UNDERSTANDING THE MECHANISM OF

NERVE GROWTH FACTOR SIGNALING DURING

PERIPHERAL INFLAMMATION

By

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Title of Study: UNDERSTANDING THE MECHANISM OF NERVE GROWTH
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Abstract:
Scope and Method of Study:
The purpose of this study is to understand the role of nerve growth factor (NGF) signaling in the development and maintenance of peripheral inflammation. Dermal-epidermal separation technique, western blot analysis, confocal microscopy, quantitative image analysis, and qualitative PCR were used to evaluate the phasic expression of NGF in the epidermis of an inflammatory animal model. Pharmacological inhibition and pain behavioral studies were implemented for determining the involvement of NGF in the expression of dorsal root ganglion (DRG) glutaminase during the process of peripheral inflammation and nociception.
Findings and Conclusions:
Cold thermolysin epidermal-dermal separation technique was found to be useful for evaluating the alteration in the expression of inflammatory mediators like IL-6 and neurotrophins such as NGF during inflammation in epidermal tissue. As the separation takes place at a cold temperature (4°C), the integrity of protein and mRNA is maintained, allowing us to evaluate the temporal expression pattern. A significant elevation of NGF was observed at two different time points, indicating a biphasic expression in the epidermis of adjuvant-induced arthritic (AIA) animals. This temporal change in the expression of NGF might help in determining time-points for therapeutic intervention by peripheral inhibition of NGF signaling. We also showed that the peripheral inhibition of NGF signaling by either TrkA or Rab7GTPase attenuates the elevated glutaminase levels in DRG cell bodies during inflammation. These results indicated that the NGF signaling is involved in the process of glutamate metabolism for developing and maintaining the peripheral inflammatory process by sensitizing the primary sensory DRG neurons. Also, the peripheral inhibition of Rab7 by a receptor antagonist provided an analgesic effect in animals suffering from hyperalgesia due to AIA. Overall, the results of this study show that NGF plays a vital role in altering the expression of glutaminase in DRG neurons. Furthermore, peripheral blockade of NGF signaling via TrkA or Rab7 along with glutaminase inhibition can lead to the development of an analgesic regime for treating inflammatory pain.

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ABBREVIATIONS

AIA - adjuvant-induced arthritis

CFA - complete Freund's adjuvant

CGRP - calcitonin gene-related peptide

DAPI - 4',6-diamino-2-phenylindole; binds to DNA and emits blue fluorescence

DRG - dorsal root ganglion

EAAT - excitatory amino acid transporter

GLS - glutaminase

GLU - glutamate

GluR - glutamate receptor

GS - glutamine synthetase

-ir - immunoreactivity

NGF - nerve growth factor

NSAIDs - non-steroidal anti-inflammatory drugs

p75NTR – p75 neurotrophin receptor

PBS - phosphate buffered saline

PBS-T - phosphate buffered saline with Triton X-100

PVP – polyvinylpyrollidone

Rab7A – Rab7A GTPase

ROIs - regions of interest

RT-qPCR – Reverse transcription polymerase chain reaction

SNAT - Na+-coupled neutral amino acid transporter

SP - substance P

TrkA - tropomyosin receptor kinase A, (high affinity nerve growth factor receptor)

TRPV 1 - transient receptor potential cation channel subfamily v member 1

VGLUT - vesicular glutamate transporter

CHAPTER I

INTRODUCTION

Primary Sensory Neurons and Nociception

The dorsal root ganglion (DRG) is situated in the intervertebral foramina with proximity to the spinal cord. The DRG consist of various cells such as satellite cells, macrophages and primary sensory neurons. The primary sensory neurons are primarily responsible for transmitting peripheral information to the spinal cord and brainstem, i.e., central nervous system (CNS). They are pseudo unipolar in structure with their axons splitting into two branches and are mainly classified into two categories: nociceptive and non-nociceptive neurons. The non-nociceptive neurons respond to low threshold, non-noxious (non-painful) stimuli while the nociceptive neurons respond to high threshold, noxious (painful), high-intensity stimuli. External stimuli are converted into action potentials (AP) in primary sensory neurons with the help of specific sets of proteins (Fang, McMullan, Lawson, & Djouhri, 2005).

Initially, pain was considered to be an emotion involving pleasure and fear. However, this concept was replaced by the view that activation of a set of specialized primary afferent neurons called nociceptors are responsible for causing pain. These nociceptors detect actual or potential tissue damage and when activated may cause pain in humans and pain-related reflexes in animals (Basbaum & Kaas, 2008). Pain can be neuropathic, i.e., due to direct nerve injury or nociceptive, i.e., potential damage caused due to inflammation (Verma, Sheikh, & Ahmed, 2015). The pain originating from the skin, joints, and muscle is termed as somatic pain detected by the somatic nociceptors while the one arising from the internal organs such as colon, is referred to as visceral

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pain perceived by visceral nociceptors. These nociceptors or pain-sensing neurons are mostly small diameter with unmyelinated axons (C-nociceptors) surrounded by Schwann cells or lightly myelinated A-delta nociceptors (Aδ) (Dubin & Patapoutian, 2010). Aδ fibers conduct AP's rapidly and are responsible for rapid pain sensation while unmyelinated C-fibers mediate persistent burning or aching pain due to conduction of slow AP's (Figure.1.1). Nociceptive primary afferents are sensitized during inflammation in somatic tissues leading to an increase in neurotransmitter glutamate (GLU) and neuropeptides Substance P (SP), Calcitonin gene-related peptide (CGRP) in both DRG cell bodies and peripheral terminals. (Duggan, 1995; Galeazza et al., 1995; E. M. Hoffman & Miller, 2010; Miller, Hoffman, Sutharshan, & Schechter, 2011; Mizumura & Murase, 2015). Extensive research has unraveled the pivotal role of primary sensory neurons in the development of nociceptive processing, making the primary afferents the target of choice for treating acute and chronic pain.

Inflammation and Peripheral Sensitization

Pain and inflammation are considered debilitating but, at the same time, they are also the protective responses necessary for survival. Pain is the mechanism which provides information about the presence or threat of an injury (Verma et al., 2015). In addition to the afferent function such as conveying information to the central nervous system, the DRG primary sensory neurons, specifically C and some Aδ fibers, regulate the vascular and several tissue functions at their peripheral targets. For example, during neurogenic inflammation, tissue damage causes depolarization of the primary afferents, followed by release of neuropeptides such as SP, CGRP, and neurotransmitters such as glutamate, leading to increased vascular permeability and plasma extravasation (Holzer & Maggi, 1998). Due to this increased vascular permeability, the inflammation site recruits inflammatory cells such as T lymphocytes, mast cells, macrophages and neutrophils (Chiu, von Hehn, & Woolf, 2012) which change the chemical milieu at the site of injury/inflammation by releasing pro-inflammatory molecules such as prostaglandins, bradykinin,

histamine and serotonin. Cytokines such as interleukins (ILs) and tumor necrosis factor (TNF), and neurotrophins like nerve growth factor (NGF) are released during inflammation (Watson, Allen, & Dawbarn, 2008). These inflammatory mediators sensitize the nociceptors by reducing their threshold and increasing responsiveness (Figure 1.2).

CGRP is a neuropeptide belonging to calcitonin family. It has a critical role in mediating hypersensitivity and considered a major nociceptive marker. The calcitonin-like receptor (CLR) along with its accessory membrane protein receptor activity-modifying protein 1 (RAMP-1) is a well-characterized CGRP receptor (Cottrell et al., 2012). CGRP is considered as one of the major neuropeptides during inflammation in mediating sensory perception. SP, the other neuropeptide is an 11 amino acid peptide which is widely distributed in the central and peripheral nervous system (O'Connor et al., 2004). The high-affinity neurokinin receptor-1 (NK-1) for SP is a G-protein coupled receptor and is abundantly expressed in peripheral cells. Similar to CGRP and glutamate, SP is expressed in the cell body of primary afferents in DRG, then shipped to the target organ where it aids the inflammatory process by increasing vascular permeability and sensitizing the primary afferents (Delafoy et al., 2006; Qiao & Grider, 2007).

Nerve Growth Factor (NGF)

During the research on the growth of nerve fibers, Rita Levi-Montalcini in 1951 discovered a protein that promotes the growth of sensory and sympathetic nerves (Levi-Montalcini & Hamburger, 1951). Later this protein was termed Nerve Growth Factor (NGF) belonging to a family of structurally related proteins known as neurotrophins. The in vivo and in vitro studies about NGF deprivation and neutralizing antibodies against NGF revealed the importance of NGF for the survival of sensory and sympathetic neurons during the developmental period (Levi-Montalcini, 1987). Although sensory neurons lose their dependability on NGF for survival during adult life, sympathetic neurons do depend on NGF for sustaining their existence (Korsching & Thoenen, 1988). NGF can bind to two kinds of receptors: tyrosine receptor kinase A (TrkA) which

selectively binds to NGF and p75 neurotrophin receptor (p75NTR) which binds to all neurotrophins including NGF with low affinity. Along with the peripheral nociceptors, TrkA and p75NTR are widely expressed in multiple immune cells indicating the importance of NGF signaling in immunomodulatory activity. A mutation in the gene coding for TrkA leads to an autosomal sensory neuropathy called "congenital insensitivity to pain" (CIPA). This further demonstrates the involvement of NGF signaling in the process of nociception and inflammation. CIPA patients show insensitivity to pain with various neurological alterations and modifications in the immune system. In addition, due to disrupted immune response, the patients suffering from CIPA experience reduced wound healing and are prone to develop chronic inflammation due to recurrent infections (Melamed, Levy, Parvari, & Gelfand, 2004; Sato, Tsuboi, Kurosawa, Sugita, & Eguchi, 2004).

The mature NGF protein (NGF-β) is synthesized from intracellular post-translational cleavage of its two precursors pro-NGF (32 and 25 kD) (Figure.1.3). The mature form of NGF binds with high-affinity to TrkA and binds to its low-affinity p75NTR while the pro-NGF preferentially binds to p75NTR (Fahnestock, Yu, & Coughlin, 2004). This growth factor is not only associated with the development of the peripheral nervous system (PNS) but it is also considered an important pain mediator molecule in mature neurons (Denk, Bennett, & McMahon, 2017).

Role of Nerve Growth Factor in Inflammation

Peripheral inflammation drives peripheral sensitization and modifications in DRG neuronal cells by producing retrograde signals in nociceptive neurons. These signals, e.g., action potentials (AP's) or nerve growth factor/TrkA signaling, activate or increase transcription of the pro-nociceptive molecules, such as neurotransmitters and neuropeptides, augmenting both central and peripheral sensitization (Denk et al., 2017; Miller et al., 2011). To meet the considerable challenge of conveying information from periphery to the cell body located far away, dedicated mechanisms of retrograde NGF signaling have evolved which carry the signals generated from the

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axonal endings to the neuron's cell body (Harrington & Ginty, 2013). Following NGF engagement, TrkA forms a homodimer and is autophosphorylated (Figure 1.4). This complex is internalized by clathrin-dependent endocytosis giving rise to a signaling endosome. It has been demonstrated that the NGF signaling endosomes are multivesicular bodies (MVB's) that mediate long-range retrograde transport (Ye, Lehigh, & Ginty, 2018). These TrkA multivesicular bodies contain a small GTPase Rab7 (Saxena, Bucci, Weis, & Kruttgen, 2005). Rab GTPases are directly associated with organelle trafficking and are also linked to endosomal signaling receptors. With the help of cytoskeletal motor protein dynein, signaling endosomes are transported retrogradely to the cell body for transcription regulation (Figure.1.5). Three major signaling pathways have been described for TrkA mediated transcriptional regulation: the PLCγ pathway (phosphoinositide phospholipase C) which leads to the activation of ion channels, the ERK pathway (extracellular-signal-regulated kinase), and/or the PI3K/Akt pathway (phosphatidylinositol 3-kinase) which augment gene expression via transcription factor, Cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) (Segal, 2003). Once the NGF-TrkA signaling endosome arrives at the cell body, it can switch gene promoters on and off via the signaling mediators, leading to a change in the sensory phenotype (Mantyh, Koltzenburg, Mendell, Tive, & Shelton, 2011). NGF can also bind to p75NTR with less affinity, which lacks the catalytic domain and hence forms a heterodimer with either TrkA or sortilin to activate the downstream signaling cascade (Denk et al., 2017).

Although it is widely documented that NGF expression in epidermal cells is highly upregulated during various inflammatory states (Watson et al., 2008), the temporal expression pattern of NGF is fairly unknown. In addition, little is known about the role of Rab7 GTPase NGF TrkA signaling endosomes in glutamate metabolism during the process of peripheral inflammation.

Glutamate Metabolism During Peripheral Inflammation

It is well documented that the DRG primary afferents contain enzymes for the glutamate-glutamine cycle essential for glutamate synthesis, i.e., glutaminase 1 (GLS1) and cytosolic

aspartate aminotransferase (cAST). In the central nervous system, the production and degradation of glutamate is carried out by neurons and glial cells respectively. An identical process also occurs in the peripheral nervous system, between primary afferents and the counterparts of astrocytes; satellite cells and Schwann cells. All the primary afferents in the DRG are glutamatergic and contain the enzyme GLS1 that converts glutamine into glutamate. Glutamate is packaged by vesicular glutamate transporters (VGLUT's) into synaptic vesicles. When released it is taken up by the satellite cells and Schwann cells where it is converted into glutamine via glutamine synthetase. Glutamine is transported out of the cells by sodium-coupled neutral amino acid transporter (SNAT) 3,4 transport proteins (Miller, Douglas, & Kaneko, 1993; Miller, Richards, & Kriebel, 2002). The glutamate-glutamine cycle is crucial in maintaining glutamate levels in the DRG neurons and simultaneously removing excessive glutamate, preventing excitotoxicity (McKenna, 2007).

In the peripheral afferents, levels of glutamate increase after various types of noxious stimulations like carrageenan-induced inflammation (Dmitrieva, Rodriguez-Malaver, Perez, & Hernandez, 2004) and formalin injection, indicating its potential role in the process of neurogenic inflammation. In addition, glutamate's involvement in acute and chronic nociceptive transmission has been established in animal pain models at the peripheral terminals of DRG neurons (Miller et al., 2011). GLS1 is the primary enzyme for neurotransmitter glutamate production and is found to be elevated in peripheral terminals of DRG neurons after inflammation (Ernest Matthew Hoffman, 2009; Miller et al., 2012; Zhang, 2013). Our laboratory has been instrumental in establishing the pivotal role of GLS in nociception and neurogenic inflammation. This modulation of GLS1 in the cell soma of DRG neurons during inflammation has been attributed to the axonal transport of NGF signaling molecules (Miller et al., 2011). However, the exact molecular mechanism of this process is yet to be determined. (Figure.1.6).

Adjuvant-Induced Arthritis (AIA) Animal Model for the Study of Inflammation

Two animal models for inflammation are widely used for studying inflammatory process, carrageenan-induced inflammatory animal model and Complete Freund's Adjuvant (CFA)-Induced Arthritis animal model (AIA). Carrageenan is a polysaccharide extracted from red seaweeds and exists in three forms: Kappa, iota and lambda. The lambda (λ) carrageenan has been shown to elicit acute inflammatory response and hyperalgesia when injected into cutaneous tissue (Nicklin & Miller, 1984). Carrageenan is used to induce a transient inflammation that maximizes swelling after 5-6 hours, which decreases after 24-36 hours. CFA consists of attenuated mycobacteria in paraffin or mineral oil and when injected with PBS generates a persistent inflammatory response (Broderson, 1989). The subcutaneous injection of CFA causes swelling and inflammation which peaks after 24 hours and can persist for almost 14 days. Both these models are used for studying the process of inflammation, nociception, and for accessing the efficacy of potential anti-inflammatory medications and analgesics (Fehrenbacher, Vasko, & Duarte, 2012).

Dermal-Epidermal Separation for the Evaluation of NGF

The complex structure of skin poses difficulty for studying the interaction between the epidermal keratinocytes and intraepidermal nerve fibers. Hence, an epidermal-dermal separation technique can play an important role in unravelling both inflammation and nociception mechanisms. There are several different approaches for separating the epidermal-dermal junction: mechanical, heat, chemical and enzymatic separation. The mechanical, chemical and heat separation methods can dissociate the two layers in a short amount of time but can potentially damage the mRNA and proteins of interest (Zou & Maibach, 2018). The proteolytic enzyme thermolysin separates the epidermis from dermis efficiently at 4 °C and selectively disrupts the hemidesmosome connection. As the separation takes place in cold temperature without disrupting the epidermal structure, this technique can be utilized for evaluating the expression of neurotrophic

factors and inflammatory mediators during the process of peripheral inflammation (Gujar, Anderson, Miller, Pande, Nawani, & Das, 2020).

Summary

Release of the neurotransmitter glutamate and NGF signaling are directly associated with the development of peripheral sensitization of nociceptor terminals during inflammation. We previously reported that the immunoreactivity of the enzyme glutaminase (GLS) which converts glutamine to glutamate is increased in DRG neurons during peripheral inflammation (Miller et al., 2012). After the initiation of the inflammatory process, the DRG sensory neurons alter the expression of several proteins, mostly attributed to NGF and its high-affinity receptor, TrkA. The basal expression of GLS is not regulated by NGF (E. M. Hoffman, Zhang, Anderson, Schechter, & Miller, 2011), but during inflammation, the role of NGF/TrkA in the regulation of GLS expression needs further evaluation. In addition, an anti-NGF antibody for the treatment of inflammatory conditions such as arthritis is in its pre-clinical phase, however our understanding is limited concerning the molecular mechanism that mediates this process. For addressing these questions, we hypothesized that NGF/TrkA signaling endosomes are responsible for altering glutaminase levels in primary sensory neurons and play a critical role in the development of peripheral inflammation.

To understand the basic mechanism of NGF signaling during the process of peripheral inflammation, there is a need to evaluate NGF expression in peripheral tissue like skin during ongoing noxious stimulus. Skin consists of three completely different layers: dermis, epidermis and hypodermis and due to inflammatory processes, all these layers are infiltrated with a variety of cells (Zou & Maibach, 2018). This heterogeneity of cells poses a considerable difficulty in evaluating of neurotrophic factors and inflammatory mediators (Gujar, Anderson, Miller, Pande, Nawani, & Das, 2020). In addition, most of the intra-epidermal fibers of nociceptors terminate in the epidermis, and for studying the changes occurring in these terminals, the epidermis needs to be separated from

the dermis. ***Therefore, our first study was focused on determining the feasible dermal epidermal separation technique to detect the site-specific inflammatory mRNA and protein (Chapter 2).*** The proteolytic enzyme thermolysin (TL) is used for the dermis and epidermis separation in rat skin during carrageenan-induced inflammation and the levels of NGF and Interleukin-6 (IL-6) were determined.

Although the importance of NGF in the process of nociception is widely established and levels of NGF are upregulated after the noxious stimulus, (Woolf, Safieh-Garabedian, Ma, Crilly, & Winter, 1994) the information regarding the expression pattern of NGF during the initial progression of peripheral inflammation is unknown. ***This necessitates the need for determining the phasic changes in Nerve Growth Factor expression in rat skin during adjuvant-induced cutaneous inflammation (Chapter 3).***

The long term changes due to inflammation causes the elevation in the levels of glutaminase in the rat DRG neurons leading to increased production and release of glutamate in the peripheral terminals during inflammation (Ernest Matthew Hoffman, 2009; Miller et al., 2012; Miller et al., 1993; Miller et al., 2011; Zhang, 2013). For signals to travel from axon to cell body, activation of TrkA and its downstream signaling pathways are required. These signaling pathways are responsible for the transcriptional upregulation of proteins in the cell body (Harrington & Ginty, 2013). The actions of NGF/TrkA has been attributed to the hyperalgesic action by upregulating the expression of neuropeptides such as CGRP and SP (Frade & Barde, 1998; Skoff & Adler, 2006). The retrograde signaling of NGF/TrkA endosome is mediated by the endosomal GTPase Rab7 (Puehler et al., 2004; Saxena et al., 2005). As the NGF/TrkA signaling endosome and glutaminase both play crucial roles in the development and maintenance of inflammation, ***it will be advantageous to know if NGF signaling regulates the levels of glutaminase during peripheral inflammation (Chapter 4).***

Hypothesis and Specific Aims:

The previous studies have widely established the direct relation of NGF signaling and glutamate metabolism during the process of inflammation and nociception. However, the detailed research addressing the molecular mechanism involved in the process of nociception was needed. For this study, we hypothesized that NGF/TrkA signaling endosomes are responsible for altering glutaminase levels in primary sensory neurons during peripheral inflammation. We addressed our central hypothesis with the help of three specific aims.

Aim 1: Evaluate the easy-to-use and inexpensive technique for the separation of the epidermis from dermis to determine the expression of neurotrophins and inflammatory mediators, specifically in epidermal tissue.

Aim 2: Determine the effect of acute and chronic inflammation on the expression of NGF in the epidermis separated by thermolysin proteolytic treatment.

Aim 3: Determine the role of NGF signaling on glutaminase expression in dorsal root ganglion primary afferent neurons during inflammation by pharmacological inhibition of peripheral TrkA and Rab7GTPase.

The present data from this study demonstrate that the epidermal-dermal separation by thermolysin proteolytic enzyme is a useful technique for studying the interaction between intraepidermal nerve fibers and epidermal keratinocytes. Also, the temporal change in NGF expression might help determine the timepoints for therapeutic intervention by peripheral inhibition of NGF signaling.

The data also support the hypothesis that NGF signaling has a role in the glutamate metabolism by modulating the expression of enzyme glutaminase during peripheral inflammation.

The pharmacological inhibition of NGF associated molecules demonstrated the alternate technique for NGF signaling blockade other than anti-NGF antibodies. Also, peripherally targeting Rab7GTPase by its receptor antagonist provided the analgesic effect in inflammatory animals highlighting its potential as a therapeutic target for treating acute and chronic inflammatory pain.

Figure 1.1
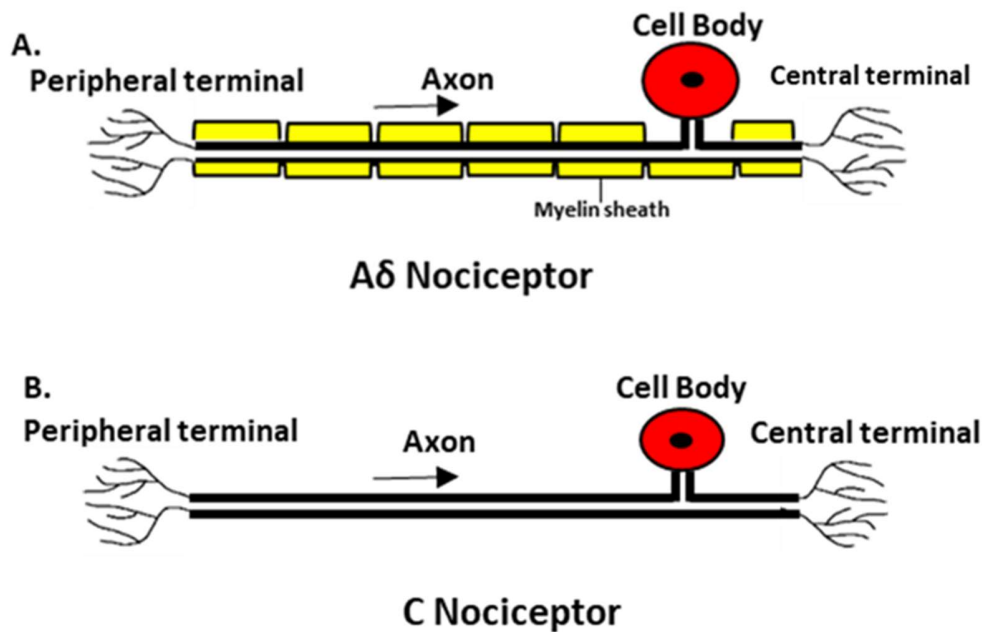
Figure 1.1 The term nociceptor is originated from the word noxious i.e. damaging or harmful. The nociceptors (cellular receptors) are nerve cell endings of pseudounipolar neurons that detects the sensation of pain and considered to have cell body and axon associated with the spinal cord and end organs like skin. **A.** Aδ nociceptors have lightly myelinated axon and conduct action potential at the rate of 20 meters/second towards CNS. **B.** C-nociceptors have unmyelinated axons and conduct action potential at the rate of 2 meters/seconds.

Figure 1.2.
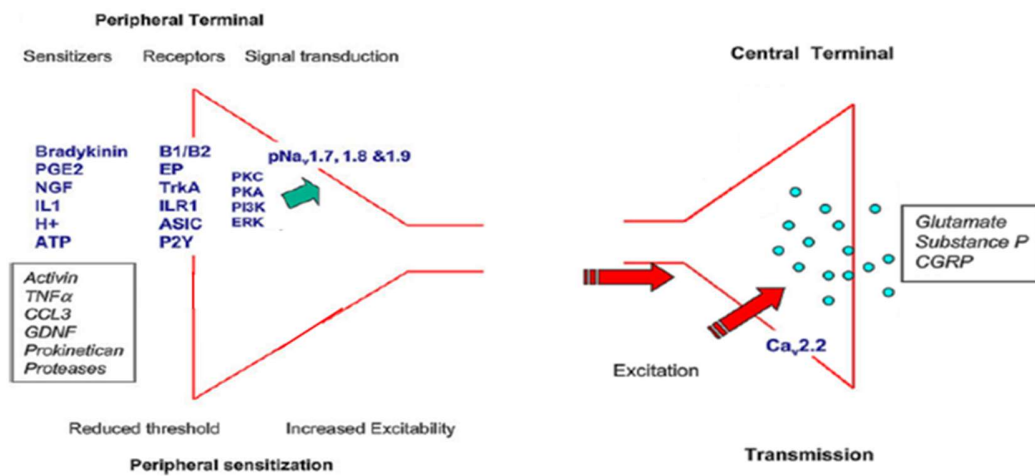
Figure 1.2. Several inflammatory mediators are released by the inflammatory cells and damaged tissues after noxious stimulus which determines the severity and extent of inflammation. Inflammatory mediators like bradykinin, histamine, serotonin, prostaglandins and neurotrophins like NGF lower the nociceptor threshold leading to peripheral sensitization. The protein receptors for inflammatory mediators are present on the nociceptors which assist in developing the peripheral sensitization. Modified from (Woolf & Ma, 2007)

Figure 1.3.
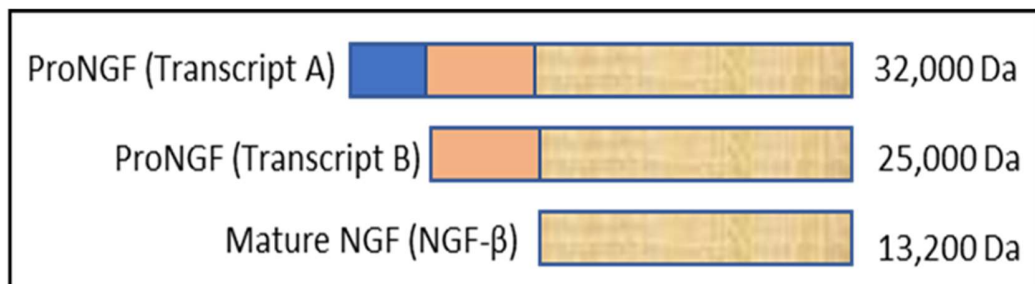
Figure 1.3. NGF is encoded by a 45kb gene with alternative splicing of two promotors and two exons. This splicing gives rise to two major and two minor variants. The two major variants get translated into NGF prepro species of 34 and 27 kD. These prepro transcripts gets modified in the endoplasmic reticulum to give Transcript A (32kD) and Transcript B (25kD). Finally, proNGF transcripts gets cleaved and forms Mature NGF (NGF -β) (Fahnestock et al., 2004).

Figure 1.4.
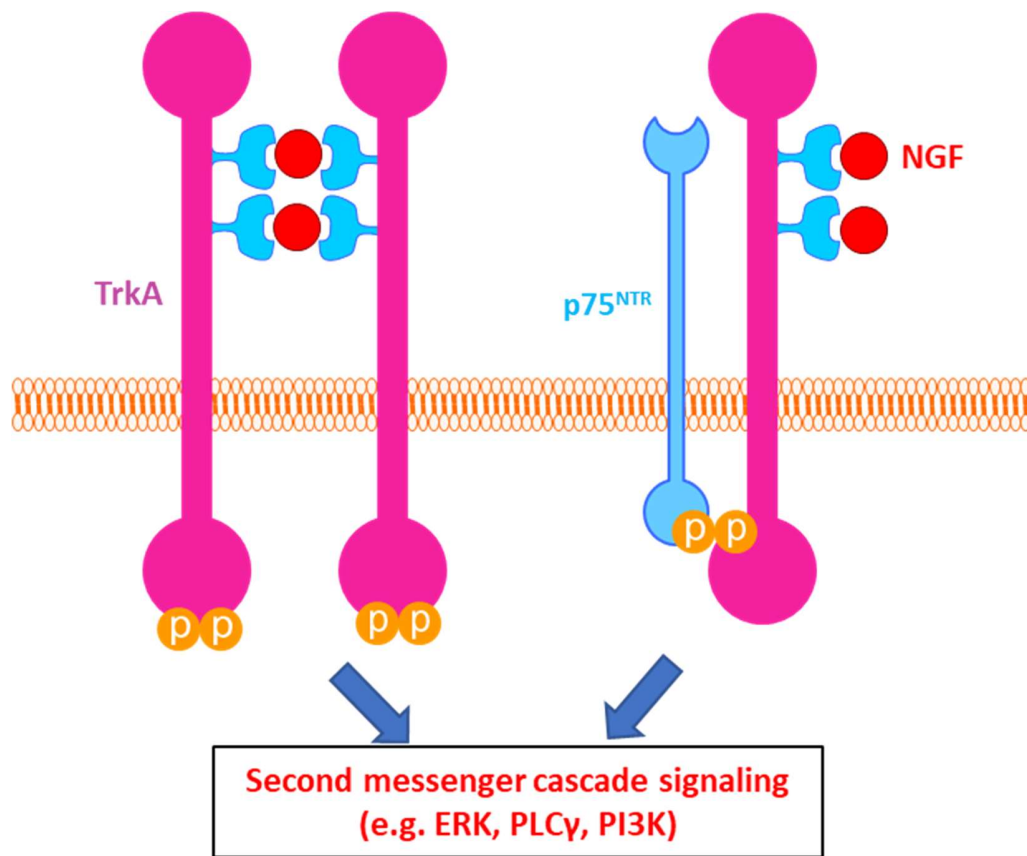
Figure 1.4. NGF can bind to either TrkA homodimer or TrkA/p75NTR heterodimer. The ligand receptor complex triggers the phosphorylation of the cytoplasmic ends leading to initiation of the second-messenger cascades of the signaling like PI3K-Akt, ERK or PLCγ. These signaling pathways are responsible for controlling the growth and survival of the primary sensory neurons (Denk et al., 2017).

Figure 1.5.
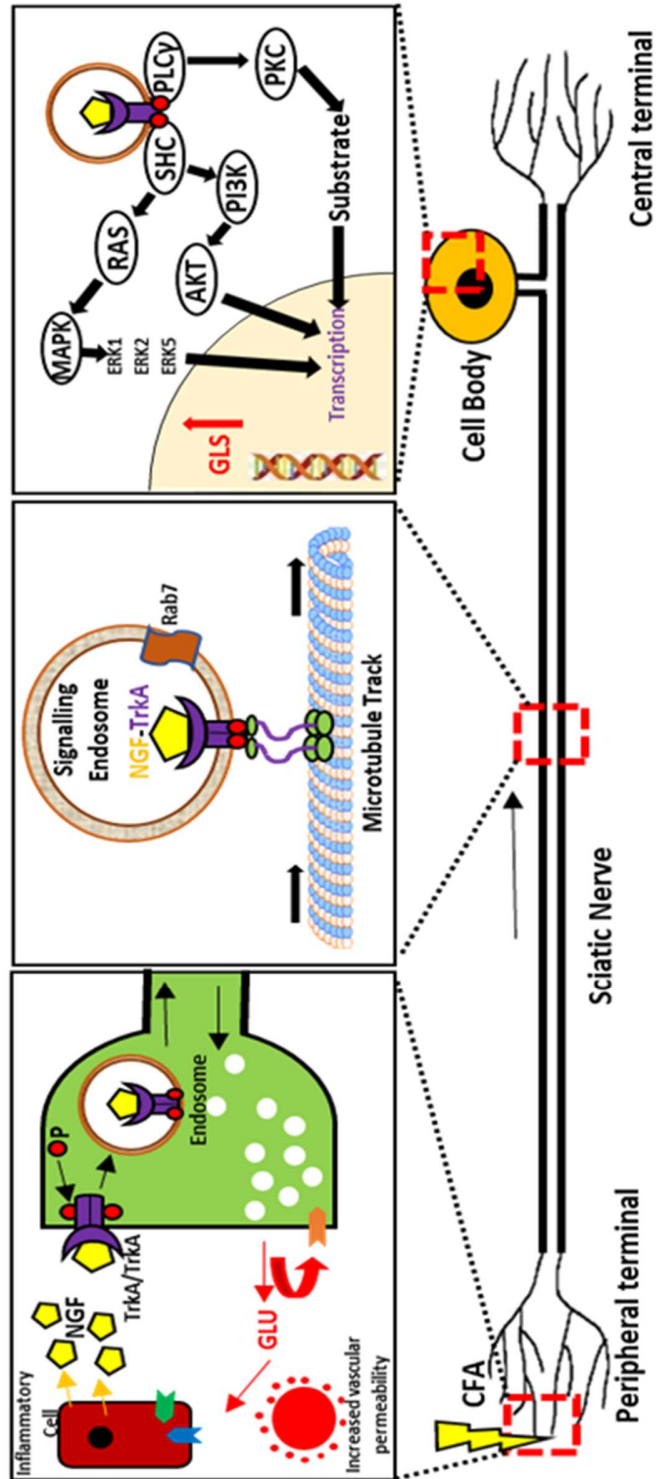
Figure 1.5. Mechanism for the sensitization of primary afferent during Antigen induced arthritis. After the noxious stimuli, peripheral cells will express NGF, which will bind to TrkA receptor present on primary sensory terminals. This NGF-TrkA complex is then retrogradely transported to cell soma. The signaling endosome is Rab7GTPase positive, which is essential for retrograde transport. In the cell body, NGF signalling can modulate the production of neuropeptides like SP, CGRP and neurotransmitter producing enzyme glutaminase (GLS).

Figure 1.6.
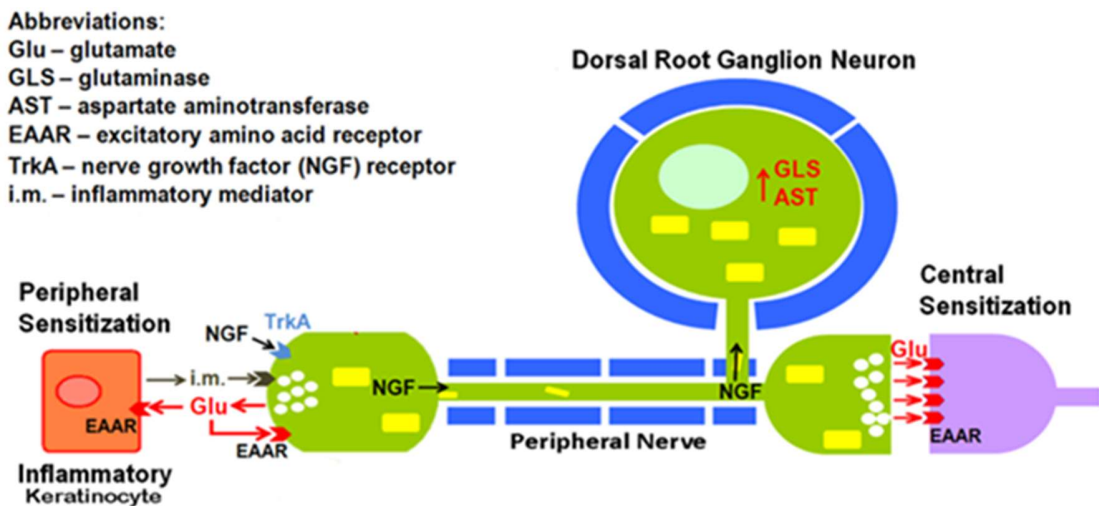
Figure 1.6. After peripheral sensitization in skin with CFA, inflammatory cells produce NGF, which binds to its specific receptor TrkA at the nerve terminal. This complex is packaged into clathrin dependent signalling endosomes and transported in retrograde manner to the DRG cell body. NGF-TrkA signalling causes changes in protein expression in DRG cell soma, e.g., upregulation of SP, CGRP and glutaminase production. This glutaminase produces glutamate, which is released in the periphery, sensitizing the nerve terminals and inflammatory cells. Modified from Miller et.al (Miller et al., 2011)

CHAPTER II

SEPARATION OF RAT EPIDERMIS AND DERMIS WITH THERMOLYSIN TO DETECT

SITE-SPECIFIC INFLAMMATORY MRNA AND PROTEIN

Abstract

Easy-to-use and inexpensive techniques are needed to determine the site-specific production of inflammatory mediators and neurotrophins during skin injury, inflammation, and/or sensitization. The goal of this study is to describe an epidermal-dermal separation protocol using thermolysin, a proteinase that is active at 4 °C. To illustrate this procedure, Sprague Dawley rats are anesthetized, and right hind paws are injected with carrageenan. Six and twelve hours after injection, rats with inflammation and naïve rats are euthanized, and a piece of hind paw, glabrous skin is placed in cold Dulbecco's Modified Eagle Medium. The epidermis is then separated at the basement membrane from the dermis by thermolysin in PBS with calcium chloride. Next, the dermis is secured by microdissection forceps, and the epidermis is gently teased away. Toluidine blue staining of tissue sections show that the epidermis is separated cleanly from the dermis at the basement membrane. All keratinocyte cell layers remain intact, and the epidermal rete ridges along with indentations from dermal papillae are clearly observed. Qualitative and real-time RT-PCR is used to determine nerve growth factor and interleukin-6 expression levels. Western blotting and immunohistochemistry are finally performed to detect amounts of nerve growth factor. This report illustrates that cold thermolysin digestion is an effective method to separate epidermis from dermis for evaluation of mRNA and protein alterations during inflammation (Gujar, 2020).

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Introduction

Evaluation of inflammatory mediators and neurotrophic factors from the skin can be limited due to the heterogeneity of cell types found in the inflamed dermis and epidermis (Choi & Di Nardo, 2018; Manti, Brown, Perez, & Piedimonte, 2017; Schakel, Schon, & Ghoreschi, 2016). Several enzymes, chemical, thermal, or mechanical techniques involving separation of the two layers or for performing cell dissociation for evaluation have been reviewed recently (Zou & Maibach, 2018). Acid, alkali, neutral salt, and heat can quickly divide the epidermis from dermis, but cellular and extracellular swelling often occur (Baumberger, Suntzeff, & Cowdry, 1942; Felsher, 1947). Trypsin, pancreatin, elastase, keratinase, collagenase, pronase, dispase, and thermolysin are enzymes that have been used for epidermal-dermal separation (Einbinder, Walzer, & Mandl, 1966; Zou & Maibach, 2018). Trypsin and other broad-scale proteolytic enzymes are active at 37–40 °C but must be monitored carefully to prevent dissociation of epidermal layers. Dispase cleaves the epidermis at the lamina densa but requires 24 h for separation in the cold (Rakhorst et al., 2006; Zou & Maibach, 2018) or short timepoints at 37°C (Tschachler et al., 2004; Zou & Maibach, 2018). A limiting feature of all these techniques is the potential disruption of tissue morphology and the loss of mRNA and protein integrity.

Skin separation method should be carried out in the cold for a short time to maintain the integrity of mRNA and associated proteins. In evaluating skin separation techniques for inflammation studies, thermolysin is an effective enzyme to separate the epidermis from dermis at cold temperatures (Zou & Maibach, 2018). Thermolysin is active at 4 °C, cleaves epidermal hemidesmosomes from the lamina lucida, and separates the epidermis from dermis within 1–3 h (Rakhorst et al., 2006; Walzer, Benathan, & Frenk, 1989; Zou & Maibach, 2018). This report aims to optimize the use of thermolysin for the separation of inflamed rat epidermis from dermis to detect mRNA and protein levels for inflammatory mediators and neurotrophic factors. Several preliminary reports have been presented (Anderson, 2017; Anderson, Miller, & Schechter, 2010;

Gujar, 2017; IBitokun, 2010; Nawani, 2011). The objective of this manuscript is to describe an optimal skin separation technique using thermolysin and demonstrate the detection of 1) markers of inflammation, 2) interleukin-6 (IL-6) mRNA, and 3) Nerve Growth Factor (NGF) mRNA and protein in the epidermis of rats with carrageenan-induced inflammation (C-II) (Fehrenbacher, Vasko, & Duarte, 2012; Vinegar et al., 1987). A preliminary report using the Complete Freund's Adjuvant model indicates that NGF mRNA and protein levels increase early during inflammation (Gujar, 2017). In mice, skin sensitization with the topical application of oxazolone causes an early rise in the IL-6 mRNA using in situ hybridization (Flint, Dearman, Kimber, & Hotchkiss, 1998). Both IL-6 and NGF have been implicated in C-II (Li et al., 2018; Sammons et al., 2000), but there have been no reports describing mRNA or protein levels for IL-6 or NGF specifically from the epidermis during the acute stages of C-II.

The thermolysin technique is inexpensive and straightforward to perform. Furthermore, thermolysin separation of the epidermis from dermis allows for mRNA, western blot, and immunohistochemical analysis of inflammatory mediators and neurotrophic factors during inflammation (Gujar, 2017). Investigators should be able to easily use this technique in both preclinical and clinical studies of skin inflammation.

Materials and Methods

This protocol follows the animal care guidelines of Oklahoma State University Center for Health Sciences IACUC (#2016-03).

1. *Carrageenan-induced inflammation (C-II)*

1.1 Anesthetize male and/or female Sprague Dawley rats (200–250 g; 8–9 weeks old) with isoflurane (or injectable anesthetic).

1.2 Check the depth of anesthesia by touching the cornea and lightly pinching the left hind paw. When the animal is appropriately anesthetized, no corneal or paw response will be observed.

1.3 Subcutaneously inject the right glabrous, hind paw with 100 µL of 1% (w/v) λ-carrageenan diluted in phosphate-buffered saline (PBS) (Hoffman & Miller, 2010).

1.3.1 Make sure that appropriate controls are used, such as naïve rats without isoflurane in this report. Preliminary studies indicate that naïve rats with or without isoflurane have the same basal expression of epidermal IL-6 and NGF.

NOTE: Naïve rats are preferred controls for inflammation studies since subcutaneous saline or PBS cause local inflammation (Crosby, Ihnat, & Miller, 2015; Hoffman, Zhang, Schechter, & Miller, 2016).

1.4 Evaluate the edema of C-II rats to demonstrate the effectiveness of the carrageenan (Figure 2.1) (Hoffman & Miller, 2010). Determine the amount of edema by measuring the hind paw metatarsal thickness with calipers.

1.5 At 6–12 h, euthanize rats with CO_2 (or injectable anesthetic overdose) and cut 1 mm x 2 mm pieces of glabrous hind paw skin with sharp scalpel. If hairy skin is used, then shave it before cutting the 1 mm x 2 mm pieces of skin.

NOTE: Make sure that the appropriate timepoints are chosen according to the specific studies.

1.6 Using microdissection forceps, transfer the skin into 1 mL of cold Dulbecco's Modified Eagle Medium (DMEM) in a microcentrifuge tube on ice and keep cold for 15–60 min.

2. *Thermolysin separation of epidermis and dermis*

2.1 Prepare and activate thermolysin.

2.1.1 Prepare a solution of thermolysin, by adding 5 mg of thermolysin obtained from Geobacillus stearothermophilus to 10 mL of PBS, at pH = 8 (concentration 500 µg/mL).

2.1.2 Prepare a 1 M solution of calcium chloride ($CaCl_2$ anhydrous) by adding 1.11 g into 10 mL of distilled H_2O.

2.1.3 To prevent autolysis of thermolysin, add 10 µL of calcium chloride to 10 mL of thermolysin solution. The calcium chloride final concentration will be 1 mM.

2.1.4 Aliquot 1 mL of activated thermolysin into 10 wells of a 24 well cell culture plate on ice.

2.2 Use thermolysin enzyme digestion to separate the epidermis from dermis.

2.2.1 Using microdissection forceps, transfer one skin sample into each well of activated thermolysin. Make sure not to immerse the skin in the thermolysin solution.

2.2.2 Gently tap the skin on the side of the well to assist in releasing the skin sample from the forceps to float on the thermolysin solution.

2.2.3 Float the skin into the thermolysin solution with the stratum corneum (outer epidermis) side up and dermis face down. It is critical that the dermis faces down, or the effective separation will not take place.

NOTE: The amount of time for thermolysin incubation must be determined empirically by the end-user. Glabrous, hind paw skin from Sprague Dawley rats (200–250 g; 8–9 weeks old) often requires 2.0–2.5 h for separation. Incubation time is expected to vary with species and age.

2.2.4 After the appropriate incubation time in thermolysin, use microdissection forceps to transfer one skin sample into a well of a 6 well cell culture plate with 7–8 mL of cold (4 °C) DMEM. This allows more room for separation of the epidermis from the dermis.

2.2.5 Immerse the skin into the DMEM.

2.2.6 Gently brush the epidermis with the forceps around the perimeter of the skin until the near-translucent epidermis is observed at the borders. If this cannot be achieved, return the skin sample to the thermolysin solution for another 15–30 min.

2.2.7 Once the epidermis noticeably separates from the dermis, then carefully hold both the epidermis and dermis with microdissection forceps and very slowly pull the epidermis from the dermis.

2.2.8 Evaluate the translucence of the isolated epidermis and make sure it is optically consistent. See Figure 2.2 for an example of a 1 mm x 2 mm sample of rat epidermis. If there is a variation in the translucence, then proper separation has not occurred.

2.3 Inactivate thermolysin using ethylenediaminetetraacetic acid (EDTA) in the separated pieces of epidermis and dermis.

CAUTION: The thermolysin that remains in the epidermis and dermis is still active and can damage the layers if not inactivated.

2.3.1 Prepare a 0.5 M EDTA stock solution. To do so, slowly add 0.93 g EDTA into 5 mL of double-distilled water. Add sodium hydroxide to the solution until it clears. Ensure that the pH of the solution is ~8.0.

2.3.2 Make a 5 mM EDTA solution in DMEM. Add 0.25 mL of 0.5 M EDTA stock solution to 25 mL of DMEM.

2.3.3 Place the separated epidermis and dermis into the 5 mM EDTA/DMEM solution at 4 °C for 30 min to deactivate thermolysin's activity.

2.4 Evaluate the epidermis with tinctorial histology (Rakhorst et al., 2006; Tschachler et al., 2004; Walzer et al., 1989).

2.4.1 Fix a portion of the epidermis in a 10% neutral formalin, 4% paraformaldehyde, or 0.25% paraformaldehyde with 0.8% picric acid solution for 1 h at room temperature (RT) with agitation.

2.4.2 Place the fixed epidermis in 10% sucrose in PBS for 1 h at RT with agitation.

2.4.3 Freeze the epidermis in a tissue embedding matrix for sectioning. Cut 14 μm cross-sections using a cryostat and thaw-mount sections onto gelatin-coated glass microscope slides.

2.4.4 Dry sections on a slide warmer and stain with a working solution of toluidine blue (TB; 10% TB in 1% sodium chloride) for 90 s. Appose coverslips with an aqueous mounting medium.

2.4.5 Observe the epidermis with brightfield microscopy at 50x–250x.

NOTE: If proper separation has occurred, the epidermis will be divided cleanly from the dermis and the five layers will be detected: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. An example of separated rat skin epidermis can be seen in Figure 2.3.

3. *Protein extraction and western blot analysis*

3.1 Perform Western blotting on the separated tissue samples using previously published protocol (Crosby, Ihnat, Spencer, & Miller, 2015).

3.2 Homogenize the epidermis in 50 μL of lysis buffer (25mM Tris HCl, pH = 7.4, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and 1% Triton X-100) containing a phosphatase and protease inhibitor cocktail.

3.3 Centrifuge samples at max speed for 15 min at 4°C and evaluate the supernatant for protein concentration using a protein assay kit.

3.4 Load equal concentrations of protein (30 μg) onto SDS gels, perform electrophoresis, and then transfer proteins to nitrocellulose or PVDF membranes.

3.5 Block membranes with 5% milk for 2 h and incubate overnight in primary antibody (mouse anti-NGF, E12, 1:1000).

3.6 Wash 3x with PBS with 0.3% tween for 10 min each and incubate with a labeled secondary antibody (e.g., alkaline phosphatase labeled rabbit anti-mouse IgG).

3.7 Use a scanning system to evaluate western blot signal (e.g., ECF substrate and an imaging platform).

4. *Immunohistochemistry*

4.1 Place tissue samples in a fixative for optimal immunoreactivity: 0.96% (w/v) picric acid and 0.2% (w/v) formaldehyde in 0.1 M sodium phosphate buffer, pH = 7.3 (Crosby, Ihnat, Spencer, et al., 2015; Hoffman, Schechter, & Miller, 2010; Hoffman et al., 2016) for 4 h at RT. Transfer to 10% sucrose in PBS overnight at 4 °C.

4.2 Perform standard immunohistochemistry on the tissue sections (Crosby, Ihnat, Spencer, et al., 2015; Hoffman et al., 2010; Hoffman et al., 2016).

4.3 Embed the epidermis from animals into a single frozen block in embedding matrix and cut 10–30 μm sections on a cryostat. Mount the sections on gelatin-coated, glass microscope slides and dry at 37 °C for 2 h.

4.4 Wash sections for three, 10 min rinses in PBS and incubate for 24–96 h in primary antisera, [e.g., mouse anti-NGF (E12, 1:2000)] and rabbit anti-protein gene product 9.9 (PGP 9.5, 1:2000) diluted in PBS containing 0.3% (w/v) Triton X-100 (PBS-T) PBS-T with 0.5% bovine serum albumin (BSA) and 0.5% polyvinylpyrrolidone (PVP).

4.5 After primary antiserum incubation, rinse sections three times for 10 min in PBS and incubate 1 h at RT in Alexa Fluor 488 donkey anti-rabbit IgG (1:1000) and Alexa Fluor 555 donkey anti-mouse IgG (1:1000) diluted in PBS-T.

4.6 Rinse sections three times in PBS for 10 min and affix coverslips with non-fading mounting medium to retard fading of immunofluorescence.

5. *RNA isolation and cDNA synthesis*

5.1 Perform standard reverse transcriptase polymerase chain reaction (RT-PCR) on the skin samples (Crosby, Ihnat, Spencer, et al., 2015). Isolate total RNA using a phenol, guanidine isothiocyanate solution.

5.2 Carry out complementary DNA synthesis by Moloney murine leukemia virus reverse transcriptase.

5.3 Use the following primer sequences for NGF and IL-6 amplification:

NGF (Sense) - GTGGACCCCAAACTGTTTAAGAAACGG

NGF (Antisense) – GTGAGTCCTGTTGAAGGAGATTGTACCATG

IL-6 (Sense) - GCAATTCTGATTGTATGAACAGCGATGATGC;

IL-6 (Antisense) – GTAGAAACGGAACTCCAGAAGACCAGAG

5.4 Compare the levels of NGF and IL-6 mRNA to β-actin housekeeping gene:

β-ACTIN (Sense) - TGCGTGACATTAAAGAGAAGCTGTGCTATG

β-ACTIN (Antisense) – GAACCGCTCATTGCCGATAGTGATGA

5.4 Evaluate with qualitative RT-PCR using thermal cycler and quantitative reverse transcription PCR (qRT-PCR) using a qRT-PCR system.

Results

Carrageenan injection into the rat hind paw caused classic symptoms of inflammation such as redness and edema (Fehrenbacher et al., 2012; Vinegar et al., 1987). The swelling of the hind paw was measured with mechanical calipers (Hoffman & Miller, 2010). A baseline value of the paw thickness was obtained for each rat before carrageenan treatment and measured again at 6 h and 12 h. Paw thickness was increased significantly compared to the baseline values (Figure 2.1).

Thermolysin incubation of the rat glabrous hind paw skin produced a sheet of epidermis. Brightfield microscopy was used to evaluate the effectiveness of thermolysin separation of the epidermis and dermis (Figure 2.2). The layers of the epidermis could be determined while focusing through the sheet at higher magnification. Toluidine blue staining of epidermal cross-sections showed that the epidermis was separated from the dermis at the basement membrane (Figure 2.3). The epidermal rete ridges (epidermal pegs) along with the indentations from dermal papillae were intact. All keratinocyte cell layers were observed.

Western blotting of thermolysin-separated epidermis produced consistent results indicating stable protein levels during the technique at 4 °C. Very little NGF protein was detected in naïve rat epidermis, but NGF protein levels were upregulated (250%) after 6 h of C-II compared to naïve animals (Figure 2.4). After 12 h of C-II, NGF levels were reduced compared to 6 h but remained elevated (55%) relative to controls. Immunohistochemistry for NGF in the separated epidermis provided reliable immunostaining and confirmed the results from Western blots (Figure 2.5). NGF-immunoreactivity (ir) was not detected in naïve control epidermis, but at 6 h C-II, there was NGF-ir in most of the keratinocytes of the stratum granulosum and stratum lucidum. A few cells for the stratum spinosum were NGF-immunoreactive (IR) at 6 h C-II. At 12 h C-II, NGF-ir occurred in

keratinocytes of the stratum granulosum and stratum lucidum with some cells in stratum granulosum intensely NGF-IR. At timepoints was NGF-ir detected in stratum basale or stratum corneum. PGP9.5-IR intraepidermal, varicose nerve fibers were present in the separated epidermis from naïve and C-II rats (Figure 2.5).

Qualitative RT-PCR demonstrated that good quality mRNA from thermolysin-separated epidermis was obtained (Figure 2.6A, Figure 2.7A). NGF mRNA expression in epidermis during C-II was significantly elevated (>3-fold) at 6 h compared to naïve rats, using actin as a housekeeping gene for quantitative real-time PCR (Figure 2.6B). At 12 h, NGF mRNA returned to baseline levels (Figure 2.6B). Using actin as a housekeeping gene for quantitative RT-PCR, IL-6 mRNA in epidermis during C-II was significantly elevated (>6-fold) at 6 h compared to naïve rats (Figure 2.7B). At 12 h, IL-6 mRNA levels dropped significantly from the 6 h amounts but remained elevated (2-fold) compared to naïve rats (Figure 2.7B).

Discussion

The study determined that the epidermis of rat hind paw glabrous skin was easily separated from dermis using thermolysin (0.5 mg/mL) in PBS with 1 mM calcium chloride at 4 °C for 2.5 h. Histological evaluation indicated that the epidermis was separated from the dermis at the basement membrane and that the epidermal rete ridges were intact. Thermolysin is an extracellular metalloendopeptidase produced by Gram-positive GeoBacillus thermoproteolyticus (van den Burg & Eijsink, 2013). Its activity is stable at 4 °C but is functional over a wide range of temperatures (Matthews, 2011; van den Burg & Eijsink, 2013; Walzer et al., 1989). This enzyme has been used extensively for protein chemistry (Matthews, 2011; van den Burg & Eijsink, 2013), but several groups have shown its application for skin separation into epidermal and/or dermal sheets (Glade et al., 1996; Hybbinette, Bostrom, & Lindberg, 1999; Rakhorst et al., 2006; Walzer et al., 1989; Zou & Maibach, 2018). Walzer et al. were the first to report epidermal-dermal separation of human

skin using thermolysin at 4 °C (250–500 µg/mL for 1 h). With light and electron microscopy, separation was determined to occur at the epidermal basement membrane between laminin and the bullous pemphigoid antigen site (Walzer et al., 1989).

Furthermore, hemidesmosomes, the attachments of basal keratinocytes to the basement membrane, were disrupted selectively (Gragnani, Sobral, & Ferreira, 2007; Walzer et al., 1989). Rakhorst et al. compared thermolysin (4 °C, 500 µg/mL, overnight) to dispase for epidermal-dermal separation of rabbit buccal mucosa (Rakhorst et al., 2006). Thermolysin was incomplete in separating the mucosal epidermis from dermis signifying that differences may occur for species, incubation time, solution composition (no $CaCl_2$ to prevent thermolysin autolysis) (van den Burg & Eijsink, 2013), source of thermolysin, and/or site-specific differences indicated from other studies (Glade et al., 1996; Hybbinette et al., 1999; Walzer et al., 1989). End-users of the current protocol should make sure to use fresh thermolysin and always include calcium chloride but also should be aware of these potential limitations.

Although some investigators have used thermolysin at 37 °C (Gragnani et al., 2007; Hybbinette et al., 1999), users of this protocol should be mindful to keep skin tissue at 4 °C to preserve the stability of protein and mRNA. We used DMEM at 4 °C as a solution for skin prior to and after thermolysin separation because of its usefulness in maintaining cells in culture (Sato & Kan, 2001), and it has been used previously for skin separation with thermolysin (Germain et al., 1993; Glade et al., 1996; Hybbinette et al., 1999; Michel et al., 1999; Rakhorst et al., 2006). However, Walzer et al. used sterile PBS supplemented with 200 µG/mL streptomycin, 200 U/mL penicillin, and 2.5 µg/mL fungizone (Walzer et al., 1989), whereas others have used different media (e.g., keratinocyte culture media free of epidermal growth factor) followed by PBS rinsing (Gragnani et al., 2007).

In the protocol, thermolysin separation was performed in 500 μg/mL thermolysin and 5 mM calcium chloride in PBS (pH = 8), similar to the original method (Walzer et al., 1989). DMEM has been used as a solution for thermolysin separation at 4 °C (overnight) (Rakhorst et al., 2006), and HEPES buffer has been used effectively with 500 μg/mL thermolysin solution at 37 °C for 2 h (Gragnani et al., 2007). However, we did not explore how culture medium or other buffers affects thermolysin's activity for epidermal-dermal separation. Calcium chloride is an important addition to decrease autolysis of thermolysin (Fassina et al., 1986; van den Burg & Eijsink, 2013). The deletion of this step may lead to incomplete cleavage of the epidermis from dermis (Rakhorst et al., 2006).

The size of the skin sample appears to influence the time needed for thermolysin incubation and the effectiveness of enzymatic cleavage of the epidermis from dermis. Investigators need to evaluate the appropriate sample size and incubation time for their tissues. Floating the samples on the thermolysin solution with the epidermis facing upward is important for optimal enzyme effectiveness (Walzer et al., 1989). As noted earlier, the site of action for thermolysin is at the keratinocyte hemidesmosomes and basement membrane (Gragnani et al., 2007; Walzer et al., 1989; Zou & Maibach, 2018); therefore, thermolysin works inward from the edges of the skin. From our experience, the skin edges separate earlier than the middle of the sample, and it is important to allow enough time for complete enzymatic cleavage. When cleavage is complete, the epidermis should pull away easily from the dermis. If still attached, tugging on the layers may cause portions of dermis to separate with the epidermis.

A limitation of the thermolysin technique is the time required. Increasing the thermolysin concentration beyond 500 μG/mL does not decrease the time for separation and there is poor preservation of the epidermis at higher concentrations (Walzer et al., 1989). Epidermal-dermal separation methods have been reviewed recently (Zou & Maibach, 2018), and many methods take 30–60 min at 20–40 °C. Heat (50–60 °C) separation of skin occurs quickly (30 s to 10 min), but

proteins and mRNA are known to degrade quickly at such high temperatures. Alternatively, sodium thiocyanate (2 N) at RT may be an acceptable rapid separation technique (5 min) (Felsher, 1947; Zou & Maibach, 2018), but protein and mRNA integrity have not been studied with this method (Felsher, 1947). The cold thermolysin method was chosen for the preservation of protein and mRNA, but there were no direct comparisons made between protein and mRNA integrity using other techniques.

In the present study, cold thermolysin digestion is demonstrated to be an effective method to separate the epidermis from the dermis for evaluation of mRNA and protein alterations during inflammation. During carrageenan-induced inflammation, NGF mRNA and protein levels and IL-6 mRNA levels were elevated at 6 h, returning close to baseline by 12 h. With immunohistochemistry, NGF immunoreactivity was increased in keratinocytes at 6 h and 12 h. An advantage of the thermolysin method is the ability to perform site-selective analysis. For example, the increased production of NGF and IL-6 in the current study is from keratinocytes, since dermal cells are excluded from the assays. This method allows for insight into the location and mediator types for the sensitization of primary afferent terminals (Petho & Reeh, 2012). In addition, this method allows for a better understanding of the time course associated with neurotrophin production, and uptake and transport of neurotrophin in primary afferents during inflammation (Denk, Bennett, & McMahon, 2017; Djouhri, Dawbarn, Robertson, Newton, & Lawson, 2001).

Figure 2.1.
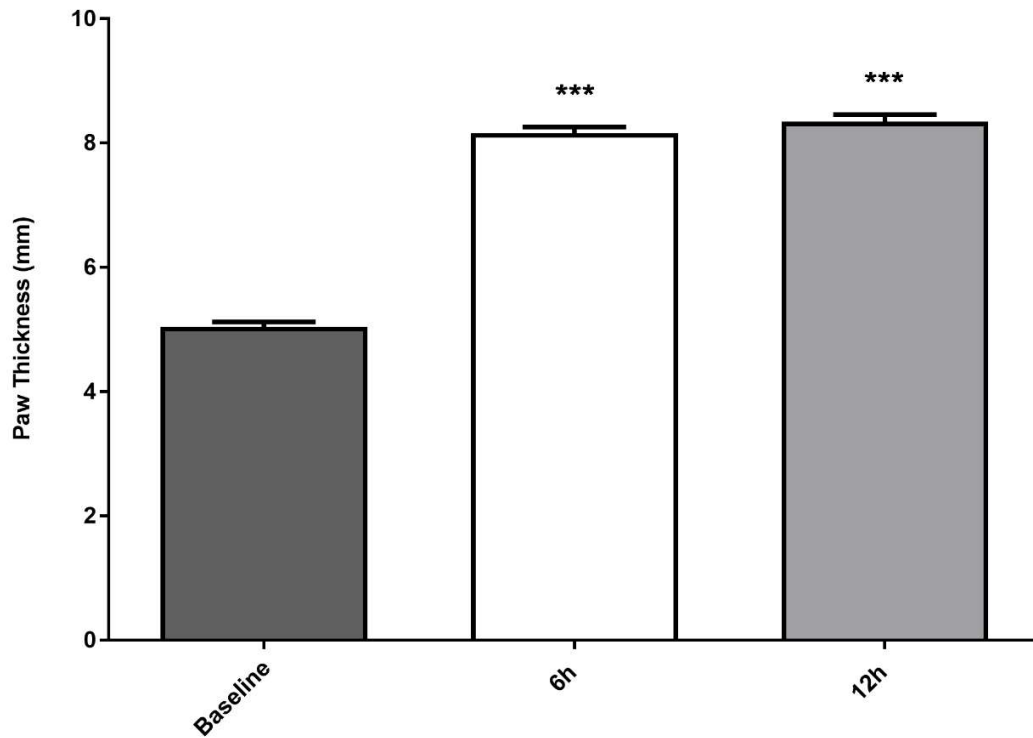
Figure 2.1: **Carrageenan injection produces paw edema.** A baseline value was obtained for each rat prior to carrageenan treatment. After 6 h and 12 h of treatment, paw thickness increased significantly compared to baseline values. The results are expressed as the SEM with six rats per treatment (*p < 0.05, **p < 0.01, ***p < 0.001; student's t-test, unpaired, two-tail, was performed at each timepoint).

Figure 2.2.
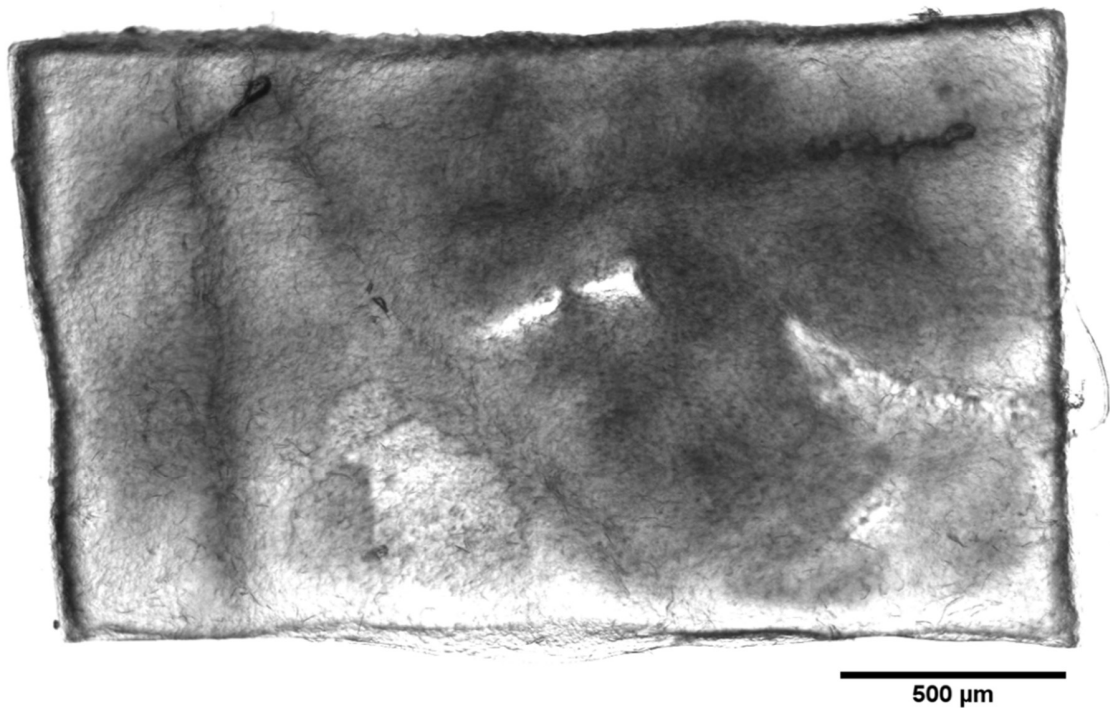
Figure 2.2: **Thermolysin produces a sheet of epidermis**. Brightfield microscopy revealed a translucent epidermal sheet approximately 1 mm x 2 mm in size. The layers of the epidermis could be determined while focusing through the sheet at higher magnification. Scale bar = 500 µm.
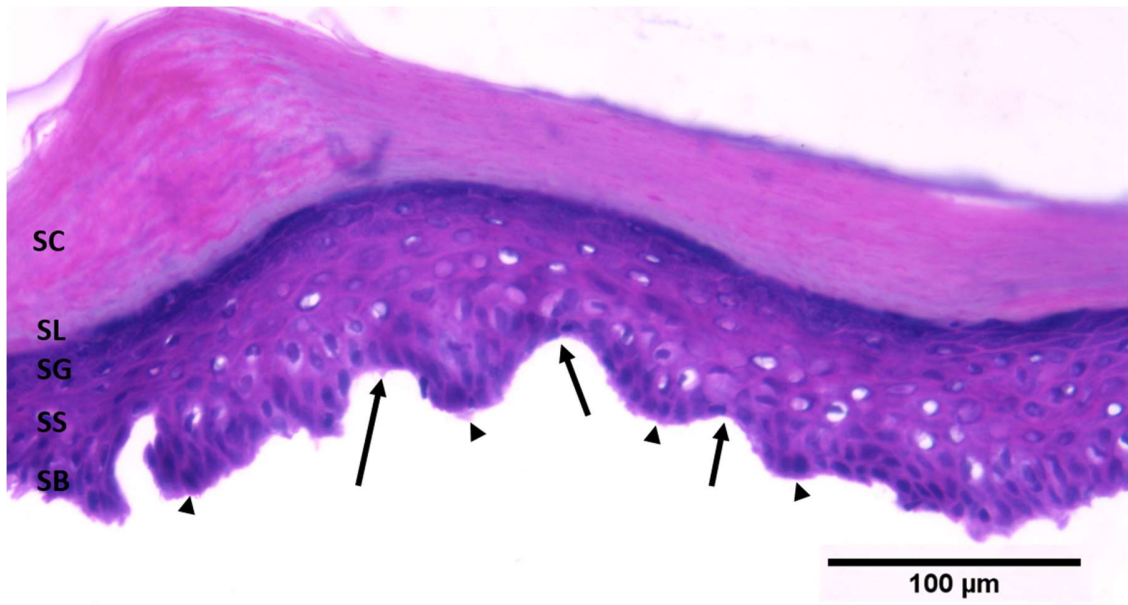
Figure 2.3.

Figure 2.3: **Toluidine blue staining of the epidermis.** Brightfield microscopy determined that thermolysin caused an effective separation of the epidermis from dermis. Epidermal rete ridges (epidermal pegs; arrowheads) were observed along with indentations from dermal papillae (arrows). All keratinocyte cell layers were intact. SB: stratum basale, SS: stratum spinosum, SG: stratum granulosum, SL: stratum lucidum, SC: stratum corneum. Scale bar = 100 µm.

Figure 2.4.
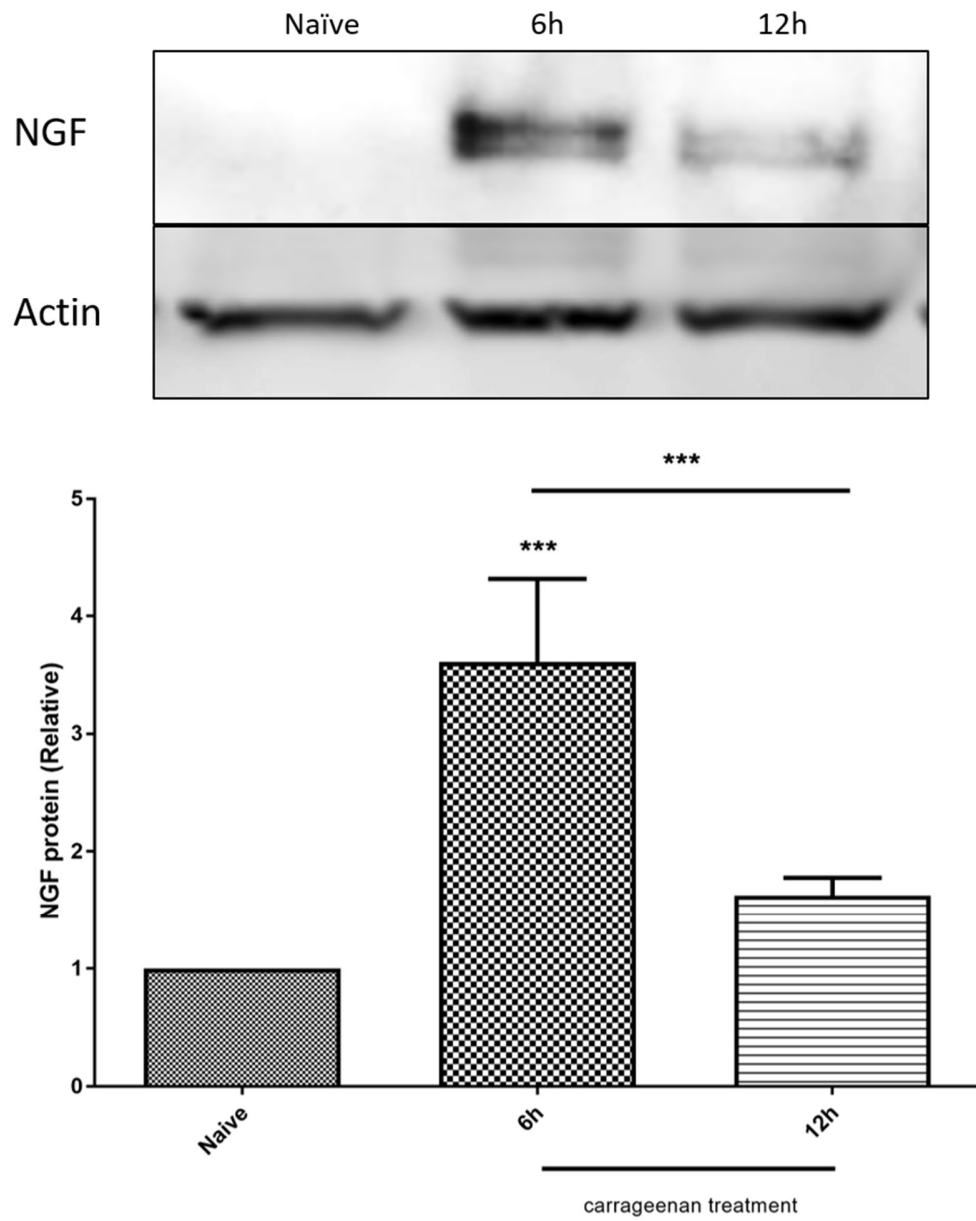
Figure 2.4: **NGF protein expression in epidermis during carrageenan-induced inflammation.**

NGF levels were increased (250%) after 6 h of inflammation compared to naïve animals. After 12 h, the levels were reduced compared to 6 h but were elevated (55%) in contrast to naïve animals. The results are expressed as the SEM with three rats per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; student's t-test, unpaired, two-tail was performed at each timepoint)

Figure 2.5
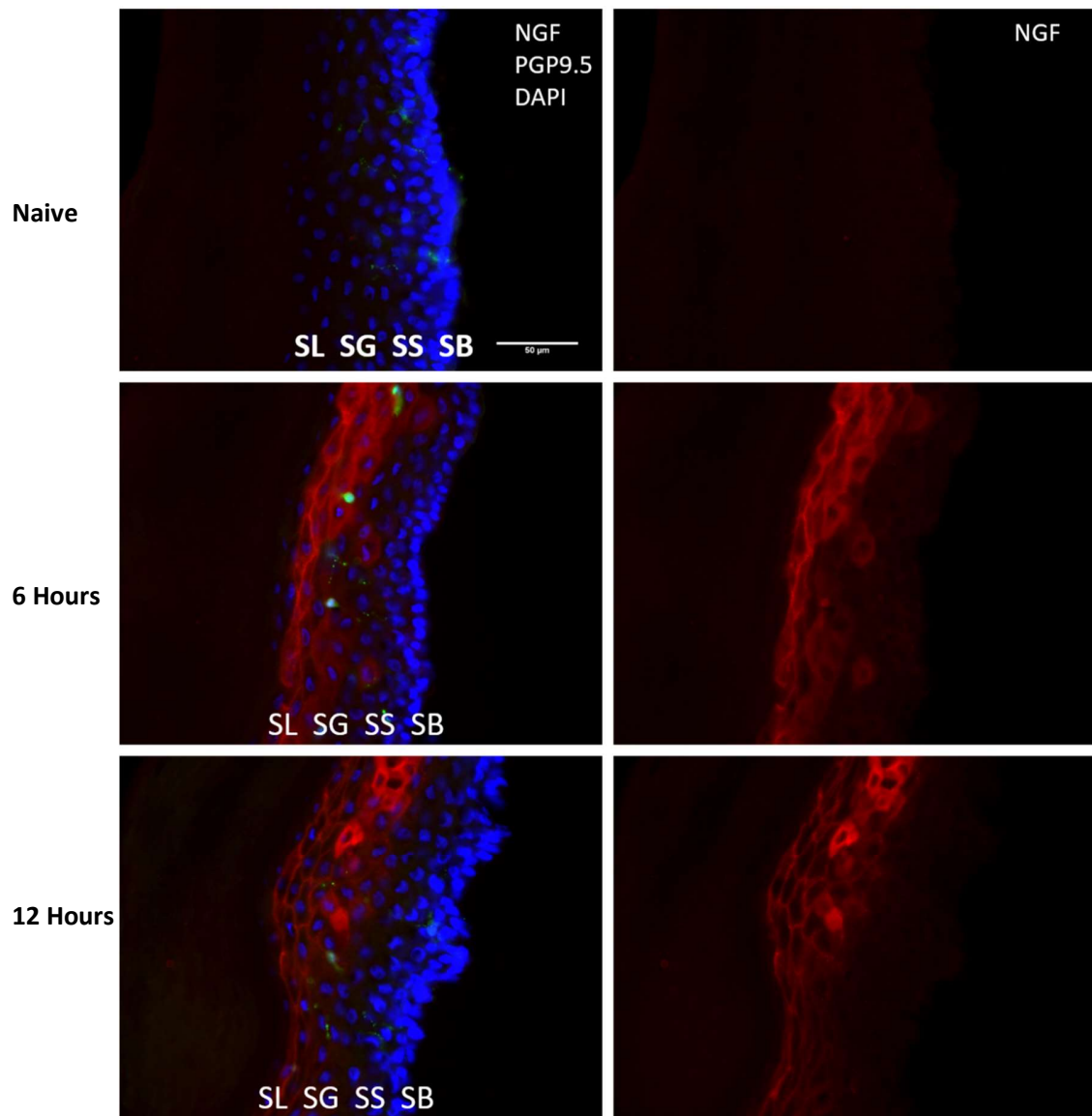
Figure 2.5: **NGF and PGP9.5 immunoreactivity (ir) during C-II**. Columns A,B,C show NGF-ir, PGP9.5-ir, and DAPI nuclear staining, whereas columns A_1-C_1 show only NGF-ir. In all images, stratum corneum is towards the left and stratum basale towards the right. NGF-ir was not detected in naïve control epidermis (A, A_1). At 6 h C-II (B,B_1), NGF-ir was present in most keratinocytes of the stratum granulosum (short arrows) and stratum lucidum (long arrows). A few cells for the stratum spinosum were NGF-ir at 6 h C-II (large arrowheads). At 12 h C-II (C,C_1), NGF-ir occurred in keratinocytes of the stratum granulosum and stratum lucidum (long arrows) with some cells in stratum granulosum intensely NGF-ir (short arrows). At time-pointint was NGF-ir detected in stratum basale or stratum corneum. PGP9.5-ir intraepidermal nerve fibers were present in the separated epidermis from naïve and C-II rats (small arrowheads, A-C). SB: stratum basale, SS: stratum spinosum, SG: stratum granulosum, SL: stratum lucidum. Scale bar = 50 μm.

Figure 2.6.

A

Naïve   6h   12h

NGF

Actin

***

B

carrageenan treatment
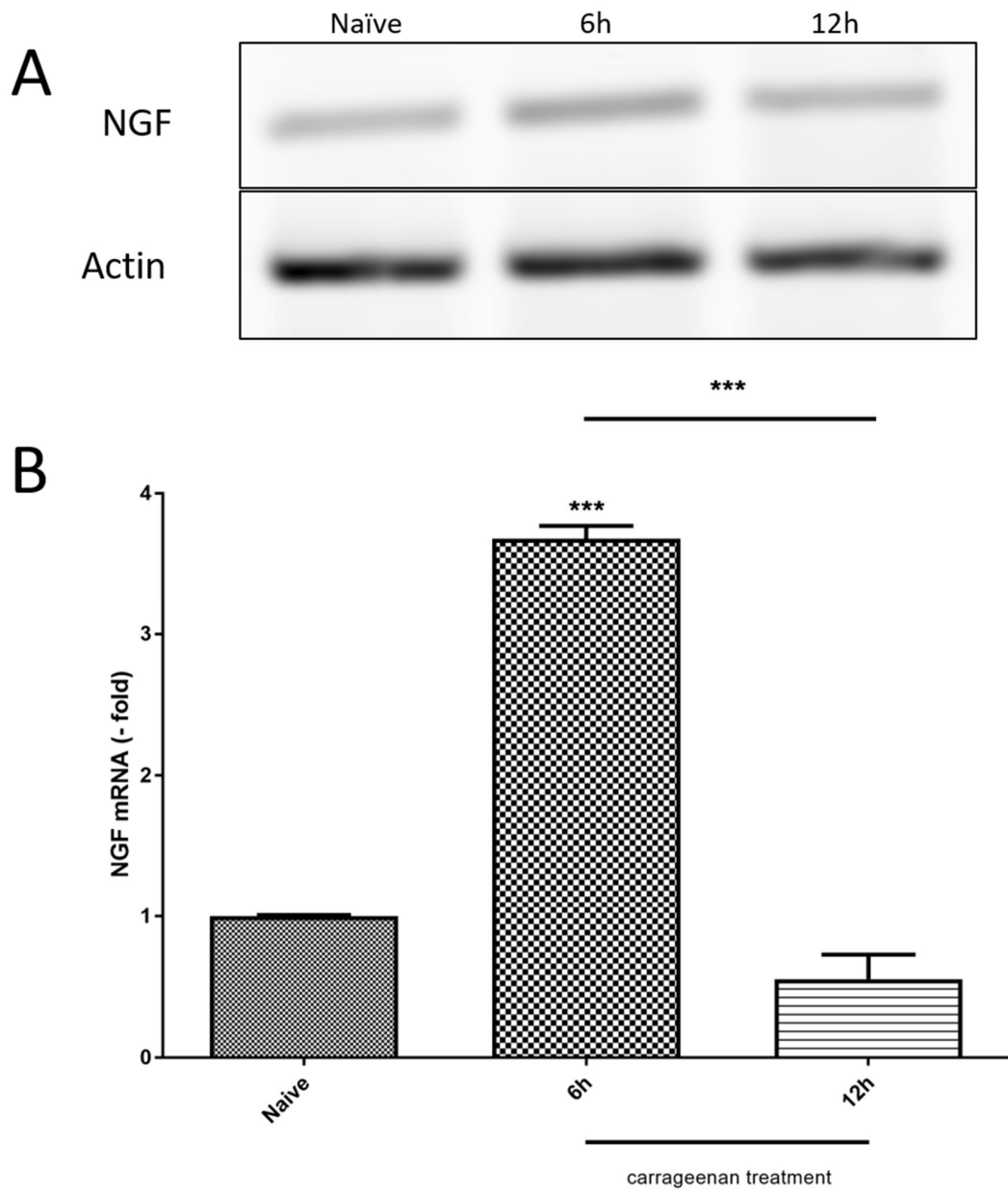
Figure 2.6: NGF mRNA during carrageenan-induced inflammation. NGF mRNA expression in thermolysin-separated epidermis during C-II was evaluated by qualitative PCR (**A**) and quantitative real-time PCR (**B**). Qualitative mRNA blots (A) for NGF and actin demonstrated that there was good quality mRNA that could be evaluated during inflammation. NGF mRNA expression in epidermis during C-II was evaluated by quantitative real time PCR using actin as a housekeeping gene (B). NGF mRNA was significantly elevated (>3-fold) after 6 h of C-II compared to naïve untreated rats, but levels returned to baseline at 12 h (B). Results are expressed as the SEM with three rats per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; student's t-test, unpaired, two-tail was performed at each timepoint).

Figure 2.7.
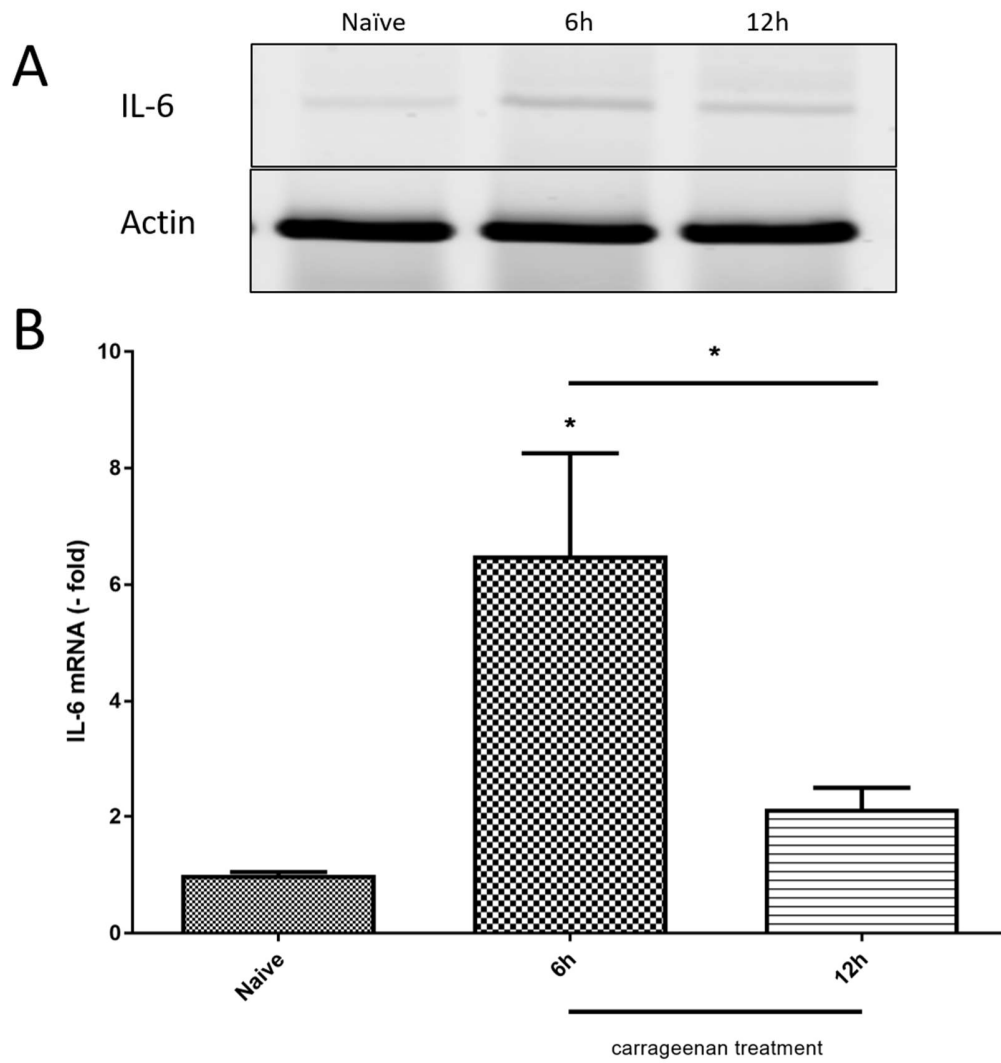
**Figure 2.7**: **IL-6 mRNA during carrageenan-induced inflammation**. IL-6 mRNA expression in thermolysin-separated epidermis during C-II was evaluated by qualitative PCR (**A**) and quantitative real-time PCR (**B**). Qualitative mRNA blots (A) for IL-6 and actin showed there was good quality mRNA for evaluation during inflammation. IL-6 mRNA expression in epidermis during C-II was evaluated by quantitative real-time PCR using actin as a housekeeping gene (B). IL-6 mRNA was significantly elevated (>6-fold) after 6 h of C-II compared to naïve untreated rats (B). At 12 h, IL-6 mRNA levels were reduced significantly from 6 h levels but remained elevated (2-fold) compared to naïve rats (B). Results are expressed as the mean S.E.M. with two rats per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, student's t-test, unpaired, two-tail was performed at each timepoint).

CHAPTER III

PHASIC CHANGES IN NERVE GROWTH FACTOR EXPRESSION DURING

ADJUVANT-INDUCED CUTANEOUS INFLAMMATION

Abstract

Chronic inflammatory diseases are considered as the most significant cause of death across the world. Current treatments for inflammatory diseases are limited, as we have little understanding of the biological factors involved in early-stage disease progression. Nerve growth factor (NGF) is a neurotrophic factor directly associated with different inflammatory and autoimmune diseases like osteoarthritis, multiple sclerosis, and rheumatoid arthritis. It has been shown that NGF levels are significantly upregulated at the site of inflammation and play a crucial role in the development of a robust inflammatory response. However, little is known about NGF's time course expression during the initial progressive phase of inflammation. The goal of this study is to determine the temporal expression pattern of NGF in rat skin during Adjuvant-Induced Arthritis (AIA). Male and female Sprague Dawley rats were randomly divided into control and Complete Freund's Adjuvant (CFA) treated groups. Levels of NGF were evaluated following unilateral AIA (150μl of CFA) at different time-points (6, 12, 24, 48, 96, and 192 hours). Peripheral inflammation due to AIA significantly upregulated the expression of NGF mRNA and protein after 6 and 96 hours showing a biphasic response. These results suggest that NGF signaling is crucial for the initiation of inflammation and contributes to the maintenance of inflammation.

Introduction

Nerve growth factor (NGF) is a protein, which regulates the maturation of developing sensory neurons in the peripheral nervous system (PNS) and acts as neurotrophin for a subset of nociceptive sensory neurons (Denk, Bennett, & McMahon, 2017). During inflammation induced by unilateral injection of complete Freund's adjuvant (CFA), the level of NGF is elevated in the skin (Woolf, Safieh-Garabedian, Ma, Crilly, & Winter, 1994). NGF is also elevated in different animal models of inflammation, including carrageenan and formalin (Mizumura & Murase, 2015). However, the expression pattern of NGF during the progressive phase of peripheral inflammation is not yet well defined.

Pain and inflammation are considered debilitating, but at the same time, they are also the protective responses necessary for survival. Pain is the mechanism that provides information about the presence or threat of an injury (Verma, Sheikh, & Ahmed, 2015). In addition to the afferent function such as conveying information to the central nervous system, the dorsal root ganglion primary sensory neurons specifically, C and some Aδ fibers, regulate the vascular and tissue function at their peripheral targets. For example, during neurogenic inflammation, tissue damage causes depolarization of the primary afferents, which releases neuropeptides such as SP, CGRP, and neurotransmitters such as glutamate, leading to increased vascular permeability and plasma extravasation (Holzer & Maggi, 1998). Due to this increased vascular permeability, the inflammation site recruits inflammatory cells such as T lymphocytes, mast cells, macrophages, and neutrophils (Chiu, von Hehn, & Woolf, 2012). These cells change the chemical milieu at the site of injury/inflammation by releasing pro-inflammatory molecules such as prostaglandins, bradykinin, histamine, and serotonin. Cytokines such as interleukins (ILs) and tumor necrosis factor (TNF), and neurotrophins like NGF are released during inflammation (Watson, Allen, & Dawbarn, 2008).

Peripheral inflammation drives peripheral sensitization and modifications in dorsal root ganglion (DRG) neuronal cells by producing retrograde signals in nociceptive neurons. Retrograde NGF signals, activate or increase transcription of the pro-nociceptive molecules, such as neurotransmitters and neuropeptides, to augment both central and peripheral sensitization (Denk et al., 2017; Miller, Hoffman, Sutharshan, & Schechter, 2011). To meet the considerable challenge of conveying information from periphery to the cell body located far away, dedicated mechanisms of retrograde NGF signaling have evolved, which carry the signals generated from the axonal endings to the neuron's cell body (Harrington & Ginty, 2013). Following NGF engagement, TrkA forms a heterodimer and is autophosphorylated. This complex is internalized by clathrin-dependent endocytosis, which gives rise to signaling endosomes. It has been shown that the NGF signaling endosomes are multivesicular bodies (MVB's) that mediate long-range retrograde transport (Ye, Lehigh, & Ginty, 2018).

Although the levels of NGF are upregulated after a noxious stimulus, the information regarding the expression pattern of NGF during the initial progression of peripheral inflammation is unknown. In this study, we aim to determine the effect of AIA in rat skin on the time course alteration in the expression of NGF. For this purpose, we employed the unilateral AIA rat model and measured the metatarsal thickness of the rat hind paw as a factor determining the severity of inflammation. The levels of NGF protein and mRNA was determined by quantitative image analysis, western blotting, and quantitative polymerase chain reaction at different time points.

Materials and Methods

1. Animals

Male Sprague-Dawley rats bred on site (350 - 450 g, n= 42) were used in this study. Animals were maintained on a 12 h light: 12 h dark cycle and provided with continuous access to food and water. These studies were performed at Oklahoma State University-Center for Health

Sciences (OSU-CHS), and all procedures were approved by the OSU-CHS Institutional Animal Care and Use Committee (Protocol # 2020-01). All necessary efforts were taken to reduce the number of animals used in this study.

2. Induction of adjuvant-induced arthritis (AIA)

For inducing unilateral inflammation of the hind paw, Complete Freund's Adjuvant (CFA; Sigma; St. Louis, MO, USA) was used. Rats (n = 36) were anesthetized with isoflurane (initially 5%, then reduced to 2.5%), and 150 µl of a 1:1 emulsion of CFA in 1X phosphate buffer saline (PBS) was injected into the plantar surface of the right hind paw. Naïve rats (n = 6) were anesthetized with the same procedure but not injected since PBS injection produces a local inflammation in the skin (Crosby, Ihnat, & Miller, 2015). Inflammation was allowed to persist for 6, 12, 24, 48, 96, and 192 hours and skin was collected after euthanizing with CO_2 asphyxiation after each time-point.

3. Hind Paw Edema and Body Weight

Metatarsal thickness was used to assess the severity of the inflammation in animals. The ipsilateral hind paw thickness was measured with a dial caliper (Mitutoyo, Japan) to the nearest 0.05 mm after each time-point before collecting the tissue. Bodyweight of each animal was also measured using electronic balance to monitor any significant changes due to inflammation.

4. Thermolysin treatment

The skin sample from the plantar surface of the right hind paw was collected after each time point in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) on ice. The skin tissues were transferred in a 0.5 mg/ml thermolysin solution (Sigma-Aldrich, St. Louis, MO, USA) and kept at 4°C for 2 hrs. After the incubation, the stratum corneum along with the epidermis was separated from the dermis, then immersed in 5mM EDTA in DMEM for 30 min

to stop the activity of thermolysin. Following the proteolytic treatment, the epidermal sections were used for RNA and protein analysis (Gujar, 2020).

5. Immunohistochemistry (IHC)

After the thermolysin treatment of the skin samples (separation of the epidermis from the dermis), the epidermal section of the skin was immersed in 0.96% (w/v) picric acid and 0.2% (w/v) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.3 (E. M. Hoffman, Schechter, & Miller, 2010; E. M. Hoffman, Zhang, Anderson, Schechter, & Miller, 2011)(Hoffman et al., 2010; Hoffman et al., 2011) for three hours at 4°C. Tissues were transferred to 10% sucrose in PBS, pH 7.3 overnight at 4°C. The epidermal section of the skin were vertically embedded in a frozen block and cut in 14 μm sections on a Leica CM 1850 cryostat (Leica Biosystems; Wetzlar, Germany). Sections were mounted onto gelatin-coated SuperFrost slides with three sections per slide. Five slides from each time point were dried at 37°C for one hour. Dried sections were rinsed with PBS three times for 10 minutes each. After washing, frozen sections were incubated with primary antibodies (1:1000) (anti-Nerve Growth Factor, Santa Cruz, TX, USA) and (1:10,000) (anti-PGP9.5, Cedarlane Labs, Burlington, Canada) for four days at 4°C (Gujar, 2020). Following the incubation in primary antibodies, sections were rinsed three times in PBS and incubated with anti-mouse Alexa Flour 555 and anti-rabbit FITC 488 for 60 min at room temperature in a dark box. After secondary antibody incubation, sections were rinsed with PBS once and incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 15 min at room temperature on a shaker. Finally, after removal of the DAPI sections were rinsed with PBS three times and coverslipped with ProLong Gold Mounting Media (Invitrogen; Carlsbad, CA, USA).

6. Quantitative Image Analysis

All images were taken with a Leica DMI 4000B confocal microscope (Resolution: 2048 x 2048)

with a 40X objective. The final images were a sequential merge of 3-10 confocal images (Resolution: 2048 x 2048) that best represented the particular field of view. The micrographs were stored as an 8-bit grayscale tiff format with the pixel intensity value ranging from 0-255. Three fields of view were captured from each epidermal section. For each field of view, three filters were used to detect each fluorophore: FITC, TRITC, and DAPI. All the images were analyzed in ImageJ (National Institute of Health; Bethesda, MD, USA) by using a fixed box with area 7392 μm^2 to select the epidermal region of interest (ROI). Once all ROIs for a given image were selected and added to the ROI manager, the area (in μm^2) and mean gray values were measured and exported for subsequent statistical analysis.

7. RNA isolation and Quantitative Real-time PCR

Total RNA was isolated and purified from the epidermal tissue of naïve and treated rats at different time points after CFA injection using Trizol solution (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) synthesis was performed by M-MLV Reverse transcriptase (Promega, Madison, MI, USA). Quantitative real-time PCR (qRT-PCR) was performed using the ABI StepOne™ system (Applied Biosystems, Foster City, CA, USA). SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for the detection of NGF mRNA, and GAPDH RNA was used as an internal reference for NGF. Primer sequences used for NGF and GAPDH were as follows:

NGF-F: 5'-GTGGACCCCAAACTGTTTAAGAAACGG-3'

NGF-R: 5'-GTGAGTCCTGTTGAAGGAGATTGTACCATG-3'

GAPDH-F: 5'- GAACCACGAGAAATATGACAACTCCCTCAAG-3'

GAPDH-R: 5'- GCAGTGATGGCATGGACTGTGG-3'

The results of the PCR analysis were expressed as threshold cycle (CT), which were used to determine the target gene mRNA in relation to the reference gene mRNA. ΔCT indicated the difference between the number of cycles necessary to detect the PCR products for target and reference genes. ΔΔCT was the difference between the naïve animal group, and the AIA group and finally the data were expressed as $2^{-\Delta\Delta CT}$ which represents the relative amount of target mRNA present in the CFA treated sample to the naïve animal group.

8. Western blot analysis

After the thermolysin treatment, the epidermal tissue was homogenized with lysis buffer (25mM Tris HCl pH-7.4, 150 mM NaCl, 151 1mM EDTA, 5% glycerol and 1% Triton X-100) containing protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO, USA). Samples were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and total protein concentration was evaluated using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were normalized to 50 μg/ml of total protein, suspended in 10mM Tris Base, 1mM EDTA, 2.5% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue and boiled at 100 °C for 10 min. Samples and Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) were separated using 12% TGX™ FastCast™ Acrylamide Solutions (Bio-Rad Laboratories, Hercules, CA, USA). Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA) was used to transfer the proteins onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% Carnation milk in Tris-buffered saline Tween (TBST, 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) at room temperature. After rinsing in TBST, the membranes were incubated overnight at 4 °C with NGF antibody at 1:1000 in TBST/5% milk. Membranes were washed in TBST and incubated in secondary alkaline phosphatase labeled anti-mouse and anti-rabbit IgG (Promega; Madison, WI, USA) at 1:1000 dilution for 120 min. The ECF substrate was used on a

Typhoon 9410 Variable Mode Imager for western blot images. The images were analyzed by ImageJ (National Institute of Health; Bethesda, MD, USA).

9. Statistical Analysis

Student t-test was performed on all data sets using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego California USA). P values less than 0.05 were considered significant for all tests. The data presented in the graph are group means $\pm$ SEM.

Results

Changes in Hind Paw Edema and Body Weight

The metatarsal thickness of the ipsilateral hind paws was significantly increased ($p < 0.001$) in animals treated with CFA as compared to the control untreated animals. The increased thickness was observed at all the seven-time points with the highest peak at 48 hours (Figure 3.1A). Bodyweight of animals after CFA treatment was not significantly different from the untreated animals at any of the seven-time points (Figure 3.1B).

Change in NGF-immunoreactivity (-ir) During Inflammation

For determining the levels of NGF protein, double labeling of NGF and PGP9.5 was performed on epidermal sections of rat hind paw. DAPI was used as a nuclear stain. The images (Figure 3.2A) indicate that the immunoreactivity of NGF after 6h and 96h of inflammation was comparatively higher with that to the control animals. The results from quantitative image analysis indicate that the NGF immunoreactivity was significantly higher after 6h of inflammation ($p < 0.001$) compared to the control sample. The immunoreactivity was reduced after 24 hours but increased significantly after 96h of treatment ($p < 0.001$), indicating a biphasic response. After 192 hours, the immunoreactivity was found to be lower as compared to the 96h sample ($p < 0.001$).

PGP 9.5 immunoreactivity in intraepidermal nerve fibers was qualitatively similar at all timepoints similar to the results in our other studies (Anderson, 2010; Gujar, 2020) (Figure 3.2).

NGF expression shows biphasic response during peripheral inflammation

To evaluate the effect of acute and chronic peripheral inflammation on NGF expression in rat epidermis, we determined the expression of NGF mRNA and protein by quantitative reverse transcription PCR and western blot analysis, respectively. Levels of NGF mRNA were significantly upregulated after 6 hours of AIA, with a decrease after 24 hours and a second spike after 48 hours. The NGF protein level showed a similar biphasic response with two peaks after 6 hours and 96 hours of peripheral inflammation. Data shown as mean ± SEM, compared with respective controls by Student's t-test (n = 6 per group)

Discussion

The unilateral AIA resulted in a significant increase in the metatarsal thickness of the ipsilateral hind paws indicating a robust inflammatory process, as reported previously (Ernest Matthew Hoffman, 2009; Zhang, 2013). The injection of CFA induces the release of several inflammatory mediators leading to peripheral and central sensitization (Ji, Xu, & Gao, 2014) confirming that the AIA rat model is appropriate for studying the process of chronic inflammatory pain (Burma, Leduc-Pessah, Fan, & Trang, 2017). The present study corroborates the contribution of NGF in the development and maintenance of peripheral inflammation. We found that the levels of NGF protein were significantly upregulated after six hours of noxious stimulus and decreased after twenty-four hours. The levels again increased after ninety-six hours, showing a biphasic response.

As inflammation is converted from acute to chronic, it maintains its distinct characteristics, such as increased vascular permeability, vasodilation, and macrophage migration (Pahwa & Jialal, 2018). After initiation of inflammation in the periphery, both central and peripheral nervous

systems exhibit significant changes that lead to altered sensory inputs and processing, for instance, enhanced excitability of primary afferent neurons (Lamb, Zhong, Gebhart, & Bielefeldt, 2006). Skin, especially epidermis, is densely innervated by specialized nerve ending of sensory afferent neurons that express and release neuropeptides. SP and CGRP specific receptors are located on the epidermal keratinocytes, and during inflammation, SP can stimulate epidermal keratinocytes to produce and release neurotrophins such as Nerve Growth Factor (NGF) and inflammatory mediators such as interleukin-1β (IL-1β)(Gibbins, Wattchow, & Coventry, 1987; Kemeny, von Restorff, Michel, & Ruzicka, 1994; Shi, Wang, Clark, & Kingery, 2013; Viac et al., 1996). Collectively, these results support the release of the neurotransmitter glutamate and NGF signaling are directly associated with the development of peripheral sensitization of nociceptor terminals during inflammation.

Additionally, we confirmed that the mRNA levels of NGF are mirrored with that of the protein levels, showing two peaks. Previous work has shown the rapid increase in NGF protein and mRNA levels in different inflammatory models such as carrageenan, formalin, complete Freund's adjuvant and turpentine oil (McMahon, Bennett, Priestley, & Shelton, 1995; Mizumura & Murase, 2015; Oddiah, Anand, McMahon, & Rattray, 1998; Woolf et al., 1994). The primary source of NGF is attributed to certain inflammatory cells like mast cells (Leon et al., 1994), macrophages (Heumann et al., 1987), lymphocytes, and eosinophils (Mantyh, Koltzenburg, Mendell, Tive, & Shelton, 2011) whereas the source in the previous study is epidermal keratinocytes. This modulation of NGF expression during peripheral damage is likely mediated by either neuropeptide released from cutaneous nerves or by cytokines typically involved in tissue damage like IL-1β, IL-6 and TNF-α (Dallos et al., 2006; Lindholm, Heumann, Meyer, & Thoenen, 1987; Marz, Heese, Dimitriades-Schmutz, Rose-John, & Otten, 1999; Safieh-Garabedian, Poole, Allchorne, Winter, & Woolf, 1995; Woolf, Allchorne, Safieh-Garabedian, & Poole, 1997). Intraepidermal nerve fibers

labeled with PGP 9.5 were consistent across the temporal course of AIA indicating their potential for promoting and responding to inflammation and their presumptive uptake of the elevated NGF.

The immunohistochemical image analysis and Western blotting showed the two peaks of NGF protein, but there is a disparity in the percent change levels (Immunohistochemical image analysis; 6h – 20%, 96h – 15% and Western blotting; 6h – 40%, 96h – 30%). These variations may exist due to the change in the protein conformation and target accessibility in immunohistochemistry compared to Western blotting (Uhlen et al., 2016). Although the NGF antibody (E-12) produced by Santa Cruz Biotechnology Inc. has specificity for both the mature (13kDa) and pro-NGF (27 and 35kDa) forms (Edwards, Selby, Garcia, & Rutter, 1988), our Western blotting protocol was only able to detect the pro-form, and we failed to demonstrate the lower molecular weight NGF forms. This might be due to the specific tissue processing, e.g., thermolysin separation, and Western blotting conditions we employed for this study. The pro-NGF is formed by the alternate splicing of the NGF gene, which is cleaved into a mature form (Seidah et al., 1996). The Western blotting results are in accordance with the previous studies on the rat retina during optic nerve crush (Mesentier-Louro et al., 2017). We also observed some variations in the percent change of mRNA levels as compared to the protein levels. These changes may be the result of the change in expression levels of the housekeeping gene, which could be due to variations in biological conditions or differences in specific experimental conditions (Li & Shen, 2013; Turabelidze, Guo, & DiPietro, 2010). Taking this into consideration, we tested several housekeeping genes like β-actin, GAPDH, 18s rRNA gene and found that the mRNA expression of GAPDH has the minimum variation (data not shown) in the skin of control and AIA rats.

Conclusion

The present result indicates that the biphasic increase in the expression of NGF occurs after 6h and 96h, hence playing a pivotal role in the phasic progression of the inflammatory process.

This temporal change in NGF expression during the peripheral inflammation may be useful to determine the timing of therapeutic interventions like anti-NGF antibodies for the treatment of diseases like osteoarthritis and rheumatoid arthritis. Chronic inflammatory conditions in humans can undergo amplified active and preclinical or quiescent stages (Bezuidenhout & Pretorius, 2020; Conti et al., 2012; Helmchen et al., 2005; Wenink et al., 2009) and effective anti-NGF therapy may require specific temporal dosings. Further studies will be required to assess the NGF levels during the chronic time-points of inflammation to fully understand the role of NGF signaling during peripheral sensitization and determining the novel therapeutic targets.

Figure 3.1
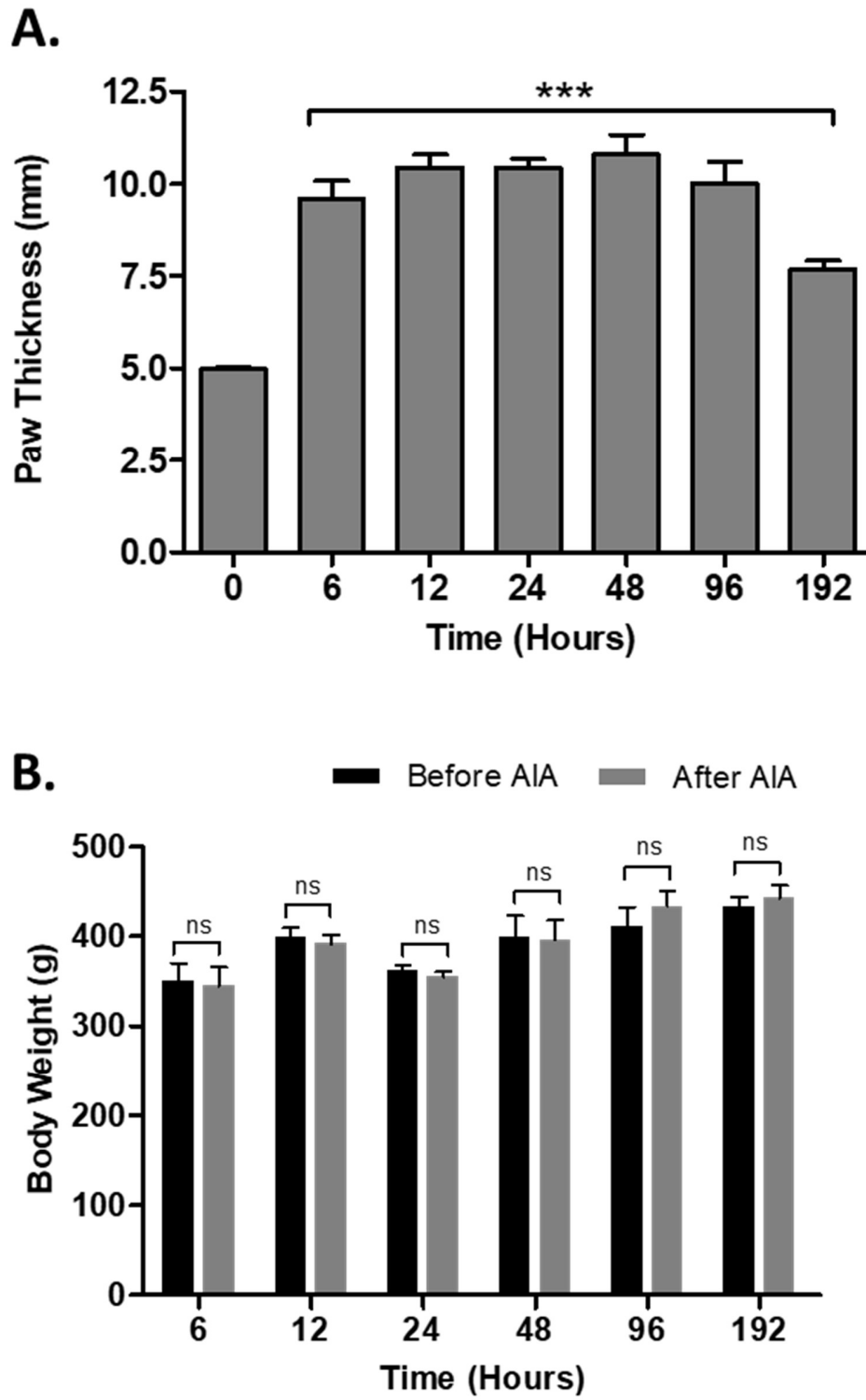
Figure 3.1. Effect of peripheral inflammation on hind paw edema and body weight. (A) Analysis of hind paw edema by measurement of the metatarsal thickness before and after CFA treatment at different time points (n = 8 per group). AIA caused a significant increase in the metatarsal thickness of animals treated with CFA at all time points. (B) Bodyweight of animals treated with CFA was not significantly different at any time point as compared to the untreated animals (n = 7 per group). Data are shown as mean $\pm$ SEM. ***$p < 0.001$, compared with respective controls by Student's t-test.

Figure 3.2

58
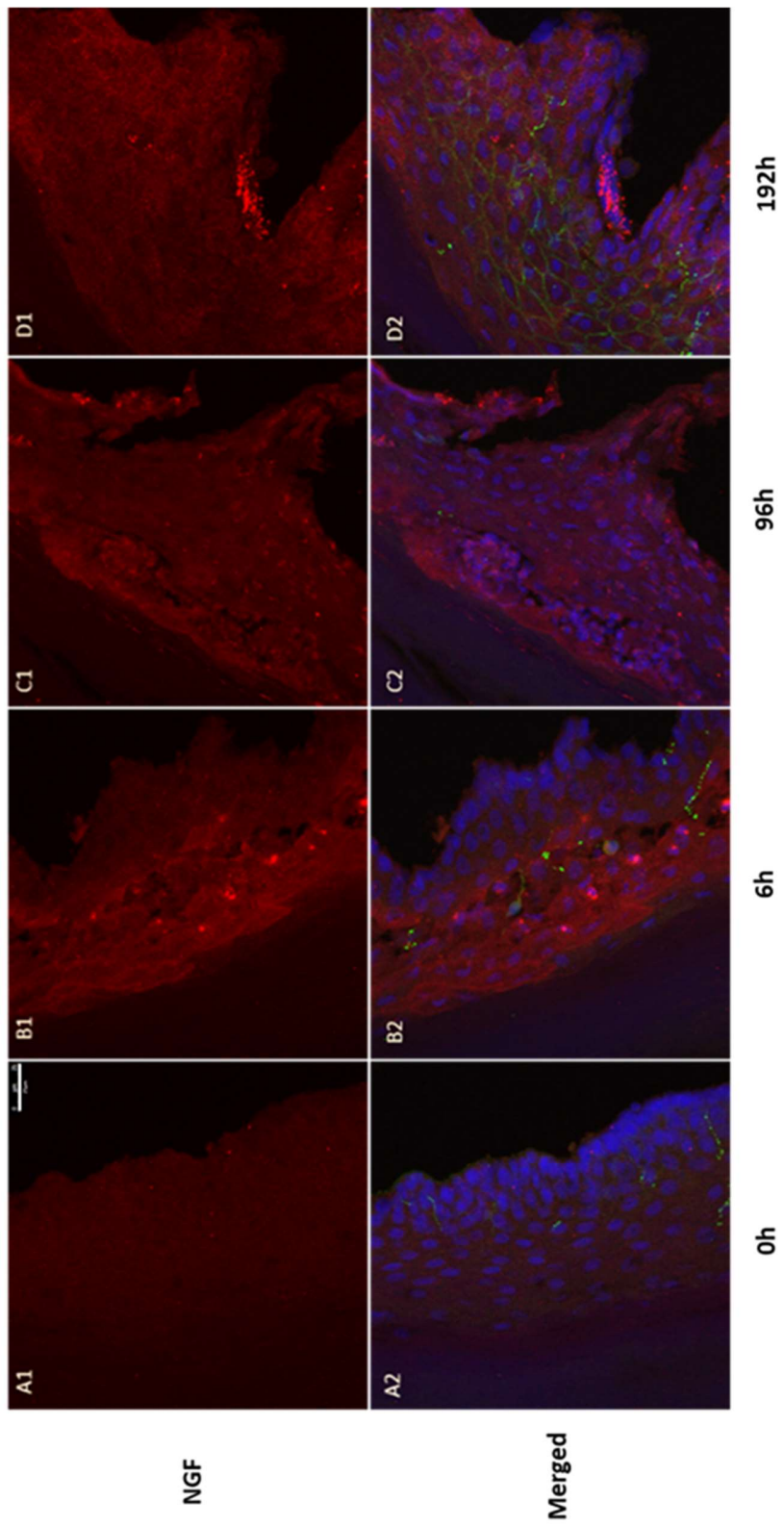
Figure 3.2: Effect of peripheral inflammation on the immunoreactivity of nerve growth factor (NGF) in rat epidermis. (A1-D1) NGF fluorescence (red); (A2-D2) Merged images of NGF (red), PGP 9.5 (green), and DAPI (blue) in the rat epidermis. Representative images of rat epidermis at 0h (A1-A2), 6h (B1-B2), 96h (C1-C2), and 192h (D1-D2) after adjuvant-induced arthritis (AIA). Scale bar = 25μm (n = 6 per group).

Figure 3.3
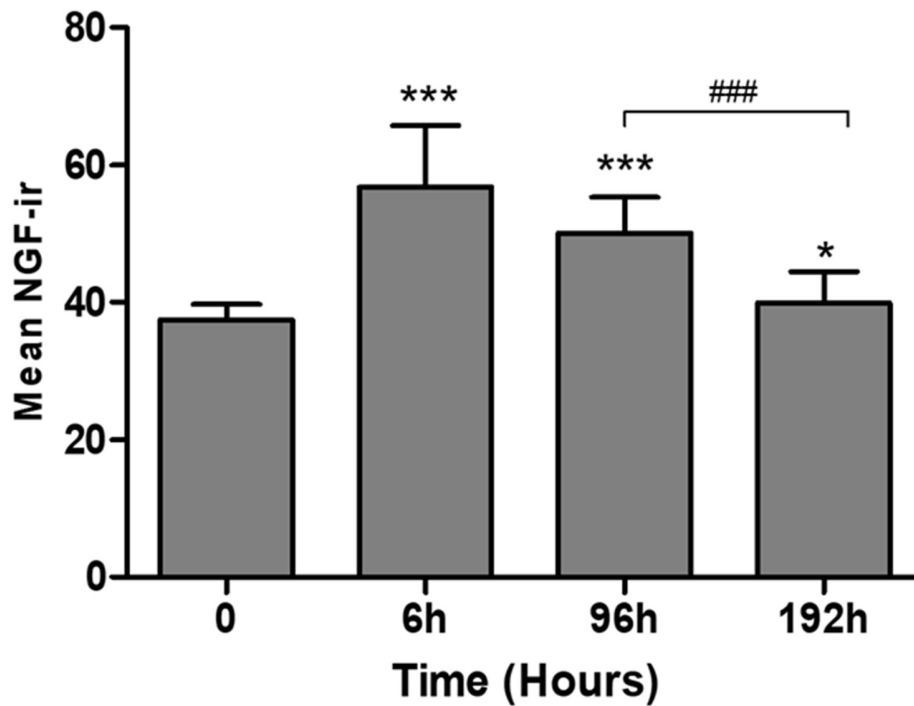
Figure 3.3. Effect of peripheral inflammation on the immunoreactivity of nerve growth factor (NGF) in rat epidermis. Quantitative image analysis shows the NGF-ir to be significantly higher after 6h, 96h, and 192h as compared to the control animal (*$p < 0.05$, ***$p < 0.001$). However, the NGF-ir after 192h is reduced significantly as compared to 96h of inflammation (###$p < 0.001$). Data are shown as mean ± SEM, compared with respective controls by Student's t-test (n = 6 per group)

Figure 3.4.
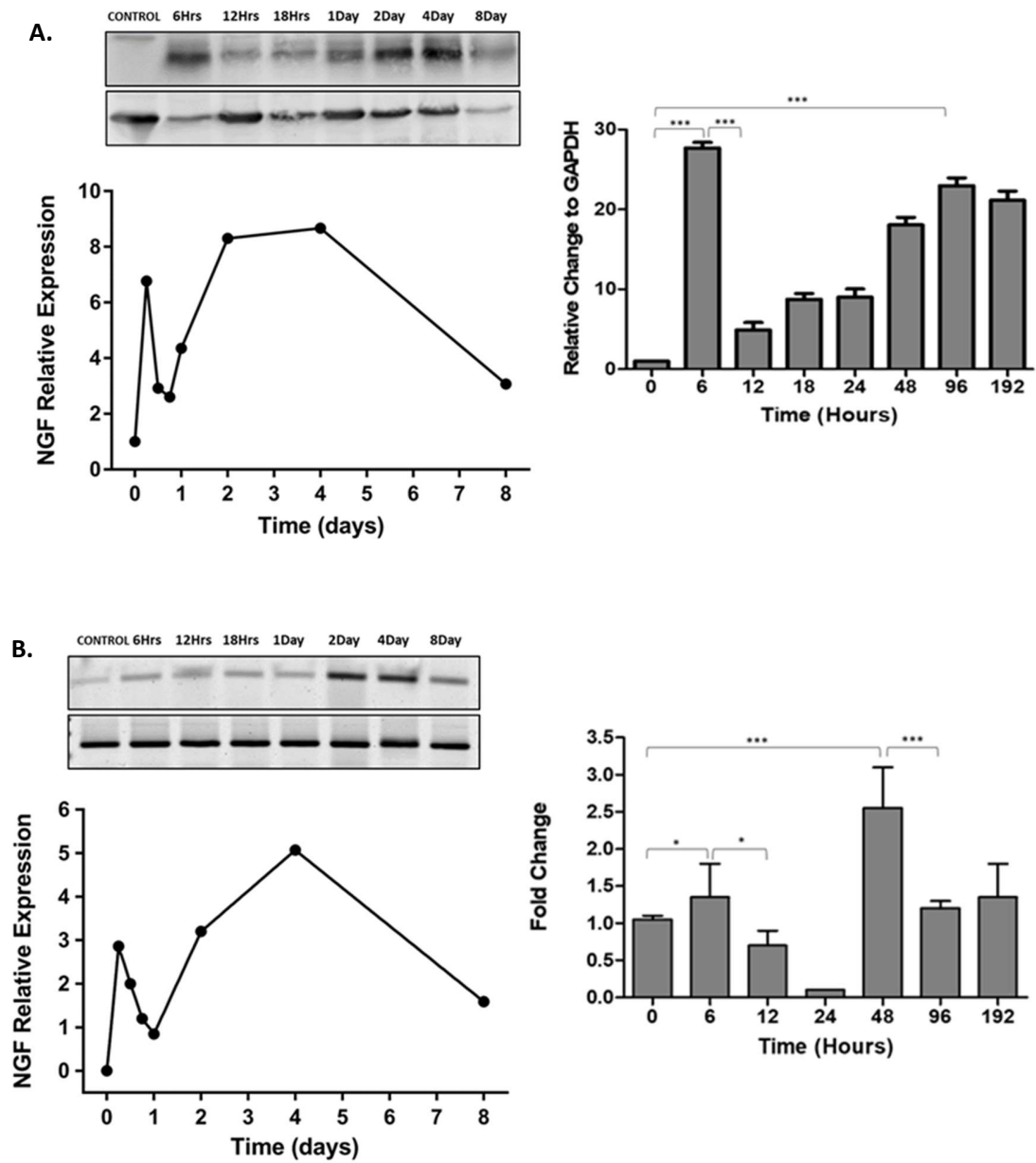
Figure 3.4. NGF expression shows biphasic response during peripheral inflammation in rat epidermis. (A) Western blot analysis of NGF protein expression under control and AIA conditions. The data was normalized against GAPDH housekeeping protein and represented as relative change. (B) Comparison of NGF mRNA expression levels in control and CFA treated rats by quantitative real-time PCR at various time points. Data are represented as fold change against GAPDH. Bars represent means ± SEM, compared with respective controls by Student's t-test (n = 6 per group)

CHAPTER IV

RETROGRADE NERVE GROWTH FACTOR TROPHIC SIGNALING MODULATES THE

EXPRESSION OF GLUTAMINASE IN DORSAL ROOT GANGLION NEURONS DURING

PERIPHERAL INFLAMMATION

Abstract

Glutamate functions as the major excitatory neurotransmitter for primary sensory neurons and has a crucial role in sensitizing peripheral nociceptor terminals producing sensitization. Glutaminase (GLS) is the synthetic enzyme that converts glutamine to glutamate. GLS-immunoreactivity (-ir) and enzyme activity are elevated in dorsal root ganglion (DRG) neuronal cell bodies during chronic peripheral inflammation, but the mechanism for this GLS elevation is yet to be fully characterized. It has been well established that, after nerve growth factor (NGF) binds to its high-affinity receptor tropomyosin receptor kinase A (TrkA), a retrograde signaling endosome is formed. This endosome contains the late endosomal marker Rab7GTPase and is retrogradely transported via axons to the cell soma located in DRG. This complex is responsible for regulating the transcription of several critical nociceptive genes. Here, we show that this retrograde NGF signaling mediates the expression of GLS in DRG neurons during the process of peripheral inflammation. We disrupted the normal NGF/TrkA signaling in adjuvant-induced arthritic rats by pharmacological inhibition of TrkA or blockade of Rab7GTPase, which significantly attenuated the expression of GLS in DRG cell bodies. These results indicate that NGF/TrkA signaling is crucial for the production of glutamate and has a vital role in the

development of neurogenic inflammation. Our pain behavioral data suggest that Rab7GTPase can be a potential therapeutic target for treating peripheral inflammatory pain.

Introduction

The involvement of the Nerve growth factor (NGF) is very well documented in the development of the peripheral nervous system and in determining the phenotype of sensory and sympathetic neurons. In addition, NGF levels increase during various inflammatory conditions and are involved in inducing pain and hyperalgesia (Levi-Montalcini & Hamburger, 1951; Woolf, 1996; Woolf, Safieh-Garabedian, Ma, Crilly, & Winter, 1994). NGF can bind to its high-affinity receptor, tropomyosin receptor kinase A (TrkA) and its low affinity, p75 neurotrophin receptor (p75NTR). The NGF secreted by resident and inflammatory cells and keratinocytes in injured tissue acts via TrkA receptors present on primary sensory nerve endings and participates in peripheral sensitization. Hence, NGF/TrkA signaling is considered as a primary target for the development of pain therapeutics (Hirose, Kuroda, & Murata, 2016) leading to the development of anti-NGF antibodies and TrkA antagonists (McMahon, Bennett, Priestley, & Shelton, 1995; Shelton, McMahon, Winslow, & Qao, 1995). However, for these analgesics to be effective with the least possible side effects, the detailed mechanisms involved in peripheral sensitization need to be evaluated further.

For blocking NGF signaling, several drugs have been developed such as NGF protein inhibitors (Anti-NGF antibodies), NGF/TrkA binding inhibitors and TrkA inhibitors (Ghilardi et al., 2010; Lane et al., 2010; Ueda et al., 2010; Urschel, Brown, & Hulsebosch, 1991). TrkA is present on the cell membranes of various cell types, including primary sensory neurons (intraepidermal nerve fibers), immune and skin cells. After the binding of NGF to TrkA, the complex undergoes dimerization and autophosphorylation on the axonal terminals of primary sensory neurons, followed by endocytosis either in a clathrin-dependent or an independent manner.

The signaling endosome is retrogradely transported to the cell bodies in dorsal root ganglion (DRG). Before the long-range transport is initiated, the TrkA positive endosomes accumulate the small GTPase from the Rab family, i.e., Rab7 GTPase in the peripheral terminal (Saxena, Bucci, Weis, & Kruttgen, 2005). Rab GTPase's are the molecules that determine whether the endosome should travel retrogradely to the cell body for signal transduction or degradation in lysosomes. Once the TrkA signaling endosome associates with Rab7, it moves retrogradely and initiates the downstream signaling for the expression of different inflammatory mediators and neurotransmitters (Delcroix et al., 2003; Hirose et al., 2016). Therefore, in this study, we used TrkA and Rab7 pharmacological inhibitors to block the NGF signaling during adjuvant-induced arthritis (AIA) to determine the effect of NGF on glutaminase (GLS) levels in DRG neurons and pain behaviour in AIA animals.

All DRG neurons are glutamatergic as they utilize glutamate to convey information from their peripheral and central terminals. Previous studies linked the peripheral release of glutamate with the process of nociception and sensitization of primary afferent DRG neurons (E. M. Hoffman & Miller, 2010). Levels of glutamate are elevated in the synovial fluid and epidermis of patients with arthritis and gold-induced skin inflammation, respectively (McNearney, Speegle, Lawand, Lisse, & Westlund, 2000; Nordlind, Johansson, Liden, & Hokfelt, 1993). The primary afferents convert glutamine to glutamate via the enzyme GLS. This GLS is activated regionally by calcium and phosphate and hence also known as Phosphate-activated Glutaminase (PAG) (Wallace & Dawson, 1993). We previously reported that the level of GLS is elevated significantly in DRG cell bodies during chronic inflammation, and peripheral inhibition of GLS reduces sensitization and pain associated with inflammation (Ernest Matthew Hoffman, 2009; E. M. Hoffman & Miller, 2010; K.E. Miller, 2010; K. E. Miller et al., 2012).

After the initiation of the inflammatory process, the DRG sensory neurons alter the expression of proteins such as SP and CGRP, mostly attributed to NGF and its high-affinity

receptor, TrkA. Although, under normal conditions, the basal expression of GLS is not regulated by NGF (E. M. Hoffman, Zhang, Anderson, Schechter, & Miller, 2011), while during the process of inflammation, the role of NGF/TrkA in the regulation of GLS expression needs further evaluation. To identify NGF signaling associated molecules, we evaluated the immunoreactivity of NGF, pTrkA and Rab7 in the ligated sciatic nerve after AIA. The goal of this study was to explore the involvement of NGF signaling in the process of neurogenic inflammation by evaluating the levels of GLS in DRG neurons by inhibiting TrkA and Rab7 during peripheral inflammation. The selective TrkA inhibition in the periphery will block the formation of NGF/TrkA complex, while the inhibition of Rab7 will disrupt the NGF/TrkA long-range retrograde signaling, hence affecting the alteration in DRG cell bodies. We used GW441756 (TrkA inhibitor) and CD1067700 (Rab7 inhibitor) to block the NGF signaling in the periphery and evaluated the expression of GLS in DRG cell bodies. We observed that inhibition of both TrkA and Rab7 individually attenuated the levels of GLS, thereby confirming the involvement of NGF retrograde signaling in the glutamate metabolism. We also established the potential of Rab7 as a therapeutic target for treating pain as Rab7 blockade showed reduced pain behavior in arthritic animals.

Material and Method

1. Animals

Sprague-Dawley rats (350 – 450g, N = 99) bred and housed in the OSU-CHS animal facility were used in this study. Animals were maintained on a 12 h light: 12 h dark cycle and provided with continuous access to food and water. These studies were performed at Oklahoma State University-Center for Health Sciences (OSU-CHS), and all procedures are approved by the OSU-CHS Institutional Animal Care and Use Committee. All the procedures were performed according to theNational Institute of Health and International Association for the Study of Pain guidelines (Zimmermann, 1983).

2. Adjuvant-induced Arthritis (AIA) and Sciatic nerve ligation

For inducing unilateral inflammation of the hind paw, Complete Freund's Adjuvant (CFA; Sigma; St. Louis, MO, USA) was used. Rats (n = 12) were anesthetized with isoflurane (initially 5%, then reduced to 2.5%) and 150 µl of a 1:1 emulsion containing CFA and sterile phosphate-buffered saline (PBS) was injected into the plantar surface of the right hind paw. Rats were allowed to recover on a warm towel and then placed back into their cages. After 6hrs of CFA treatment, animals were anesthetized again, and the right sciatic nerve was exposed and ligated with non-absorbable silk before the trifurcation, 5mm below the sciatic notch (Puehler et al., 2004). Sham animals were used as a control group in which the same surgery was performed, but no CFA injection was given.

3. Adjuvant-induced Arthritis (AIA) and Pharmacological Interventions

Rats were anesthetized prior to all injections, and AIA was induced by injecting 150 µl of CFA into the plantar surface of the right hind paw. TrkA inhibitor GW441756 (Tocris Biosciences, Bristol, UK) and Rab7 receptor antagonist CD1067700 (Cayman, MI, USA) were dissolved in 5% DMSO in phosphate-buffered saline (PBS) and injected as 20nmol/20µl and 100nmol/20µl respectively in the plantar surface of the right hind-paw. Besides the injection, the inhibitors were also applied topically onto skin with the help of filter paper soaked in DMSO and inhibitor. The inhibitors were injected and topically applied once before 30 mins of CFA treatment and two times: 12 hours and 36 hours after CFA treatment. Rats with no treatment were used as controls since PBS produces a local acute inflammation (Crosby, Ihnat, & Miller, 2015).

4. Immunofluorescence

The animals (n = 9) were perfusion fixed with a fixative containing 0.96% (w/v) picric acid and 0.2% (w/v) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.3 (E. M. Hoffman, Schechter, & Miller, 2010; E. M. Hoffman et al., 2011). The sciatic nerve and the L4 and L5 DRG's

were collected from the perfusion fixed rats and immersed in the same fixative for four hours at room temperature. The tissues were transferred to a 10% sucrose solution in PBS (pH 7.3) and incubated at 4°C overnight. The next day, tissues were embedded and frozen in Lipshaw embedding matrix and cut in 14 μm sections on a Leica CM 1850 cryostat (Leica Biosystems; Wetzlar, Germany). The slices were collected on gelatin-coated slides and dried on a slide warmer at 37°C for 1 hour. After rinsing the slides with 1X PBS, the slides were incubated with primary antibodies mentioned in Table 1 for four days at 4°C (E. M. Hoffman et al., 2010). The slides were washed three times with 1X PBS and incubated with respective fluorescent-labeled secondary antibodies (Table 1) and incubated for 60 mins at room temperature in the dark. Finally, after incubation in secondary antibodies, the slides were rinsed thrice with 1X PBS and coverslipped with ProLong Gold Mounting Media (Invitrogen; Carlsbad, CA, USA) for image analysis.

Tissue	Primary Antibodies	Dilutions	Secondary Antibodies	Dilutions
Sciatic Nerve	NGF Anti-mouse (E-12, Santa Cruz, TX, USA)	1:1000	Donkey anti-mouse Alexa Flour 555 (Invitrogen; Carlsbad, CA, USA)	1:1000
	pTrkA Anti-rabbit (4168S, Cell signaling, MS, USA)	1:2000	Donkey anti-rabbit FITC 488 (Invitrogen; Carlsbad, CA, USA)	1:1000
	Rab7 Anti-rabbit (55469-1-AP, Proteintech, Rosemont, IL, USA)	1:2000	Donkey anti-rabbit FITC 488 (Invitrogen; Carlsbad, CA, USA)	1:1000
L4 and L5 DRG	GLS Anti-rabbit (Norman Curthoys, Colorado State University, Ft. Collins, CO, USA)	1:1000	Donkey anti-rabbit FITC 488 (Invitrogen; Carlsbad, CA, USA)	1:1000

Table.4.1: Details of Primary and secondary antibodies used in Immunofluorescence studies.

5. Western Blot analysis

The sciatic nerve and L4, L5 DRG's were homogenized in lysis buffer (25mM Tris HCl pH-7.4, 150 mM NaCl, 151 1mM EDTA, 5% glycerol and 1% Triton X-100) added with protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO, USA) for 5 mins with a handheld homogenizer and incubated on ice for 10 mins. The supernatant was collected in a fresh tube after centrifugation

of homogenized sample at 14,000 rpm for 15 mins at 4°C. Total protein was estimated by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples (20-50 µg/ml of total protein) were dissolved in loading buffer (10mM Tris Base, 1mM EDTA, 2.5% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and boiled at 100 °C for 10 min. The sciatic nerve protein samples were separated on 12% Gel, and DRG samples were separated on 7.5% Gel (TGX™ FastCast™ Acrylamide Solutions, Bio-Rad Laboratories, Hercules, CA, USA) along with Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) by SDS -PAGE and blotted on to nitrocellulose membrane in Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA).

TrkA Inhibition study: The membranes were incubated with 5% Carnation milk in Tris-buffered saline Tween (TBST, 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) at room temperature for 1 hour and incubated overnight at 4°C in primary antibody against GLS (Table 2). After washing with 1XTBST thrice (20 mins each), the membranes were incubated in alkaline phosphatase labeled anti-rabbit IgG (Promega; Madison, WI, USA) secondary antibodies at 1:1000 dilution for 120 min. After washing with 1XTBST three times for 20 mins each, the ECF substrate was used on a Typhoon 9410 Variable Mode Imager for taking the western blot images. The images were analysed by ImageJ (National Institute of Health; Bethesda, MD, USA).

Sciatic nerve ligation and Rab7 inhibition study: After the electrophoretic transfer, the membranes were rinsed with Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, 0.05%, pH 7.5) and incubated in Revert Total Protein Stain (Li-Cor) for 5 mins and rinsed with Revert wash solution and blocked with 5% Carnation milk in TBS at room temperature for 1 hour. All membranes were incubated overnight at 4°C with primary antibodies against NGF, pTrkA, Rab7, and GLS (Table 2) diluted in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). After washing with TBST four times (5 mins each), the membranes were incubated for one hour at room temperature in IRDye 800, and IRDye 680 labeled Donkey anti-rabbit and anti-mouse

secondary antibodies (1:20,000). The membranes were rinsed with TBS (no Tween) followed by image acquisition on the Odyssey CLx Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE, USA). The images were normalized against the total protein or total TrkA, and analysis was performed on Image Studio Lite software (Li-Cor Biosciences, Lincoln, NE, USA).

Study/Tissue	Primary Antibodies	Dilutions	Secondary Antibodies	Dilutions
Sciatic Nerve Ligation/Sciatic nerve	NGF Anti-Mouse (E-12, Santa Cruz, TX, USA)	1:1000	IRDye 800CW Donkey anti-mouse (Li-Cor, Lincoln, NE, USA)	1:20,000
	pTrkA Anti-rabbit (4168S, Cell signalling, MS, USA)	1:2000	IRDye 800CW Donkey anti-rabbit (Li-Cor, Lincoln, NE, USA)	1:20,000
	Rab7 Anti-rabbit (55469-1-AP, Proteintech, Rosemont, IL, USA)	1:2000	IRDye 800CW Donkey anti-rabbit (Li-Cor, Lincoln, NE, USA)	1:20,000
	Total TrkA Anti-rabbit (2505S, Cell signalling, MS, USA)	1:2000	IRDye 800CW Donkey anti-rabbit (Li-Cor, Lincoln, NE, USA)	1:20,000
TrkA Inhibition/L4, L5 DRG	GLS Anti-rabbit (Norman Curthoys, Colorado State University, Ft. Collins, CO, USA)	1:1000	AP labeled Anti-rabbit IgG (Promega; Madison, WI, USA)	1:1000
Rab7 Inhibition/L4, L5 DRG	GLS Anti-rabbit (Norman Curthoys)	1:1000	IRDye 800CW Donkey anti-rabbit (Li-Cor, Lincoln, NE, USA)	1:20,000

Table.4.2: Details of Primary and secondary antibodies used in western blot studies.

6. Quantitative Reverse Transcription PCR

DRG's were collected in liquid nitrogen and stores at -80°C until further use. Total RNA was extracted using Trizol solution (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed using M-MLV Reverse transcriptase (Promega, Madison, MI, USA), and Quantitative real-time PCR (qRT-PCR) was performed using the ABI StepOne™ system (Applied

Biosystems, Foster City, CA, USA). GAPDH was used as an internal reference with primer sequence:

forward 5'- GAACCACGAGAAATATGACAACTCCCTCAAG-3',

reverse 5' GCAGTGATGGCATGGACTGTGG-3'.

The primer sequences for GLS were

forward 5'- GGGTCTGTTACCTAGCTTGGAAGATTTGC-3',

reverse 5'- GAGTTAATCTTAACATATCCATACACT-3'.

The data were expressed as $2^{-\Delta\Delta CT,}$ which represents the relative amount of target mRNA present in the treated sample to the naïve animal group.

7. Pain behavior

The animals (n = 45) were tested for pain behavior by determining the mechanical thresholds and thermal latencies after the initiation of inflammation with CFA. Before the testing started, rats were familiarized in the behavioral room by handling them for at least three days (twice every day for one hour). The baseline values were recorded for three days (-3, -2, -1 day) before the initiation of inflammation. On the day of treatment (day 0), the values were recorded before the CFA injection. All behavioral testing was performed at the same time (late afternoon, around 3 PM) every day to avoid any variation in animal responses. A total of 9 readings were obtained, including four baseline testing (Day -3, -2, -1 and 0) and five after CFA treatment (Day 1, 2, 3, 4, and 5). Mechanical threshold values were obtained using a Dynamic Plantar Aesthesiometer (Ugo Basile, Gemonio, Italy). The maximum force was set to 50 g with the ramp rate of 5 g/sec. Thermal latencies were measured using a Plantar Test apparatus (Ugo Basile, Gemonio, Italy) with the maximum cut off value set at 55°C and the intensity was set at 55 mW/cm^2. All the tests were

performed by placing the animal in the acrylic apparatus, and readings were obtained once the exploratory behavior ceased. For both mechanical threshold and thermal latency, five readings at intervals of 5 mins were recorded from the ipsilateral hind paw of each rat.

8. Statistical Analysis

All the data were subjected to Student t-test using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego California USA). P values less than 0.05 were considered significant for all tests. The data presented in the graph are group means $\pm$ SEM.

Results

Effect of AIA on levels of NGF, pTrkA, and Rab7 in sciatic nerve

For determining the levels of proteins associated with NGF signaling in the sciatic nerve during peripheral inflammation, we ligated the sciatic nerve of adjuvant-induced arthritic animals. The ligation obstructed the axonal transport allowing us to evaluate the levels of NGF, pTrkA, and Rab7. Representative images of the ipsilateral sciatic nerve showed a qualitative increase in the immunoreactivity of NGF, pTrkA, and Rab7 (Figure 4.1). Western blot data detected the band for pro-NGF form at 27 KDa, confirming previous studies (Chapter 2 and 3). The NGF-ir was elevated in the sciatic nerve of AIA animals after the initiation of inflammation (Figure 4.1A). After 48 hours of AIA, the levels of pTrkA, compared to the total TrkA, were found to be significantly higher (**p = 0.0061) in the sciatic nerve with ligation (Figure 4.2B). Quantitative western data indicated a significant increase in the immunoreactivity of Rab7 in the sciatic nerve of CFA treated animals compared to the sham animals (Figure 4.2C).

Effect of TrkA inhibition on GLS expression during AIA

The levels of GLS were evaluated after 48 hours in the L4 and L5 DRG's of rat treated with CFA and selective TrkA inhibitor (GW441756). Almost all the DRG neurons were immunoreactive

for GLS, as reported in the previous studies (E. M. Hoffman et al., 2010; K. E. Miller et al., 2012; K. E. Miller, Douglas, & Kaneko, 1993; K. E. Miller, Richards, & Kriebel, 2002). The GLS-ir was elevated in the animals treated with CFA as compared to naïve rats. TrkA pharmacological inhibition with selective TrkA inhibitor attenuated the GLS-ir in the DRG neurons compared to the neurons of CFA treated animals (Figure 4.3). The western blot data indicated a significant reduction in the levels of GLS in the DRG cell bodies of animals treated with the TrkA inhibitor compared to AIA animals (Figure 4.4A). We also evaluated the levels of GLS mRNA in DRG's to determine the transcriptional changes due to NGF signaling blockade via TrkA inhibition. The GLS mRNA levels of TrkA inhibited rats were significantly diminished compared to the CFA–alone group (Figure 4.4B).

Effect of Rab7 inhibition on GLS expression and pain behavior during AIA

The peripheral inhibition of Rab7GTPase during CFA-induced inflammation decreased the GLS-ir in the L4 and L5 DRG's. The quantitative western blot data show the elevation of GLS after peripheral inflammation confirming previous findings (Ernest Matthew Hoffman, 2009; K. E. Miller et al., 2012; K. E. Miller et al., 1993; Z. Zhang, 2013). After the Rab7 inhibition, the GLS levels were attenuated significantly in the DRG's of AIA rats (Figure 4.5).

Pain behavior was determined by measuring the mechanical thresholds and thermal latencies in hind paws of animals after injecting and topically applying the CFA and Rab7 receptor antagonist. The reduction in paw withdrawal force and thermal withdrawal latency was interpreted as the presence of mechanical and thermal hyperalgesia, respectively. The mechanical threshold values for animals treated with CFA were significantly reduced compared to naïve animals starting at 24 hours and remained reduced until day 5 ($p = <0.001$), as reported previously (Z. Zhang, 2013). The treatment with Rab7 inhibitor along with CFA (Group: CFA + Inhibitor) significantly reduced the mechanical hyperalgesia as compared to CFA only group after Day 1 ($p < 0.001$), Day 2 ($p <$

0.01), Day 3 (p < 0.05) and Day 4 (p < 0.01) (Figure 4.6). Similarly, the thermal withdrawal latencies were increased significantly in animals treated with CFA and Inhibitor as compared to the animals treated with only CFA. The reduced thermal hyperalgesia was observed after Day 1 (p < 0.01), Day 2 (p < 0.05), Day 3 (p < 0.05) and Day 4 (p < 0.05) in rats treated with Rab7 receptor antagonist with CFA (Figure 4.7).

Discussion

Levels of NGF, pTrkA and Rab7 increases during AIA in sciatic nerve

Our study showed that the unilateral injection of CFA in rat's hind paw elevates the levels of NGF, pTrkA, and Rab7 in the sciatic nerve. These results confirmed the previous findings of the involvement of NGF/TrkA axis in the development of peripheral inflammation (Chapter 1, (E. M. Hoffman et al., 2011; Mesentier-Louro et al., 2017; Woolf, Allchorne, Safieh-Garabedian, & Poole, 1997; Woolf et al., 1994). Levels of NGF are elevated in the cerebrospinal fluid and synovial fluid of patients suffering from inflammatory and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, respectively (Aloe, Tuveri, Carcassi, & Levi-Montalcini, 1992; Falcini et al., 1996). Different animal models of induced arthritis show increased NGF expression (Manni & Aloe, 1998; Woolf, 1996). Neuropeptides like SP, CGRP, and cytokines like IL-6, IL-β are released after the noxious stimuli and are primary mediators of regulating the inflammatory process. This release of inflammatory mediators might elevate the NGF basal levels (Steiner, Pfeilschifter, Boeckh, Radeke, & Otten, 1991; Woolf et al., 1997).

The NGF antibody we used in this study can bind to mature (13kDa) and pro-NGF (27 and 35kDa) forms (Edwards, Selby, Garcia, & Rutter, 1988) simultaneously, hence we cannot differentiate between the two forms in the immunohistochemistry data. Our western blot analysis also failed to detect the mature NGF (β-NGF) while the pro- NGF form gave a prominent band at

27kDa. The method of tissue processing and western blotting we employed in this study might be why we were not able to detect mature NGF.

TrkA is the high-affinity receptor for NGF and is expressed in various types of cells, including peripheral sensory neurons. After binding to NGF on the cell membrane, TrkA is activated and phosphorylates the tyrosine residues present on the cytoplasmic domain, thus converting from TrkA to pTrkA (Huang & Reichardt, 2003; Kaplan, Martin-Zanca, & Parada, 1991). Therefore, for determining the expression of TrkA during the process of inflammation, we used the pTrkA antibody and normalized its expression with total TrkA. After the initiation of inflammation and disrupting the NGF trafficking by sciatic nerve ligation, we found that the levels of pTrkA compared to total TrkA were significantly increased in the sciatic nerve. Previous studies demonstrated that the inflammatory stimulation significantly elevates TrkA expression in the immunologic cells like monocytes, macrophages, B lymphocytes, and T lymphocytes (Minnone, De Benedetti, & Bracci-Laudiero, 2017) therefore some of the TrkA inhibition may occur among these cells, also. In addition, the TrkA immunoreactivity and phosphorylation are shown to be strongly upregulated in the lumbosacral DRG's of animals with cyclophosphamide (CYP)-induced cystitis (Qiao & Vizzard, 2002), but we did not attempt to detect these in the DRG in the current study.

We also found the levels of Rab7GTPase increased in the sciatic nerve along with NGF and pTrkA. The Rab family of GTPase is crucial for organelle transport and is responsible for regulating different processes involved in membrane trafficking pathways (Saxena et al., 2005). The endosomal GTPase Rab7 is functionally distinct from other members of the Rab superfamily and has been implicated in the transport of late endosomes (Feng, Press, & Wandinger-Ness, 1995). After the endocytosis of NGF/TrkA complex, the signaling endosome travels retrogradely to the cell bodies to initiate signal transduction. Before the initiation of retrograde transport, the TrkA positive endosomes undergo maturation forming Rab5GTPase positive early endosomes to

Rab7GTPase positive late endosomes (Deinhardt et al., 2006). Furthermore, a mutation in Rab7 leads to the defective axonal transport and dysregulated NGF/TrkA signaling in DRG neurons, suggesting Rab7's pivotal role in the trafficking of TrkA positive endosomes (K. Zhang et al., 2013).

Selective TrkA inhibition attenuates GLS expression during peripheral inflammation

GLS plays a crucial role in the glutamine-glutamate cycle. Several studies documented the immunoreactivity of GLS in all sizes of DRG neurons (K. E. Miller et al., 2012; K. E. Miller et al., 1993). Our lab previously showed that GLS-immunoreactivity (-ir) and enzyme activity are elevated in DRG neuronal cell bodies during chronic peripheral inflammation (K. E. Miller et al., 2012), but the mechanism for this GLS elevation has not been fully characterized. In this study, we found that the NGF signaling blockade in the periphery via selective TrkA pharmacological inhibition attenuated the levels of GLS in the DRG during AIA, confirming the interplay between NGF signaling and glutamate metabolism (E. M. Hoffman et al., 2011). During the inflammatory process, elevated amounts of GLS are anterogradely transported to the peripheral nerve terminals causing an increase in glutamate production. The elevated glutamate production and release is responsible for sensitizing the primary sensory afferents, thereby regulating nociceptive transmission (Z. Zhang, 2013). The data from this study implicate that the NGF signaling inhibition eventually attenuates the glutamate production in the periphery leading to maintenance in nociceptive sensitization.

TrkA inhibition indicates other potential ways of inhibiting NGF signaling apart from anti-NGF antibodies. NGF has a high affinity for TrkA receptors and a low affinity for p75 neurotrophin receptors present at the axonal terminal. Binding of NGF to TrkA promotes endocytosis and the formation of the signaling endosomes. These NGF/TrkA signaling endosomes are structurally and molecularly defined as multivesicular bodies. For eliciting the transcriptional modulation in the cell

soma, NGF/TrkA endosomes undergo long-range retrograde travel from the distal axons to the DRG cell bodies for initiating subsequent downstream intracellular signaling (Ascano, Bodmer, & Kuruvilla, 2012; Harrington & Ginty, 2013; Wehrman et al., 2007). TrkA activation elevates the expression of SP and CGRP, leading to the sensitization of nociceptors during inflammation (Bullock et al., 2014; O'Connor et al., 2004). Therefore, selective TrkA inhibition can decrease inflammation and sensitization, thus reducing the pain behavior in conditions with a prominent inflammatory process like arthritis (Ashraf, Bouhana, Pheneger, Andrews, & Walsh, 2016) (Nwosu, Mapp, Chapman, & Walsh, 2016). In addition, pharmacological inhibition of TrkA can also be used for studying the mechanisms involved in the trafficking of NGF signaling endosomes during the process of nociception.

Peripheral inhibition of Rab7GTPase decreases the GLS expression and provides analgesia

Our study shows the pharmacological inhibition of small GTPase Rab7 via receptor antagonist in the periphery attenuates the GLS levels in DRG cell bodies of primary sensory neurons after the initiation of inflammation. Previous studies have linked the Rab7 with the TrkA positive endosomes in neurons and retrograde transport from the axon to the neuronal cell body (Ginty & Segal, 2002; K. Zhang et al., 2013). This makes Rab7 as a potential target for disrupting the NGF signaling. The primary function of the Rab7 is to regulate the maturation of early endosomes into late endosomes and are associated with the fusion and clustering (Girard et al., 2014). As Rab7 interacts with TrkA in the multivesicular bodies, Rab7 inhibition leads to an excess of TrkA to be accumulated in the endosomes and hindrance of TrkA endosomal trafficking (Saxena et al., 2005). We postulate that the inhibition of NGF/TrkA signaling via Rab7 is responsible for modulating the levels of GLS in DRG, hence suggesting the potential mechanism behind the regulation of glutamate metabolism during peripheral inflammation.

The findings of this study also provide evidence for the analgesic potential of Rab7 inhibition, as the Rab7 receptor antagonist CD1067700 reduced pain behavior associated with the adjuvant-induced inflammation. This offers a novel therapeutic strategy for alleviating arthritic pain. We found the mechanical and thermal hyperalgesia were reduced significantly after Day 1 in groups treated with Rab7 inhibitor. As the long-range NGF/TrkA retrograde signaling takes a considerable amount of time to reach the DRG cell bodies and exert transcriptional changes, the early analgesic effect might be due to the interaction of Rab7 to various other pain-related molecules such as μ-opioid receptors and calcium-/calmodulin-dependent protein kinase 4. These studies suggest Rab7's interaction with different molecules related to pain processing might be necessary for the development of nociceptive processes (Kallenborn-Gerhardt et al., 2017). In addition, the involvement of Rab7 in the hereditary sensory neuropathies (HSN) and an autosomal dominant inherited disorder causing peripheral neuropathy, Charcot-Marie-Tooth (CMT) provides a rationale to study this small GTPase closely.

Conclusion

In the adjuvant-induced animal model of persistent inflammation, we have demonstrated that the peripheral inhibition of TrkA and Rab7 can modulate the expression of GLS in DRG, thus affecting the primary afferent's sensitization process. Our data suggest the Rab7GTPase inhibitor CD1067700 provides an analgesic effect in arthritic animals via NGF signaling blockade. Further studies directed towards Rab7 are necessary to understand the trafficking of NGF signaling endosome during the process of inflammation and nociception. We predict that such information on the NGF/TrkA axis can provide potential therapeutic targets for treating chronic pain.
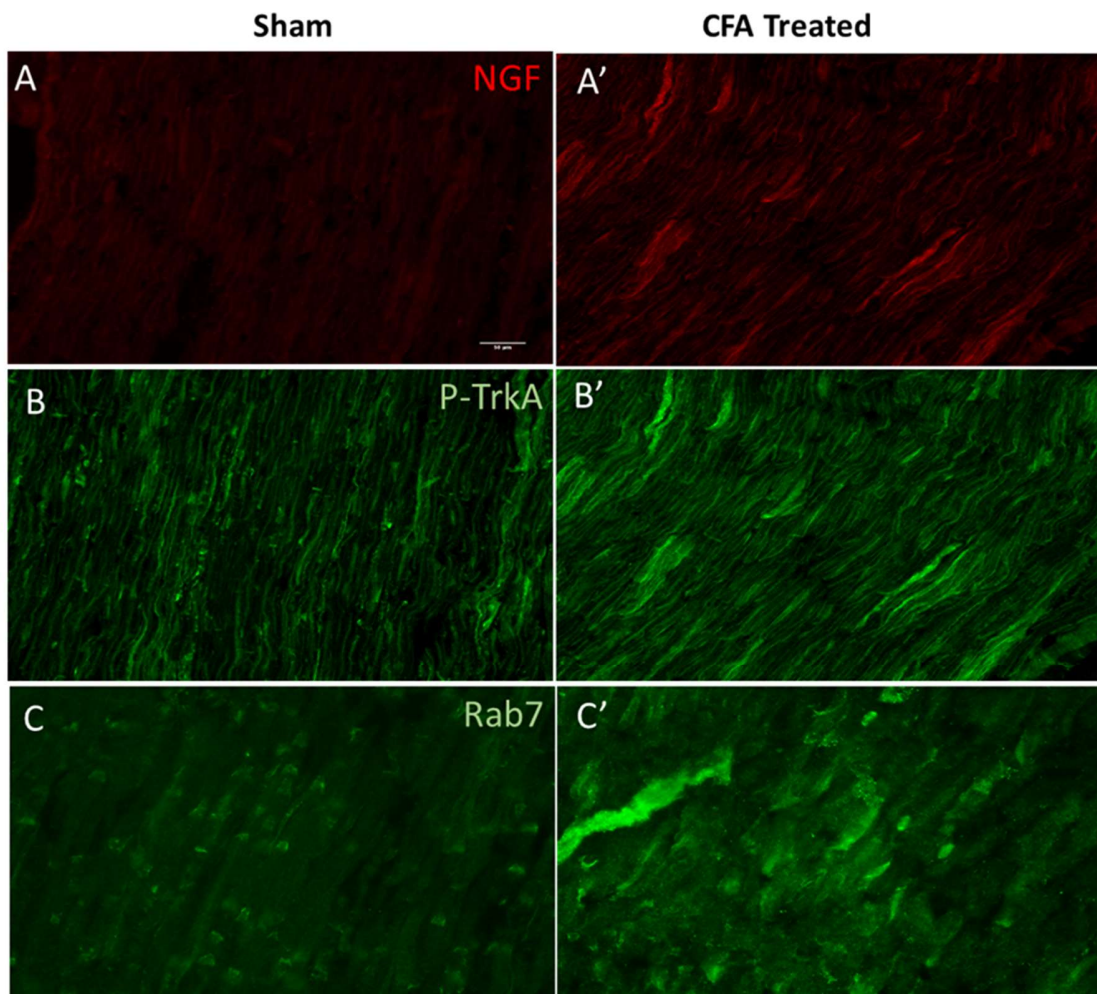
Figure 4.1

Figure 4.1. Peripheral inflammation elevates the levels of NGF, pTrkA, and Rab7 GTPase in ligated sciatic nerve. Increased levels of NGF (A-A'), pTrkA (B-B') and Rab7 (C-C') were detected in sciatic nerve of CFA treated animals as compared to sham animals as determined by qualitative immunohistochemistry. Scale bar = 50 μm, (n=3).

Figure 4.2

(A) NGF

NGF

Total Protein

%Change Normalized to Total Protein

150

100

50

0

*

Naive   CFA Treated

(B) P-TrkA

pTrkA

Total TrkA

%Change Normalized to Total TrkA

2500

2000

1500

1000

500

***

Naive   CFA Treated

(C) Rab7

Rab7

Total Protein

%Change Normalized to Total Protein

110
108
106
104
102
100
98
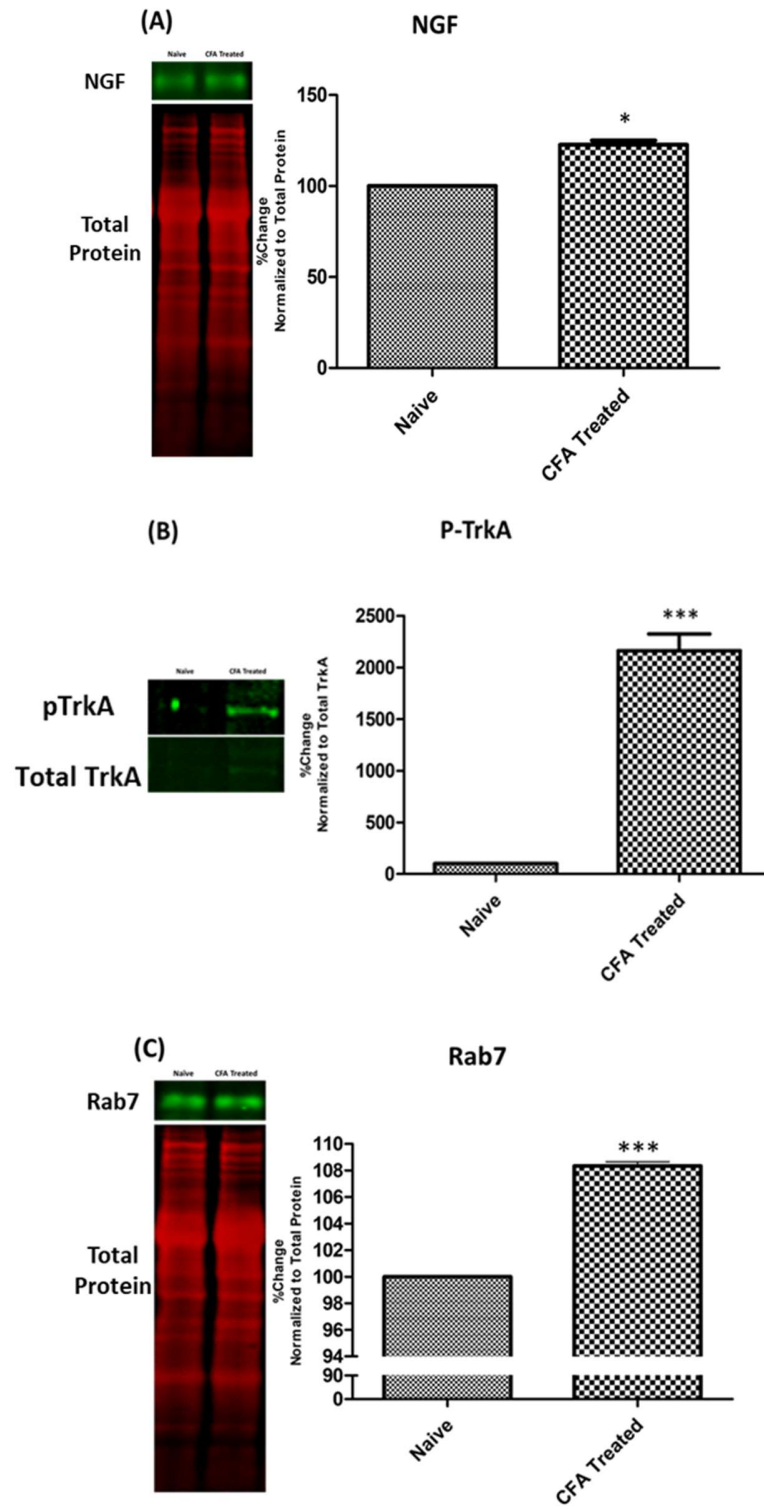96
94
90
0

***

Naive   CFA Treated

Figure 4.2. Peripheral inflammation elevates the levels of NGF, pTrkA and Rab7 GTPase in sciatic nerve after ligation. Increased levels of NGF (A), pTrkA (B) and Rab7 (C) were detected in sciatic nerve of CFA treated animals as compared to Naïve rats after 48 hrs of inflammation as determined by fluorescence western blot. The NGF and Rab7 data were normalized by total protein while pTrkA data was normalized against total TrkA. $*P < 0.05$, $**p < 0.01$, $***p < 0.001$. (n=3)
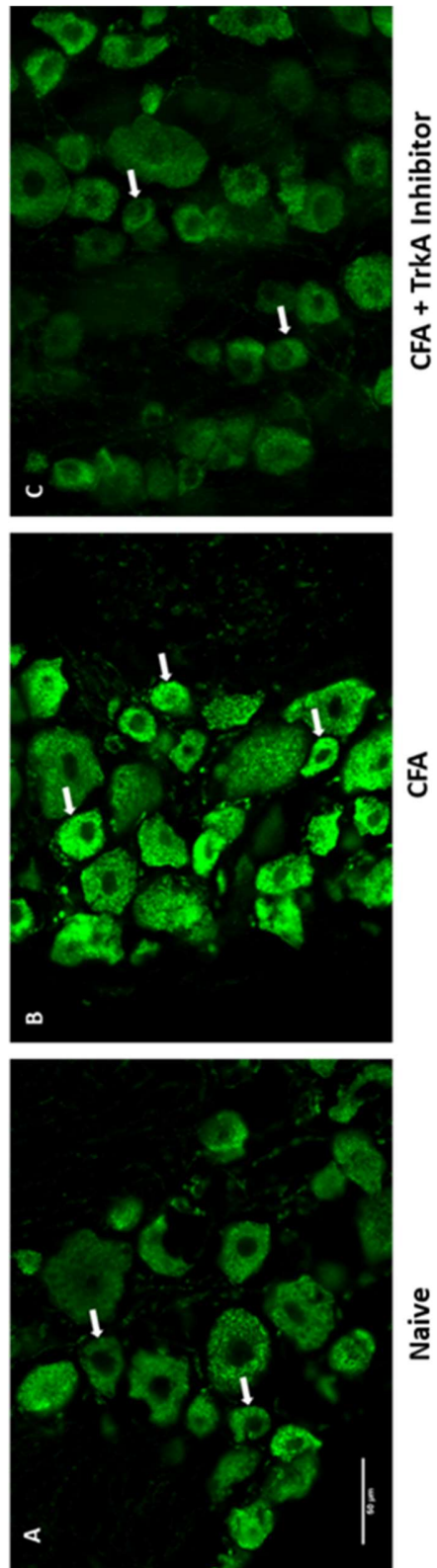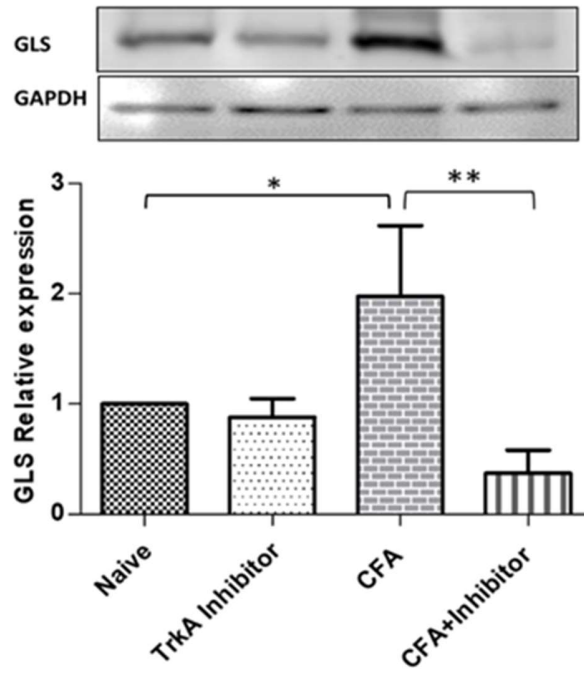
Figure 4.3

Figure 4.3. Selective TrkA inhibition attenuates GLS levels in DRG during peripheral inflammation: Representative images showing GLS immunoreactivity (green). A: Expression of GLS in DRG of Naïve animal. B: GLS expression in DRG of AIA animal after 48h of inflammation. C: GLS expression in animal treated with CFA and selective TrkA inhibitor (GW441756). Note the expression of GLS in small diameter neurons (white arrows) in animals treated with CFA is higher as compare to the animals treated with TrkA inhibitor along with CFA. Scale bar = 50 μm, was applied to all photomicrographs. (n=3)

Figure 4.4
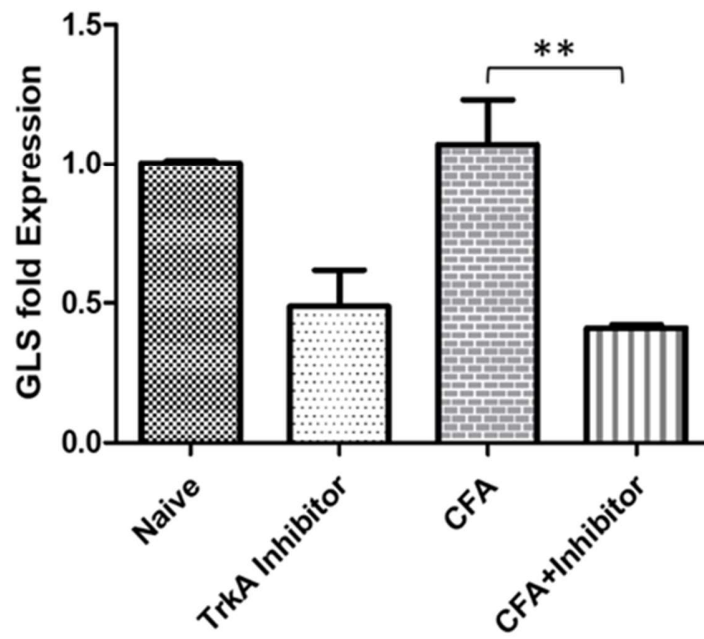
Figure 4.4. Selective inhibition of TrkA in the periphery during AIA, changes the expression of GLS expression in rat DRG neurons after 48hrs of inflammation. (A) The levels of GLS protein are upregulated in animals treated with CFA as compared to naïve rats but decreased significantly in the group treated with TrkA inhibitor and CFA as determined by quantitative western blot analysis. (B) The GLS mRNA analysis showed that peripheral TrkA inhibition decreased GLS mRNA in both control and CFA treated rats as determined by qRT-PCR. $*P < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's t-test; n=3).

Figure 4.5

(A)

	Control	CFA	CFA + Inhibitor

GLS

Total
Protein

(B)

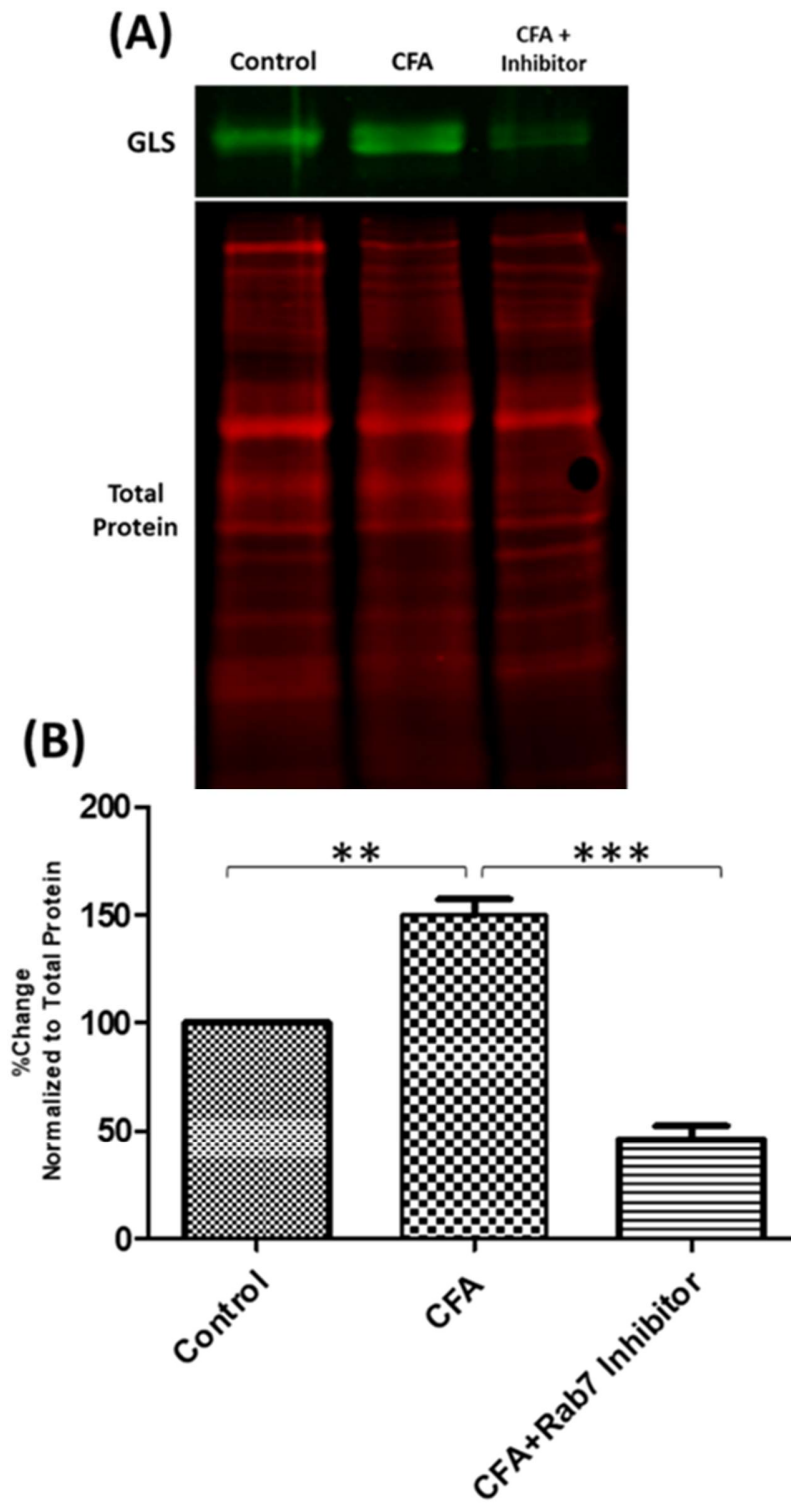**Figure 4.5. Inhibition of Rab7 in the periphery during adjuvant-induced arthritis (AIA), changes the expression of GLS in rat DRG neurons after 48hrs of inflammation.** (A) Image showing GLS bands (66 and 68 kDa) and total proteins after western blotting. (B) The expression of GLS is significantly reduced in the group CFA+Inhibitor as compare to CFA after normalizing with total protein. *P < 0.05, **p < 0.01, ***p < 0.001. (n=4)

Figure 4.6

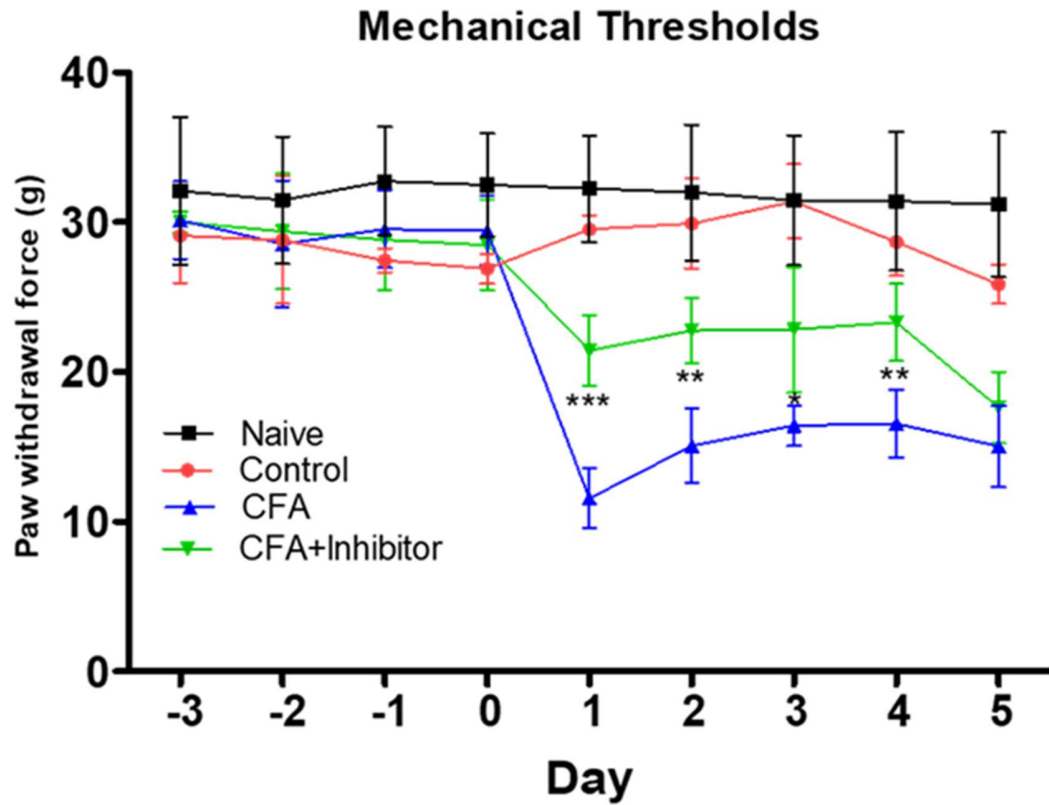**Figure 4.6. Mechanical thresholds from rat hind paws after inhibition of Rab7 in the periphery during adjuvant-induced arthritis (AIA).** Inhibition of Rab7 in the periphery during AIA (CFA+Inhibitor), caused significant increase in paw withdrawal force from days 1 through 4 as compared to the CFA group. Data are graphed as means (±SD). *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$. (Student's t-test; n=5)

Figure 4.7

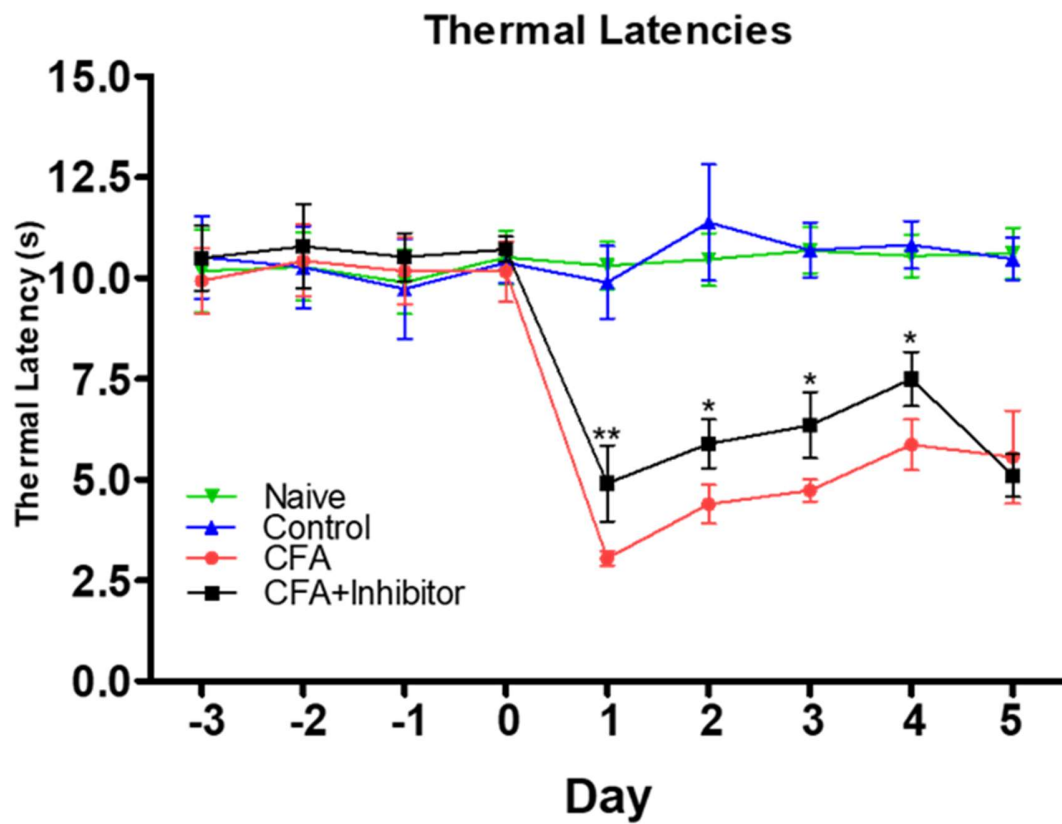**Figure 4.7. Thermal latencies from rat hind paws after inhibition of Rab7 in the periphery during adjuvant-induced arthritis (AIA).** Inhibition of Rab7 in the periphery during AIA (CFA+Inhibitor), caused significant increase in the thermal latencies from days 1 through 4 as compared to the CFA group. Data are graphed as means (±SD). *P < 0.05, **p < 0.01, ***p < 0.001. (Student's t-test; n=5)

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Conclusion

Chronic inflammatory diseases and chronic pain are considered as the most significant causes of death across the world. The main symptom of chronic inflammation is chronic pain, which is a debilitating condition. Pain is a protective mechanism that allows us to avoid injury and assist in the healing process. When this protective pain is converted into chronic pain, it becomes a distinct entity altogether and can be considered as an independent condition instead of an injury accompanying phenomenon. The primary analgesic drugs currently used for treating chronic pain are non-steroidal anti-inflammatory drugs and opioids, but these medications are accompanied by numerous side effects and must be used in combinations with non-pharmacologic strategies (Mantyh, Koltzenburg, Mendell, Tive, & Shelton, 2011). Therefore, finding new targets for treating chronic pain with minimal or no side effects remains the top priority of researchers working in nociception. In 2001, Woolf et al. stated in their review article that "It is largely believed that mechanism-based treatments, rather than disease- or diagnosis-based treatments, hold the key to the development of new successful therapies for pain" (Woolf & Max, 2001). The main objective of this study was based on the Woolf et al. statement, the study of the molecular mechanisms of NGF signaling underlying the process of peripheral inflammation by addressing the hypothesis that NGF/TrkA signaling endosomes are responsible for altering glutaminase levels in primary sensory

neurons during peripheral inflammation. Nerve growth factor (NGF) initially was identified as the molecule crucial for the development of sympathetic and primary sensory neurons, but later it was found that the NGF and its receptor TrkA are involved in the development of inflammatory hyperalgesia (Woolf, Safieh-Garabedian, Ma, Crilly, & Winter, 1994). This made NGF a prime target for investigation in the field of inflammation and pain. Several studies documented the positive effect of NGF signaling blockade in controlling acute and chronic pain (Mantyh et al., 2011). Although anti-NGF strategies have proven effective in alleviating inflammatory hyperalgesia, the underlying mechanism behind its effectiveness is yet to be fully elucidated. Based on this information and my hypothesis, I had the following specific aims:

Aim 1: To evaluate the easy to use and inexpensive technique for the separation of the epidermis from dermis for determining the expression of neurotrophins and inflammatory mediators, specifically in epidermal tissue.

Aim 2: Determine the effect of acute and chronic inflammation on the expression of NGF in the epidermis separated by thermolysin proteolytic treatment.

Aim 3: Determine the role of NGF signaling on glutaminase expression in dorsal root ganglion primary afferent neurons during inflammation by pharmacological inhibition of peripheral TrkA and Rab7GTPase.

For determining the expression of molecules like inflammatory mediators and neurotrophins involved in the development of peripheral inflammation in the skin, there has been an unmet need of an inexpensive and easy to use epidermal-dermal separation technique. Evaluation of proteins specifically in the skin is challenging due to the heterogeneity of cell types present in the tissue during inflammation. These issues were addressed in chapter 2 (Figure 5.2). I used proteolytic thermolysin digestion to separate the epidermis from the dermis for the evaluation of NGF and IL-6 protein and mRNA expression during carrageenan-induced inflammation.

Different techniques are available for separating the epidermis from dermis by using acids, alkali, or enzymes like keratinase, collagenase, pronase, dispase, and thermolysin (Einbinder, Walzer, & Mandl, 1966; Zou & Maibach, 2018). As I wanted to evaluate the alterations in the expression of inflammatory mediators and neurotrophins, the separation technique had to be carried out in cold temperatures to protect the protein and mRNA from degradation. The thermolysin separation provides favorable conditions for studying the proteins and enzymes expression in the epidermis without hampering the downstream processing. It also allows the study of the interaction of epidermal cells with the intraepidermal nerve fibers during neurogenic conditions.

The data from this study (Chapter 3, Figure 5.2) shows the elevation in the levels of NGF during a long-term inflammatory process (AIA) confirming the previous findings (Minnone, De Benedetti, & Bracci-Laudiero, 2017; Sofroniew, Howe, & Mobley, 2001; Woolf et al., 1994). During AIA, the temporal expression of NGF showed two peaks, one after 6 hours and second after 96 hours. This biphasic response of NGF represents its involvement in the phasic progression of neurogenic inflammation. In addition, the change in NGF expression at different time points suggests the potential role in the conversion of acute to chronic inflammation. Further investigations are needed to understand how NGF is expressed in the inflamed tissue and which cellular and molecular mechanisms are involved in the peripheral inflammatory process.

The previous studies from our lab demonstrated the significant increase in the levels of glutaminase in DRG neurons during inflammation implicating its response to and role in the process (Ernest Matthew Hoffman, 2009; E. M. Hoffman, Zhang, Schechter, & Miller, 2016; Miller et al., 2012; Miller, Douglas, & Kaneko, 1993; Zhang, 2013). In this study, I evaluated the cellular and molecular mechanism contributing to the glutaminase alteration in DRG neurons (Chapter 4, Figure 5.3, 5.4 and 5.5). The peripheral inhibition of NGF signaling by blocking TrkA and Rab7 attenuated the glutaminase levels in DRG neurons during AIA, affecting the glutamate metabolism involved in peripheral sensitization. Further studies directed towards Rab7 are necessary to

understand the trafficking of NGF signaling endosome during the process of inflammation and nociception.

Although the anti-NGF human monoclonal antibody for treating inflammatory conditions like osteoarthritis is in clinical trials (Schmelz et al., 2019; Tive et al., 2019), the exact molecular mechanism behind the efficacy of these antibodies is not yet fully elucidated. Besides, the use of human monoclonal antibodies carries a significant risk of immunogenic reactions. Our data suggest an alternate way of blocking NGF signaling via peripheral inhibition of Rab7 as it provided analgesic effect in AIA animals. With further understanding of the Rab7 in the peripheral inflammatory diseases, we predict that Rab7 can be a potential therapeutic target for treating chronic pain.

Future Directions

The epidermal-dermal separation technique using the thermolysin enzyme (Chapter 2) paved the way for studying the alterations occurring in the epidermal tissue during peripheral inflammatory diseases. The technique will also assist in understanding the detailed molecular mechanisms of interaction between the intraepidermal nerve fibers and epidermal keratinocytes. Although numerous studies were performed on NGF's functional role in different processes like neural development, transcriptional modulation, and inflammatory conditions, future studies are required to understand the cellular process behind the expression and release of NGF during disease conditions (Table 5.1). We also found that NGF is elevated primarily in keratinocytes in the outermost layer of the epidermis (stratum lucidum and stratum granulosum). Hence, studying the type of cells expressing NGF can provide insight into the alteration of cellular process occurring due to inflammation (Table 5.1).

The temporal NGF study showed a biphasic expression of NGF in the peripheral tissue during the AIA inflammatory process (Chapter 3). The two peaks of NGF protein and mRNA in

the skin indicate the cellular and molecular transformation occurring between the intraepidermal nerve fibers and peripheral tissue, pointing towards the conversion of acute to chronic inflammation. Further studies directed towards understanding the conversion process from acute to chronic conditions are needed, which will provide the opportunity for the development of novel therapeutic drugs for treating chronic diseases (Table 5.1). Earlier we documented that during the peripheral inflammation, GLS shows a biphasic expression in the DRG cell bodies. We believe that the first peak of NGF (Chapter 3) elevates the expression of GLS in DRG and GLS is shipped out in the periphery for increasing the production of glutamate. The possibility that the increased glutamate production in return contributing to the second peak of NGF cannot be ruled out. Hence, future studies addressing the crosstalk between phasic changes of GLS and NGF are needed.

NGF is implicated in the development of hyperalgesia by sensitizing the primary sensory neurons, and the results of this study point out its involvement in the regulation of glutamate metabolism in DRG neurons (chapter 4). We showed that the peripheral inhibition of Rab7GTPase, an endosomal marker, can lead to the disruption of NGF signaling and attenuation of the expression of glutaminase, which is a major glutamate producing enzyme. Besides, the Rab7 peripheral inhibition also provided an analgesic effect in animals suffering from adjuvant-induced arthritis. Therefore, the possibility of Rab7 as a novel therapeutic target for treating chronic pain cannot be ruled out, and future studies directed towards its involvement in the process of inflammation will be performed (Table 5.1). Simultaneously, our lab has documented the analgesic properties of glutaminase peripheral inhibition; hence a combined approach of blocking Rab7 and glutaminase during the process of peripheral inflammation will be performed. Our lab has shown that the level of the other glutamate producing enzyme Aspartate aminotransferase (AST) is also elevated in DRG neurons during the peripheral inflammation. Taken together, our future studies will include determining the effect of NGF on the expression of AST. These studies could lead in the direction of finding a potential cure for chronic inflammatory diseases.

Figure 5.1

Figure 5.1. Summary of Chapter 2 findings: The small diameter primary sensory neuron is shown. The peripheral terminal of the sensory neuron innervates the skin especially epidermis and the central terminal terminates in the spinal cord dorsal horn. After the noxious stimulus with the carrageenan in the periphery, the expression levels of NGF and IL-6 was determined in the epidermis. For the epidermal-dermal separation, cold thermolysin technique was optimized. The levels of NGF and IL-6 were elevated after 6 hours and 12 hours of carrageenan injection.
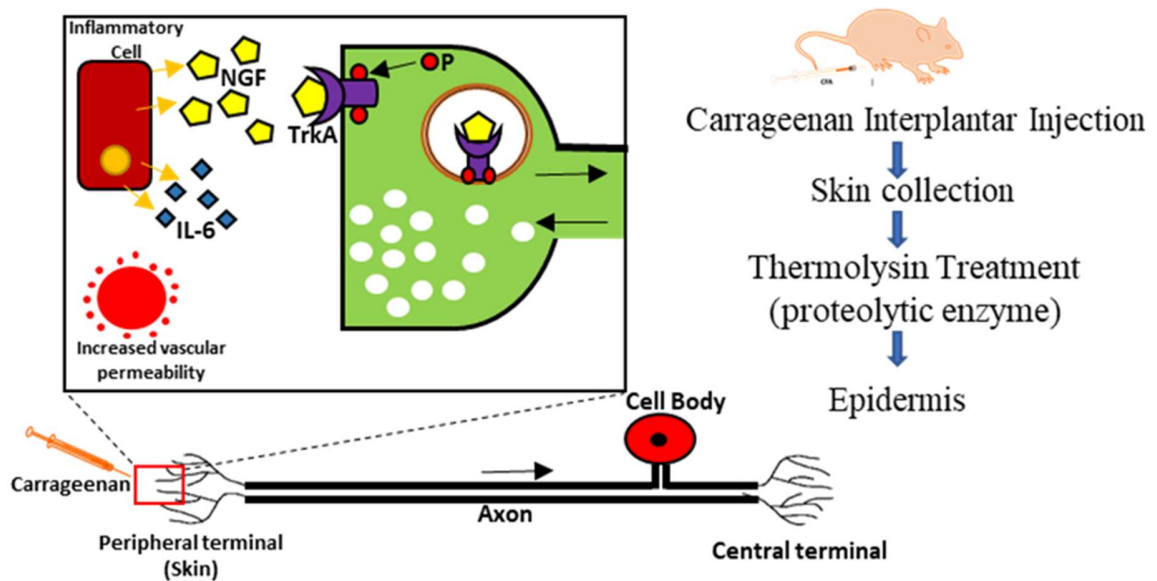
Figure 5.2

Figure 5.2. Summary of Chapter 3 findings: The small diameter primary sensory neuron is shown. The peripheral terminal of the sensory neuron innervates the skin especially epidermis and the central terminal terminates in the spinal cord dorsal horn. After the noxious stimulus with the CFA (persistent inflammatory model) in the periphery, the phasic expression level of NGF protein and mRNA was determined in the epidermal tissue. The NGF shows biphasic expression i.e. first peak after 6 hours and second peak after 96 hours of peripheral inflammation.

Figure 5.3

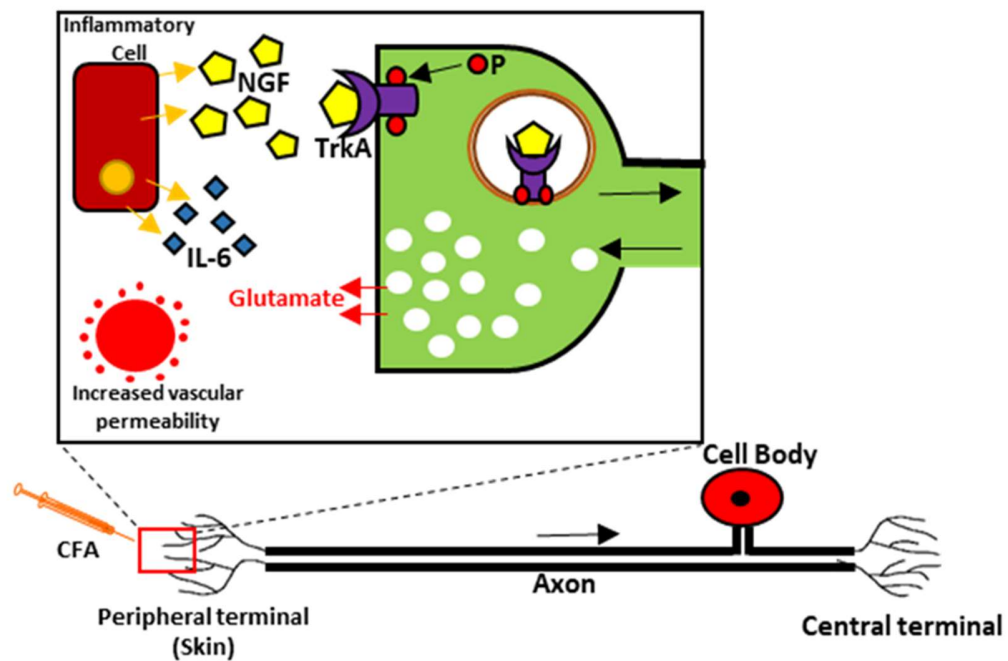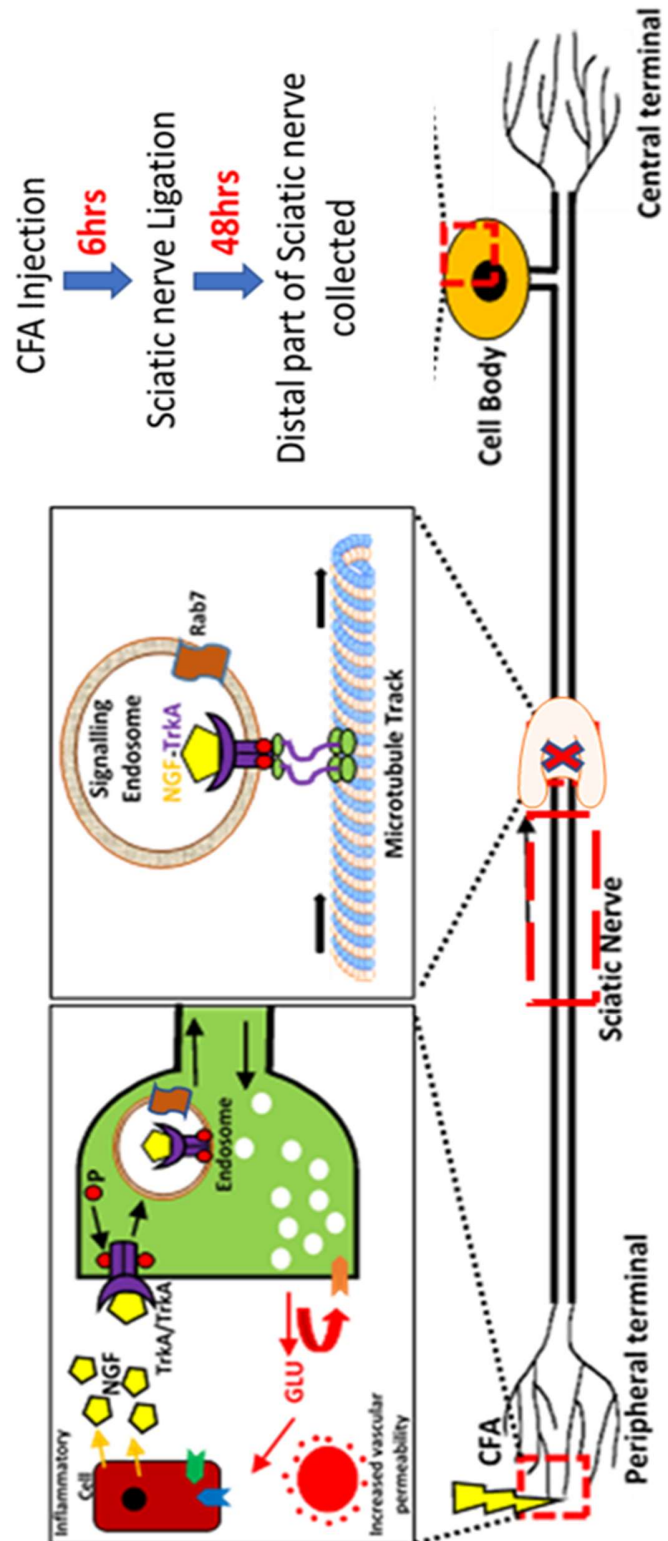**Figure 5.3. Summary of Chapter 4 findings (Sciatic nerve ligation):** The small diameter primary sensory neuron is shown. The peripheral terminal of the sensory neuron innervates the skin especially epidermis and the central terminal terminates in the spinal cord dorsal horn. After the noxious stimulus with the CFA (persistent inflammatory model) in the periphery, the sciatic nerve was ligated and levels of NGF, pTrkA and Rab7 protein and mRNA were evaluated. The sciatic nerve ligation disrupted the axonal trafficking leading to the elevated levels of NGF, pTrkA and Rab7 in the distal part of sciatic nerve.

Figure 5.4

Figure 5.4. Summary of Chapter 4 findings (TrkA pharmacological inhibition): The small diameter primary sensory neuron is shown. The peripheral terminal of the sensory neuron innervates the skin especially epidermis and the central terminal terminates in the spinal cord dorsal horn. The cell body of the primary sensory neuron is in the DRG. After the injection of CFA and TrkA selective inhibitor in the periphery, the levels of glutaminase (GLS) was evaluated in the DRG. The GLS immunoreactivity was decreased significantly after NGF signaling blockade via TrkA inhibition indicating the direct crosstalk between NGF and glutamate metabolism.
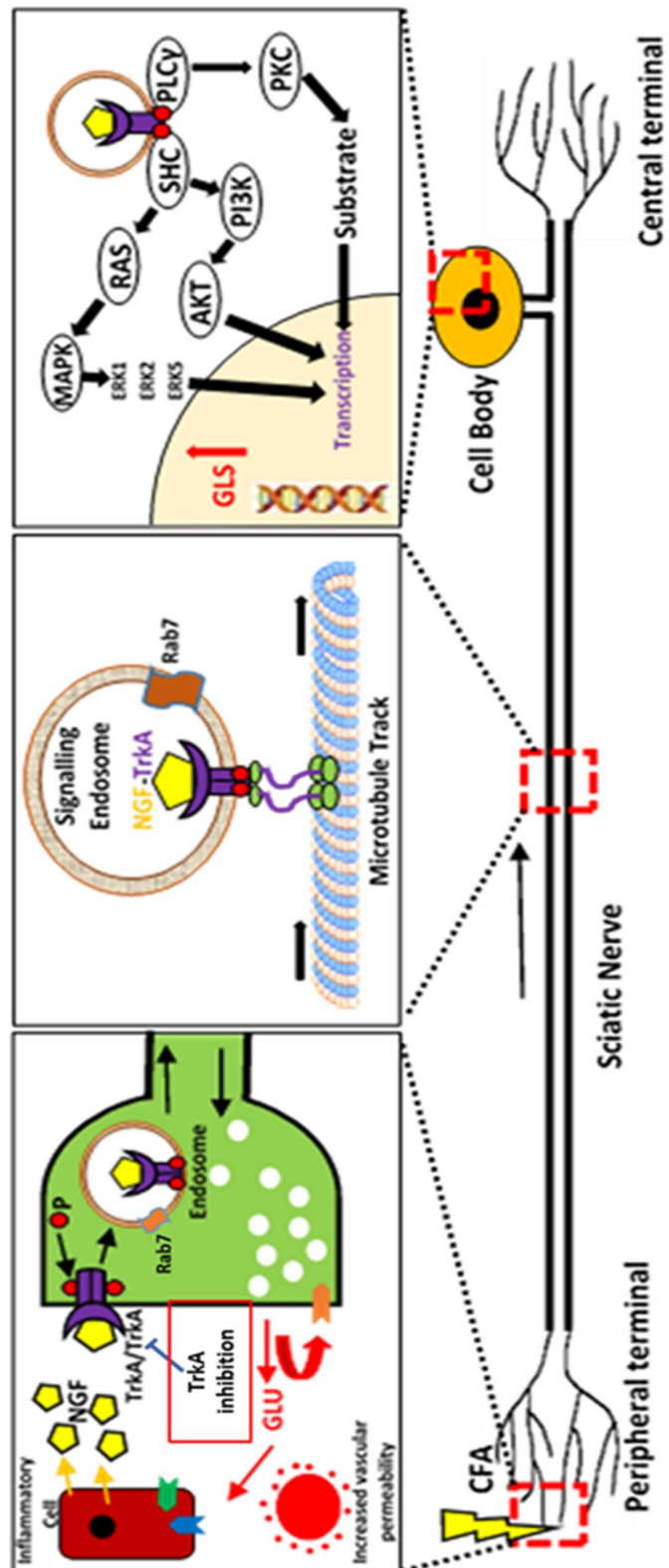
Figure 5.5
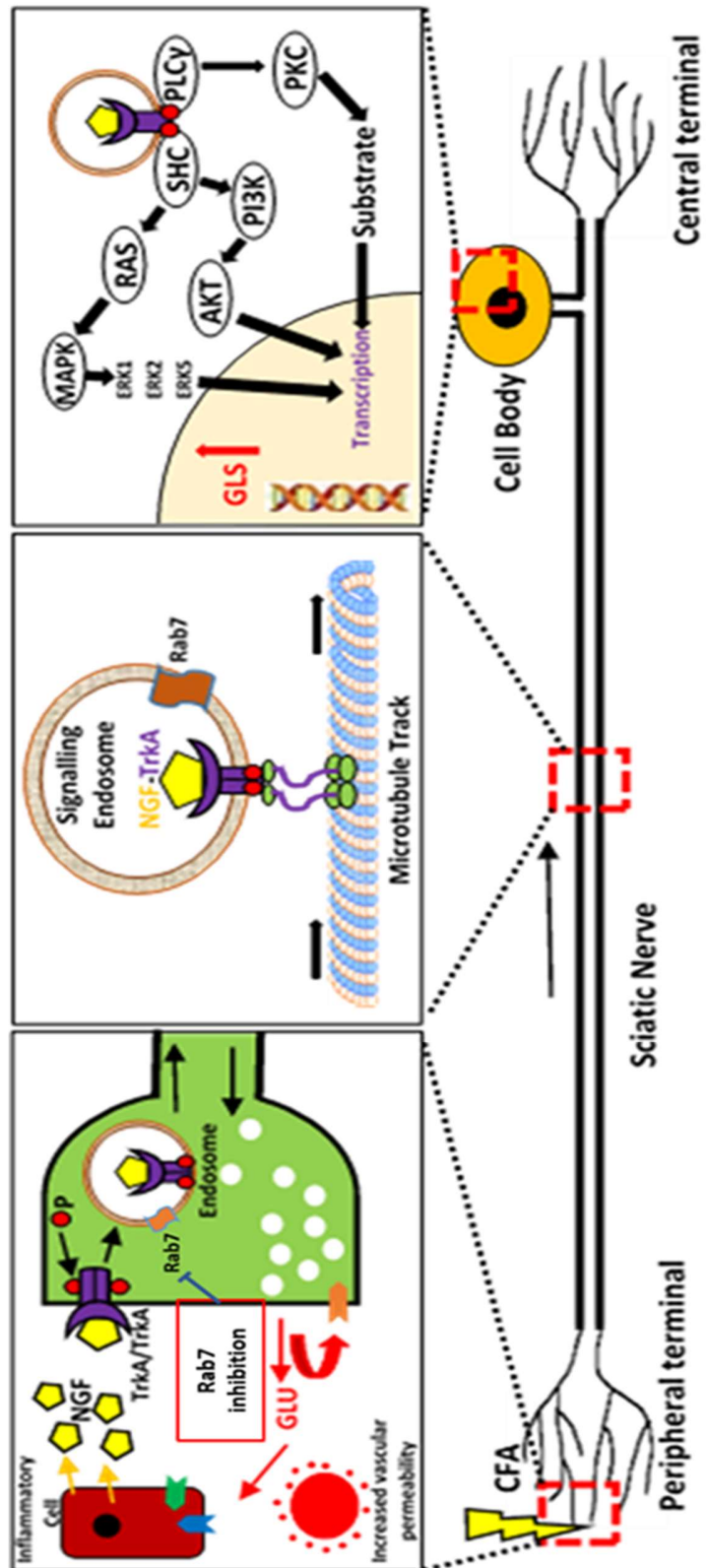
Figure 5.5. Summary of Chapter 4 findings (Rab7 pharmacological inhibition): The small diameter primary sensory neuron is shown. The peripheral terminal of the sensory neuron innervates the skin especially epidermis and the central terminal terminates in the spinal cord dorsal horn. The cell body of the primary sensory neuron is in the DRG. After the injection of CFA and Rab7 receptor antagonist in the periphery, the levels of glutaminase (GLS) was evaluated in the DRG. The GLS immunoreactivity was decreased significantly in the DRG after disrupting the NGF signaling endosome trafficking via Rab7 inhibition. The results indicate an alternate way of NGF signaling blockade and a potential therapeutic target for treating chronic inflammatory diseases.

Figure 5.6

A.

B.
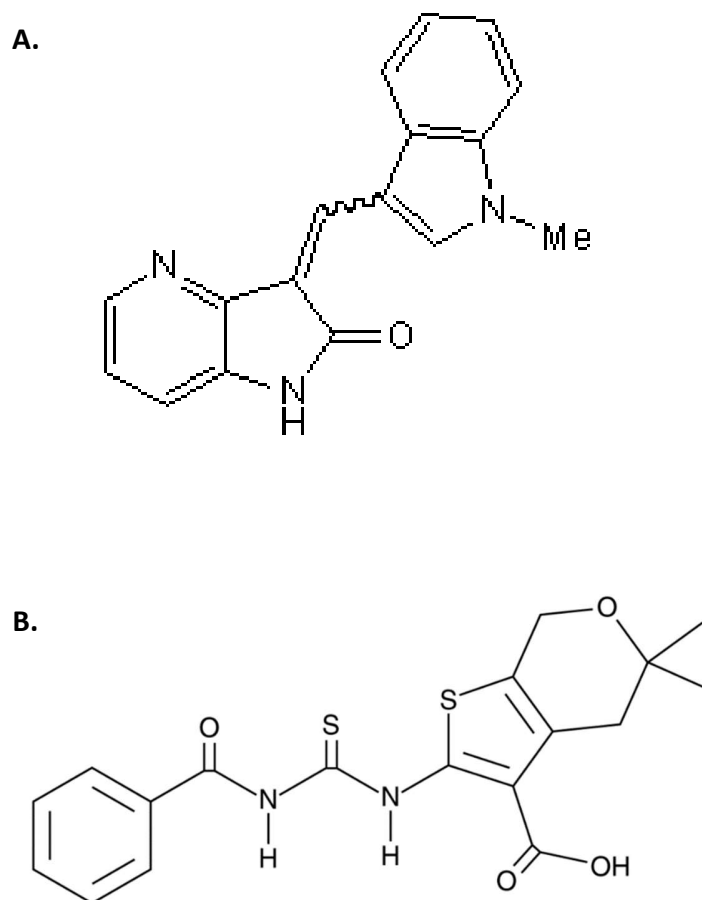
Figure 5.6. Molecular structures of TrkA and Rab7 pharmacological inhibitors: A. Potent selective TrkA inhibitor GW 441756 (Tocris Biosciences, Bristol, UK); B. Rab7 receptor antagonist CD1067700 (Cayman, MI, USA).

Findings	Significance
Chapter 2 Inexpensive and easy to use epidermal-dermal separation technique	• To study the mechanism behind peripheral neuropathies **(Diabetic Neuropathies)** • To study the interaction between skin cells and intraepidermal nerve fibres during **Skin Diseases like Psoriasis**
Chapter 3 Biphasic expression of Nerve growth Factor	• For determining the exact timepoints for **Therapeutic Intervention** by NGF signaling blockade
Chapter 4 NGF modulates GLS expression And Rab7 inhibition provide analgesic effects	• **Combination of NGF and GLS inhibition** can be used for pain management • Rab7 a **Novel potential Therapeutic Target** for treating chronic pain

Table 5.1. Translational significance of the study.

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