UNIVERSITY OF CALIFORNIA SAN DIEGO

Targeting the low-density lipoprotein receptor-related protein for treating pain

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

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Professor Jerry Yang, Co-Chair
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The thesis of Zixuan Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

AAT	alpha 1 antitrypsin
AUC	Area under curve
BCA	Bicinchoninic Acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CCI	Chronic constriction injury
CCR	Clusters of compliment-like repeats
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
EI	Enzymatically inactive
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
GFAP	Glial fibrillary acidic protein
HBSS	Hank’s Buffered Salt Solution
HPCD	(2-Hydroxypropyl)-β-cyclodextrin
IL	Interleukin
IOD	Integrated optical density
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide

LRP1	Low-density lipoprotein receptor-related protein-1
MMP-9	Matrix metalloproteases-9
NGF	Nerve growth factor
Phospho	Phosphorylated
PNL	Partial sciatic nerve ligation
PNS	Peripheral nervous system
PWT	Paw withdrawal threshold
RAP	Receptor-associated protein
RIPA	Radioimmunoprecipitation assay
RT-qPCR	Quantitative Reverse transcription polymerase chain reaction
SCPs	Schwann cell precursors
SFM	Serum-free medium
SGC	Satellite glia cells
sLRP1	Shed LRP1
SNL	Tight ligation of spinal nerve
SP	Substance P
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen activator
Tris	Tris(hydroxymethyl)aminomethane
TRPV1	Transient receptor potential vanilloid 1 receptor
α2M	α2-Macroglobulin

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ABSTRACT OF THE THESIS

Targeting the low-density lipoprotein receptor-related protein for treating pain

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The low-density lipoprotein receptor-related protein-1 (LRP1) is an endocytic and cell signaling receptor, highly involved in innate immunity. Although activation of membrane-bound LRP1 results in anti-inflammatory activity in vitro and in vivo, and genetic deletion of LRP1 in Schwann cells increase neuropathic pain, the ability of LRP1 agonists to alleviate pain is unknown. Thus, we tested whether a novel small peptide, SP16, derived from alpha-1-anti-trypsin and binds LRP1 with high affinity, could regulate pain states in three distinct mice models. Initially, we determined that SP16 robustly activates cell signaling in PC12 cells via an LRP1 dependent manner. SP16 also

increased neurite length and expression of regenerative genes in cultured adult primary sensory neurons. In response to intraplantar capsaicin, SP16, two SP16 analogs (A2-5, A15) and a well-known LRP1 activator, enzymatically inactive (EI)-tissue plasminogen activator (tPA) significantly reduced paw licking time compared to vehicle. SP16 blocked the development of tactile allodynia after partial sciatic nerve ligation ($P<0.05$). The underlying mechanism includes limited recruitment of CD11b+ inflammatory cells ($P<0.01$) into the nerve injury site and reduced levels of Toll-like receptor-4 (TLR4) ($P<0.05$). In injured DRGs, SP16 reduced the presence of CD11b+ cells and GFAP, suggesting that inflammatory and satellite cell activation was inhibited. Following intraplantar formalin injection, SP16-treated mice showed reduced responses in phase 1 ($P<0.01$) and delayed the onset of phase 2 ($P<0.05$). We conclude that SP16 inhibits acute nociceptive, inflammatory, and neuropathic pain-related behaviors, suggesting that LRP1 agonists have uniquely broad anti-inflammatory activities that minimize the development of pain states.

Chapter 1

Background Information

1.1 The peripheral nervous system

In the human nervous system, the brain and spinal cord constitute the central nervous system (CNS), with other parts being associated with the peripheral nervous system (PNS), including spinal nerves, cranial nerves, and peripheral ganglia which lie outside the brain and spinal cord. The peripheral nervous system consists of both sensory and motor nerves and ganglions. There are three types of cells in PNS: neuronal cells, glial cells, and stromal cells[1]. Within the nerve, the individual nerve fibers (myelinated and unmyelinated) are grouped in bundles called fascicles (**Figure 1.1**). Each fascicle is enveloped by a highly specialized structure called epineurium, the connective tissue that surrounds nerve fibers, and endoneurial connective tissue in fascicles to protect the fibers and maintain homeostasis[2]. Inside the fascicle, there are fibroblasts, connective tissue, blood vessels, resident macrophages, and Schwann cells[3]. Endoneurial fibroblasts are derived from the neural crest and Schwann cell precursors[4], they account for more than half of the nerves' non-neuronal cells population[5] and secrete the extracellular matrix protein collagen[6]. Macrophages in the fiber often come from blood monocytes[7], they are an important part of the cellular response to peripheral nerve injury. Within the region containing degenerating axons, they are recruited in significant numbers in the first few days[8]. Among these components, we will especially focus on Schwann cells due to their important role in nerve repair and regeneration.

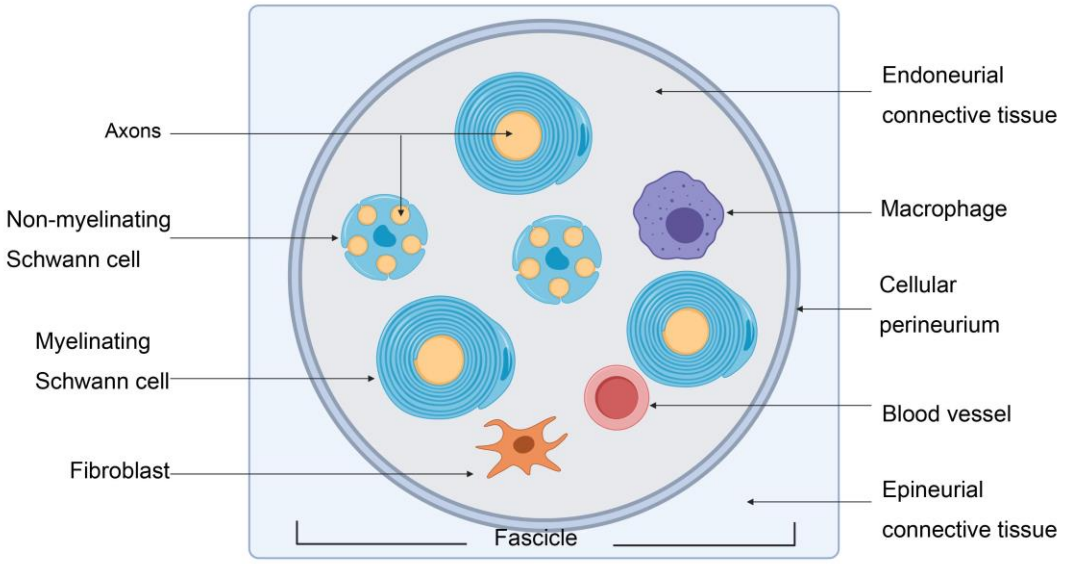

Figure 1.1- The diagram of transverse section of peripheral nerve, showing the components of a fascicle. Created with Biorender.

1.2 Schwann cells

Schwann cells are a type of glial cells that are unique to the peripheral nerve system, they help to form the myelin sheath in myelinated axons also release a variety of growth factors to help nerve regeneration and remyelination when the nerve is injured[9]. Schwann cells originate from neural crest cells (**Figure 1.2**). Migrating neural crest cells can transform to Schwann cell precursors (SCPs), which are the gila cells of embryonic day E12-13 (mouse), SCPs can develop to immature Schwann cells which are glia cells appear later than SCPs, from E17-18 (mouse) to about the time of birth[6]. There are two major phenotypes of Schwann cells: myelinating Schwann cells and non-myelinating

(ensheathing) Schwann cells. The development from immature Schwann cells to myelinating or non-myelinating Schwann cells is determined by which axons they randomly associate with[6]. Myelinating Schwann cells wrap around axons greater than 1μm in diameter in the mature peripheral nerve system, produce myelin for axons. The myelin can electrically insulate the axonal membrane and dramatically increase the speed at which the nerve impulse propagates[10]. Non-myelinating Schwann cells loosely embrace Remak fibers. The Remak fibers have small axons that include the C fiber nociceptors, the postganglionic sympathetic fibers, and some of the preganglionic sympathetic and parasympathetic fibers. The Remak fibers can be viewed as highly plastic for the “first responders,” providing protective sensibility to a neighboring denervated region[11].

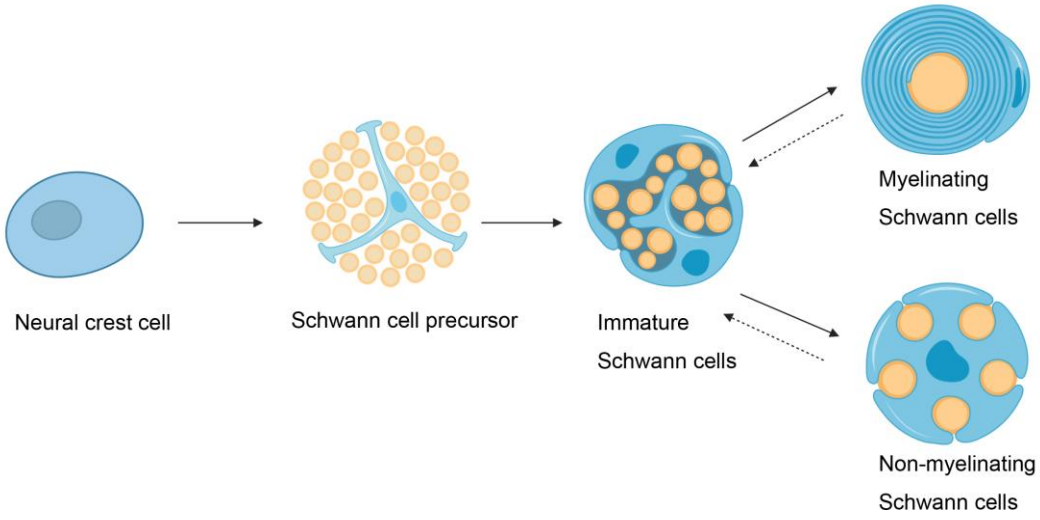

Figure 1.2 - Schwann cell lineage. Schematic illustration of the Schwann cells development. Dashed arrows indicate that the development is reversible, mature myelinating and non-myelinating Schwann cells can revert to a phenotype similar to that of the immature Schwann cells in response to injury. Created with BioRender.

After traumatic nerve injury, Schwann cells are activated before central glia and release various inflammatory mediators. These mediators are important for the recruitment of immune cells, including neutrophils, macrophages, dendritic cells, and T and B lymphocytes, to the site of nerve injury[12]. The secretion of matrix metalloproteases-9 (MMP-9) by activated Schwann cells causes the breakdown of the blood-nerve barrier, which promotes the recruitment of immune cells[13]. Released cytokines and chemokines during this process coordinate the cellular response to nerve injury can directly alter the sensory transduction properties of nociceptive afferent axons and cause ongoing activity[10]. Schwann cells also undergo phenotype changes after peripheral nerve injury to support axon outgrowth. They lose their myelinating phenotype and dedifferentiate, become repair cells to promote nerve regeneration[14].

Schwann cell proliferation is also a crucial step for Schwann cell migration and nerve regeneration after nerve injury[15]. In the normal state, glial fibrillary acidic protein (GFAP) is suppressed in myelinating Schwann cells but retained in non-myelinating Schwann cells[16]. Following nerve injury, Schwann cells change their phenotypes to become repair cells, myelinating Schwann cells begin to express GFAP, the upregulation of GFAP can be detected days after the damage[17]. Schwann cells also proliferate and migrate with axons[18]. The production of GFAP by astrocytes also increased following injury[19], In the GFAP-null mice, the loss of GFAP led to a significant reduction in Schwann cell proliferation, the delayed regeneration could also be observed[20].

1.3 The low-density lipoprotein receptor-related protein-1 (LRP1)

The low-density lipoprotein receptor-related protein-1 (LRP1) is a member of the low-density lipoprotein (LDL) family, this is a family of receptors with multiple functions, ranging from lipid and lipoprotein metabolism to neuronal regeneration and survival. LRP1 is one of the largest receptors in the LDL family, it is a multifunctional endocytic cell-surface receptor that could bind and internalize a diverse array of ligands[21]. It participates in many physiological and pathological processes ranging from lipid and lipoprotein metabolism to neuronal regeneration and survival[22]. The LRP1 expression is ubiquitous in the human body and can be found in the nervous system, it is expressed by many kinds of cells including neurons and astrocytes[23], LRP1 expression in Schwann cells plays an important role in injury response. LRP1 is dramatically increased in Schwann cells after peripheral nerve injury[24].

LRP1 is a 600-kDa membrane glycoprotein, it is synthesized as a single polypeptide chain but is cleaved into two units. The C-terminal 85-kDa light chain (alpha chain) comprises the transmembrane domain which anchors LRP1 to the cell membrane and cytoplasmic domain (**Figure 1.3**). The N-terminal 515-kDa heavy chain (beta chain) locates outside the cell and contains several ligand-binding domains, they are called clusters of compliment-like repeats (CCR).[21, 25]. These binding sites could recognize more than one hundred structurally and functionally diverse ligands such as

α_2-Macroglobulin (α_2M), tissue-type plasminogen activator (tPA)[26], receptor-associated protein (RAP) ,and matrix metalloproteinases (MMP-13, -2 and -9)[27].

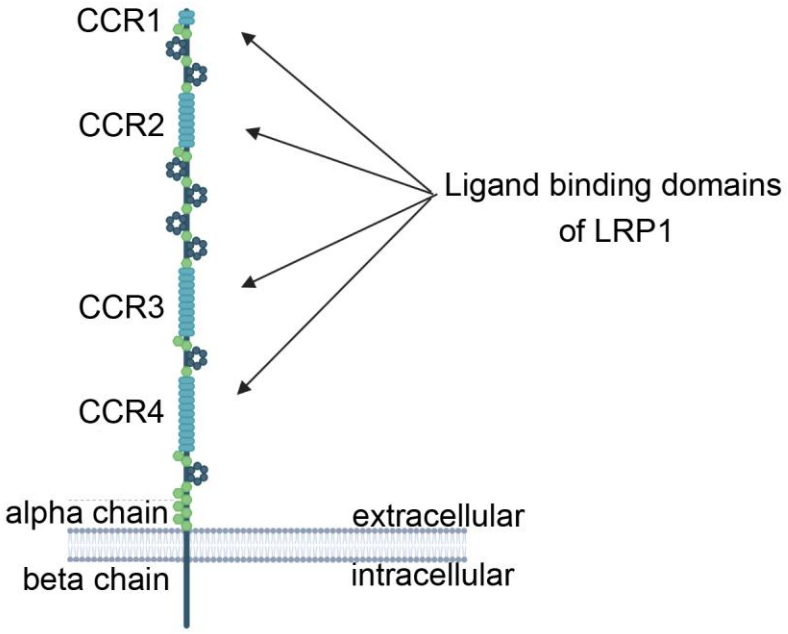

Figure 1.3 - Schematic illustration of LRP1. The alpha chain contains the intracellular and transmembrane regions to anchor LRP1, beta chain contains four ligand-binding domains. Created with BioRender.

1.4 The role of LRP1 in nerve injury and neuroinflammation

LRP1 functions in receptor-mediated endocytosis and cell signaling, It is also a mediator and regulator in neuroinflammation.

In the central nervous system, the conditional forebrain LRP1 knock-out in mice led to neuroinflammation in the brain. Microglia and astrocytes were activated in the hippocampus after LRP1 deletion, expression levels of the pro-inflammatory cytokines like interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 were elevated[28]. Silencing of LRP1 in the brain of the APP/PS1 mice exacerbated inflammatory response via TLR4-mediated NF-κB and MAPK signaling pathway and increased neuronal loss[29].

In the peripheral nervous system, in the injured sciatic nerves of rats, the β-chain was truncated while intact α-chain of LRP1 was shed from the Schwann cells, this process eliminated anti-inflammatory membrane-bound LRP1 and generate soluble pro-inflammatory shed LRP1 (sLRP1)[30]. The injection of sLRP1 in the rat sciatic nerve prior to chronic constriction injury (CCI) antagonizes proinflammatory cytokine expression. In cultures of Schwann cells, astrocytes, and microglia, sLRP1 inhibited TNF-α–induced activation of phosphorylated (phospho)-38 and phospho -ERK[31].

The term Wallerian degeneration has been used to describe the response follow the traumatic injury in the nervous system, including the dedifferentiation of Schwann cells, the break-down of the myelin sheath, and the up-regulation and down-regulation of genes. The activation of the transcription factor c-Jun in Schwann cells could control the phenotype of denervated Schwann cells and works as a global regulator of Wallerian degeneration[32].

In the earlies stage of injury, LRP1 induces c-Jun phosphorylation, indicating Schwann cells LRP1 functions as an injury detection receptor in peripheral nerve system[33].

After a sciatic nerve crush injury, the immunopositivity of LRP1 increases in Schwann cells, *in vitro* studies also indicate the LRP1-dependent cell signaling could promote the survival of Schwann cells in response to serum deprivation or TNF-α[24]. LRP1 also promotes Schwann cell migration which is important for sustaining axons and nerve regeneration[34]. Thus, targeting LRP1 to active Schwann cells may be a new method to treat neuropathic pain.

1.5 Neuropathic pain in the peripheral and central nervous system

Neuropathic pain is caused by a lesion or disease of the somatosensory system, it is generally chronic, patients typically experience burning or electrical-link sensations, and pain resulting from non-painful stimulations[35]. According to a population-based survey, neuropathic pain has a prevalence of 5.1% in the general population[36]. The pathogenic mechanisms focused mainly on the role of neurons in the past[37]. Following nerve damage, both central and peripheral sensitization plays an important role in neuropathic pain mechanisms[35], including the abnormal signal transduction as well as homosynaptic and heterosynaptic sensitization[38]. Besides the changes in the activities of neurons, accumulating evidence suggests that non-neuronal cells such as glial cells are also critical in the pathogenesis and resolution of pain[39]

Depending on the impaired nerve, neuropathic pain can be classified into two categories: central neuropathic pain and peripheral neuropathic pain. They share some

common mechanisms like pathologic active or sensitized nociceptors and persistent inflammatory reactions[40]. Central neuropathic pain can result from any type of injury to the central nervous system and syrinx formation in the spinal cord or brainstem. It is challenging to distinguish central neuropathic pain from peripheral neuropathic pain in patients who are neurologically impaired, central neuropathic pain often occurs months or years after the original central nervous system damage[41]. Following the spinal cord injury, microglia accumulation occurs, they produce pro-inflammatory markers such as brain-derived neurotrophic factor (BDNF), chemokines, and cytokines, causing increased numbers of microglia cells, and excess neuronal excitability[42,42b]. Peripheral neuropathic pain associates with damage that affects the peripheral nervous system. Patients with neuropathic pain exhibit a variety of pain-related sensory symptoms and signs[43]. The cell bodies of the primary sensory neurons are located in dorsal root ganglia (DRG)[44], peripheral sensitization comes from ongoing DRG activity and oscillations. The DRG neurons after nerve injury undergo an increase in the intrinsic electrogenic properties which result in hyperexcitability and develop into neuropathic pain. Among all the important ion channels, including sodium, potassium, calcium channels, the upregulation of voltage-gated sodium channels plays a major role in hyperexcitability[45]. Macrophages are recruited in response to peripheral nerve injury, they phagocytose leukocytes and remove dying tissue debris in Wallerian degeneration. Schwann cells also undergo remarkable changes, synthesize a range of potent biological molecules, including nerve growth factor (NGF), TNF-α, IL-1β, IL-6.

1.6 SP16, a peptide agonist to LRP1

Based on the known structure-activity relation of the LRP1 binding site, a series of short peptides ranging from 11 to 38 amino acids in length were synthesized. Based on several *in vitro* assays, a small peptide containing 17 amino acids was chosen. After modification, the peptide was designated as SP16 (Ac-VKFNKPFVFLNleIEQNTK-NH2). SP16 is derived from a serine protease inhibitor, known as SERPINs. SP16 mimics the binding of the endogenous SERPIN like alpha 1 antitrypsin (AAT). *In vitro,* SP16 binds with high affinity to LRP1, SP16 significantly inhibited NF-kB activation induced by LPS or Gp96 in monocytes in comparison with a scrambled control peptide or vehicle solution. The treatment with an LRP1 blocking antibody significantly reduced the anti-inflammatory signal, indicating that SP16 functions as an LRP1 agonist[46].

Previous studies have shown that some LRP1 agonists could promote the neurite outgrowth and ERK cell signaling activation in sensory neurons[47], but these effects can be blocked by an LRP1 competitive antagonist, receptor associate protein (RAP)[27], which indicated the LRP1 dependent pathway. Besides sensory neurons, in primary Schwann cells, LRP1 ligand matrix metalloproteinases-9 (MMP9) induced the phospho -Akt and phospho -ERK cell signaling. When the cells were treated with RAP, the LRP1 ligand did not activate cell signaling[34]. All these data confirmed the LRP1-dependent activity.

Because SP16 has been shown to regulate Akt and NFkB cell signaling in macrophage-like cells via an LRP1 dependent manner[46], we hypothesize that SP16 activates cell signaling in sensory neurons as an LRP1 agonist. Considering that LRP1 could promote Schwann cells adhesion and migration and the important role LRP1 plays in neuropathic pain, SP16 may also be able to treat neuropathic pain as an LRP1 agonist. Thus, we designed experiments to determine the bioactive effects of SP16 in the peripheral nerve.

Chapter 2

Materials & Experimental Methods

2.1 Animals

Male Sprague Dawley rats (170-200g; 8-12 weeks old) and C57BL/6J mice (25 g; male and female, 8-12 weeks old) were purchased from Envigo and Jackson Laboratory, respectively. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. All rats and mice were housed with a 12 h: 12 h light: dark cycle with ad libitum access to food and water.

2.2 Cell signaling analysis

Rat PC12 cells were from ATCC (CRL-1721). PC12 cells were maintained in DMEM (high glucose; Gibco) containing 10% heat-inactivated FBS (Gibco), 5% heat-inactivated horse serum (HyClone), penicillin (100 units/ml) and streptomycin (1mg/ml) in 6-well plates that were pre-coated with 0.01 mg/ml type IV collagen (Sigma-Aldrich). Cells were transferred to serum-free medium (SFM) 4 hours prior to adding effectors, and then treated with SP16 (50 or 500 ng/mL); NGF (50 ng/mL); or vehicle (PBS) for 10 min. In some cases, cells were pre-incubated with the competitive antagonist of LRP1, GST-RAP (150 nM) for 15 min. Cells were rinsed with ice-cold PBS and proteins were extracted in RIPA buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Roche Diagnostics). After 30 min on ice, lysates

were centrifuged at 15,000 x g for 5 min, supernatant collected and stored at -20C. Equal amounts of protein from cell lysates (20 µg), as determined by BCA Protein Assay (Thermo Scientific), were subjected to 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk and then incubated with primary antibodies that target phospho-ERK1/2 (1:1000, Cell Signaling Technology, catalogue no.9101L). or total-ERK1/2 (1:1000, Cell Signaling Technology, catalogue no.9102L). Immunoblots were developed using Radiance, Radiance Q, and Radiance Plus chemiluminescent substrates (Azure Biosystems) and imaged using the Azure Biosystems c300 digital system.

2.3 Neurite outgrowth in primary cultures of adult DRG neurons

Primary DRG neurons were isolated from adult male Sprague Dawley rats and cultured as previously described by us (Yoon et al., 2013). The DRGs were stripped of their roots and collected in Hank's Buffered Salt Solution (HBSS) on ice. DRGs were enzymatically digested and approximately 4000 DRG neurons were plated in each well of a 12-well tissue culture plate (Thermo Scientific, Logan, UT). All DRG neurons were cultured at 37°C in 5% CO2 for 54 hours in DMEM/F12 containing 2% B27 and 1%FBS with vehicle or SP16 (0-500 ng/mL) added every 24 hours. Primary cultured DRG neurons were imaged by phase contrast and the viability of cells was assessed by

Trypan blue. Primary DRG neurons were cultured, fixed in 4% paraformaldehyde, and immunofluorescence was performed using a mouse anti-βIII-tubulin (1:250, Promega, Fitchburg, WI) primary antibody, and then with Alexa Fluor-488 anti-mouse antibody (1:1000, Life Technologies, Carlsbad, CA) as the secondary antibody. DRG neurons were imaged at 20X and 40X manually, and the longest neurite length per cell was measured in 11 images from multiple wells and separate experiments. Approximately 222 and 144 neurons were measured in SP16 and control groups, respectively. Quantification was performed in a blinded manner. For all neurite outgrowth measurements, at least 6 individual experiments were performed in duplicate.

RT-qPCR

RNA was isolated from DRG cultures using the NucleoSpin RNA kit (MachereyNagel) and reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using TaqMan gene expression products (Thermo Fisher Scientific) for GAP-43 (Rn01474579), IL-1b (Rn00676333) and GAPDH (Rn99999916_s1). The relative change in mRNA expression was calculated using the 2ΔΔCT method with GAPDH mRNA as an internal normalizer.

2.4 Intraplantar formalin and capsaicin models

Male mice (N=14) were acclimated to the behavior testing facility for at least 60 min. Mice were randomized into two groups SP16 (2 µg/g) or vehicle (H20) were administered subcutaneously. After one hour, 20µL of 2.5% formalin was injected subcutaneously into the plantar area of the left hind paw. Immediately after formalin injection, mice are placed in a Plexiglas box (22x22x14 cm), and two observers, blinded to treatments, recorded the total amount of time mice spent on licking, shaking, holding ,and flinching the left hind paw every 5 minutes over a 60 minute period. To quantify the formalin response, activity during phase 1 (0-10 min) and phase 2 (20-40 min) were examined separately.

For the capsaicin studies, male (n=29) and female (n=23) mice were acclimated to the behavior testing facility for at least 60 min. Capsaicin was dissolved in a 20% (2-Hydroxypropyl)-β-cyclodextrin (Sigma) solution. This vehicle concentration solubilized the capsaicin and did not induce a behavioral response when administered alone. One hour prior to intraplantar injections, vehicle, SP16 (2 µg/g; s.c.) or enzymatically inactive tPA (EI-tPA; 2 µg/g i.v.) were administered. Subsequently, 10µL of 2µg/µL capsaicin solution was injected into the plantar area of the left hind paw. Immediately after capsaicin injection, mice were placed in a plexiglass box (22x22x14 cm), and two observers, blinded to treatments, recorded the time spent licking, shaking, holding, and flinching the left hind paw over 10 min.

2.5 Neuropathic pain models

Mice (n=30) were randomly assigned to two different groups: SP16 (2 µg/g; s.c. 100 µl) and vehicle (H20 s.c. 100 µl). Mice were treated 60 min prior to partial nerve ligation (PNL) and then daily at least 1 hour prior to behavior testing.PNL studies were performed as we previously described and modified for mice[48]. Male mice were anesthetized with 3% isoflurane (Vetone) in 1.5L/min oxygen (Praxair) and maintained with 2.5% isoflurane. An incision was made along the long axis of the femur. The sciatic nerve was exposed at mid-thigh level by separating the biceps femoris and the gluteus superficialis and then carefully cleared of surrounding connective tissue. A 9–0 nylon suture (Ethicon) was inserted into the nerve and ligated so that one-third to one-half of the nerve was included. The muscle and skin layers were closed using 7mm stainless steel wound clips (Reflex7, CellPoint Scientific, Inc). For behavior testing, mice were acclimated and baseline tested for one week prior to PNL. Mechanical sensitivity (tactile allodynia) was tested by applying 0.04 to 4 g Von Frey filaments (Stoelting) to the plantar surface of the ipsilateral hind paw. Filaments were presented consecutively either ascending or descending using the up-down method as previously described[49] and modified for mouse[48b]. The filament that caused paw withdrawal 50% of the time (the 50% PWT) was determined. Tactile allodynia was tested on days 2, 4, 9, 11, and 14 days following PNL. Results were averaged and subjected to statistical analysis. All experiments were performed by an investigator blinded to mouse identity.

2.6 Immunoblots of the sciatic nerve

Sciatic nerves were harvested 2 days after PNL to identify early molecular and cellular changes. Approximately 0.5 cm of the sciatic nerve was collected distal from the ligation site. Ipsilateral and contralateral nerves were collected. Nerves were lysed in RIPA buffer and equal amounts of protein (20 µg) from nerves lysates, as determined by BCA Protein Assay (Bio-Rad), were subjected to 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk and then incubated with primary antibodies that target: TLR4(CD284)/MD2 (1:1000; Biolegend cat# 117601); CD11b (1:1000; Abcam, cat# Ab133357); or Glial Fibrillary Acidic Protein (GFAP) (1:1000; Dako, cat# Z0334). Primary antibodies were detected with HRP-conjugated species-specific secondary antibodies (Cell Signaling Technology). Immunoblots were developed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific), and the Protec Ecomax X-ray film processor. Densitometry analysis was performed using Image J software.

2.7 Immunohistochemistry of DRGs

For IHC studies, 4 µm thick DRG tissue sections were stained with antibodies to cd11b (1:4500). Slides were stained on a Ventana Discovery Ultra (Ventana Medical Systems). Antigen retrieval was performed using CC1 (tris-based; pH 8.5) for 40 minutes at 95oC. The primary antibody CD11b (Abcam, catalogue no. ab133357) was incubated

with the slides for 32 minutes at 37 oC. The secondary antibody (HRP-coupled goat anti-rabbit; OmniMap system; Ventana) was incubated on the sections for 12 min at 37℃.

Antibodies were visualized using diaminobenzidine as a chromagen followed by hematoxylin as a counterstain. Slides were rinsed, dehydrated through alcohol and xylene, and cover-slipped. Light microscopy was performed using a Leica DFC420 microscope with Leica Imaging Software 2.8.1(Leica Biosystems).
Integrated optical density analysis was performed using Image J software.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc.). All results are expressed as the mean ± S.E.M. Comparisons between two groups were performed using two-tailed unpaired or paired T-tests. When we compared greater than two groups, one-way ANOVA was performed. Measurements of inflammatory pain, in which we collected multiple observations in individual mice over time, were analyzed by repeated-measures ANOVA with a Schefe post hoc test. $P<0.05$ was considered statistically significant.

Chapter 3

In vitro cell signaling and neuron sprouting assays of SP16

3.1 SP16 demonstrates bioactivity in PC12 cells and primary cultured adult DRG neurons.

To determine whether SP16 has similar bioactive effects with other LRP1 ligands, we designed experiments *in vitro*.

First, we used PC12 cells for the cell signaling assay. We treated PC12 cells with vehicle or SP16 for 10 minutes and pretreated them with RAP for 15 minutes in some wells. Nerve growth factor (NGF) was also used to treat PC12 cells for 10 minutes and a cell signaling control.

The immunoblots of cell lysates showed that SP16 robustly induced phosphor -ERK and phospho -Akt cell signaling compared to vehicle-treated cells (**Figure 3.1A**). But in the cells treated with SP16 and RAP, the effects of SP16 were blocked with RAP, indicating that SP16 induced the cell signaling through an LRP1 dependent pathway.

Primary adult rat DRG neurons were cultured with vehicle or SP16 daily, 96 hours later, more abundant cell bodies can be observed in SP16 treated group, the neuronal network was also more extensive and dense compared to vehicle-treated group (**Figure 3.1B**).

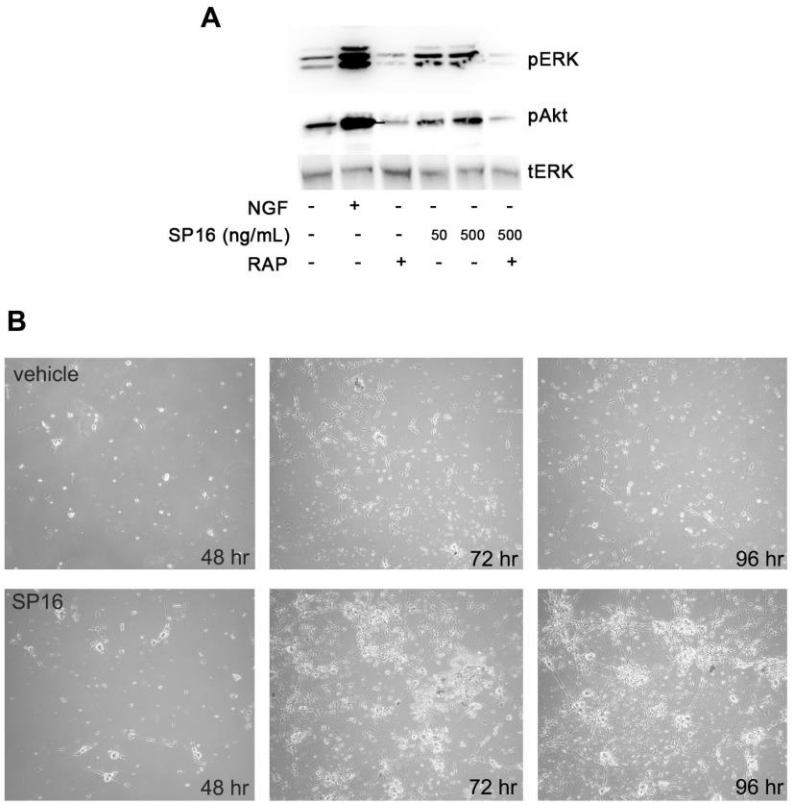

Figure 3.1- The LRP1 ligand, SP16 demonstrates bioactivity in PC12 cells and primary cultured adult DRG neurons. (**A**) Immunoblot analysis to detect phopsho-ERK1/2, phospho-Akt ,and total ERK1/2. PC12 cells treated with vehicle or SP16 (50 or 500 ng/mL) for 10 min and in some wells, pretreated with RAP (150 nM) for 15 min. NGF (50 ng/mL) for 10 min served as a cell signaling control. Equal amounts of protein lysates (20 µg) were loaded per lane. (**B**) Phase contrast images of primary adult DRG neuron cultures over time. Cultures were treated with vehicle or SP16 (100 ng/mL) daily for 96 hours. Note extensive neuronal networks and abundant neuronal cell bodies in cultures over time in the SP16 treated cells.

3.2 SP16 promotes neurite sprouting and expression of regenerative associated genes

Since the last experiment showed that after 96 hours of culture with SP16, primary adult DRG neurons had more cell bodies and a more extensive network, we wanted to see if SP16 could also promote the neurite sprouting.

To help to quantify the length of neurites, we used immunofluorescence to detect class III beta-tubulin (βIII-tubulin) in neurons. βIII-tubulin is a microtube protein, normally expressed in cells of neuronal origin[50]. Images of neurite sprouting revealed a greater extension of neurites in Sp16 treated cultures after 54 hours. The quantification of the longest neurites showed that SP16 significantly increased the length of neurites (***P < 0.005) (**Figure 3.2A, B**). In separate cultures, quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to measure mRNA levels of GAP-43 and IL-1β after 24 hours. GAP-43 is a major constituent of the growth cone upregulated during peripheral nervous system regeneration, also enables neurons to sprout new terminals[51]. IL-1β is a pro-inflammatory cytokine, it works as a mediator of inflammatory response[52].

The results of RT-qPCR showed that SP16 significantly induced the GAP-43 level (*P < 0.05) (**Figure 3.2C**). IL-1β also increased significantly, but the addition of RAP

blocked the induction, which proved the LRP1 dependent activities (*P **<** 0.05) (**Figure 3.2D**).

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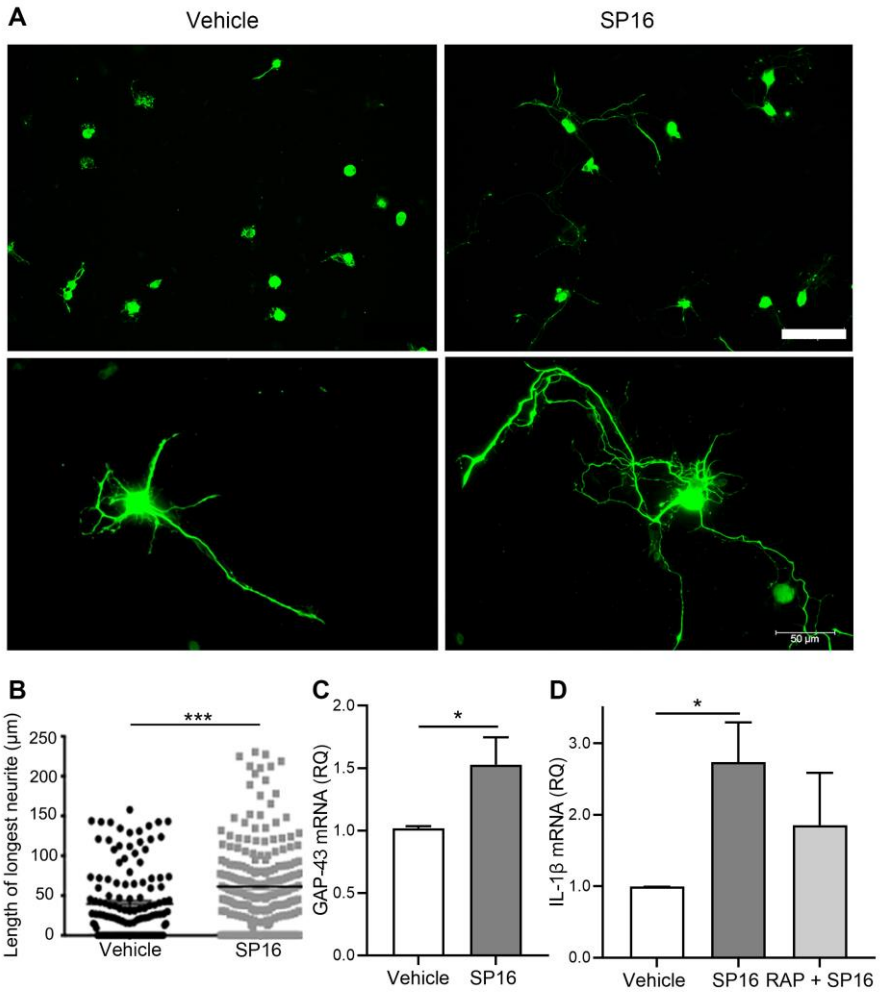

Figure 3.2 - SP16 promotes neurite length and expression of regenerative associated genes that are LRP1 dependent. (**A**) Representative image of immunofluorescence to detect bIII-tubulin in primary cultured adult DRG neurons in control and SP16 (500 ng/mL) treated cells. Upper panel, scale bar 100 µm. Lower panel, scale bar 50 µm. Note extensive neurite length in SP16 treated neurons. (**B**) Quantification of immunofluorescence in control (n=144 neurons) and SP16 (n=222 neurons) obtained from 11 distinct cultures. SP16 significantly increased maximum neurite length compared to controls (***P<0.005). (**C**) Primary cultured adult DRG neurons were treated with vehicle or SP16 (500 ng/mL) for 24 hours. RT-qPCR was performed to compare mRNA levels for GAP-43 (n=8 independent studies). (**D**) Primary cultured adult DRG neuron were treated with vehicle, SP16 (500 ng/mL) or SP16 (500 ng/mL) + RAP (150 nM) for 24 hours. RT-qPCR was performed to compare mRNA levels for IL-1b (n=5-8 independent studies). Data are expressed as mean ± SEM.

3.3 Acknowledgements

The cell signaling data in figure 3.1 were collected by Stefano Martellucci, PhD, a postdoctoral fellow in the Campana Lab.

The neurite sprouting data in figure 3.2 were collected by Alicia Van Enoo, a previous graduate student in the Campana Lab,

Chapter 4

The effects of SP16 in pain models

Our previous studies showed the bioactivity of SP16 in sensory neurons *in vitro*, based on those results, we hypothesized that SP16 may be able to regulate acute or neuropathic pain. To study the pain in animals, it is crucial to use the right pain model and an appropriate method to evaluate the pain level in animals. We used three distinct models to test the bioactive effects of SP16 in treating different kinds of pain.

4.1 Capsaicin test

The first pain model we performed was the capsaicin test. Capsaicin test also involves peripheral nociception. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a pungent chemical derived from hot peppers, when applied to the skin or injected into adult rats, it evokes an initial violent pain reaction and subsequent long-lasting desensitization to chemically-induced pain. Previous studies demonstrated that capsaicin depolarized cell bodies of dorsal root ganglion neurons and their axons[53].

Capsaicin was injected subcutaneously in the left hind paw and the amount of time the mice spent on elevating, licking, shaking the injected paw in five minutes was recorded. Capsaicin binds with the transient receptor potential vanilloid 1 receptor (TRPV1) as an agonist[54]. TRPV1 activation leads to a burning sensation and the increased sensitivity to heat and mechanical stimulation, promoting the allodynia and hyperalgesia[55]. These responses are triggered by the release of some proinflammatory

substances like substance P (SP) and calcitonin gene-related peptide (CGRP) from nerve endings[56].

Based on the structure of capsaicin (**Figure 4.1**), pure capsaicin is a hydrophobic compound, it is almost insoluble in cold water (28.93 mg/L at 25 °C). Due to the volume limit of subcutaneous hind paw injection and the response threshold of mice, the concentration of capsaicin in solution needs to reach 2μg/μL.

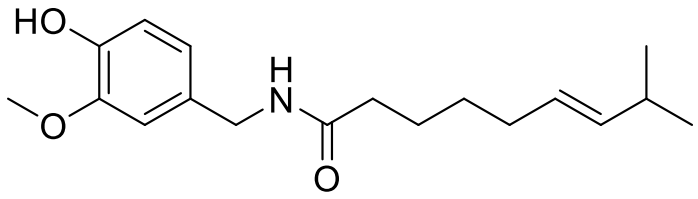

Figure 4.1 - Structural formula of capsaicin (8-methyl-N-vanillyl-6-nonenamide). Created with ChemDraw.

In previous literature about capsaicin test, dimethyl sulfoxide (DMSO)[53] or alcohol with Tween 80[57] [58]were used to increase the solubility of capsaicin in water. We have tried to use these organic solvents to dissolve capsaicin, but we found out that the vehicle alone will also cause obvious paw licking or lifting response in mice. Considering that the response caused by the vehicle may interfere with our evaluation of the analgesic effect of SP16, we chose a solubilizer to help dissolve capsaicin.

(2-Hydroxypropyl)-β-cyclodextrin (HPCD) is a derivative of β-cyclodextrin and has been used in improving the aqueous solubility of a variety of compound[59] and toxicology study shows that it is well-tolerated in mice[60]. It is a cyclic compound containing seven D-(+)-glucopyranose subunits, the CH_2 groups. and ether linkages face the hollow

interior of the configuration, form a hydrophobic cavity to interact with capsaicin molecule, the hydroxyl group face outside and form the hydrophilic exterior to interact with water (**Figure 4.2**).

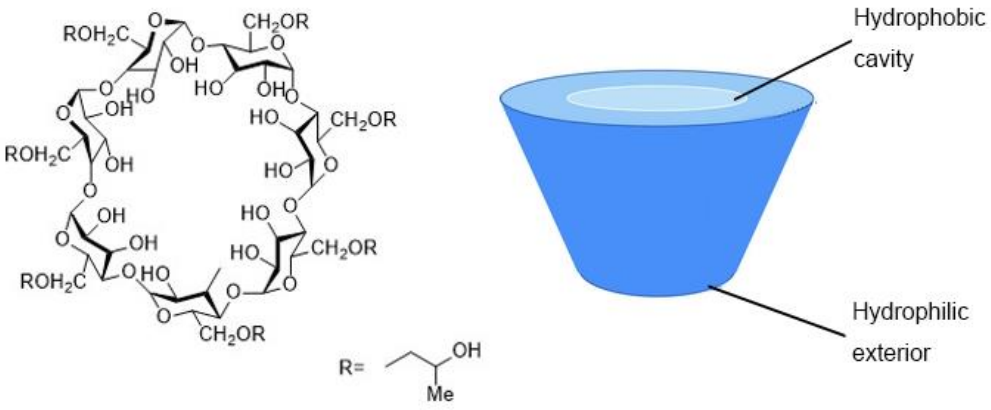

Figure 4.2 – The structure of (2-Hydroxypropyl)-β-cyclodextrin (HPCD). The hydrophobic cavity and hydrophilic exterior increased the solubility of capsaicin in an aqueous environment. Created with ChemDraw.

Since it is novel to use LRP1 agonists in pain models, we also tested a known LRP1 ligand, tissue-type plasminogen activator (tPA). It is a serine protease and major activator of the fibrinolytic system[61]. TPA can bind with LRP1 and activate pERK cell signaling in neuronal cells through LRP1 dependent pathway[62]. In our study, we used the enzymatically inactive tPA, in which the enzyme active site is muted, but it interacts with the LRP1 receptor equivalently to enzymatically active tPA to trigger signal transduction[63].

.We used both male and female mice in the capsaicin test. Mice were administrated SP16 or tPA or vehicle one hour prior to injection with capsaicin. Then

capsaicin (20μg/mouse) was injected subcutaneously into the left hind paw. Immediately after injection, a blinded observer began to count the total time of mice licking, shaking ,and holding the injected paw for 10 minutes. The capsaicin was dissolved in 20% HPCD solution, the HPCD solution was tested for subcutaneous hind paw injection and the response time of mice is very short compared to capsaicin solution. Surprisingly, males mice seem to be more sensitive to capsaicin compared to females (**P **<** 0.01) (**Figure 4.3A**). According to the results, both EI-tPA and SP16 treatment significantly reduced the total time of mice licking, shaking, and holding the paw in both male (**P **<** 0.05) (**Figure 4.3B**) and female (***P **<** 0.01) (**Figure 4.3C**) mice compared to the vehicle group.

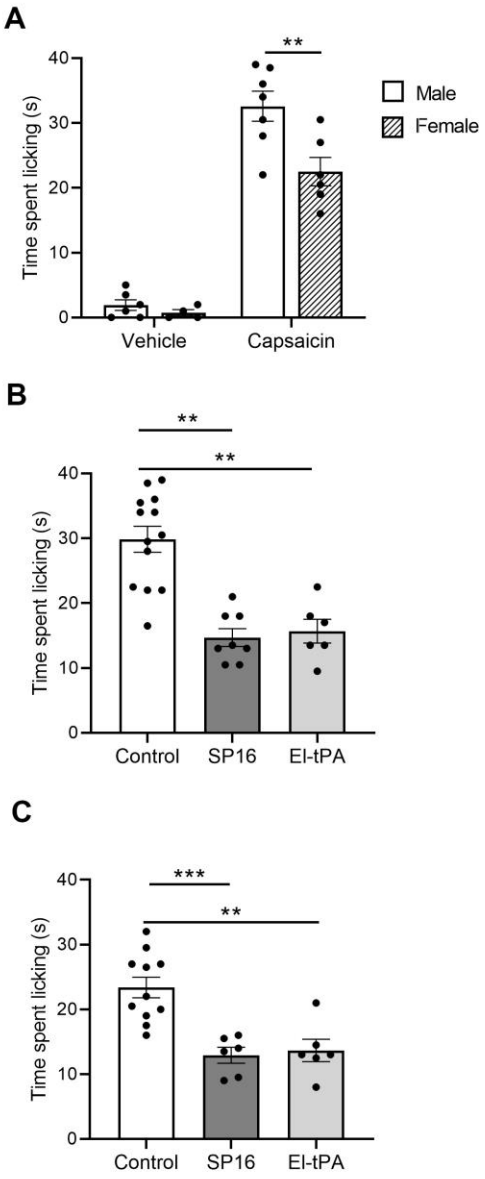

Figure 4.3 - Systemic LRP1 ligand administration attenuates acute spontaneous pain induced by intraplantar capsaicin. (**A**) Pain-related behavior (time spent licking) increased in both male and female mice after intraplantar injection of capsaicin (20 µg) when compared to vehicle (cyclodextrin; 20%). Male mice were more sensitive in their response than females (**P<0.01). Data are mean SEM (n=6 mice/group). (**B**) Preemptive administration of LRP1 ligands, SP16 (50 µg/mouse) or EI-tPA (50 µg/mouse) in male mice block capsaicin-induced acute pain over 5 minutes (**P<0.01). Data are expressed as mean ± SEM (n=6-13/group). (**C**) Preemptive administration of LRP1 ligands, SP16 (50 µg/mouse) or EI-tPA (50 µg/mouse) in female mice block capsaicin induced acute pain over 5 minutes (***P<0.001). Data are expressed as mean ± SEM (n=6-11/group).

Though SP16 has been tested to be an LRP1 agonist with high affinity, there are still some disadvantages of SP16. For example, the pharmacokinetics of SP16 is limited, it can easily be metabolized and excreted thus cannot maintain high plasma concentration after a single dose. The half-life of SP16 is 3.3 hour[46] and the solubility of SP16 in plasma is also limited. To improve the pharmacokinetics and activity, SP16 was optimized. We used the capsaicin test to test two of the generated analogs (**Figure 4.4A**). The first one is A15, the proline in SP16 was removed, the arginine head was added and the peptide is cyclized. The second one is A2-5, it is also cyclized and norleucine was removed for better absorption, hydrophilic amino acid was added for solubility and activity.

Due to the limit stock of the peptide analog and the solubility differences between SP16, A15, and A2-5, we were not be able to use the same concentration for these three peptides. All peptides were injected subcutaneously around the neck one hour prior to capsaicin injection. For SP16, we administrated 50μg before the capsaicin test, but for A15 and A2-5, we administrated 2.7μg and 5μg respectively. Though the dose of A2-5 was 10 fold less than SP16, A2-5 could significantly reduce the licking time and there is no significant difference between the SP16 group and A2-5 group (*P **<** 0.05). The average licking time in the A15 group was also lower than the vehicle group though there was no significance (**Figure 4.4B**). The effects of SP16 optimized analogs in the capsaicin test implied the possible higher potential and bioactivity compared to SP16.

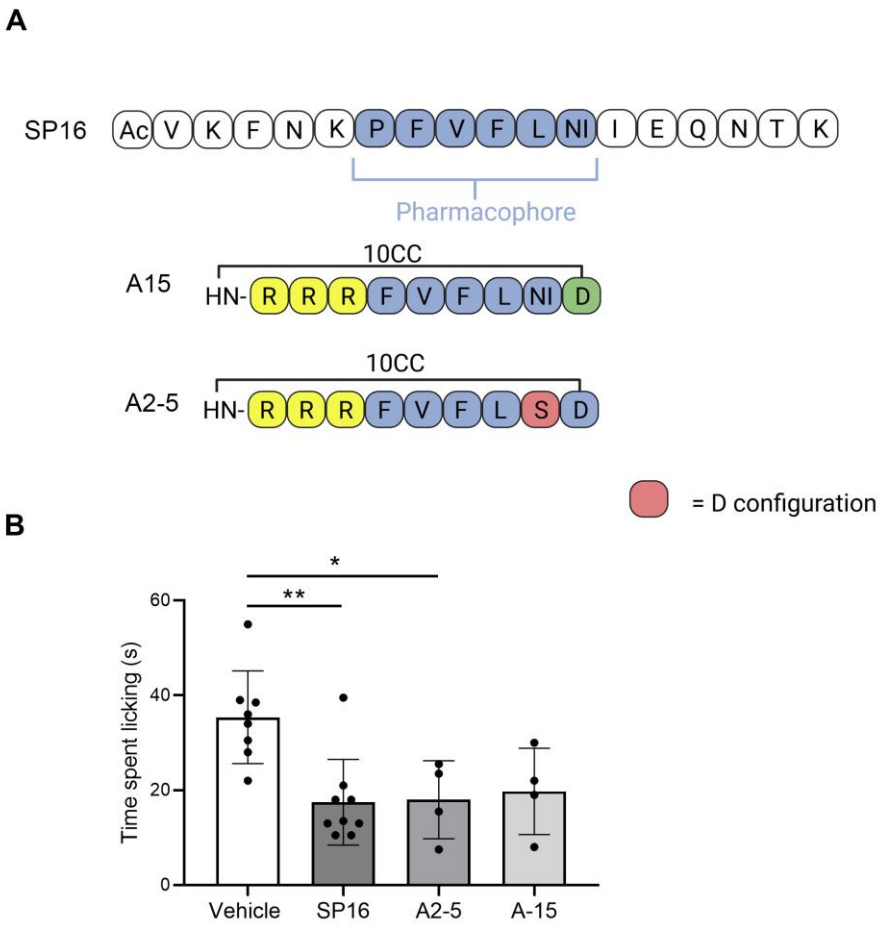

Figure 4.4 – Systemic SP16 analogs administration attenuates acute spontaneous pain induced by intraplantar capsaicin. (**A**) The optimized analogs (A15, A2-5) of SP16. (**B**) Preemptive administration of peptide analogs, SP16 (50 µg/mouse) or A2-5 (5 µg/mouse) in male mice block capsaicin-induced acute pain over 5 minutes (**P<0.01) (*P<0.05). No significant difference between the A-15 and vehicle group. Data are expressed as mean ± SEM. (n=4-9/group).

4.2 Partial sciatic nerve ligation

Partial sciatic nerve ligation (PNL) is also known as Seltzer model, it is a widely used model for neuropathic pain. The sciatic nerve will be exposed at a high-thigh level; l/3-1/2 of the nerve thickness was trapped in the ligature (**Figure 4.5**). After the surgery, animals will show the signs of spontaneous pain and allodynia to mechanical stimulation[48a]. Compared to other neuropathic pain models like chronic constriction injury (CCI), which comprises 3 to 4 loose ligations of the sciatic nerve; and tight ligation of the spinal nerve (SNL), PNL is a model that induces tactile allodynia for more than 3 weeks[48a].

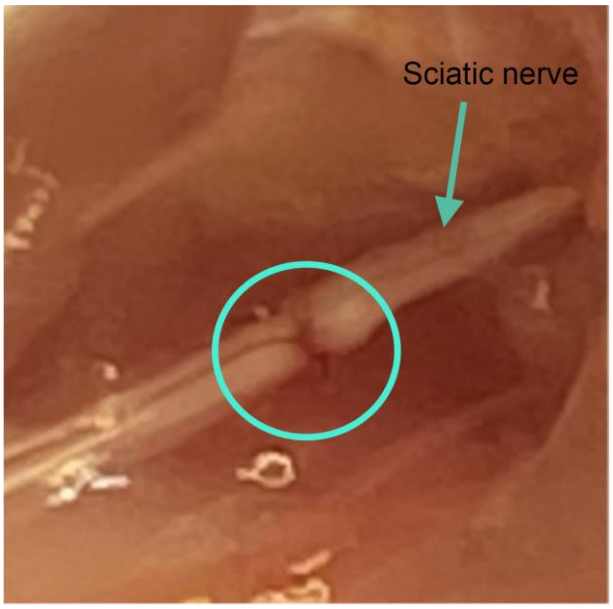

Figure 4.5 – Partial sciatic nerve ligation in a mouse. A 9-0 nylon suture is used to tie off 1/2-1/3 of the sciatic nerve. A double knot is applied to make tight ligatures and cause pain.

To evaluate and measure the level of mechanical allodynia caused by PNL, we used von Frey hairs ranging from 0.04 grams to 4 grams. The 50% paw withdrawal threshold (PWT) was determined by the Chaplan method[49]. The hair was presented perpendicular to the plantar surface with sufficient force to cause slight buckling against the paw and held for approximately 3-5 s. A positive response was recorded if the paw sharply flinched. All measurements started with the same von Frey hair, if the animal responds, move to a stiffer filament; if there is no response, move to a softer filament. Animals will be tested nine times at a five-minute interval or until the hair strength reaches the upper or lower limit.

The mice were acclimated to the room and apparatus for the Von frey test for several days, then baseline tested for a week prior to PNL surgery. All mice have similar 50% PWTs at the beginning of the study (2.21±0.37g). After the PNL surgery, a decrease in 50% PWT indicates “a painful” response or tactile allodynia. In vehicle-treated mice, reduced PWTs were observed, as anticipated. Four days after the surgery, the mice in the control group developed more significant allodynia while SP16 prevented the development and maintenance of neuropathic pain-related behaviors, the 50% PWTs in SP16 treated group is significantly higher (*$P < 0.01$) than in the control group (**Figure 4.6A**). After day four post-surgery, the mice in the control group were recovering from allodynia and the effect of SP16 got weaker, until two weeks after the surgery, the 50% withdrawal thresholds in the two groups were similar again.

To better understand the molecular and cellular mechanism underlying the effects of SP16 in pain-related behaviors, the sciatic nerves were collected on day two and day seven post-surgery. The first marker we chose is CD11b. CD11b is a widely used marker for inflammation, it is expressed by inflammatory cells including macrophages which play an important role in peripheral nerve injury and repair[64], it regulated the adhesion and migration of macrophages to mediate the response to inflammation. In the sciatic nerve samples collected on day two post-surgery, CD11b level was examined by immunoblot (**Figure 4.6B**). Densitometric analysis revealed a ten-fold increase in cd11b+ cells in injured nerve compared to contra nerves (**P<0.01), which was significantly reduced by treatment with SP16 (* P<0.05) (**Figure 4.6C**). These findings provide evidence for the anti-inflammation effect of SP16. In samples collected on day seven post-surgery, there is no significant difference between the SP16 treated group and the vehicle group which suggests that SP16 delayed the infiltration of macrophages or activation of resident macrophages in the nerve (**Figure 4.7A, B**).

The second protein marker that interests us is toll-like receptor 4 (TLR4). It is a transmembrane protein and a member of the toll-like receptor (TLR) family that are involved in innate immunity[65]. It is most well known for its function in lipopolysaccharide (LPS) recognition, but recent studies revealed its irreplaceable role in neuropathic pain. TLR4 is expressed by microglia[66] and is crucial for glia activation signals[67]. TLR4 is critical for pain induction after nerve injury[67] and the administration of TLR4 antagonist suppresses the neuropathic pain in sciatic nerve injured mice[68].

We performed immunoblot analyses on day two and day seven post-surgery nerve lysates to examine TLR4 expression. On day two post-surgery, the TLR4 level in injured sciatic nerve samples significantly increased compared to uninjured contralateral nerves (***P < 0.005). SP16 treatment significantly reduced the TLR4 upregulation to the uninjured level (***P < 0.005) (**Figure 4.6D, E**). On day seven, there is no significant difference in CD11b expression in all three groups, but the TLR4 level still showed a similar pattern to day two: vehicle-treated injured nerve samples significantly upregulated compared to SP16 treated group (*P < 0.05) and uninjured nerves (**P < 0.01) (**Figure 4.7A, C**). Studies have shown that the expression of TLR4 increases in sciatic nerves after nerve injury. *In vitro*, TLR4 helped cultured Schwann cells proliferate, migrate and prevent Schwann cells apoptosis[69]. However, *in vivo* studies showed TLR4 knocked-out mice had less myelin loss and less pro-apoptotic gene expression after nerve injury[70], activated TLR4 in Schwann cells also promote the infiltration of macrophages[71]. The immunoblots of TLR4 on day two and day seven post-surgery in the SP16 group suggest that daily SP16 treatment alleviates the TLR4 activation caused by traumatic injury response. The reduction of TLR4 on day seven in the SP16 group is less significant compared to day two post-surgery, may result from the subsequent decrease in LRP1 level after injury[24].

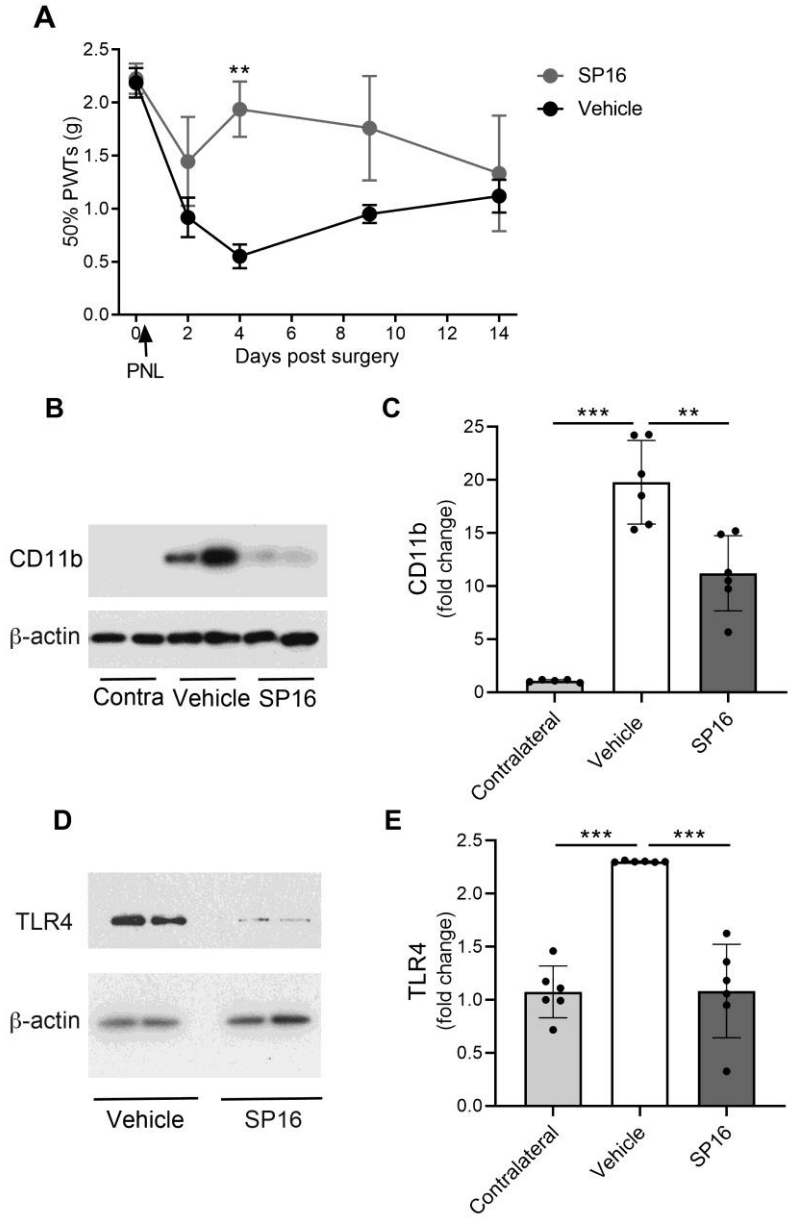

Figure 4.6- Continuous systemic SP16 treatment blocks the development of neuropathic pain and inflammatory cell recruitment after PNL. (**A**) Tactile allodynia is induced by PNL and sustained for 14 days. SP16 (50 µg) delivered daily and subcutaneously prevented the development of tactile allodynia that was significant on Day 4 (**P<0.01). Data are expressed as mean ± SEM (n=6-8 mice/group). Immunoblot analysis of sciatic nerve collected immediately distal to the ligation site two days after PNL in vehicle and SP16 treated C57BL6 mice. SP16 treatment reduced inflammatory cell infiltration (CD11b) (**B**) and activation of innate immunity (TLR4) (**D**) into the nerve. Densitometric analysis of CD11b (**C**) and TLR4 (**E**) levels in vehicle or SP16 treated PNL nerves. Data are expressed as mean ± SEM (n=6 mice/group).

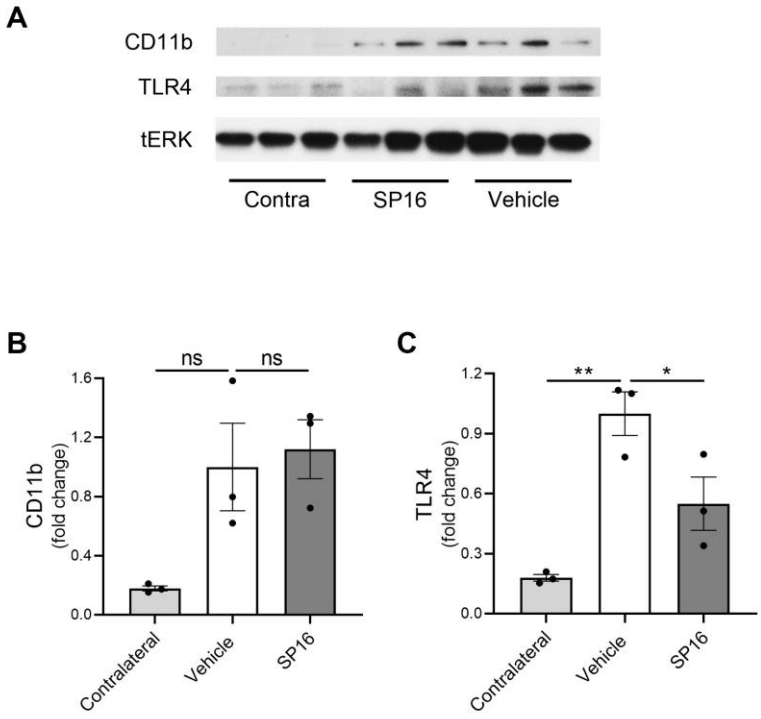

Figure 4.7 - Continuous systemic SP16 treatment reduces TLR4 until day seven post-surgery.(**A**) Immunoblot analysis of sciatic nerve collected immediately distal to the ligation site seven days after PNL in vehicle and SP16 treated C57BL6 mice. Densitometric analysis of CD11b (**B**) and TLR4 (**C**) levels in vehicle or SP16 treated PNL nerves.SP16 significantly reduced TLR4 on day seven compared to the vehicle group (*P＜0.05). There is no significant difference between vehicle and SP16 treated in CD11b level on day seven. Data are expressed as mean ± SEM (n=3 mice/group).

The L3-L5 DRGs were also collected on day two post-surgery. The quantification of DRGs by immunohistochemistry analysis showed that $CD11b^+$ inflammatory cells were abundantly recruited and/or expressed in DRGs in vehicle treated mice and consistent with pain behavior outcomes. However, in the DRGs of SP16 treated mice, there were very few $CD11b^+$ cells observed (**Figure 4.8A**), the quantification of the integrated optical density (IOD) revealed this significant downregulation (*P **<** 0.05) (**Figure 4.8B**). Glial fibrillary acidic protein (GFAP) is the intermediate filament in mature astrocytes[72], it is thought to help maintain mechanical strength, as well as the shape of cells. It is also involved in the functions of astrocytes, which are important in regeneration, synaptic plasticity, and reactive gliosis[73]. In DRG, the cell bodies of sensory neurons are wrapped by satellite glia cells (SGC), previous studies have shown that SGC respond to injury in the sciatic nerve or DRG by upregulating the GFAP immunoreactivity[74], our study showed consistent results. SGCs underwent division after nerve injury, functional connections between SGCs through gap junctions also increased[74b], and changes in gap junction coupling between SGCs can change nociception[75]. SP16 decreased the expression of GFAP in DRG neurons indicating a lesser activation of SGCs and less change in nociception. (***P **<** 0.005) (**Figure 4.8C**).

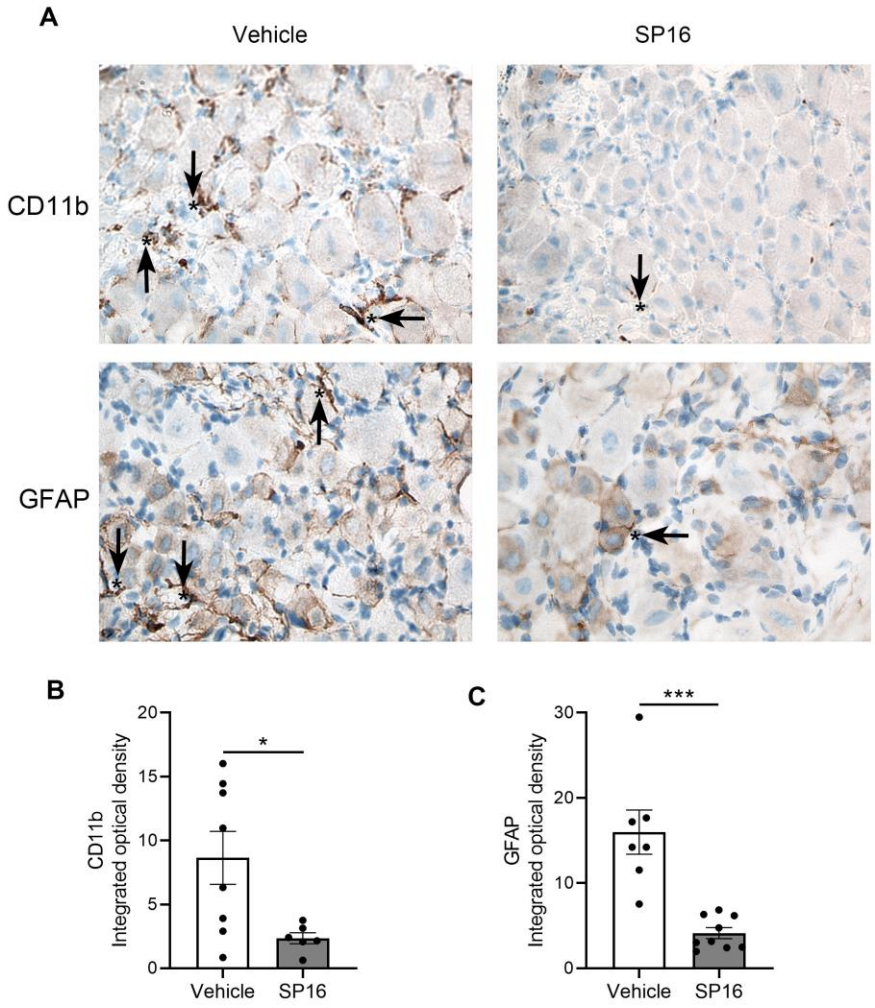

Figure 4.8 - Inflammatory cell recruitment into the DRG after PNL is reduced by SP16 treatment. (**A**) Transverse sections of L4 DRG immunostained for GFAP after vehicle or SP16 treatment two days post PNL. Note abundant brown immunoreactivity identifying CD11b (black arrows) in between neuronal cell bodies and close to blood vessels in injured vehicle treated DRG. CD11b immunoreactivity in SP16 treated DRGs is minimal. Nuclei are stain with hematoxylin (blue). GFAP immunoreactivity was heavily localized to satellite cells surrounding large and small neuronal cell bodies (black arrows) in injured vehicle-treated DRGs. SP16 treatment showed no immunoreactivity in satellite cells surrounding large-diameter neurons, and minimally in small-diameter neurons. Nuclei are stain with hematoxylin (blue). Quantification of (**B**) CD11b and (**C**) GFAP immunoreactivity in vehicle and SP16 treated injured DRGs (n=6-8 fields in 3 mice). Data are expressed as mean ± SEM.

4.3 Formalin test

Formalin is the aqueous solution of 37% (weight/ weight) formaldehyde. Formalin test is a predominantly used test for nociception for mice and rats. We chose the formalin test as an inflammatory pain model for SP16. Unlike traditional nociception tests like the hot-plate method which is based on the phasic stimulus of high intensity, injured tissue caused by formalin could generate moderate and continuous pain which allows us to assess the response in a longer phase[76].

Similar to the capsaicin test, formalin was also injected subcutaneously into the hind paw of mice. After injection, the total time of the animal holding, licking, biting, and shaking the injured paw was recorded. The response shows a first phase and the second phase. The first phase is caused predominantly by the C-fiber activation due to peripheral stimuli, the second phase is involved with the inflammatory reaction in peripheral tissue and functional changes in the dorsal horn of the spinal cord[76]. Formalin test was widely used to screen analgesic and anti-inflammatory pain therapeutics.

SP16 and vehicle (50μg/mouse) were injected subcutaneously around the neck of the mice one hour prior to formalin injection. Immediately after formalin was injected subcutaneously in the left paw, the mice were observed by a blinded treatment observer for 50 minutes, the total time of paw elevating, licking, shaking was recorded. Compared to the vehicle group, the first acute nociceptive phase was reduced significantly in the

SP16 group (**P **<** 0.01) (**Figure 4.9A, B**), indicating that SP16 alleviated the pain caused by nociceptive stimuli and may modulate the sensory fiber activity. The peak of the second phase in the SP16 treated group was late for around five minutes compared to the vehicle group, suggesting that SP16 delayed the inflammation, the duration of the (**Figure 4.9A, C**).

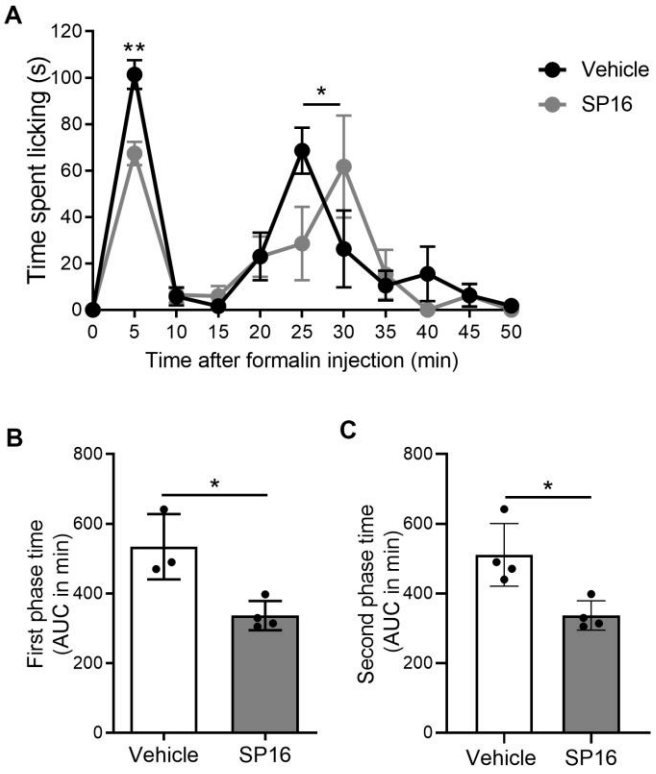

Figure 4.9 -. SP16 modulates sensory fiber activity and delays the onset of inflammation. (**A**) Time course of formal induced paw licking in C57BL6 mice. Vehicle or SP16 was given one hour prior to formalin injection into the hind paw. SP16 affects both the first acute nociceptive phase and the second inflammatory phase of the formalin test. (**B**) SP16 significantly reduced the duration of the first phase. (**C**) SP16 significantly delays the duration of the second phase (*P<0.05; **P<0.001). Data are expressed as mean ± SEM (n=5-6 mice/group).

4.4 Acknowledgements

All the peptides (SP16, A15, A2-5) were provided by Cohava Gelber, PhD, MBA and Dana Austin, MS and, Cyrille Gineste, PhD (protein chemist) from Serpin Pharma.

All the formalin test data in figure 4.8 were collected by Alicia Van Enoo and Dr. Wendy Campana.

All the surgical methods, behavior tests, and immunoblot skills were taught to me by Haylie Romeo and Alicia Van Enoo.

DRG immunohistochemistry was carried out by Don Pizzo at the histology core in the Department of Pathology.

Chapter 5

Conclusions & Future Directions

4.1 Conclusions

In vitro studies showed that a novel and innovative LRP1 ligand, SP16, is highly bioactive in sensory neurons. SP16 induced phospho -ERK and phospho -Akt through LRP1 dependent pathway, confirmed its role as an LRP1 ligand. In adult rat DRG neurons, SP16 not only improved the viability of cultured neurons, but also promoted neurite sprouting. These results are consistent with previous studies about LRP1 ligands.

In three distinct pain models, including acute, inflammatory, and neuropathic pain, LRP1 ligands blocked the development of pain-related behaviors, which suggests a novel role of LRP1 ligands in pain regulation and their potent anti-inflammatory activity. The three pain models we performed were all previously demonstrated and widely performed.

Immunohistochemistry analysis showed that LRP1 agonists limit inflammatory cell recruitment into both sciatic nerves and DRGs after traumatic nerve injury. This is important in pain regulation because in many types of neuropathies, macrophages recruitment into sciatic nerves and DRGs is important for pain regulation[77].

Our findings are consistent with previous studies showing that the membrane-bound LRP1 supports anti-inflammatory activities and SP16's role as an LRP1 agonist.

In murine macrophages, LRP1 promotes the anti-inflammatory phenotype[78]. In leukocytes, LRP1 binds apoptotic cells that produce an anti-inflammatory signal[79]. SP16

significantly inhibited NF-κB activation induced by LPS, after added the treatment of LRP1 blocking antibody, the anti-inflammatory signal of SP16 was significantly reduced, these results proved that SP16 functions as an LRP1 agonist[46]. In other *in vivo* models, SP16 treatment significantly decreased mortality in the endotoxemia model induced by LPS, a single SP16 dose in mice receiving myocardial ischemia-reperfusion injury reduced the infarct size and recruitment of leukocytes to the injury site compared to a scrambled peptide control, cardiomyocytes also showed greater survival[46]. In our PNL model, the recruitment of CD11b+ cells was reduced by SP16, this inflammatory cell population includes monocytes, macrophages, and granulocytes, we hypothesize that SP16 reduces inflammatory cells that include a diverse cell population. We propose that the anti-inflammatory activities of LRP1 are operational in nerve injury and are directly related to the regulation of pain-related behaviors, suggesting a novel role of LRP1 agonists in preventing the development of chronic pain.

LRP1 is a ubiquitous receptor and it is abundantly present in glia and neurons and in both PNS and CNS. Both EI-tPA and SP16 appear to cross the blood-brain-barrier, thus direct regulation of nociceptive cells in the spinal cord, brain as well as in periphery are possible. We observed the GFAP induction was reduced by SP16 after PNL surgery because GFAP is related to the activation of satellite cells which are involved in inflammatory cell hypersensitivity[80], it appears that LRP1 activation antagonizes glia immune interactions that contribute to pain. In the capsaicin model, both TRPV1 (receptor of capsaicin) and LRP1 locate on small-diameter neurons, that may de-activate other receptor systems. Recently, it has been shown that intraplantar injection of

capsaicin induces spinal astrocytes in the superficial laminae. Specifically, Hes5+ spinal astrocytes in superficial laminae appear to play a central role in gating brainstem descending control of hypersensitivity[81]. Further studies to determine the key cellular targets for regulating pain processing by LRP1 agonists in the PNS and CNS are underway.

There have been clues that LRP1 may be a potential target for treating pain because of its role in inflammation, glia cell activation, and myelination[82]. Our results provide evidence that LRP1 agonists prevent the development of distinct pain states that implicate both inflammatory cell regulation and regulation of DRG neurons. Fully elucidating the molecular and cellular role of LRP1 agonists in pain processing related to painful peripheral neuropathies will be an important goal.

4.2 Future directions

Our project demonstrated the bioactivity in cell signaling, neurite sprouting, and pain regulation. Peptides with higher affinity to LRP1 and activity can be developed based on SP16. More studies of SP16 and analogs are underway to extend the efficacy of the peptides. Future studies will elucidate the analgesic versus the anti-inflammatory nature of the LRP1 agonist peptides and their translational potential.

4.2.1 Optimization of SP16

As described in the capsaicin test part, the SP16 has some disadvantages that can be improved. The analog peptides A15 and A2-5 we tested in the capsaicin test came from two first and second-generation analog sets respectively. Computer-aided drug design helped us to understand the interaction between SP16 and the receptor. The pharmacophore model revealed that the proline only created a turn in the structure and did not interact with the receptor, norleucine and phenylalanine pointed to one direction while leucine and valine pointed to the opposite direction. To improve these, in the first generation of the analog set, proline was removed, cyclization was performed to lock the peptide in its “best” conformation and different sizes of the ring were attempted. Arginine head was also explored. According to *in vitro* tumor necrosis factor-alpha (TNF-α) enzyme-linked immunosorbent assay (ELISA), A15 was the most potent peptide.

Based on the first-generation analog sets, all the best features identified were combined in the second analog sets. Head-to-tail cyclization was used and norleucine was removed for better absorption. The hydrophilic amino acid serine was also inserted in different positions in the pharmacophore to increase solubility and activity. A2-5 was one of the most active peptides based on TNF-α inhibition and NF-κB activation effects. A2-5 only had half of the SP16’s size but it was more stable and much more active according to *in vitro* studies.

Start from A2-5, there was a third generation of the analog set, the context of the head-to-tail cyclization was modified, the sequence of amino acids in pharmacophore was also modified to figure out the structure-activity-relationship. An analog with a novel pharmacophore sequence called A3-14 was tested to be the most active peptide in the third set *in vitro*, but we have not had the chance to test it *in vivo*.

For future work, the *in vitro* study needs to be completed to test the stability of the peptides when exposed to key digestive proteases and plasma proteases. We also need to have a better understanding of the in vivo efficacy and pharmacokinetics in rodents. More formulation of the peptides can also be developed to achieve more administration routes, for example, transdermal, oral, or topical delivery. One more round of optimization may be performed if needed.

4.2.2 Follow-up research of SP16

Phase 1 clinical trial of SP16 shows safety in healthy volunteers, a single subcutaneous injection of SP16 up to 0.200 mg/kg (max dose of 12 mg) was well-tolerated, suggesting the translational potential of SP16[83]. In the future, we need more studies to have a better understanding of the mechanisms of SP16 and determine the molecular and cellular pathways activated and deactivated by LRP1 agonists during neuroinflammation and pain. Though our results showed that SP16 regulates three different kinds of pain, we could not prove that SP16 achieves these effects by binding with LRP1 receptors on Schwann cells, studies using Schwann cell LRP1 knock-out

mice were planned. We also need further studies to determine whether SP16 is a potential therapeutic for painful peripheral neuropathy such as chemotherapy-induced neuropathy. In the next step of the clinical trial, efficacy or pharmacodynamics in humans also need to be tested.

Appendix

REFERENCES

1. Menorca, R. M. G.; Fussell, T. S.; Elfar, J. C., Nerve physiology: mechanisms of injury and recovery. *Hand Clin* **2013,** *29* (3), 317-330.

2. Stewart, J. D., Peripheral nerve fascicles: Anatomy and clinical relevance. *Muscle & Nerve* **2003,** *28* (5), 525-541.

3. Jessen, K. R.; Mirsky, R.; Lloyd, A. C., Schwann Cells: Development and Role in Nerve Repair. *Cold Spring Harbor Perspectives in Biology* **2015,** *7* (7).

4. Joseph, N. M.; Mukouyama, Y.-s.; Mosher, J. T.; Jaegle, M.; Crone, S. A.; Dormand, E.-L.; Lee, K.-F.; Meijer, D.; Anderson, D. J.; Morrison, S. J., Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. *Development* **2004,** *131* (22), 5599-5612.

5. Schubert, T.; Friede, R. L., The Role of Endoneurial Fibroblasts in Myelin Degradation. *Journal of Neuropathology & Experimental Neurology* **1981,** *40* (2), 134-154.

6. Jessen, K. R.; Mirsky, R., The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* **2005,** *6* (9), 671-682.

7. Epelman, S.; Lavine, Kory J.; Randolph, Gwendalyn J., Origin and Functions of Tissue Macrophages. *Immunity* **2014,** *41* (1), 21-35.

8. Perry, V. H.; Brown, M. C.; Gordon, S., The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. *Journal of Experimental Medicine* **1987,** *165* (4), 1218-1223.

9. Chen, G.; Zhang, Z.; Wei, Z.; Cheng, Q.; Li, X.; Li, W.; Duan, S.; Gu, X., Lysosomal exocytosis in Schwann cells contributes to axon remyelination. *Glia* **2012,** *60* (2), 295-305.

10. Lemke, G., Neuregulin-1 and Myelination. *Science's STKE* **2006,** *2006* (325), pe11.

11. Griffin, J. W.; Thompson, W. J., Biology and pathology of nonmyelinating Schwann cells. *Glia* **2008,** *56* (14), 1518-1531.

12. Calvo, M.; Dawes, J. M.; Bennett, D. L. H., The role of the immune system in the generation of neuropathic pain. *The Lancet Neurology* **2012,** *11* (7), 629-642.

13. Shubayev, V. I.; Angert, M.; Dolkas, J.; Campana, W. M.; Palenscar, K.; Myers, R. R., TNFα-induced MMP-9 promotes macrophage recruitment into injured peripheral nerve. *Molecular and Cellular Neuroscience* **2006,** *31* (3), 407-415.

14. Scheib, J.; Höke, A., Advances in peripheral nerve regeneration. *Nature Reviews Neurology* **2013,** *9* (12), 668-676.

15. Chen, Y. Y.; McDonald, D.; Cheng, C.; Magnowski, B.; Durand, J.; Zochodne, D. W., Axon and Schwann Cell Partnership During Nerve Regrowth. *Journal of Neuropathology & Experimental Neurology* **2005,** *64* (7), 613-622.

16. Jessen, K. R.; Morgan, L.; Stewart, H. J.; Mirsky, R., Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. *Development* **1990,** *109* (1), 91-103.

17. Zhang, S.-J.; Wu, W.-L.; Yang, K.-Y.; Chen, Y.-Z.; Liu, H.-C., Phenotypic changes of Schwann cells on the proximal stump of injured peripheral nerve during repair using small gap conduit tube. *Neural Regen Res* **2017,** *12* (9), 1538-1543.

18. Cheng, C.; Zochodne, D. W., In vivo proliferation, migration and phenotypic changes of Schwann cells in the presence of myelinated fibers. *Neuroscience* **2002,** *115* (1), 321-329.

19. Garrison, C. J.; Dougherty, P. M.; Kajander, K. C.; Carlton, S. M., Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Research* **1991,** *565* (1), 1-7.

20. Triolo, D.; Dina, G.; Lorenzetti, I.; Malaguti, M.; Morana, P.; Del Carro, U.; Comi, G.; Messing, A.; Quattrini, A.; Previtali, S. C., Loss of glial fibrillary acidic protein (GFAP) impairs Schwann cell proliferation and delays nerve regeneration after damage. *Journal of Cell Science* **2006,** *119* (19), 3981-3993.

21. Neels, J. G.; van den Berg, B. M. M.; Lookene, A.; Olivecrona, G.; Pannekoek, H.; van Zonneveld, A.-J., The Second and Fourth Cluster of Class A Cysteine-rich Repeats of the Low Density Lipoprotein Receptor-related Protein Share Ligand-binding Properties*. *Journal of Biological Chemistry* **1999,** *274* (44), 31305-31311.

22. Rohlmann, A.; Gotthardt, M.; Hammer, R. E.; Herz, J., Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *The Journal of Clinical Investigation* **1998,** *101* (3), 689-695.

23. Moestrup, S. K.; Gliemann, J.; Pallesen, G., Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell and tissue research* **1992,** *269* (3), 375-82.

24. Campana, W. M.; Li, X.; Dragojlovic, N.; Janes, J.; Gaultier, A.; Gonias, S. L., The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. *J Neurosci* **2006,** *26* (43), 11197-11207.

25. Makarova, A.; Bercury, K. K.; Adams, K. W.; Joyner, D.; Deng, M.; Spoelgen, R.; Koker, M.; Strickland, D. K.; Hyman, B. T., The LDL receptor-related protein can form homo-dimers in neuronal cells. *Neuroscience Letters* **2008,** *442* (2), 91-95.

26. Willnow, T. E.; Orth, K.; Herz, J., Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *Journal of Biological Chemistry* **1994,** *269* (22), 15827-15832.

27. Strickland, D. K.; Gonias, S. L.; Argraves, W. S., Diverse roles for the LDL receptor family. *Trends in Endocrinology & Metabolism* **2002,** *13* (2), 66-74.

28. Liu, Q.; Trotter, J.; Zhang, J.; Peters, M. M.; Cheng, H.; Bao, J.; Han, X.; Weeber, E. J.; Bu, G., Neuronal LRP1 Knockout in Adult Mice Leads to Impaired Brain Lipid Metabolism and Progressive, Age-Dependent Synapse Loss and Neurodegeneration. *The Journal of Neuroscience* **2010,** *30* (50), 17068-17078.

29. He, Y.; Ruganzu, J. B.; Zheng, Q.; Wu, X.; Jin, H.; Peng, X.; Ding, B.; Lin, C.; Ji, S.; Ma, Y.; Yang, W., Silencing of LRP1 Exacerbates Inflammatory Response Via TLR4/NF-κB/MAPKs Signaling Pathways in APP/PS1 Transgenic Mice. *Molecular Neurobiology* **2020,** *57* (9), 3727-3743.

30. Brifault, C.; Gilder, A. S.; Laudati, E.; Banki, M.; Gonias, S. L., Shedding of membrane-associated LDL receptor-related protein-1 from microglia amplifies and sustains neuroinflammation. *Journal of Biological Chemistry* **2017,** *292* (45), 18699-18712.

31. Gaultier, A.; Arandjelovic, S.; Li, X.; Janes, J.; Dragojlovic, N.; Zhou, G. P.; Dolkas, J.; Myers, R. R.; Gonias, S. L.; Campana, W. M., A shed form of LDL receptor–related protein–1 regulates peripheral nerve injury and neuropathic pain in rodents. *The Journal of Clinical Investigation* **2008,** *118* (1), 161-172.

32. Arthur-Farraj, Peter J.; Latouche, M.; Wilton, Daniel K.; Quintes, S.; Chabrol, E.; Banerjee, A.; Woodhoo, A.; Jenkins, B.; Rahman, M.; Turmaine, M.; Wicher, Grzegorz K.; Mitter, R.; Greensmith, L.; Behrens, A.; Raivich, G.; Mirsky, R.; Jessen, Kristján R., c-Jun Reprograms Schwann Cells of Injured Nerves to Generate a Repair Cell Essential for Regeneration. *Neuron* **2012,** *75* (4), 633-647.

33. Flütsch, A.; Henry, K.; Mantuano, E.; Lam, M. S.; Shibayama, M.; Takahashi, K.; Gonias, S. L.; Campana, W. M., Evidence that LDL receptor-related protein 1 acts as an

early injury detection receptor and activates c-Jun in Schwann cells. *Neuroreport* **2016,** *27* (18), 1305-1311.

34. Mantuano, E.; Inoue, G.; Li, X.; Takahashi, K.; Gaultier, A.; Gonias, S. L.; Campana, W. M., The Hemopexin Domain of Matrix Metalloproteinase-9 Activates Cell Signaling and Promotes Migration of Schwann Cells by Binding to Low-Density Lipoprotein Receptor-Related Protein. *The Journal of Neuroscience* **2008,** *28* (45), 11571.

35. Colloca, L.; Ludman, T.; Bouhassira, D.; Baron, R.; Dickenson, A. H.; Yarnitsky, D.; Freeman, R.; Truini, A.; Attal, N.; Finnerup, N. B.; Eccleston, C.; Kalso, E.; Bennett, D. L.; Dworkin, R. H.; Raja, S. N., Neuropathic pain. *Nature Reviews Disease Primers* **2017,** *3* (1), 17002.

36. Bouhassira, D.; Lantéri-Minet, M.; Attal, N.; Laurent, B.; Touboul, C., Prevalence of chronic pain with neuropathic characteristics in the general population. *PAIN* **2008,** *136* (3).

37. Ji, R.-R.; Kohno, T.; Moore, K. A.; Woolf, C. J., Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends in Neurosciences* **2003,** *26* (12), 696-705.

38. Campbell, J. N.; Meyer, R. A., Mechanisms of Neuropathic Pain. *Neuron* **2006,** *52* (1), 77-92.

39. Ji, R.-R.; Chamessian, A.; Zhang, Y.-Q., Pain regulation by non-neuronal cells and inflammation. *Science* **2016,** *354* (6312), 572.

40. Baron, R., Peripheral neuropathic pain: from mechanisms to symptoms. *Clin J Pain* **2000,** *16* (2 Suppl), S12-20.

41. Watson, J. C.; Sandroni, P., Central Neuropathic Pain Syndromes. *Mayo Clinic Proceedings* **2016,** *91* (3), 372-385.

42. (a) Viswanath, O.; Urits, I.; Burns, J.; Charipova, K.; Gress, K.; McNally, A.; Urman, R. D.; Welschmeyer, A.; Berger, A. A.; Kassem, H.; Sanchez, M. G.; Kaye, A. D.; Eubanks, T. N.; Cornett, E. M.; Ngo, A. L., Central Neuropathic Mechanisms in Pain Signaling Pathways: Current Evidence and Recommendations. *Advances in Therapy* **2020,** *37* (5), 1946-1959; (b) Tang, Y.; Liu, L.; Xu, D.; Zhang, W.; Zhang, Y.; Zhou, J.; Huang, W., Interaction between astrocytic colony stimulating factor and its receptor on microglia mediates central sensitization and behavioral hypersensitivity in chronic post ischemic pain model. *Brain Behav Immun* **2018,** *68*, 248-260.

43. Baron, R.; Maier, C.; Attal, N.; Binder, A.; Bouhassira, D.; Cruccu, G.; Finnerup, N. B.; Haanpää, M.; Hansson, P.; Hüllemann, P.; Jensen, T. S.; Freynhagen, R.; Kennedy, J.

D.; Magerl, W.; Mainka, T.; Reimer, M.; Rice, A. S. C.; Segerdahl, M.; Serra, J.; Sindrup, S.; Sommer, C.; Tölle, T.; Vollert, J.; Treede, R.-D.; German Neuropathic Pain Research, N.; the, E.; consortia, N., Peripheral neuropathic pain: a mechanism-related organizing principle based on sensory profiles. *Pain* **2017,** *158* (2), 261-272.

44. Chaban, V. V., Peripheral sensitization of sensory neurons. *Ethn Dis* **2010,** *20* (1 Suppl 1), S1-6.

45. Chung, J. M.; Chung, K., Importance of Hyperexcitability of DRG Neurons in Neuropathic Pain. *Pain Practice* **2002,** *2* (2), 87-97.

46. Toldo, S.; Austin, D.; Mauro Adolfo, G.; Mezzaroma, E.; Van Tassell Benjamin, W.; Marchetti, C.; Carbone, S.; Mogelsvang, S.; Gelber, C.; Abbate, A., Low-Density Lipoprotein Receptor–Related Protein-1 Is a Therapeutic Target in Acute Myocardial Infarction. *JACC: Basic to Translational Science* **2017,** *2* (5), 561-574.

47. Yoon, C.; Van Niekerk, E. A.; Henry, K.; Ishikawa, T.; Orita, S.; Tuszynski, M. H.; Campana, W. M., Low-density Lipoprotein Receptor-related Protein 1 (LRP1)-dependent Cell Signaling Promotes Axonal Regeneration*. *Journal of Biological Chemistry* **2013,** *288* (37), 26557-26568.

48. (a) Seltzer, Z. e.; Dubner, R.; Shir, Y., A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **1990,** *43* (2), 205-218; (b) Poplawski, G.; Ishikawa, T.; Brifault, C.; Lee-Kubli, C.; Regestam, R.; Henry, K. W.; Shiga, Y.; Kwon, H.; Ohtori, S.; Gonias, S. L.; Campana, W. M., Schwann cells regulate sensory neuron gene expression before and after peripheral nerve injury. *Glia* **2018,** *66* (8), 1577-1590.

49. Chaplan, S. R.; Bach, F. W.; Pogrel, J. W.; Chung, J. M.; Yaksh, T. L., Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods* **1994,** *53* (1), 55-63.

50. Person, F.; Wilczak, W.; Hube-Magg, C.; Burdelski, C.; Möller-Koop, C.; Simon, R.; Noriega, M.; Sauter, G.; Steurer, S.; Burdak-Rothkamm, S.; Jacobsen, F., Prevalence of βIII-tubulin (TUBB3) expression in human normal tissues and cancers. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **2017,** *39* (10), 1010428317712166.

51. Benowitz, L. I.; Routtenberg, A., GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends in Neurosciences* **1997,** *20* (2), 84-91.

52. Dinarello, C. A., Biologic Basis for Interleukin-1 in Disease. *Blood* **1996,** *87* (6), 2095-2147.

53. Sakurada, T.; Katsumata, K.; Tan-No, K.; Sakurada, S.; Kisara, K., The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. *Neuropharmacology* **1992,** *31* (12), 1279-1285.

54. Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **1997,** *389* (6653), 816-824.

55. Geppetti, P.; Nassini, R.; Materazzi, S.; Benemei, S., The concept of neurogenic inflammation. *BJU International* **2008,** *101* (s3), 2-6.

56. Julius, D.; Basbaum, A. I., Molecular mechanisms of nociception. *Nature* **2001,** *413* (6852), 203-210.

57. (a) Gamse, R., Capsaicin and nociception in the rat and mouse. *Naunyn-Schmiedeberg's Archives of Pharmacology* **1982,** *320* (3), 205-216; (b) Schwartz, E. S.; Lee, I.; Chung, K.; Chung, J. M., Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. *PAIN* **2008,** *138* (3), 514-524.

58. Maia, J. L.; Lima-Júnior, R. C. P.; Melo, C. M.; David, J. P.; David, J. M.; Campos, A. R.; Santos, F. A.; Rao, V. S. N., Oleanolic acid, a pentacyclic triterpene attenuates capsaicin-induced nociception in mice: Possible mechanisms. *Pharmacological Research* **2006,** *54* (4), 282-286.

59. Shewale, B. D.; Sapkal, N. P.; Raut, N. A.; Gaikwad, N. J.; Fursule, R. A., Effect of hydroxylpropyl-beta-cyclodextrin on Solubility of Carvedilol. *Indian J Pharm Sci* **2008,** *70* (2), 255-257.

60. Gould, S.; Scott, R. C., 2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): a toxicology review. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **2005,** *43* (10), 1451-9.

61. Castellino, F.; Ploplis, V., Structure and function of the plasminogen/plasmin system. *Thrombosis and haemostasis* **2005,** *93 4*, 647-54.

62. Martin, A. M.; Kuhlmann, C.; Trossbach, S.; Jaeger, S.; Waldron, E.; Roebroek, A.; Luhmann, H. J.; Laatsch, A.; Weggen, S.; Lessmann, V.; Pietrzik, C. U., The Functional Role of the Second NPXY Motif of the LRP1 β-Chain in Tissue-type Plasminogen Activator-mediated Activation of N-Methyl-D-aspartate Receptors*. *Journal of Biological Chemistry* **2008,** *283* (18), 12004-12013.

63. Das, L.; Banki, M. A.; Azmoon, P.; Pizzo, D.; Gonias, S. L., Enzymatically Inactive Tissue-Type Plasminogen Activator Reverses Disease Progression in the Dextran

Sulfate Sodium Mouse Model of Inflammatory Bowel Disease. *The American Journal of Pathology* **2021,** *191* (4), 590-601.

64. Chen, P.; Piao, X.; Bonaldo, P., Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury. *Acta Neuropathologica* **2015,** *130* (5), 605-618.

65. Xu, Y.; Jagannath, C.; Liu, X.-D.; Sharafkhaneh, A.; Kolodziejska, K. E.; Eissa, N. T., Toll-like Receptor 4 Is a Sensor for Autophagy Associated with Innate Immunity. *Immunity* **2007,** *27* (1), 135-144.

66. Lehnardt, S.; Massillon, L.; Follett, P.; Jensen, F. E.; Ratan, R.; Rosenberg, P. A.; Volpe, J. J.; Vartanian, T., Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proceedings of the National Academy of Sciences* **2003,** *100* (14), 8514-8519.

67. Tanga, F. Y.; Nutile-McMenemy, N.; DeLeo, J. A., The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (16), 5856-5861.

68. Bettoni, I.; Comelli, F.; Rossini, C.; Granucci, F.; Giagnoni, G.; Peri, F.; Costa, B., Glial TLR4 receptor as new target to treat neuropathic pain: Efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* **2008,** *56* (12), 1312-1319.

69. Zhang, H.; Shao, Z.; Zhu, Y.; Shi, L.; Li, Z.; Hou, R.; Zhang, C.; Yao, D., Toll-Like Receptor 4 (TLR4) Expression Affects Schwann Cell Behavior in vitro. *Scientific Reports* **2018,** *8* (1), 11179.

70. Morzaev, D.; Nicholson, J. D.; Caspi, T.; Weiss, S.; Hochhauser, E.; Goldenberg-Cohen, N., Toll-like receptor-4 knockout mice are more resistant to optic nerve crush damage than wild-type mice. *Clinical & Experimental Ophthalmology* **2015,** *43* (7), 655-665.

71. Ye, W.; Liu, X.; Bai, Y.; Tang, N.; Wu, G.; Wang, X.; Cheng, J.; Liu, L., Sepsis Activates the TLR4/MyD88 Pathway in Schwann Cells to Promote Infiltration of Macrophages, Thereby Impeding Neuromuscular Function. *Shock* **2021,** *55* (1), 90-99.

72. Eng, L. F., Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. *Journal of Neuroimmunology* **1985,** *8*, 203-214.

73. Middeldorp, J.; Hol, E. M., GFAP in health and disease. *Progress in Neurobiology* **2011,** *93* (3), 421-443.

74. (a) Chudler, E. H.; Anderson, L. C.; Byers, M. R., Trigeminal ganglion neuronal activity and glial fibrillary acidic protein immunoreactivity after inferior alveolar nerve crush in the adult rat. *Pain* **1997,** *73* (2), 141-149; (b) Zhang, H.; Mei, X.; Zhang, P.; Ma, C.; White, F. A.; Donnelly, D. F.; Lamotte, R. H., Altered functional properties of satellite glial cells in compressed spinal ganglia. *Glia* **2009,** *57* (15), 1588-1599.

75. Jasmin, L.; Vit, J.-P.; Bhargava, A.; Ohara, P. T., Can satellite glial cells be therapeutic targets for pain control? *Neuron Glia Biol* **2010,** *6* (1), 63-71.

76. Tjølsen, A.; Berge, O.-G.; Hunskaar, S.; Rosland, J. H.; Hole, K., The formalin test: an evaluation of the method. *Pain* **1992,** *51* (1), 5-17.

77. Zhang, L.; Xie, W.; Zhang, J.; Shanahan, H.; Tonello, R.; Lee, S. H.; Strong, J. A.; Berta, T.; Zhang, J.-M., Key role of CCR2-expressing macrophages in a mouse model of low back pain and radiculopathy. *Brain, Behavior, and Immunity* **2021,** *91*, 556-567.

78. May, P.; Bock, H. H.; Nofer, J.-R., Low density receptor-related protein 1 (LRP1) promotes anti-inflammatory phenotype in murine macrophages. *Cell and tissue research* **2013,** *354* (3), 887-889.

79. Donnelly, S.; Roake, W.; Brown, S.; Young, P.; Naik, H.; Wordsworth, P.; Isenberg, D. A.; Reid, K. B. M.; Eggleton, P., Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. *Arthritis & Rheumatism* **2006,** *54* (5), 1543-1556.

80. Hanani, M.; Spray, D. C., Emerging importance of satellite glia in nervous system function and dysfunction. *Nature Reviews Neuroscience* **2020,** *21* (9), 485-498.

81. Kohro, Y.; Matsuda, T.; Yoshihara, K.; Kohno, K.; Koga, K.; Katsuragi, R.; Oka, T.; Tashima, R.; Muneta, S.; Yamane, T.; Okada, S.; Momokino, K.; Furusho, A.; Hamase, K.; Oti, T.; Sakamoto, H.; Hayashida, K.; Kobayashi, R.; Horii, T.; Hatada, I.; Tozaki-Saitoh, H.; Mikoshiba, K.; Taylor, V.; Inoue, K.; Tsuda, M., Spinal astrocytes in superficial laminae gate brainstem descending control of mechanosensory hypersensitivity. *Nature Neuroscience* **2020,** *23* (11), 1376-1387.

82. García-Fernández, P.; Üçeyler, N.; Sommer, C., From the low-density lipoprotein receptor-related protein 1 to neuropathic pain: a potentially novel target. *Pain Rep* **2021,** *6* (1), e898-e898.

83. Wohlford, G. F.; Buckley, L. F.; Kadariya, D.; Park, T.; Chiabrando, J. G.; Carbone, S.; Mihalick, V.; Halquist, M. S.; Pearcy, A.; Austin, D.; Gelber, C.; Abbate, A.; Van Tassell, B., A phase 1 clinical trial of SP16, a first-in-class anti-inflammatory LRP1 agonist, in healthy volunteers. *PLOS ONE* **2021,** *16* (5), e0247357.