18 protein were cloned and revealed to share 65% homology in sequence (Ushio,

Namba et al. 1996). The expression of IL-18 has been evident in a number of cell types including macrophages (Kim, Talanian et al. 1998), microglia (Prinz and Hanisch 1999), dendritic cells (Stoll, Jonuleit et al. 1998), kupffer cells (Matsui, Yoshimoto et al. 1997), keratinocytes (Stoll, Muller et al. 1997), osteoblasts (Udagawa, Horwood et al. 1997), astrocytes (Conti, Park et al. 1999) and intestinal epithelial cells (Pizarro, Michie et al. 1999).

The activity of IL-18 is transduced via an IL-18 receptor (IL-18R) complex, which is comprised of a binding chain, IL-18R α , and a signalling chain, IL-18R β . Both the binding chain and signalling chain of IL-18R are required for IL-18 signalling (Born, Thomassen et al. 1998). Upon binding of IL-18 to IL-18R α on the target cells, IL-18R β is engaged to form a high-affinity complex, which recruits IL-1 receptor-related protein-activating kinase (IRAK) and Tumor necrosis factor receptor-associated factor-6 (TRAF-6) that subsequently phosphorylate nuclear factor kappa B (NF κ B)-inducing kinase (NIK), leading to an ultimate activation of NF κ B (Dinarello 1999).

IL-18 has been demonstrated to function as a pro-inflammatory cytokine and exerts physiological roles in a variety of immunomodulatory activities (Biet, Loch et al. 2002), which include host defences against several of infections. Indeed, IL-18 has been evident to play an essential role in fighting viral (Fujioka, Akazawa et al. 1999, Choi, Dinarello et al. 2001), bacterial (Bohn, Sing et al. 1998, Mastroeni, Clare et al. 1999), fungal (Kawakami, Qureshi et al. 1997, Qureshi, Zhang et al. 1999) and protozoa (Meyer Zum Buschenfelde, Cramer et al. 1997) infections via induction of host-derived interferon- γ , which subsequently activate clearance of foreign antigen by involving molecules such as nitric oxide. Apart from its physiological role, IL-18

has also been associated to induce several inflammatory disorders and their disease progression, which includes the rheumatoid arthritis (Tanaka, Harigai et al. 2001, Yamamura, Kawashima et al. 2001), multiple sclerosis (Wildbaum, Youssef et al. 1998, Balashov, Rottman et al. 1999), and inflammatory bowel disease (Furuya, Yagihashi et al. 2002, Ishikura, Kanai et al. 2003) especially the Crohn's disease (Monteleone, Trapasso et al. 1999, Kanai, Uraushihara et al. 2003, te Velde, van Kooyk et al. 2003).

A role of IL-18 in the pain pathway has also been suggested. It was demonstrated that intraplantar injection of IL-18 results in a dose- and time-dependent peripheral mechanical hypersensitivity in naïve mice (Verri, Cunha et al. 2007). In addition, intrathecal injection of IL-18 activates microglia and astrocytes in the spinal dorsal horn and results in the mechanical allodynia in the naïve rats, via a NF κ B dependent pathway (Miyoshi, Obata et al. 2008). In the context of neuropathic pain, IL-18 and its receptor has been evident to be up-regulated in expression in the spinal dorsal horn after L5 spinal nerve ligation (Miyoshi, Obata et al. 2008). The increase expression of IL-18 was shown to be in the activated microglia and that of IL-18R in the astrocytes, which peaks at 3 days and 7 days post-operation, respectively. Moreover, the IL-18 and IL-18R neutralising antibodies, which block the IL-18 signalling following the nerve injury, results in a reversal in the nerve injury-induced mechanical allodynia (Miyoshi, Obata et al. 2008). At the periphery, IL-18 expression was low in the sciatic nerve of the naïve animal, which is dramatically increased after sciatic nerve injury (Menge, Jander et al. 2001). The increase in IL-18 expression after the peripheral nerve injury was resulted from the strong increased in IL-18 expression level in the infiltrated macrophages at the injured site (Menge, Jander et al. 2001). Together, IL-18 expression has been suggested to be

up-regulated, both centrally and peripherally, after nerve injury and both central and peripheral injection of IL-18 results in nociceptive behaviours in the naïve animals. Thus, the proinflammatory cytokine IL-18 may play pivotal roles, both centrally and peripherally, in the initiation and/or maintenance of the neuropathic pain.

CD40 molecule, TNF receptor superfamily member 5 (CD40, upregulated by 1.58 folds at 30 minutes time point)

CD40 molecule belongs to the tumour necrosis factor receptor superfamily because of its homology with the TNF receptor (Smith, Farrah et al. 1994). It is an ubiquitously expressed type I transmembrane protein that is shown to be expressed on a variety of cell types including macrophage, basophils, eosinophils, dendritic cells, fibroblasts and B and T lymphocytes (Schonbeck and Libby 2001). CD40 binds to its trimeric ligand CD40L, also known as GP39 or CD154, and subsequently triggers complex downstream signalling cascade involving activation of multiple pathways including the NF κ B (Pype, Declercq et al. 2000), phosphatidylinositol 3-kinase (PI3K) (Deregibus, Buttiglieri et al. 2003) and signal transducers and activators of transcription-3 (STAT3) (Ahmed-Choudhury, Williams et al. 2006).

The first documented biological function of CD40 upon CD40L binding is the immunoglobulin synthesis and DNA recombination by the B cells (Zan, Cerutti et al. 1998). Since the advance in the understanding of the CD40 functional role, it was also evident that CD40/CD40L signalling results in release of proinflammatory cytokines in a number of cell types and subsequently contribute to inflammation. Indeed, the CD40/CD40L interaction was shown to induce production of various

cytokines including IL-8, IL-12, TNF- α and macrophage inflammatory protein (MIP)-1 α in the dendritic cells (Caux, Massacrier et al. 1994, Cella, Scheidegger et al. 1996). In addition, ligation of CD40 on monocytes has been shown to induce secretion of IL-1 β , IL-6, IL-8, IL10 TNF- α and MIP-1 α (Grewal and Flavell 1998). Furthermore, CD40 and CD40L interaction signals to activate macrophages (Andrade, Portillo et al. 2005) and is essential for the subsequent nitric oxide (NO) and IL-12 production by macrophages (Imaizumi, Kawabe et al. 1999, Reichmann, Walker et al. 2000).

Aberrant expression of CD40 has been associated in multiple sclerosis, in which the majority of CD40 positive and CD154 positive cells found in the brains of MS patients were macrophage/microglia/B cells and CD4+ T cells, respectively, and interfering the interactions between CD40 and CD40L was suggested to disrupt MS disease progression (Gerritse, Laman et al. 1996). CD40 signalling has also been reported to play a pivotal role in another autoimmune inflammatory disease, rheumatoid arthritis (RA). It was demonstrated that activating CD40 in the synovial cells from RA patients resulted in elevated expression of IL-1 α , IL-1 β and TNF- α , which in turn exacerbate the synovial inflammation in RA (Harigai, Hara et al. 2004)

CD40 has been implicated in the neuropathic pain. CD40-CD40L interaction between microglia and CD4+ lymphocytes has been evident to be pivotal for the cell-to-cell communication (Grace, Rolan et al. 2011), and the microglial CD40 is essential in maintenance of mechanical hypersensitivity in the spinal nerve L5 transection model of neuropathic pain (Cao, Palmer et al. 2009). It was shown in the study that CD40 knock out mice exhibited less mechanical hypersensitivity

compared to the wild type mice that underwent the surgery on post-operative day 5 (Cao, Palmer et al. 2009). It was later demonstrated that CCL2 production and the increase in CGRP expression that is often observed after nerve injury was reduced in the CD40 knock out animal underwent spinal nerve L5 transection compared to the wild type. It was hence suggested the microglial CD40 may contribute to the onset and maintenance of neuropathic pain after spinal nerve transection injury through an increase in CGRP expression and CCL2 synthesis (Malon, Maddula et al. 2011).

Transforming growth factor β 3 (Tgfb3, downregulated by 2.08 folds at 30 minutes time point)

Transforming growth factor β 3 (TGF- β 3) is an anti-inflammatory cytokine belongs to the transforming growth factor β superfamily, which is comprised from the TGF- β s, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), inhibins and activins (Herpin, Lelong et al. 2004). TGF- β s are one of the most versatile cytokines, which are involved in a variety of cellular events in the development, maintenance of homeostasis and disease ((Kingsley 1994, Unsicker and Kriegstein 2002)). There are three mammalian TGF- β isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. Although little information is available about TGF- β 3, it is evident to be expressed in a variety of cells including macrophage, dorsal root ganglionic neurons, Satellite cells and Schwann cells (Unsicker, Flanders et al. 1991, Bottoms, Howell et al. 2010).

TGF- β 3 binds to its receptors, type I/II/III TGF- β receptors, with high affinity to exert its physiological functions. Binding of TGF- β 3 to the Type I and II TGF- β receptors induce receptors assembly into a heteromeric complex, which is comprised

of two TGF- β type I and two TGF- β type II receptors. Upon activation, type II TGF- β receptors phosphorylate the type I TGF- β receptors, and such phosphorylation is essential for subsequent TGF- β signalling (ten Dijke and Hill 2004). Unlike Type 1 and type II TGF- β receptors are the integral transmembrane proteins, type III TGF- β receptor is an extracellular proteins that has been suggested to act as a negative regulator of TGF- β signalling (Chu, Li et al. 2011). The TGF- β receptors induced downstream signalling pathway can be receptor-regulated Smads (R-Smads) dependent (Schmierer and Hill 2007) or non-Smad dependent signalling (Aomatsu, Arao et al. 2011).

Functionally, TGF- β 3 has been shown to play an important role involved in wound healing and cutaneous scarring and has been suggested to be an anti-scarring agent (Shah, Foreman et al. 1995). Moreover, it is shown to exhibit anti-inflammatory effect and slow the progression of intervertebral disks degeneration *in vitro* (Tramullas, Lantero et al. 2010, Abbott, Purmessur et al. 2013). Furthermore, it has also been suggested to act as a negative regulator in the pain pathway. Indeed, it was demonstrated that increasing the TGF- β signalling in the transgenic mice by depleting TGF- β negative regulator, bone morphogenetic protein and activin membrane-bound inhibitors (BAMBI), results in reduction in acute pain to the thermal, mechanical, and chemical stimuli (Tramullas, Lantero et al. 2010). In the context of neuropathic pain, it was evident that the mechanical allodynia was attenuated when TGF- β signalling was enhanced by knocking out its corresponding negative regulator (Tramullas, Lantero et al. 2010), and the δ -opioid receptor signalling pathway had been suggested to be the attenuation mechanism.

Chloride channel, voltage-sensitive 7 (Clcn7, downregulated by 1.98 folds at 24 hours time point)

Chloride channel, voltage-sensitive 7 (ClC7) is a member of the voltage-gated chloride channels family (ClCs). To date, there are 9 members in the ClCs family and they can be divided into two groups depending on their subcellular localisations and functions: Plasma membrane expression (ClCKa, ClCKb, ClC1 and ClC2 ion channels) and intracellular organelle expression (ClC3-7 proton-coupled chloride transporters) (Graves, Curran et al. 2008).

Lysosomes are the cellular organelles harbouring hydrolytic enzymes that are important for breaking down cellular debris and unwanted materials in the cells. Efficient acidification of lysosomes and maintaining low luminal pH are crucial for effective function, as the hydrolytic enzymes are activated by the acidic pH (~ pH4.5). The acidification of the lysosomes is largely mediated by V-type protein ATPases that metabolise ATP and actively transport protons into the lysosome lumens. ClC7 has been evident to play a crucial role in balancing the transmembrane voltage generated during the process for net pumping to occur (Mindell 2012). ClC7 deficient mice was shown to display severe lysosomal diseases, neurodegeneration in the nervous system and died within 7 weeks (Kornak, Kasper et al. 2001). In addition, failure to acidify the lysosome due to ClC7 disruption has also been associated with human disease such as Alzheimer's (Majumdar, Capetillo-Zarate et al. 2011).

Prokineticin receptor 2 (Prokr2, downregulated by 1.93 folds at 30 minutes time point)

Prokineticin receptor 2 is a G-protein coupled receptor which binds to secreted proteins, prokineticins (Lin, Bullock et al. 2002). Human mutations in Prokineticin receptor 2 and its ligand has been associated with Kallmann syndrome (Dode, Teixeira et al. 2006, Sinisi, Asci et al. 2008, Sarfati, Guiochon-Mantel et al. 2010), which is characterised by failure to commence or delayed puberty. To date, two subtypes of Prokineticin receptor have been identified, Prokineticin receptor 1 and Prokineticin receptor 2, which share 85% sequence homology with a higher sequence conservation at the C terminus (Kaser, Winklmayr et al. 2003). The prokineticin receptor 2 is expressed in neurons of the central nervous system, peripheral nervous system and the immune cells including macrophages (Martucci, Franchi et al. 2006, Negri, Lattanzi et al. 2009). Intriguingly, most of the dorsal root ganglion neurons expressing prokineticin receptors also co-express transient receptor potential vanilloid receptor-1 (TRPV1) (Hu, Zhang et al. 2006).

Prokineticin receptors bind to Gq, Gi and Gs and subsequently evoke a number of different cellular responses. Indeed, activation of prokineticin receptors has been shown to couple to Gq protein and subsequently stimulate an intracellular calcium response. The Prokineticin receptor 2 exhibit different affinities for its nature ligands (Prokineticin-2 > Prokineticin-1 > Prokineticin 2-β) and the binding of different ligands dictates the potency of its subsequent calcium response (Chen, Kuei et al. 2005). Moreover, prokineticin receptors activation has also been shown to induce downstream phosphorylation of the p44/p42 mitogen-activated protein kinase (MAPK) and increase the Akt phosphorylation by coupling to Gi proteins (Chen,

Kuei et al. 2005, Keramidas, Faudot et al. 2008) Furthermore, activated Prokineticin receptors are suggested to induce cAMP accumulation possibly via Gs protein-mediated signalling(Chen, Kuei et al. 2005) .

Prokineticin receptor 2 exhibits the highest affinity to Prokineticin 2 (PROK2) among all of its nature ligands. PROK2 has been heavily implicated in the pain pathway and is suggested to be an inflammatory cytokine-like molecule. Indeed, mice injected with PROK2 at the intraplantar surface showed hyperalgesia to thermal nociceptive stimuli. In addition, PROK2 knock out mice exhibits impaired pain sensation to the thermal and chemical noxious stimuli without affecting the inflammatory response to capsaicin (Watson, Lilley et al. 2012). Since many sensory neurons that express Prokineticin receptors also express TRPV1, it was thus suggested that TRPV1 may plays a role in the PROK2 signalling downstream to Prokineticin receptors, leading to the nociceptor sensitisation without affecting the inflammatory response (Watson, Lilley et al. 2012).

CHAPTER 5:
GENERAL DISCUSSION AND CONCLUSION

5.1. Discussion

Neuropathic pain remains a major clinical challenge, severely afflicting up to 8% of the adult population in the UK (England, Wagner et al. 2011). Unlike acute pain that is often relieved after the primary cause has been resolved, neuropathic pain usually remains as a life-long condition and can greatly affect patients' quality of life. In addition, the lack of effective therapeutic treatment for neuropathic pain, largely due to the fact that the underlying mechanism is incompletely understood, hinders the effective management of persistent neuropathic pain.

In the hope to identify the targets for better neuropathic pain analgesic development, there is a growing interest in the field of pain research in the recent years attempting to identify the 'common' gene that is dis-regulated in the sensory neurons in different animal models of neuropathic pain. As a result of this, *vgf* was found to be one of the common genes that were up-regulated among different models by different research groups independently. It was found to be upregulated in a number of experimental nerve injury models of neuropathic pain (Costigan, Befort et al. 2002, Wang, Sun et al. 2002, Valder, Liu et al. 2003, Maratou, Wallace et al. 2009, Riedl, Braun et al. 2009) and also the disease-induced neuropathy models of neuropathic pain (Maratou, Wallace et al. 2009). *vgf* was shown to be commonly up-regulated in at least 6 different animal models of neuropathic pain, it is hence hypothesised that VGF may play an important role in initiating and/or maintenance of neuropathic pain, rather than involved in the pathophysiological responses to a nerve lesion. Indeed, there is growing evidence in the literature which implicates the VGF-derived peptides in roles in the pain pathways, particularly the LQEQ-19 (Riedl, Braun et al. 2009),

TLQP-21(Rizzi, Bartolomucci et al. 2008) and TLQP-62 (Moss, Ingram et al. 2008) peptides.

However, the cellular and molecular mechanism of VGF-derived peptide signalling in neuropathic pain had not been well established and the precise roles of VGF-derived peptides in the development and/or maintenance of neuropathic pain remain unclear to date. The works presented in this PhD project attempted to examine the mechanisms of action of the VGF-derived peptides, followed by more focussed research on elucidating the role of TLQP-21 and its receptor in the pain pathway. The project provides an inside into the potential molecular mechanisms of TLQP-21 signalling pathways and the major findings will be summarised and discussed in the following sections.

5.1.1. Summary of data

Depolarisation of the sensory neurons resulting from direct nerve injury or disease-associated neuropathy leads to the release of the VGF-derived peptides from the dense vesicles of the sensory neurons. Through a pathway that is not yet fully characterised, the signal is conveyed to result in an increase in nociception. In order to dissect the pathway underlying VGF-derived peptides signalling in the pain pathways, it was essential to distinguish the target cells of the VGF-derived peptides. Do all the VGF-derived peptides target the same cell type? Or do they function through different cell types by associated with different receptors/binding partners? The specific cells that the VGF-derived peptides target and exert their functional effects remained unknown, and hence were investigated in **Chapter 2**. The peripheral sensory neurons are pivotal for nociception detection. Since VGF is

released from the terminus of the sensory neurons, it was speculated that the VGF-derived peptide could act on the primary sensory neurons and hence transduce the nociceptive signals. I have hence tested the effect of VGF-derived peptides (LQEQ-19 and TLQP-21) on the primary DRG neurons by calcium imaging in **figure 2.2**. Though the possibility that the peptides activate the sensory neurons in a calcium-independent pathway cannot be rule out, the data suggested that LQEQ-19 and TLQP-21 do not act on the sensory neuron, at least, via the calcium-dependent manner. It is established now that neurons are not the only cells involved in the pain pathway. Indeed, neuronal cells, glial cells and immune cells are suggested to all play part in the development of neuropathic pain (Scholz and Woolf 2007). In addition, macrophages are shown to infiltrate to the injured nerve, DRG and spinal cord after peripheral damage and depletion of macrophage has been shown to attenuate neuropathic pain development. I next tested the effects of the VGF derived peptides on the bone-marrow derived primary macrophage. It was found that one of the VGF-derived peptides, TLQP-21 specifically acted on primary macrophage in a calcium dependent manner as evident in **figure 2.3**. 100nM TLQP-21 elicited an immediate and transient increase in the intracellular calcium, but not the scrambled control. Furthermore, TLQP-21-induced calcium increase in the primary macrophage was dose-dependent. As established in **figure 2.5**, primary macrophages responded to TLQP-21 at concentration as low as 10nM and the number of cells responded to TLQP-21 plateaued at 100nM concentration. Interestingly, TLQP-21 desensitised the macrophage to further TLQP-21 stimulation, as it was shown in **figure 2.6**, where the first application of TLQP-21 elicited an increase in the intracellular calcium, but not with the second stimulation. The desensitisation was observed even after a one-hour time interval was allowed between the two TLQP-21 applications. Whilst TLQP-21 induced profound desensitisation upon the second treatment, it was shown in **figure**

2.7 that pre-treatment with another VGF-derived peptide, TLQP-62, which harbours TLQP-21 at its N-terminus, did *not* desensitise the cells to the subsequent TLQP-21 stimulation.

Having demonstrated that TLQP-21 acted on primary macrophage in a calcium-dependent manner, some intriguing questions were raised. Was the calcium response a result of influx of extracellular calcium or a release of intracellular calcium stores? Do polarised macrophages respond to the TLQP-21 peptide differently? I have demonstrated in **figure 2.8** that the TLQP-21-induced calcium response was still observed when the extracellular calcium was omitted in the extracellular solution. This implied that the source of calcium of the calcium response is likely to be a release of calcium from the intracellular stores such as ER or mitochondria. Moreover, I have demonstrated in **figure 2.9** that different subsets of macrophages responded to TLQP-21, in terms of calcium response, with a similar percentage of cells responding to the peptide. Indeed, the percentages of responding cells to TLQP-21 for different macrophage subsets were not significantly different. This is, to our knowledge, the first evidence of a VGF- derived peptide acting on macrophages. Interestingly, other VGF-derived peptides, LQEQ-19 and TLQP-62, did not induce an increase in intracellular calcium levels in cultured macrophages, suggesting a specific action of TLQP-21 on macrophages. I next investigated further the effect of TLQP-21-stimulated macrophages in the pain pathway in an *in vivo* study and demonstrated in **figure 2.13** that TLQP-21-stimulated macrophage intraplantar injection induced a mechanical hypersensitivity *in vivo* up to POD3 on the ipsilateral paw but not the contralateral paw, suggesting TLQP-21-activated macrophage elicited the pain-like behaviours in the animals.

As demonstrated in **chapter 2**, where repetitive TLQP-21 treatments desensitised the primary macrophages to the subsequent TLQP-21 stimulation, I hence speculated that this is a receptor-mediated desensitisation event. Fortuitously, Dr. Mahmood Ayub identified a potential receptor, GC1qR, for TLQP-21 in our laboratory. Therefore, in **chapter 3**, I have focused on verifying if GC1qR is the receptor for TLQP-21 on the primary macrophages. I investigated this in **figure 3.5**, using two GC1qR neutralising antibodies in calcium imaging, and demonstrated that blockade of GC1qR by pre-treatment with anti-GC1qR antibodies significantly decreased the number of cells responding to TLQP-21. This is confirmed by the *in vitro* siRNA knock down of GC1qR, where that the percentage of cells responding to TLQP-21 was reduced in the primary macrophages in **figure 3.13**. However, heterologously expression of GC1qR in HEK293 cells failed to elicit an increase in intracellular calcium upon TLQP-21 stimulation in **figure 3.17** and **figure 3.18**. Nevertheless, the possibility that the HEK293 cells do not express certain effector molecules required for TLQP-21-induced-GC1qR-dependent calcium response cannot be ruled out. In this chapter, I have also demonstrated *in vivo* that application of GC1qR neutralising antibody significantly reduced the mechanical hypersensitivity in the PSNL animal model of neuropathic pain in **figure 3.21**. Despite the fact that the effect was transient and lasted for only 24 hours, the study provides us with preliminary evidence of the involvement of GC1qR in the development of neuropathic pain. Nonetheless, the experiment will benefit from further optimisation of antibody delivering methodologies and antibody concentrations to draw a more conclusive result. In addition, I have also confirmed that *vgf* expression was indeed upregulated in the animal model chosen in the *in vivo* study in **figure 3.19**.

The precise mechanism of transmitting the activation signal upon binding of TLQP-21 to GC1qR in the macrophages and how the downstream signalling contribute to the development/maintenance of neuropathic pain requires further investigation. Nevertheless, the preliminary data of macrophage-conditioned media eliciting an increase in calcium response in the primary DRG neurons in **figure 2.10** raises some important questions:

1. Was there an aberrant release of cytokines from the macrophages upon TLQP-21 stimulation which in turn stimulated the sensory neurons?
2. Did TLQP-21 stimulation induce changes in the gene expression in the primary macrophages and what were the subsequent consequences of the expression alternation?

In order to answer these questions, I decided **in chapter 4**, to investigate the downstream signalling pathway of TLQP-21 in the macrophage using PCR based technologies such as qRT-PCR and PCR array. I have also employed a microarray study to allow genomic-wide searches in the changes in gene expression in the macrophages upon TLQP-21 stimulation. I demonstrated in **figure 4.7** that incubating primary macrophages with TLQP-21 for long hours induced a morphological change to an elongated bipolar shape. In addition, I also showed that 30 minutes treatment of TLQP-21 on macrophages induced 22 genes upregulation and 12 genes downregulated in expression. 3 hours treatment time caused higher expression in 5 genes and 15 genes reduced in expression. Lastly, 24 hours of TLQP-21 treatment on the primary macrophage changed the expression of a total 44 genes, in which 19 were upregulated and 25 were downregulated.

5.1.2. Possible mechanism of VGF-derived peptides in the pain pathway

While there is growing interest in the literature, beginning to elucidate and implicate the roles of VGF-derived peptides in the pain pathway and the underlying mechanisms, large gaps still remain. The works presented in this thesis have investigated the involvement of TLQP-21 and its receptor GC1qR on the primary macrophages in the pain pathway.

Summing all the results presented in this study, the following scenario seems to emerge for the mechanism of action of TLQP-21 in the pain pathway (see **figure 5.1** for schematic diagram). In the physiological condition, TLQP-21 is part of the soup of molecules that is produced by the sensory neurons. The VGF pro-protein is proteolytically cleaved by prohormone convertase upon synthesis and stored in the large dense secretory vesicles of the nociceptor terminals. Upon nerve injury, by direct nerve damage or disease associated neuropathy, the noxious signals are transduced into depolarizing currents by the specialized receptors and channels that gathered on the nociceptor terminals. The depolarisation of the sensory neurons subsequently leads to the release of stored VGF-derived peptides from the primary afferent via the regulated secretory pathway into the extracellular space. Thus, the VGF derived peptides, including TLQP-21 (as well as LQEQ-19 and TLQP-62 and other functional peptides) are released at the periphery and centrally. The VGF-derived peptide TLQP-21 subsequently relays the signal by acting on the infiltrated macrophages at the periphery, at least partly, via GC1qR. Other VGF-derived peptides may possibly activate other cells types or acts on sensory neurons in an autocrine manner. However, the functions of other VGF-derived peptides still awaiting further study.

Nevertheless, the released TLQP-21 at the periphery activates the macrophages by binding to its cell surface receptor and induces an increase in intracellular calcium by releasing calcium from the internal stores such as ER (which could possibly be IP₃ mediated) or mitochondria. Although GC1qR has been shown in this study to be the receptor for TLQP-21, it is possible that the calcium elicited was not directly downstream to GC1qR, as GC1qR has been shown to associate with multiple binding partners. Therefore, the release of calcium from intracellular stores may be elicited by the unknown GC1qR associated proteins/channels. Future experiments could be carried out to immunoprecipitate the associated proteins of GC1qR using purified macrophage membrane proteins. This will provide us further evidence of whether GC1qR is associated with any other membrane proteins in macrophages and subsequent functional testing will reveal if these associated proteins/channels are involved in the TLQP-21-induced calcium response in macrophages. Moreover, the release of intracellular calcium from the internal store could be further refined to reveal the origins of the calcium sources. Specific inhibitors could be employed in conjunction with the calcium imaging, to show which intracellular organelles released the calcium store upon TLQP-21 stimulation. Nonetheless, the increase in intracellular calcium consequently leads to changes in gene expressions in the macrophages, which include upregulation of some proinflammatory cytokines (e.g. IL-18) and cytokine receptor (e.g. prokinectin receptor 2). At the same time, the expression of some anti-inflammatory mediator such as TGF- β 3 is reduced in expression. The changes in gene expressions of these genes may consequently lead to a change in the amount of pro-inflammatory/anti-inflammatory cytokines, chemokines and mediators to be produced/released, which may transduce the signals by acting on adjacent cells, such as the damaged Schwann cells or sensory neurons.

The functional outcomes downstream of TLQP-21 signalling after the calcium response still remain to be further elucidated. Do the stimulated-macrophages exert a neuroprotective effect? Or do they contribute to the Wallerian degeneration and thus exacerbate the nerve damage? Could it also be possible that they modulate the neuron excitability by releasing certain factors that in turn bind to the sensory neuron and subsequently change its surface receptors expressions as seen in the microglia-mediated excitation of GABAnergic neurons? These intriguing questions still need to be investigated to gain a full picture of how TLQP-21 signals via its receptor, GC1qR, on macrophages at the periphery upon nerve injuries. Nonetheless, the TLQP-21-stimulated macrophages undergo morphological changes, which could possibly increase their mobility as it has been demonstrated that elongated shape of macrophage are associated with increase in chemotaxis. Could these macrophages migrate from the periphery to the central nervous system upon TLQP-21 stimulation, since the blood-nerve barrier is disrupted upon nerve damage (as indicated by Isami K *et al.*, peripheral macrophages could infiltrate to the spinal cord in a TRPM2-dependent mechanism (Isami, Haraguchi et al. 2013))?

This also implies that the macrophages at the periphery and migrated microphage at the central nervous system could have dual effects both peripherally and centrally. Indeed, two opposite effects of TLQP-21 seem to take place in the animal models: on one hand the peripheral hyperalgesic effect of TLQP-21 facilitates the nociception at the periphery, on the other hand TLQP-21 administration centrally inhibits pain at specific concentration. Does TLQP-21 potentiate the central circuits centrally via infiltrating macrophages, thus macrophages bearing the TLQP-21 receptor could play a dual role at the peripheral and central circuits? Are the differential effects of TLQP-21 at the periphery and central loci solely

macrophage-mediated, or could it be that other cell types are involved to achieve the differential functional outcome? All these interesting questions now remain as intriguing pieces of puzzles to be completed by future experiments.

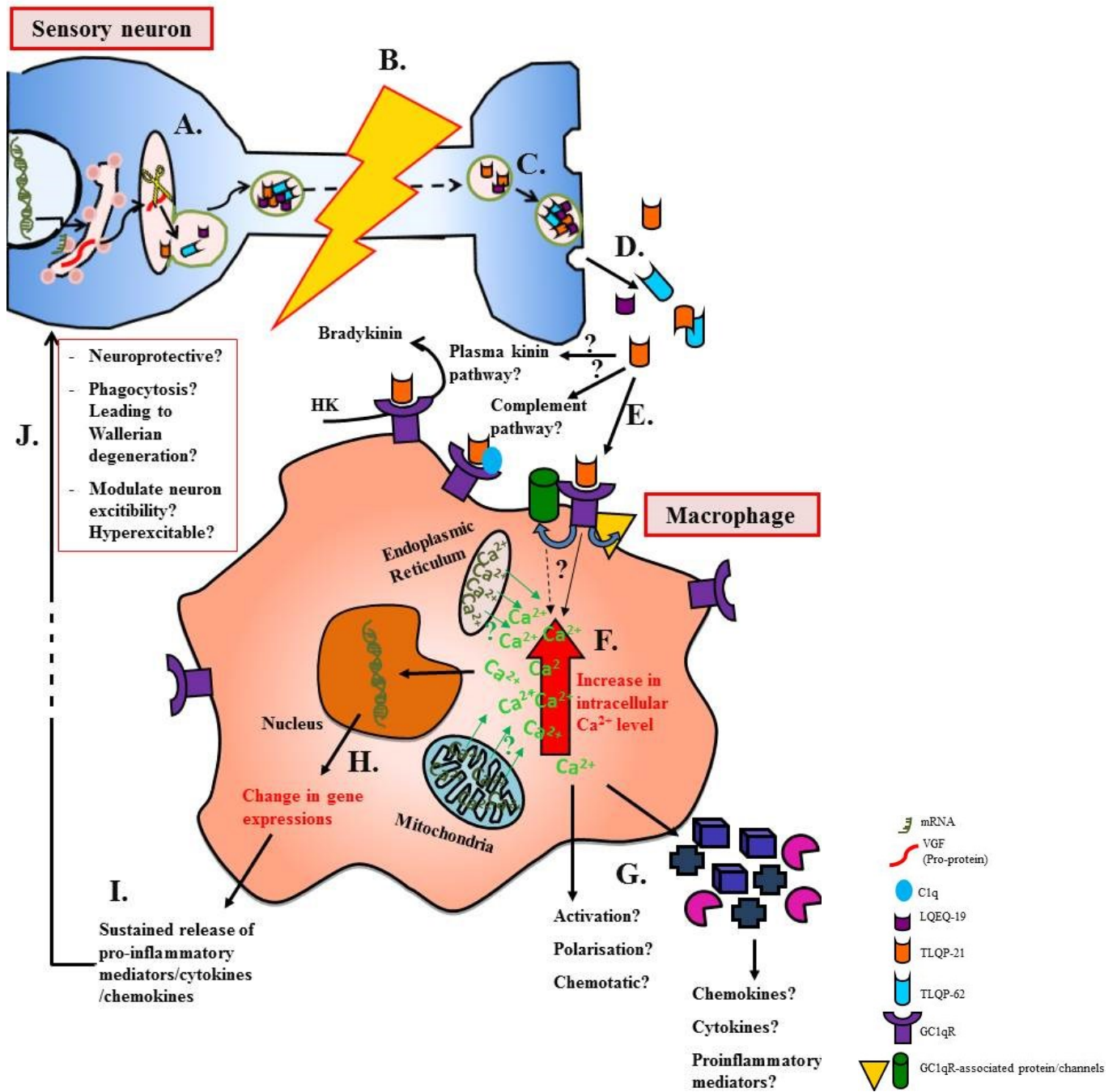


Figure 5. 1 Schematic diagram demonstrating the proposed mechanism of action of VGF-derived peptide, TLQP-21, in the development of neuropathic pain.

5.1.3. Crosstalk of the complement cascade and the pain pathway

The presented work in this thesis highlighted that VGF-derived peptide, TLQP-21, acts on the primary macrophage via the complement receptor GC1qR. The TLQP-21 and GC1qR association subsequently leads to an increase in the intracellular calcium levels, which relay to the downstream signalling pathway and ultimately a morphological change and possibly functional changes in the primary macrophage. It was surprising, and intriguing at the same time, that a complement receptor was implicated here to be involved in the signal transduction of the pain pathway. In fact, the crosstalk between the complement system and the pain pathway has recently been established in the literature and a number of growing evidences had suggested its involvement in the initiation and/or maintenances of neuropathic pain.

Complement activation is a pivotal host defence system that is essential for the innate immunity to eliminate the pathogens in an organism. However, the lack of complement signalling or prolonged/disregulated complement activations are the causes of numerous human diseases. Inappropriate activation of the complement cascade has been implicated in a diverse neurodegenerative disorders such as Multiple sclerosis (Nataf, Carroll et al. 2000, Mead, Singhrao et al. 2002), Alzheimer's (Shen and Meri 2003) and Huntington's disease (Singhrao, Neal et al. 1999). Complement system has first been implicated in the neuropathic pain by Twining et al. in 2004. It was demonstrated blockade of the complement activation by complement inhibitor, soluble complement receptor type 1 (sCR1), prevents the development of mechanical allodynia in the sciatic inflammatory neuropathy model (SIN) of neuropathic pain (Twining, Sloane et al. 2004). Independently, Li et al. also demonstrated in the partial sciatic nerve ligation model that inhibiting the

complement activation impaired the neuropathic pain development up to 19 days (Li, Peake et al. 2007). Interestingly, it was found in several subsequent studies that a number of major complement components were dysregulated in different animal models of neuropathic pain. It was shown that C1q α , C1q β , C1q γ , C1r, C1s, C2, C3, C4, C7 were up-regulated in the Spinal nerve ligation model of neuropathic pain (Levin, Jin et al. 2008). In addition, C3 mRNA was shown to be expressed at higher level in the ipsilateral DRGs of the spare nerve injury model (Vega-Avelaira, Geranton et al. 2009). Moreover, the increased in C3 expression has also been confirmed at protein level in the PSNL model, which is shown to be up-regulated from 6 hours to 7 days and the time scale of C3 protein up-regulation is correlated to the mechanical and thermal hypersensitivity behaviours in the animals (Li, Peake et al. 2007).

GC1qR has been demonstrated to be a positive regulator in the complement activation in the platelet cells. Platelet stimulation by mechanical mean increases the surface GC1qR expression, and the activated platelets increase C1q and C4d deposition, which is accompanied by a subsequent increase in C4d and C3a generation in the plasma fluid. In addition, it is demonstrated that the C4 activation in the platelets is signalled via GC1qR in a C1q-dependent manner. Truncated form of GC1qR fails to initiate C4 activation and GC1qR neutralising antibodies also inhibit C4d deposition on platelets to some extent (Peerschke, Yin et al. 2006). Although GC1qR has been implicated in activating complement in the platelets, its role in activating complement pathway in other cell types has not yet been fully characterised. As mentioned, complement activation has also been reported in the DRG and the injured nerves following peripheral nerve injury in the animal models of neuropathic pain (Li, Peake et al. 2007, Levin, Jin et al. 2008, Vega-Avelaira,

Geranton et al. 2009). Whether this is a GC1qR-dependent activation, possibly via VGF-derived peptides, still await to be further elucidated. Nevertheless, macrophage seems to play an important role in bridging such crosstalk between the complement and pain pathways. As it is demonstrated that inhibiting complement activation in the neuropathic pain animal models prevented the neuropathic pain development (Twining, Sloane et al. 2004, Li, Peake et al. 2007), and depletion of complement prior to nerve damage reduced macrophage infiltration into the injured sciatic nerve and the activation of macrophages was reduced as well in the sciatic nerve crush injury model (Dailey, Avellino et al. 1998). Furthermore, systemic macrophage depletion has been shown to delay progression and reduce hyperalgesia in the animal models of neuropathic pain (Liu, van Rooijen et al. 2000, Mert, Gunay et al. 2009). These implicate macrophages in the neuropathic pain development, and the underlying mechanism involves, at least partly, the activation of complement system and infiltration of macrophages to the injured nerve.

Taking all this evidence together, a new hypothetical pathway to the neuropathic pain development seems to emerge. Upon peripheral nerve damage (either by direct nerve injury or indirect disease-induced damage), complement activation, possibly from the damaged Schwann cells or injured nerve itself, induces the infiltration of macrophages into the damaged nerve and DRG. The injured nerve releases mediators, including VGF-derived peptides, upon depolarisation. VGF-derived peptides TLQP-21 signals to the infiltrated macrophage via its surface receptor GC1qR, inducing an increase in intracellular calcium and subsequent activation of the cells. Macrophages further amplify this damage by releasing harmful substances such as proinflammatory cytokines (e.g. IL-18), increasing receptors for proinflammatory mediators (e.g. prokinectin receptor 2) and reducing

anti-inflammatory molecules to be released (e.g. TGF- β 3). The exacerbated damage thus leads to an intensified nociception to be detected. In addition, complement activation could also disrupts the blood-nerve barrier and hence recruits peripheral circulating macrophages into the central nervous system (Isami, Haraguchi et al. 2013), where the VGF-derived peptide TLQP-21 could exert pro-nociceptive or analgesic effect depending on the local concentration of the peptide in the spinal cord (Rizzi, Bartolomucci et al. 2008). The summation of the peripheral sensory neuron hyperexcitation and the central mechanism of modulation and plasticity thus dictate the aberrant nociception in the neuropathic pain.

5.2. Concluding remarks

In conclusion, the present research demonstrated the involvement of the VGF-derived peptides TLQP-21 in pain modulation, through a mechanism that, at least partly, engages GC1qR receptor on the macrophages. The proposed mechanism of action of TLQP-21, through GC1qR, in the macrophages provides a novel insight into the comprehension of the neuropathic pain development. Additionally, the understanding of the mechanism underlying TLQP-21 signalling in the macrophages during neuropathic pain development opens the innovative therapeutic approaches and sheds light for novel development of new analgesics.

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Appendix

TLQP-21 30mins vs. Scrambled TLQP-21 30mins			
Gene Symbol	Entrez ID	P-Value	Fold Change
Ccna2	114494	0.001421213	3.46518799
Ptp4a1	29463	0.002452578	2.974659315
Etv1	362733	0.008545756	2.421056845
RGD1305110	305579	0.006189354	2.348058575
Dld	298942	0.003221764	2.3379795
Scand1	362252	0.008031736	2.2820735
Clr	312705	0.000814958	2.037630688
Jrk	315073	0.007172351	1.809726439
Cenpm	315164	0.001694829	1.785974829
Cd40	171369	0.004601085	1.584336052
Gtpbp10	312054	0.003977217	1.569666496
Orc4	295596	0.009730391	1.442373536
Neo1	81735	0.009919211	-1.283593412
Ccdc30	362580	0.007462439	-1.366575499
Ca2	54231	0.009295187	-1.368431261
Hs3st1	84406	0.005149167	-1.373733099
Spata2	114210	0.009825533	-1.406039162
Itpkb	54260	0.00744075	-1.434963755
Ereg	59325	0.004623608	-1.481882342
Parva	57341	0.006779057	-1.55700514
Col6a2	361821	0.002542384	-1.642900317
Cog2	690961	0.00974026	-1.684240697
Fgf9	25444	0.006200487	-1.837384626
Tyro3	25232	0.006119209	-1.848213632
Spon1	64456	0.007736186	-1.869626044
Prokr2	192649	0.001039878	-1.934603385
Stx11	292483	0.000268294	-1.958065449
LOC100912606	100912606	0.001083004	-1.98169275
Tgfb3	25717	0.002865028	-2.084087473
Tpm1	24851	0.005554108	-2.131683041
Btg2	29619	0.008323865	-2.179550854
Mcat	315173	0.007278664	-2.302865345
Igsf1	302822	0.000667076	-2.511811543

Fam168a	361614	0.003894542	-2.641294948
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Table A.1 Table listing genes that are differentially expressed after 30 minutes of TLQP-21 treatment when compared to that treated with scrambled TLQP-21.

The genes that increases the expression by more than 1.5 folds after TLQP-21 treatment are highlighted in pink, and those decreases the expression by more than 1.5 folds are highlighted in blue. 12 and 22 genes are shown to be significantly upregulated and downregulated, respectively, by more than 1.5 folds after 30 minute of TLQP-21 treatment compared to that treated with scrambled peptide. A total of 9824 genes are not differentially expressed between the two treatment groups.

TLQP-21 3hours vs. Scrambled-TLQP-21 3hours			
Gene Symbol	Entrez ID	P-Value	Fold Change
Lamp2	24944	0.006249104	3.629120603
Mrfap1	282585	0.001693708	3.008905743
Slc24a6	498185	0.002389034	2.872103079
Mcm6	29685	0.001829167	2.658046214
Alox5ap	29624	0.005946373	2.651306946
Slc25a16	361836	0.001133329	2.364588137
MGC94190	288616	0.009365843	2.354536598
Map1lc3b	64862	0.008172609	2.343820763
Mettl7a	315306	0.008439126	2.298627774
Ino80c	291737	0.006670217	2.277473648
Il18	29197	0.008592154	2.162657192
Abhd16a	361796	0.002447063	1.963125362
Arfgef2	296380	0.008276522	1.924408858
Htr2b	29581	0.009513113	1.735989671
Hddc2	361462	0.00445582	1.595229329
LOC689074	689074	0.008474265	-1.547285884
RGD1305422	303885	0.008990158	-1.751815559
Snhg11	362256	0.007973788	-1.966648931
Pcdh7	360942	0.008175499	-2.083417247
Srd5a2	64677	0.008929625	-2.540330003

Table A.2 Table listing genes that are differentially expressed after 3 hours of TLQP-21 treatment when compared to that treated with scrambled TLQP-21.

15and 5 genes are shown to be significantly upregulated and downregulated, respectively, by more than 1.5 folds after 3 hours of TLQP-21 treatment compared to that treated with scrambled peptide. A total of 9839 genes are not differentially expressed between the two treatment groups.

TLQP-21 24hours vs. Scrambled TLQP-21 24hours			
Gene Symbol	Entrez ID	P-Value	FoldChange
Fam76a	362618	0.005727266	3.811282024
Tmem219	308986	0.005625957	3.1552834
Klc1	171041	0.006013083	3.034474986
Spats2	300221	0.001701526	2.940853979
Zc3h13	305955	0.007269521	2.662649774
Dlgap4	286930	0.005330207	2.630163272
Apaf1	78963	0.001404337	2.525491004
Sik1	59329	0.00620099	2.510881287
Pik3ip1	305472	0.009675336	2.475857758
Hdlbp	64474	0.001672525	2.460231905
Serpinb6	291085	0.004530024	2.403154489
Ube3b	687633	0.006196031	2.378028051
Pop7	288564	0.007878522	2.189060437
Prpf38a	298374	0.007309862	2.137690164
Fgl2	84586	0.009068705	1.954638056
Fam100a	302941	0.009710433	1.846931593
Mrpl48	293149	0.003528557	1.84237579
Clk4	287269	0.007667579	1.791775232
Carkd	361185	0.008761791	1.791399672
Trappc21	292074	0.009061335	1.726548319
Etnk2	360843	0.006229538	1.694995831
LOC100912002	100912002	0.007249681	1.688407413
Dgkq	100361138	0.001893537	1.680135901
Klf10	81813	0.007633356	1.528017862
RGD1565411	363000	0.008388717	1.523194546
LOC690734	690734	0.004795433	-1.312594991
P2ry1	25265	0.007002704	-1.433480536
Kcne3	63883	0.008568304	-1.451927492
LOC100125364	100125364	0.006375943	-1.527931869
Itpkb	54260	0.004498244	-1.541020717
Cckbr	25706	0.008168725	-1.645407099
Fam82a1	313840	0.005471273	-1.655304817
Hip1r	81917	0.008824254	-1.728609254
Oprm1	25601	0.007507652	-1.746020971
Adamts12	294809	0.004293169	-1.859243979

Clcn7	29233	0.00327625	-1.984155535
Als2cl	316017	0.008394751	-2.095622081
Atat1	361789	0.006259233	-2.117569921
Fgg	24367	0.009621803	-2.175724782
Trmt2a	287953	0.001424427	-2.254584811
Tulp1	309900	0.001186429	-2.267362405
Lmo7	361084	0.004093187	-2.472343441
Prkcg	24681	0.004338107	-2.485596452
Dlgap1	65040	0.007054121	-2.893080413

Table A.3 Table listing genes that are differentially expressed after 3 hours of TLQP-21 treatment when compared to that treated with scrambled TLQP-21.

25 and 19 genes are shown to be significantly upregulated and downregulated, respectively, by more than 1.5 folds after 24 hours of TLQP-21 treatment compared to that treated with scrambled peptide. A total of 9817 genes are not differentially expressed between the two treatment groups.