Title

Orofacial Pain Operant Analysis with Concomitant Investigation of Peripherally Restricted Cannabinoids and Nav1.8 Axonal Accumulation for the Potential Treatment of Neuropathic Pain

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Orofacial Pain Operant Analysis with Concomitant Investigation of Peripherally Restricted Cannabinoids and $Na_V1.8$ Axonal Accumulation for the Potential Treatment of Neuropathic Pain

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Oral Biology

By

Yatendra Mulpuri

ABSTRACT OF THE DISSERTATION

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By

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Doctor of Philosophy in Oral Biology

University of California, Los Angeles 2015

Professor Igor Spigelman, Chair

The primary emphasis of this dissertation project was to study the pathogenesis of neuropathic pain, and to develop new treatment strategies targeting primary sensory neurons of the pain pathway. Chronic neuropathic pain is debilitating and is maladaptive. Current treatment options are moderately effective with serious side-effects. Translational progress of novel drugs is impeded by side-effects, which is partly due to reliance on inaccurate methods of pain evaluation in animals. Our first step was to develop a novel behavioral assay for quantitation of spontaneous pain in an animal model. Results from this study showed that rats with trigeminal neuropathic pain had reduced drinking efficiency in an orofacial operant assay, whereas control rats maintained normal levels throughout testing.

The second step of this project was to investigate novel peripherally restricted cannabinoids (PRCBs) for the effective treatment of neuropathic pain. Our initial testing in a rat model of sciatic nerve entrapment (SNE) revealed that, systemic administration of a PRCB (0.3 mg/kg) completely suppressed signs of mechanical allodynia between two and three hours. Based on

this information, we conducted a preliminary pharmacokinetic study that showed peak plasma drug levels at 2 hrs after administration with negligible traces of drug in the brain and cerebro-spinal fluid (CSF). Subsequently, we tested these novel PRCBs for CNS side-effects due to central activation of cannabinoid receptor subtype 1 (CB1R). Systemic and oral administrations at therapeutic and higher doses showed complete lack of CNS side-effects. These side-effects prevailed after systemic administration of centrally acting cannabinoid, HU-210 in the same group of animals. Site specific administrations and further evaluation in orofacial operant assay confirmed that anti-allodynic actions of PRCB are mainly mediated at the periphery. Our in vitro data showed that PRCBs are full agonists at CB1R and partial agonists at CB2R. Co-administration of PRCB (1 mg/kg i.g.) with receptor specific antagonists revealed that anti-allodynic actions are largely mediated by CB1R. Since tolerance is of main concern with repeated use of chronic pain medications, we tested the anti-allodynic efficacy of PRCB during repeated oral administrations (1 mg/kg, i.g.) and found no appreciable tolerance after two weeks of testing. Western blot analysis showed stable levels of CB1R in the L4-L5 dorsal root ganglia (DRG) of these animals after repeated testing.

The last research aim of this project was to investigate the axonal translocation of $Na_V1.8$ mRNA after peripheral nerve injury. The hyperexcitability of afferent nociceptors is induced by axonal accumulation of certain ion channels and the role of $Na_V1.8$ sodium channel is highly implicated. Therefore, we investigated the axonal translocation of $Na_V1.8$ mRNA. Our qPCR data showed abnormal accumulation of $Na_V1.8$ mRNA along with $Na_V1.6$ and $Na_V1.9$ mRNAs in the injured infraorbital nerve. Since the 3' untranslated region (UTR) is essential for sub-cellular localization of mRNAs, we did cloning and sequencing of $Na_V1.8$ mRNA 3' UTR. Results showed previously annotated short 3' UTR and an alternative long UTR that is produced as a result of alternative splicing and alternative polyadenylation.

The current research work demonstrated the use of orofacial operant assay for the evaluation of ongoing or spontaneous pain in a rat model of trigeminal neuropathic pain. In addition, our extensive pre-clinical testing of PRCBs for the evaluation of therapeutic efficacy, may aid in the development of these novel drugs as future therapeutics. In conjunction, the newly identified 3' UTR variant of $Na_V1.8$ mRNA may represent the pathological basis for abnormal axonal accumulation of $Na_V1.8$ mRNA after peripheral nerve injury.

The dissertation of Yatendra Mulpuri is approved.

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CONTENTS

LIST OF ABBREVIATIONS

IASP – Inernational association for study of pain

SP – Substance P

CGRP – Calcitonin gene related peptide

DH – Dorsal horn

VH – Ventral horn

TRP – Transient receptor potential

FDA – Federal drug administration

CFA – Complete Freund ’s Adjuvant

NK – Neurokinin

DRG – Dorsal root ganglia

TRG – Trigeminal root ganglia

SCN – Sodium Channel

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

NMDA - N-methyl-D-aspartate

NR – NMDA receptor

MAPK – Mitogen activated protein kinase

BDNF – Brain derived neurotrophic factor

PCR – Polymerase chain reaction

cDNA – complementary DNA

DC – Direct current

PrNMI - 4-{2-[-(1E)-1[(4-propylnaphthalen-1-yl) methylidene]-1H-inden-3-yl]ethyl} morpholine

CRPS – Complex regional pain syndrome

dsRNA – Double stranded ribonucleic acid

shRNA – short hairpin RNA

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

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Meyer, E.M. Long, V., Tsuchida, R., Mill, D., Ebreo, C., **Mulpuri, Y**., Fanselow, M.S. and Spigelman, I. Increased voluntary alcohol consumption in a rat model of stress enhanced fear learning. Society for Neuroscience abstracts, 2010; San Diego, CA (Poster presentation)

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Mulpuri, Y., Marechek, A., Malkin, D., Shiner, C., Hirt, E., Gilliam, A.F., Maitra, R., Snyder, R., Spigelman, I., Seltzman, H.H. Peripherally restricted CB1 receptor agonists for treatment of neuropathic pain. Society for Neuroscience Abstracts, 173.21, 2012; New Orleans, LY (Poster presentation)

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Chapter I - Introduction

Introduction

Pain is essential for the survival of the organism as it acts as a warning signal to the individual about the potential environmental danger. The International Association for the Study of Pain (IASP) describes Pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Acute pain as a result of tissue injury is treated successfully and mechanisms underlying acute nociception are well understood. Sometimes an unrelieved acute pain can become chronic and maladaptive. Chronic pain serves no biological importance to the individual as it has no defined end point and is very difficult to manage because the underlying mechanisms are poorly understood. Generally, chronic pain syndromes of neuropathic origin have been described as more severe because lesions directly involve components of the somatosensory nervous system [1]. Depending on the site of lesion, neuropathic pain can be classified as central or peripheral. Central neuropathic pain most commonly results from spinal cord trauma, multiple sclerosis or stroke[2]. Frequent causes of peripheral neuropathy are direct damage of peripheral nerves as a result of accidents or iatrogenic procedures, metabolic disturbances due to diabetes or drugs and viral infections such as herpes zoster and HIV [3,4]. Current treatments for neuropathic pain are moderately effective with serious side-effects.

The current thesis work is primarily focused on the behavioral, pharmacological and molecular aspects of peripheral neuropathy. The pathophysiological mechanisms and treatment options described here are pertinent to neuropathy of peripheral origin, although some occasional references have been made to indicate central nervous system changes.

1.1 Neuropathic pain:

According to definition by IASP, neuropathic pain is defined as “pain caused by a disease or lesion of the somatosensory nervous system”.

1.2 Signs and symptoms:

Two most common signs of neuropathic pain along with ongoing pain are allodynia and hyperalgesia. Allodynia is defined as “pain due to a non-painful stimulus” and hyperalgesia as “increased pain from a stimulus that usually elicits pain”. As a result of nerve injury, maladaptive changes take place in the nervous system both centrally and peripherally leading to the development of pain in the nerve innervation territory and beyond. Allodynia due to light touch is described as dynamic mechanical allodynia and due to innocuous pressure as static mechanical allodynia. Hyperalgesia is evoked by a noxious pressure stimulus. Allodynia and hyperalgesia are also observed in acute and other chronic pain conditions in addition to neuropathic pain, but the underlying mechanisms differ significantly [5]. Dynamic mechanical allodynia is shown to be mediated by low-threshold Aβ fiber neurons, responsible for carrying fast tactile sensations from the periphery. Selective blocking of A fiber input resulted in the disappearance of dynamic mechanical allodynia, but persistent burning pain is noticed, possibly due to C-fiber activation [6]. In some cases, dynamic mechanical allodynia can also be mediated by unmyelinated low threshold C-tactile fibers, sensors of gentle stroking [7]. Another form of mechanical allodynia which occurs in response to pin pressure is termed as punctate allodynia and is determined by activity of Aδ and C fibers [8]. Punctate allodynia in animal models and humans can be evoked by stimulation with a monofilament. In contrast to allodynia, hyperalgesia is mediated by sensitized nociceptors, which include both Aδ and C-fibers [9]. Deep mechanical hyperalgesia is most commonly seen in patients with traumatic nerve injuries and diabetic neuropathies, suggesting the primary role of C-fiber activity [10,11]. Pain due to moderately cold stimulus is

referred to as cold allodynia and is a common finding in patients suffering from neuropathic pain. The perception of innocuous and noxious cold stimuli is mediated by Aδ and C fibers. The character of cold allodynia varies depending on the neuropathy condition such as aching and burning pain in small fiber neuropathy, and pins and needles sensation in chemotherapy induced peripheral neuropathy. Hyperalgesia to heat is mostly seen in chronic inflammatory conditions and few types of neuropathic pain [12]. Hyperactivity of C or Aδ fibers is thought to be the reason for heat hyperalgesia observed in neuropathic pain patients [13].

1.3 Neuropathic pain prevalence and impact on patient's quality of life:

Atleast, 20 million adults in the United States suffer from peripheral neuropathy every day [14]. Neuropathic pain itself is not a life threatening condition in contrast to cancer and heart disease. The morbidity associated with it can negatively affects patients quality of life and results in lost productivity of individual. The economic burden of chronic pain as estimated in 2010 for United States alone ranged $560 to $635 billion which is greater than health care expenditure for diabetes, cancer and heart disease [15].

1.4 Pathophysiology of neuropathic pain:

Evidence from previous studies on neuropathic pain conclusively shows that hyperexcitability of primary afferent neurons is the primary reason for the induction and maintenance of neuropathic pain. Increased excitability after initial trauma due to altered expression and function of ion channels and receptors, nociceptor sensitization by pro-inflammatory mediators, and exaggerated transmitter release from peripheral and central nerve terminals of primary afferent neurons are some of the important mechanisms underlying neuropathic pain

pathogenesis. As a result of nerve damage, environmental changes that impact neuronal structure, function and biochemical properties enable neurons to develop maladaptive plasticity.

1.4.1 Ectopic activity of primary afferents:

Hyperexcitability of primary afferents is primarily attributed to the reduced threshold for their activation and is often referred to as sensitization of peripheral neurons [16]. This phenomenon has been demonstrated particularly for Aδ and C-fiber nociceptive neurons both in human studies and animal models of neuropathic pain, because continuous activity of these neurons is necessary to maintain ongoing pain states [17,18]. Although sensitization of primary afferents of skin and joint nociceptors are responsible for continuous pain in inflammatory conditions, mechanisms underlying neuropathic pain considerably vary. Impulses arising from the portion of a neuron other than peripheral nerve terminals are often referred to as ectopic impulses and this type of abnormal activity has been documented for several painful peripheral neuropathy conditions [19,20]. Ectopic impulses can originate from neuromas [21], areas of demyelination along the nerve [22] or from the somatosensory ganglia [23] . Ectopic activity of C-fiber and A-δ neurons is responsible for maintaining spontaneous pain and exaggerated response to painful thermal and mechanical stimuli; however, this underlying mechanism cannot explain tactile allodynia where an innocuous stimulus can induce a painful response. Battery of evidence indicates that a painful response to an innocuous tactile stimulus is largely mediated by touch sensing Aβ fibers, not sensitized nociceptors [9,24]. Few studies have described a cause for the change in the phenotype of Aβ fibers and some of the important findings are de novo expression of peptides of nociceptive neurons such as substance P and CGRP [25,26], change in the mRNA expression of several hundred genes [27,28] and sprouting of Aβ afferents to lamina II region of dorsal horn [29].

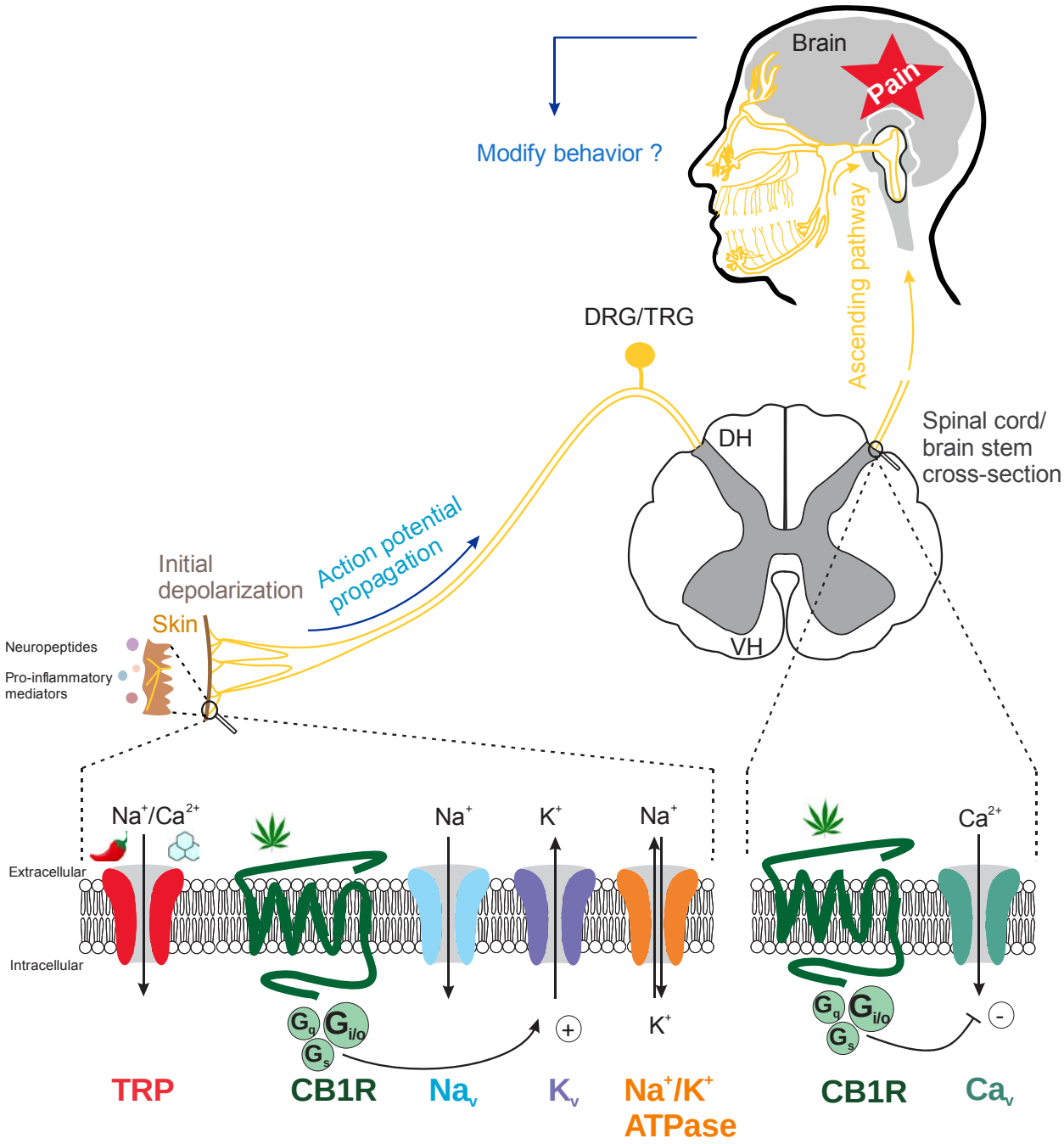


Figure 1.1: Schematic view of afferent pain pathway.
Transduction of sensory stimulus by receptors (Ex. TRPV1) in the peripheral nerve terminals of skin or oral mucosa results in the initial depolarization of neuronal membrane and activates adjacent voltage gated sodium and calcium channels. Action potential is propagated along the axon due to continuous activity of voltage gated sodium and potassium channels and reaches the cell body and central nerve terminals. Depolarization induced neurotransmitter release facilitates synaptic communication with second order neurons of spinal or medullary dorsal horn and integrates with ascending pathways of the

central nervous system. DH: Dorsal horn; VH: Ventral horn; DRG/TRG: Dorsal/Trigeminal root ganglia; TRP: Transient receptor potential; CBR1: Cannabinoid receptor subtype I; Na_V: Voltage gated sodium channel; K_V: Voltage gated potassium channel; Ca_V: Voltage gated calcium channel.

Important molecular players of neuropathic pain (1.4.2-1.4.14):

1.4.2 Transient receptor potential (TRP) channels:

Classified into 6 subfamilies based on peptide sequence homology – Canonical (C), Vanilloid (V), Melastatin (M), Polycystin (P), Mucolipin (ML) and Ankyrin (A). Subtypes of TRP channels, TRPV1-4, TRPA1 and TRPM8 have been described as transducers of mechanical, thermal and chemical stimuli in DRG neurons [30]. Among them, TRPV1 and TRPA1 channel subtypes gained wider attention because of their important role in physiological and pathological pain processing. TRPV1 is a divalent cation selective channel and is activated by heat and capsaicin. The sensory qualities of TRPV1 activation are described as burning, itching and stinging. Changes in the expression and function of TRPV1 (transcriptional and translational regulation, port-translational modifications and altered trafficking) have been reported in animal models of inflammatory and neuropathic pain [31]. TRPV1 may contribute to neuropathic pain symptoms by altering sensory thresholds of peripheral nerve terminals, inducing ectopic activity in injured and uninjured axons, and modulating neuropeptide and transmitter release [32]. Increased expression of TRPV1 has been reported in trigeminal nerve fibers of burning mouth syndrome patients [33]. Both agonists and antagonists have been described as effective in treating some of the symptoms of neuropathic pain. Potent agonists produce pain relief by desensitizing the receptor and antagonists act by blocking the receptor function [34]. Treatment strategies targeting TRPV1 such as capsaicin patches and creams have been FDA approved for the treatment of post-herpetic neuralgia, although some unwanted side-effects have been reported.

The role of TRPA1 in sensory neurons is mainly attributed to chemosensation [35]. Few studies have shown that TRPA1 is necessary for maintenance of cold hypersensitivity in response to nerve injury and mechanical hyperalgesia after CFA induced inflammation [36,37]. Similar role of TRPM8 in cold allodynia after nerve injury has also been reported [38,39].

1.4.3 Neuropeptides:

Released by peripheral and central terminals of nociceptive neurons and cause neurogenic inflammation.

a. Substance P (SP):

Member of the tachykinin neuropeptide family and its endogenous receptor is the neurokinin (NK1) receptor. Widely expressed in nociceptive sub-population of somatosensory neurons [40] and has a potent function in pain transmission [41]. So far, there is no satisfactory evidence about the direct role of SP/NK1 in neuropathic pain pathogenesis. Although, it has been reported that Aβ fibers started to express SP after peripheral nerve injury and its de novo expression correlates with the maintenance of tactile allodynia in neuropathic pain [26]. In addition, repeated topical application of capsaicin desensitizes primary afferent terminals by depleting neurons of SP [42]. These findings from the above studies suggest that SP plays an auxiliary role in the maintenance of peripheral and central sensitization in neuropathic pain conditions.

b. Calcitonin Gene Related Peptide (CGRP):

It is a 37 amino acid neuropeptide and is primarily localized to C and Aδ fibers [43]. It is a potent vasodilator [44]. CGRP exerts its activity by binding to the membrane surface receptor dimer, calcitonin receptor like receptor (CL) and receptor activity-modifying protein (RAMP) [45]. CGRP acts as an active mediator of neurogenic inflammation during migraine attacks and oral

administration of CGRP receptor antagonists in humans aborted migraine development with sustained pain free responses up to 24 hours [46]. Similar to SP, de novo expression of CGRP in low threshold Aβ fibers after peripheral nerve injury has been reported [26]. Intrathecal administration of CGRP8-37 increased withdrawal threshold bilaterally in rats with unilateral experimental neuropathy and the effect was reversed by the co-administration of naloxone (μ-opioid receptor antagonist), suggesting its role in pain inhibition is centrally mediated [47].

c. Galanin:

Expressed in peptidergic population of small diameter DRG neurons [48] and galanin like immunoreactivity has been observed in Lamina II region of spinal dorsal horn [49]. The role of galanin in neuropathic pain pathogenesis is still unclear as conflicting results have been reported about the galanin expression after peripheral nerve injury [50–52]. Given that galanin hyperpolarized postsynaptic dorsal horn neurons [53], and over expression of galanin in transgenic mice produced analgesic effects [54], it appears that basal inhibitory tone in the dorsal horn circuitry is partly regulated by galanin expressing neurons.

1.4.4 Voltage gated sodium channels (Na_V):

Neurons differ from many other cell types having a unique property, electrical excitability and the role of voltage gated sodium channels cannot be ignored. Na_V subtypes expressed in PNS neurons, $Na_V1.3$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are considered prime targets for neuropathic pain treatment because of altered expression & function in rodent neuropathy models and human clinical studies on pathological pain [55–58]. Even though the basal expression of $Na_V1.3$ in DRG neurons is very low, peripheral nerve injury induced a dramatic increase in the expression of $Na_V1.3$ mRNA and protein [55]. Moreover, shRNA mediated knockdown of

$Na_V1.3$ mRNA in DRG neurons attenuated nerve injury induced neuropathic pain [59]. Studies on inherited channelopathies in humans such as erythromelalgia and paroxysmal extreme pain disorder (PEPD) identified the role of $Na_V1.7$ in underlying pathogenesis [60,61]. Both, knockout and in vivo knockdown of $Na_V1.7$ in DRG neurons alleviated inflammation induced mechanical allodynia and thermal hyperalgesia [62,63]. Interestingly, peripheral nerve injury induced mechanical allodynia is aborted only when $Na_V1.7$ expression is knocked out in both sensory and sympathetic neurons [64]. The same study reported that deletion of $Na_V1.7$ expression in sensory neurons alone had no effect on neuropathic pain behaviors. The role of $Na_V1.7$ in sympathetic neurons and its relation to neuropathic pain development is yet to be identified. It has been reported that $Na_V1.8$ expression in DRG and TRG neurons is decreased after peripheral nerve injury and abnormally expressed proximal to the site of injury [57,65–67]. In fact, antisense and shRNA mediated knockdown of $Na_V1.8$ mRNA alleviated experimentally induced neuropathic pain in rat models [68,69]. It remains to be investigated if axonally localized $Na_V1.8$ mRNA has any specific role in the maintenance of neuropathic pain symptoms. Previous studies suggest that $Na_V1.9$ plays an important role in the development of inflammatory and bone cancer pain [70]. Recently, mutations of SCN11A gene (encoding $Na_V1.9$) have been reported in human patients with inherited painful peripheral neuropathy disorders [71].

1.4.5 Potassium channels:

Approximately 80 genes in mammals encode for the potassium channel family, serving diverse physiological roles [72]. Potassium channels play an important role in regulating resting membrane potential and repolarization during action potential propagation in neurons [73]. Subtypes of potassium channels include voltage gated potassium channels (K_V), ion activated potassium channels, two pore potassium channels and inward rectifiers [74]. Among different

subtypes, voltage gated potassium channels are key determinants of firing frequency and spike duration in neurons. A decrease in the potassium channel activity results in increased membrane excitability of neurons. Due to their important role in regulating membrane hyperexcitability of neurons, subtypes of K_V were proposed as suitable targets for inflammatory and neuropathic pain treatment [75]. Rasband et al showed that ligation of L5/L6 spinal nerves resulted in a dramatic decrease in the expression of K_V1 subtype potassium channels in DRG neurons [76]. Rescuing nerve injury induced downregulation of $K_V1.2$ in the injured DRG resulted in the attenuated development and maintenance of spinal nerve ligation induced neuropathic pain [77]. Potassium channels expressed in the non-neuronal cells like satellite glial cells (Kir 4.1, inwardly rectifying potassium channel) maintain K^+ ion buffering and are able to influence the level of neuronal excitability [78]. In fact, dsRNA mediated knockdown of Kir 4.1 potassium channel subunit in satellite glial cells of rat trigeminal ganglion resulted in pain like behaviors similar to those observed in rat models of trigeminal neuropathic pain.

1.4.6 Voltage gated calcium channels (Ca_V):

They form major pathway for voltage dependent calcium entry into neurons. Depending on voltage dependence for their activation, Ca_V are divided into low voltage activated (LVA) and high voltage activated (HVA) calcium channels [79]. HVA channels constitute diverse group of Ca_V family. Based on their pharmacology and functional properties they are further sub-divided into L-type ($Ca_V1.1$-4), P/Q- type ($Ca_V2.1$), N-type ($Ca_V2.2$), R-type ($Ca_V2.3$) and T-type ($Ca_V3.1$-3.3) [80,81]. All of the above subtypes have been shown to be expressed in CNS & PNS neurons [82] but N-type and T-type Ca_V are considered as prime targets for the development of novel analgesics [83,84]. N-type Ca_V are highly expressed at presynaptic terminals and regulate calcium entry into the synapse in response to incoming action potentials, mediating synaptic

transmitter release from the afferent terminals in the spinal dorsal horn [85]. The role of N-type Ca_V in pathological pain processing has been confirmed in a knockout mouse model where a spinal nerve ligation failed to alter mechanical withdrawal thresholds and thermal withdrawal latencies ipsilateral to the injury [86]. Intrathecal administration of Xen2174, an indirect N-type Ca_V inhibitor in cancer patients with intractable pain showed initial promising results [87].

T-type calcium channels constitute another important class of Ca_V and are considered key targets for the pathological pain treatment because of low voltage dependence of activation and intrinsic role in regulating membrane excitability [88]. Previous studies implicate the role of $Ca_V3.2$ and not other T-type calcium channels in the pathophysiology of neuropathic and inflammatory pain [89,90]. Another exciting finding from the previous study is that the T-type calcium channel current density is upregulated in DRG neurons after neuropathy induction. Currently there are no $Ca_V3.2$ selective inhibitors available but compounds with modest peripheral T-type inhibition reverse experimental neuropathic pain [91] and are effective in rodent models of paclitaxel and vincristine induced peripheral neuropathy [92].

1.4.7 Glutamate Receptors:

Glutamate receptors exist as both ionotropic (AMPA, NMDA and kainate) and metabotropic (Group I-III) subtypes. The role of all the above receptors in the modulation of pain transmission and their significance in pathological pain processing to some extent has been described previously [93]. Among them, NMDA subtype receptors received greater attention because of their critical role in pain transmission and synaptic plasticity. Studies on animal models of neuropathic pain suggest that NMDA receptor mediated synaptic plasticity plays a crucial role in the development of central sensitization [94–96]. NMDA receptors are composed of different subunits (NR1, $NR2_{A-D}$ & $NR3_{A-B}$) and their functional properties are determined by the subunit

composition [97]. NMDA receptors with NR2A subunits are located at the synaptic sites while those harboring NR2B localize extrasynaptically [98,99]. NR1 subunits are distributed in all laminae of the spinal cord where as NR2B subunit expression is restricted to superficial dorsal horn [100]. Under resting conditions, ion flow through the NMDA receptor is blocked by the Mg^{+2} ion plug. Presynaptic release of glutamate & glycine and postsynaptic membrane depolarization expels Mg^{+2} ion from the channel, allowing passage of monovalent and divalent cations [101]. NMDA receptor activity is important for synapse formation and alterations in synaptic strength (long term potentiation) in the spinal dorsal horn [102]. It has been shown that stimulation of Aβ fibers by the direct microinjection of glutamate into the spinal cord caused the spread of excitation from deep to superficial layers in neuropathic but not control rats and this effect was blocked by AMPA subtype glutamate receptor antagonist CNQX. This study suggests that tactile allodynia in neuropathic pain can be maintained by non-NMDA receptor subtypes [103]. Various noncompetitive NMDA receptor antagonists, MK-801, ketamine, memantine, and dextrorphan were shown to be effective in different types of rodent models of neuropathic pain [104–106]. In addition, Intrathecal application of amino-5-phosphonopentanoate (AP-5) reduced mechanical allodynia caused by spinal cord injury [107]. In spite of the fact that NMDA receptor antagonists suppressed pathological pain successfully in the pre-clinical neuropathy models, they failed to show promising results in human patients with different neuropathy disorders. Perioperative infusion of ketamine failed to prevent chronic neuropathic pain caused by thoracotomy in human patients [108]. Moreover, high doses of dextramethorphan failed to show satisfactory results on the reduction of diabetic neuropathic pain and postherpetic neuralgia pain [109]. NMDA receptor antagonists also produce CNS mediated side-effects such as hallucinations, drowsiness, restlessness, and memory and motor deficits. Targeting disease associated mechanisms rather than blocking overall function of NMDA receptors may offer desired results in the development of new drugs.

1.4.8 Opioids:

Both exogenous and endogenous opioids act through mu (μ), kappa (ƙ) and delta (δ) opioids receptors. Spinal and supraspinal actions of opioid receptors were described as reasons for potent analgesia. Opioids produce antinociception by modulating actions of K^+ and Ca^{+2} ion channels through downstream signaling pathways. Previous studies showed that levels of opioid receptors are downregulated in the spinal cord dorsal horn following partial or complete transection of the sciatic nerve [110]. Furthermore, morphine blocked static allodynia after systemic administration and not after spinal administration in nerve ligation models [111]. These findings suggest the lack of expression of opioid receptors at the spinal level and maintenance of anti-allodynic actions by higher pain centers such as rostroventral medulla (RVM), periaqueductal pray (PAG), thalamus and amygdala. Central targeting of opioid receptors for chronic pain management can be problematic because patients develop CNS mediated side-effects such as sedation, nausea, and respiratory depression and tolerance, which may eventually lead to opioid addiction [112].

1.4.9 Cannabinoids:

Both exogenous and endogenous cannabinoids act primarily via two cannabinoid receptors, type1 (CB1R) and type 2 (CB2R). CB1R is abundantly expressed in CNS and PNS neurons, CB2 expression is limited mostly to immune cells. CB1R expression in pain pathways is both central and peripheral. Higher pain centers with at least moderate levels of CB1R expression are thalamus, amygdala, PAG and RVM. At the periphery, CB1R is expressed in the cell bodies of somatosensory neurons and their peripheral & central nerve terminals [113]. Changes in the expression of receptors and endogenous cannabinoids in DRG neurons have been reported in different rodent models of inflammatory and neuropathic pain [114–116]. Moreover, CB1R agonists

were shown to be effective in both pre-clinical and clinical studies on neuropathic pain [117,118]. The potential problem with many of the currently available CB1R agonists is the central activation of CB1R and the associated CNS mediated side-effects, which can be circumvented by developing peripherally acting cannabinoid receptor agonists.

1.4.10 Growth Factors:

Growth factors are also called neurotrophic factors because of the essential role in the development and survival of the nervous system. These include nerve growth factor (NGF) and glial cell line derived neurotrophic factor (GDNF) family. Members of the NGF family (NGF, Brain Derived Neurotrophic Factor, Neurotrophin-3 and Neurotrophin-4) produce physiological effects via binding of cell surface tyrosine protein kinase (trk) receptors. NGF induces sensitization of primary sensory neurons through upregulation of several pain related genes such as substance P, CGRP, TRPV1, $Na_V1.8$, $Na_V1.9$ and μ-opioid receptors [119–122]. The basal levels of NGF decline initially after peripheral axotomy in the neuropathic pain model but rebound approximately two days after the injury in the satellite glial cells, promoting axonal sprouting of sympathetic neurons [123]. These findings suggest that sympathetically maintained neuropathic pain can be modulated by NGF. Antagonists of NGF signaling were shown to be effective in the pre-clinical studies of chronic inflammatory and neuropathic pain [124,125] but failed to succeed in the clinical trials on human patients with osteoarthritis pain due to serious degeneration of joints [126]. BDNF functions by altering synaptic plasticity and central sensitization in the spinal cord, and repeated intrathecal pre-treatment with anti-BDNF antibodies was able to suppress thermal hyperalgesia induced by spinal nerve ligation in mice [127,128]. NT-3 has been shown to have antagonistic effects to NGF and reduces over-expression of $Na_V1.8$ and $Na_V1.9$ sodium channels in DRG neurons under neuropathic pain states [129]. The role of NT-3 and NT-4 in neuropathic pain pathogenesis is still unclear.

Members of GDNF family include GDNF, artemin, neurturin and persephin and they initiate intracellular signaling by activating transmembrane RET (Rearranged During Transfection) receptor [130]. GDNF is expressed in the majority of non-peptidergic population of nociceptive neurons [131]. Activation of GDNF receptor has been shown to normalize abnormal expression of variety of pain related genes ($Na_V1.3$, $Na_V1.8$, P2X3, CGRP and thymidine monophosphate) after peripheral nerve injury [132,133]. These changes failed to occur in uninjured DRG neurons [134]. A single spinal administration of GDNF reversed established pain behaviors like tactile allodynia and thermal hyperalgesia after SNL and the effect was potent for 3 days [135]. Like GDNF, artemin also induced similar effects on regulating the expression of pain related genes after peripheral nerve injury except that its expression is limited to PNS. Repeated administrations of artemin produced a dose dependent normalization of neuropathic pain symptoms after spinal nerve ligation [136].

1.4.11 Inflammatory mediators:

It is well known that inflammatory mediators released at the site of injury cause sensitization of afferent terminals of nociceptors to elicit acute pain. Neuroinflammation induced by the disrupted balance of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokine expression and function in the spinal dorsal horn has been identified as the main reason for the maintenance of chronic pain states under neuropathy conditions [137]. Changes in the expression levels of IL-1β, TNF-α and IL-10 have been reported in the rodent models of neuropathic pain [138–140]. Some of the common mechanisms mediated by the inflammatory cytokines as a consequence of peripheral nerve damage are – sensitization of central terminals of nociceptors by releasing substance P [141], increased excitation of spinal dorsal horn neurons [142], enhancement of glutamatergic synaptic transmission in lamina I & II neurons [143], increased

release of chemokines (CCL2) [144] and IL-10 mediated attenuation of nuclear factor kappa B activity (results in diminished production of pro-inflammatory cytokines) [137].

1.4.12 Sympathetically maintained neuropathic pain:

It has been noticed in some neuropathic pain syndromes like complex regional pain syndrome (CRPS) that, patients exhibit signs of autonomic dysfunction with skin blood flow, temperature, sweating and trophic abnormalities in the extremity [145]. These findings have been further confirmed when human patients with nerve end neuromas reported exaggerated pain response after intradermal injection of norepinephrine [146]. The exact mechanism behind the sympathetically maintained pain is still unclear, although it has been reported that basket like sympathetic sprouting takes place in the DRG after peripheral nerve injury [147]. However, this phenomenon failed to occur in the trigeminal ganglion [148].

1.4.13 Microglia role in neuropathic pain:

Microglia are considered immune cells of the nervous system because of the expression of a repertoire of immune mediators like chemokines and cytokines. The role of microglia in neuropathic pain behavior became evident when intense proliferation was spotted in the spinal dorsal horn after peripheral nerve injury [149]. Later on it was identified that activation of C-fiber afferents alone was sufficient to induce microglial activation in the spinal cord [150]. It has remained unclear how neuroglial interactions in the spinal cord take place until Tsuda et al., demonstrated that P2X4R expression on the glial surface is important to initiate the cascade of events necessary for microglial proliferation [151]. These findings were further confirmed by injecting P2X4R stimulated microglia intrathecally into naïve rats and induced tactile allodynia similar to that seen in neuropathic rats [152]. The signaling events between primary afferents of the dorsal horn and spinal microglia are largely mediated by members of chemokine, cytokine,

extracellular matrix molecules and protease families [153–155]. After the initiation of primary signaling events by afferent terminals, the microglial cell surface expression of P2X4R is regulated intracellularly by p38 MAPK [156]. Later studies provided convincing evidence that BDNF released from activated microglia and not from primary afferents was critical for the continuous activation of second order neurons in neuropathic pain [157]. The above findings provide satisfactory evidence that the microglial activation by afferent discharge activity of injured neurons play a crucial role in the maintenance of hypersensitive states like allodynia under neuropathy conditions.

1.4.14 Toll like receptors (TLR):

Toll like receptors are a type of transmembrane glycoproteins expressed by Schwann cells of peripheral nerves [158] and microglia in the spinal cord [159]. In addition, the expression of TLRs 3,4, and 8 in DRG and trigeminal sensory neurons has been reported [160,161]. The role of TLR in neuropathic pain became evident when Hutchinson et al demonstrated that TLR4 is important for both initiation and maintenance of microglial activation and neuropathic pain [162]. Further knockdown studies showed that TLR2 expression was necessary for both activation of microglia in the spinal cord, and maintenance of mechanical allodynia and thermal hyperalgesia after peripheral nerve injury [163]. These findings suggest that microglial activation in the maintenance of hypersensitive states like allodynia and hyperalgesia is partly mediated by TLRs.

1.5 Animal models of neuropathic pain:

Animal models to study the pathogenesis of neuropathic pain offered new insights about the underlying mechanisms including candidate molecular players, distinct anatomical pathways and behavioral correlates. These pre-clinical models also aided in the development of several

novel drugs, some of them have already made their way to clinical trials. The symptoms of neuropathic pain may vary depending on the etiology of lesion involved and different animal models have been developed to mimic the signs and symptoms of each individual condition.

1.5.1 Animal model of traumatic nerve injury:

The first animal model of neuropathic pain was developed by Patrick Wall in 1979 to study the human condition of phantom limb pain [21]. This model was called neuroma model as it involved complete transection of the sciatic nerve followed by nerve end neuroma formation. Denervation of the affected paw was confirmed by the presence of autotomy and complete absence of behavioral response to noxious stimulus. This model was not pursued for further studies because the frequency of occurrence of this condition among human population is very rare [164]. Later on, a variant of this model was proposed by Seltzer et al where traumatic nerve injury was induced by tightly ligating approximately 1/3-1/2 of the sciatic nerve fascicle [165]. This model was able to reproduce clinical signs of tactile allodynia, mechanical and thermal hyperalgesia observed in human patients with traumatic nerve injuries. One drawback of this model was the inability to consistently reproduce similar nerve injury. As a refinement to Seltzer model, chronic constriction injury (CCI) model was proposed by Bennet and Xie to impose reproducible nerve injury without complete denervation [166]. A set of four chromic gut sutures (1-2 mm apart) were placed loosely around the sciatic nerve so that they did not obstruct the blood flow through the epineurium. The following behavioral symptoms were observed 1-2 weeks following the surgery: avoidance of weight bearing on the affected hind paw, thickening and elongation of claws as a result of avoidance of grooming, thermal hyperalgesia, and hypersensitivity to cold and tactile stimuli but not to noxious mechanical stimuli. An extension of this model to the trigeminal region has been described by Vos et al. that involved CCI of infraorbital nerve [167]. Rats with CCI of infraorbital nerve exhibited increased facial grooming,

decreased exploratory behavior and nocifensive responses to Von Frey stimulation. One drawback of CCI model is that the degree of constriction cannot be standardized across multiple subjects as it involves manual tightening of sutures. To refine the degree of constriction, Mosconi and Kruger proposed the sciatic nerve entrapment (SNE) model where polyethylene cuffs (2-3) of standard size (~ 0.75 mm in diameter) were placed around the sciatic nerve [168]. Wallerian degeneration of large diameter myelinated fibers was observed one week after cuff placement and remained apparent throughout the 6 week observation period. In contrast, unmyelinated and thinly myelinated small diameter fibers were initially diminished but appeared to regenerate one week after cuff placement. Changes in the morphology of nerve fibers appeared to be more consistent after SNE than after CCI. Behavioral changes observed as a result of SNE were: guarding of the affected limb, uneven weight bearing and enhanced responses to mechanical and cold stimuli. The potential problem with all the above proposed models was that it was not possible to adequately segregate the injured neurons from the uninjured ones. To address this issue, Kim and Chung proposed spinal nerve ligation model (SNL) [169]. Sciatic nerve is formed by nerves arising from L4-L6 DRG and SNL involves tight ligation (3-0 silk) of nerves distal to L5-L6 DRG while sparing those of L4 DRG. SNL procedure requires removal of L6 spinal transverse process. No signs of autotomy were observed in these rats. Robust behavioral responses to tactile and noxious thermal stimuli were observed 1-2 days after SNL and lasting longer than 10 weeks. The magnitude of cold allodynia was much less than that seen after CCI. Later it was shown in the SNL model by selective blocking of the afferent input by dorsal rhizotomy that, the evoked pain requires input from both injured DRG neurons and uninjured afferent fibers whereas spontaneous pain can be elicited by injured DRG neurons alone [170,171]. Another model that involves ligation of one or more branches of the sciatic nerve was proposed by Decosterd and Woolf [172]. It is called the spared nerve injury (SNI) model, created by tight ligation and subsequent resection of 2-4 mm of the common peroneal

and tibial nerves while leaving the sural nerve intact. Behavioral changes following SNI were described as: avoidance of weight bearing and enhanced responses to tactile, warm and cold stimuli.

1.5.2 Animal model of diabetes induced peripheral neuropathy (DIPN):

Patients with insulin dependent diabetes develop peripheral neuropathy as one of the common side effects. Neuropathic pain due to diabetes is very difficult to manage and underlying mechanisms are not well understood. Degeneration of neuronal processes along with the associated vasculature has been identified in patients with diabetic peripheral neuropathy [173]. A rat model of diabetes induced peripheral neuropathy was developed where a single intraperitoneal injection of 75 mg/kg of streptozotocin produced progressively increasing signs of diabetes mellitus including elevated blood glucose levels (34 mM), polyuria, polydipsia and weight loss [174]. Streptozotocin produces progressive and permanent degeneration of the β cells of the pancreas with dramatic decreases in serum insulin levels. Streptozotocin induced diabetes produces mechanical and cold allodynia as well as thermal hyperalgesia. Hyperexcitability of C fibers and Aβ & Aδ fibers in response to noxious and innocuous mechanical stimuli, respectively, has been reported in this model [175,176]. Some patients with severe cases of diabetic neuropathy reach a degenerative and painless stage. Similarly, animals with streptozotocin injection develop signs of hypoalgesia one month after streptozotocin injection [177]. Moreover, animals with streptozotocin induced diabetes also have severe weight loss and overall poor health, which further complicates behavioral analysis.

1.5.3 Animal model of chemotherapy induced peripheral neuropathy (CIPN):

Cancer survivors who have chemotherapy with anti-cancer agents (cisplatin, paclitaxel, vincristine, thalidomide and Bortezomib) may develop sensory neuropathy of their lower

extremities with signs of myalgia and myopathy at higher doses [178]. Patients with CIPN report neuropathic pain as sensation of pins and needles. Patients may also report hypersensitivity to cold and tactile stimuli (cold and mechanical allodynia). Studies on CIPN using animal models described mitochondrial dysfunction in primary afferent neurons, emergence of spontaneous discharges as a result of cellular energy deficit (defective Na^+/K^+ ATPase) and degeneration of intra-epidermal nerve fibers as the important causes for peripheral neuropathy due to chemotherapy [179]. Most of the anti-cancer agents that cause peripheral neuropathy in cancer patients have been used to develop rodent models of CIPN. The exact procedure in developing a rodent model of CIPN may vary depending on the species used, type of chemotherapeutic agent administered, and route, dose and frequency of administration [180]. Generally, animals treated with anti-cancer agents develop signs of mechanical and cold allodynia along with thermal hypoalgesia in some situations.

1.5.4 Animal model of post-herpetic neuralgia (PHN):

PHN is described as pain that persists 1-3 months after the onset of rash. PHN patients mainly complain of mechanical allodynia, and burning and spontaneous pain. After primary infection, the *Varicella zoster* virus (VZV) enters a latent phase in the somatosensory ganglia. Reactivation of virus results in the production of painful lesions along the innervated dermatomes. An animal model of PHN is developed by injecting cultured CV-1 cells infected with VZV into the rat hindpaw. Subsequently, rats develop symptoms of PHN such as mechanical allodynia and thermal hyperalgesia [181]. In contrast, injection of heat inactivated virus did not result in the development of PHN.

1.5.5 Animal model of HIV neuropathy:

Patients infected with human immunodeficiency virus (HIV) or treated with antiretroviral drugs may develop peripheral neuropathy [182]. An animal model is developed either by intrathecal or systemic injection of viral coat protein of HIV, Gp120. Infection with HIV protein Gp120 results in the activation of microglia and astrocytes, release of pro-inflammatory cytokines and other inflammatory mediators in the spinal cord, evoking signs of neuropathic pain [183]. The role of microglia was confirmed when glial inhibitors fluorocitrate and CNI-1493 blocked behavioral signs of HIV neuropathy [183]. After 2-4 days of Gp120 exposure, rats developed behavioral signs of tactile allodynia, mechanical and thermal hyperalgesia [184].

1.6 Behavioral readouts in animal models of neuropathic pain:

1.6.1 Allodynia:

Described as painful response to non-painful stimulus. Similar to those seen in human patients, signs of neuropathic pain are well characterized in animal models [185]. Mechanical or tactile allodynia and cold allodynia are routinely measured in animal models. Mechanical allodynia can be static or dynamic. A static mechanical allodynia is measured by applying linearly increasing force on the plantar surface of hindpaw with regular or electronic type Von Frey hairs. Dynamic allodynia is relatively difficult to characterize in animal models as behavioral outcomes are difficult to interpret. A mild stroke on the plantar surface with a cotton wool elicits paw withdrawal response, suggestive of mechanical allodynia. It has been shown that systemic administration of morphine or amitriptyline both produced a dose dependent block of static but not dynamic allodynia in a rat model of chronic constriction injury of sciatic nerve, whereas gabapentin and pregabalin blocked both forms of allodynia [186]. It's important to

consider sedative effects of gabapentinoids whenever simple withdrawal or innate reflexes are used as objective measures of pain. Cold allodynia in animal models is measured by applying drops of acetone on the plantar surface or placing the animal on a cold plate and counting the frequency of paw withdrawals [39].

1.6.2 Hyperalgesia:

Defined as an exaggerated response to normally painful stimulus. Generally, the terms allodynia and hyperalgesia are defined for clinical use. Procedures described to record objective measures of mechanical hyperalgesia are similar to those described for static mechanical allodynia [187]. In fact, it is difficult to distinguish hyperalgesia from allodynia if same physical stimulus, for example pressure or temperature is used to elicit the behavioral response in animal models, because of the difficulty in recording sub-threshold objective measures of pain. Thermal hyperalgesia is measured by recording the latency to hindpaw withdrawal after application of radiant heat [188]. The magnitude of thermal hyperalgesia measured in animal models can vary depending on the equipment used, thus placing doubt as to the sensitivity of this behavioral measurement [189].

1.6.3 Ongoing or spontaneous pain:

Objective measures of pain for recording allodynia and hyperalgesia are based on evoked responses to innocuous or noxious stimuli, respectively. The principal complaint from neuropathic pain patients is ongoing pain either paroxysmal or spontaneous. A significant impediment to the success of new therapies for neuropathic pain management is to rely heavily on innate responses and withdrawal reflexes. Recent improvements in pre-clinical assessment of pain such as operant assays and conditioned place preference enable evaluation of ongoing pain in freely moving animals. In operant assays, animal is subjected to simultaneous pain-

reward conflict paradigm. An animal behavior modification due to this conflict paradigm is interpreted as objective measures of pain [190]. Conditioned place preference (CPP) involves association of a drug treatment of an animal in pain with a particular environment followed by association of a different environment with no drug. During the test session, an animal is given a choice of two environmental conditions and the amount of time animal spent in that environment is recorded [191]. Since the amount of time the subject is able to spend in a particular environment is based on its voluntary decision, CPP can evaluate cortical processing of pain.

1.7 Current therapies for the management of neuropathic pain:

1.7.1 Anticonvulsants:

a. Gabapentinoids:

Include gabapentin and pregabalin. Gabapentin was developed as adjunctive medication for the treatment of partial seizures and is now recommended as a first line medication for the management of neuropathic pain. Gabapentin exerts its mechanism of action by binding to α2δ subunit of Ca_V^{+2} channels [192]. Recent evidence suggests that gabapentin prevents trafficking of calcium channel complex to the surface of neuronal membrane by binding to the α2δ subunit [193]. Gabapentin exerts its analgesic effect largely through calcium channels expressed in the CNS. Evidence from previous studies suggest recommended dose of gabapentin should be 300 mg on day 1, 600 mg on day 2 and 900 mg on day 3 with subsequent titration to 1800 mg/d or 3600 mg/d if required [194]. Although gabapentin is well tolerated, side-effects such as sedation, fatigue, dizziness, tremor and headache interfere with patients daily life activities. Gabapentin is eliminated by renal excretion, and its clearance is reduced in patients with renal insufficiency. Pregabalin is a successor of gabapentin with similar biological activity and a better

pharmacokinetic profile [195]. It was approved by the FDA for the treatment of diabetic neuropathy and post-herpetic neuralgia in 2004.

b. Voltage gated sodium channel blockers:

Examples of anticonvulsants with activity on voltage gated sodium channels are lamotrigine, carbamazepine, valproate, lacosamide and topiramate. They act on α-subunit of voltage gated sodium channels to stabilize neuronal membranes and inhibit neurotransmitter release [196]. These particular types of anticonvulsants are able to decrease the hyperactivity of pain network by reducing the firing of Na^+ channels expressed along the pain pathways. They typically inhibit both TTX-sensitive and resistant neuronal sodium channels [197]. Even though phenytion and carbamazepine were both originally used to treat trigeminal neuralgia, the use of phenytion is limited because of severe side-effects. Carbamazepine is also used for the management of paroxysmal extreme pain disorder (PEPD), an inherited disorder of human condition due to gain of function mutations in $Na_V1.7$ sodium channel. To improve the side effects profile of phenytion and carbamazepine, a newer analog with reduced liver metabolism was developed called oxcarbazepine. It has been shown to have higher efficacy for the treatment of allodynia and hyperalgesia in animal models of neuropathic pain [198]. Side-effects of oxcarbazepine are comparable to that of carbamazepine [199]. Lamatrogine has been reported to be effective at treating trigeminal neuralgia, diabetes induced peripheral neuropathy and HIV neuropathy [200]. However, a large placebo controlled study conducted on lamotrigine for the treatment of diabetic neuropathy showed inconsistent effectiveness in reducing pain symptoms [201]. Topiramate is a broad spectrum anticonvulsant that seems to inhibit both voltage gated sodium and calcium channels. However clinical studies suggest that topiramate is not effective in the treatment of neuropathic pain in comparison with placebo treatment in the same trial [202]. Most common adverse events associated with topiramate treatment were diarrhea, loss of appetite and

somnolence. Lacosamide promotes slow inactivation of sodium channels in contrast to other sodium channel blockers where they mediate fast inactivation. In a placebo controlled clinical trial on diabetic neuropathy, lacosamide treatment was shown to be ineffective in comparison with placebo [203]. Adverse effects of lacosamide are similar to those of topiramate.

1.7.2 Antidepressants (ADs):

Inhibit synaptic reuptake of monoamines such as serotonin and noradrenaline and block postsynaptic α-adrenergic, H_1-histaminergic and muscarinic cholinergic receptors. Presynaptic reuptake inhibition of the monoamines by ADs will increase the levels of these in the synaptic clefts. In neuropathic pain, disinhibition of pain modulating circuits from PAG and RVM has been reported [204]. ADs enhance pain suppression by increasing the availability of monoamines at these modulating circuits. Classes of antidepressant drugs attempted for the management of neuropathic pain are tricyclic ADs, serotonin-norepinephrine reuptake inhibitors, dopamine-norepinephrine reuptake inhibitors and selective serotonin reuptake inhibitors. The mechanism of action of ADs in neuropathic pain is multimodal. ADs suppress pain transmission in diffuse noxious inhibitory control by inhibition of presynaptic uptake of serotonin and noradrenaline [205]. In addition, ADs suppress pain transmission by blocking NMDA receptors and voltage gated sodium and calcium channels [206,207]. Binding of ADs to opioid receptors has also been reported but the affinity of binding is too low [208]. Common tricyclic ADs are amitriptyline, nortriptyline, imipramine and desipramine. Amitriptyline, desipramine and imipramine block reuptake of both serotonin and norepinephrine whereas nortriptyline mediates reuptake inhibition of norepinephrine alone. Non tricyclic serotonin and norepinephrine reuptake inhibitors (SNRIs) are venlafaxine and duloxetine. Bupropion is a dopamine and norepinephrine inhibitor (DNRI). Selective serotonin reuptake inhibitors (SSRI) are fluoxetine, paroxetine and citalopram. Randomized control trials are the primary evidence for the effect of ADs in the management of

neuropathic pain. TCAs with mixed serotonergic and noradrenergic reuptake inhibition have been shown to be more effective than selective noradrenergic reuptake inhibition alone [209]. The effects of TCAs has been studied mostly in diabetic neuropathy patients and are marginally effective over other medications [210]. However, TCAs failed to ameliorate pain symptoms in HIV patients with peripheral neuropathy [211]. In general, ADs have been shown to be moderately effective in relieving painful symptoms of diabetic neuropathy. Side effects noticed with ADs are dry mouth, nausea, insomnia and tremor.

1.7.3 Opioids:

The efficacy of opioids in neuropathic pain management is not clear. Long term treatment with opioids is impeded by development of tolerance. Lower doses of opioids in combination with anticonvulsants have been shown to provide better analgesia. A placebo controlled study on diabetic neuropathy and post-herpetic neuralgia showed that sustained release morphine and gabapentin combination was more effective than administration of higher doses of individual drugs alone [212]. Opioids are contraindicated in patients with significant respiratory depression, obstructive pulmonary disease and paralytic ileus. Opioid interactions with CNS depressants have also been reported.

1.7.4 Cannabinoids:

Extracts of cannabis have been shown to be effective in the management of neuropathic pain due to multiple sclerosis and HIV [213,214]. In addition to amelioration of pain, cannabinoid use has been reported to improve spasticity and sleep disturbances [215]. However, the potential limitation with the widespread use of cannabinoids is CNS mediated side effects.

1.7.6 Local anesthetics (LAs):

LAs inhibit sodium channel activity by binding to S6 transmembrane helix of domain four of sodium channel α-subunit. LAs in the form of lidocaine patch is the most common method for the management of peripheral neuropathy. A 5% lidocaine patch was shown to be effective in suppressing all signs of peripheral neuropathy [216]. A trigeminal nerve block with high concentration of lidocaine (10%) is capable of achieving an intermediate pain relief in trigeminal neuralgia patients [217]. A randomized double blind study of two week duration reported that 5% lidocaine in the form of patches, cream, gel or spray was ineffective in amelioration of painful symptoms of trigeminal neuralgia and post-traumatic trigeminal neuropathy, suggesting that lower doses are insufficient for adequate pain relief [218]. However, a higher dose of lidocaine administration has adverse effects on cardiac and neuronal conduction.

1.7.7 Topical capsaicin:

Topical capsaicin formulations are widely used to treat local pain. A high concentration (8%) capsaicin patch was recently approved by FDA for the management of post-herpetic neuralgia and in the Europe for the treatment of non-diabetic peripheral neuropathy [219]. Capsaicin is a highly selective and potent exogenous agonist at TRPV1 and activation of TRPV1 by capsaicin results in a persistent biochemical effect, causing warming, burning, stinging or itching sensations. Capsaicin improves pain symptoms by desensitizing nociceptive neurons and repeated administrations are required to achieve adequate pain relief. A most common side-effect of capsaicin treatment is burning sensation associated with each application.

1.7.8 Surgical management:

Surgical management of neuropathic pain is done mostly for traumatic neuropathies. Common surgical procedures are nerve decompression and micro-surgical repair. Procedures

involving surgical repair of nerve are recommended within few months after injury [220]. Surgical treatment may make pain worse if patient had chronic neuropathic pain for a long time [221].

Specific Aims

Despite significant advancement in the understanding of physiology of pain processing, current treatments for neuropathic pain treatment are only moderately effective with serious side-effects. Reasons being addressed are incomplete knowledge of the pathophysiology of disease and poor translational progress from pre-clinical to clinical stage. Current methods for evaluation of neuropathic pain in animal models are largely based on withdrawal reflexes, whereas in humans, neuropathic pain is mostly spontaneous or activity related. In addition, current medications for neuropathic pain treatment are centrally acting and are often associated with CNS mediated side-effects such as somnolence, dizziness, dependence and tolerance. The current thesis work is focused on addressing the behavioral, pharmacological and molecular aspects of peripheral neuropathy..

Specific Aim 1: The most important aspect of using an animal model, either to study pathogenesis or a response to drug treatment in pre-clinical studies, is to able to predict human symptoms of neuropathic pain accurately. The current research aim is focused on developing a novel behavioral measurement that closely mimics human symptoms of peripheral neuropathy. To achieve this research aim, I adopted a rat model of trigeminal neuropathic pain with concomitant behavior evaluation using an orofacial operant assay.

Specific Aim 2: The current pharmacotherapy for neuropathic pain management is mostly based on the centrally acting drugs. Thus, potential CNS mediated side-effects. The second research aim is concentrated on the investigation of novel peripherally restricted cannabinoids for the potential treatment of neuropathic pain. Using pharmacological and behavioral approaches, I will demonstrate that a peripherally restricted cannabinoid is able to alleviate neuropathic pain symptoms without central side-effects.

Specific Aim 3: The second research aim was focused on developing a palliative remedy for chronic pain management. The pathological mechanisms underlying persistent pain are still unclear. The last research aim is focused on the investigation of $Na_V1.8$ redistribution in axons because previous studies described abnormal axonal accumulation of $Na_V1.8$ and its relation to persistent pain after peripheral nerve injury.

Chapter II – Specific Aim (SA) 1

SA1: Orofacial pain operant analysis in a rat model of trigeminal neuropathic pain

2.1 Introduction:

Previous studies describing behavioral measures in animal models of neuropathic pain have been largely concentrated on the evaluation of reflex measures and innate responses to noxious and innocuous thermal and mechanical stimuli, which may not accurately represent pain processing through an entire neuraxis, and are confounded by sedative effects of pain medications [222]. Moreover, tail reflex and hindpaw withdrawal responses can be elicited even in spinalized animals [223]. Rodent models of pain using complex behavioral assays such as operant measures have been shown to be consistent with expectations from human studies [224]. Operant measures of pain are advantageous in that the animal has control over the amount of stimulus perceived (noxious or innocuous) and can modify its behavior based on cerebral processing [225]. In addition, reflex based measurements depend on the manual delivery of stimulus and are influenced by investigator bias. An operant assay for evaluation of orofacial pain sensitivity to thermal stimulus has been described, where an animal is subjected to simultaneous pain-reward conflict paradigm [190]. The operant measures described in this method have been shown to be accurate for the evaluation of acute nociception, although similar measures failed to deliver consistent results for the evaluation of neuropathic pain in a trigeminal nerve model [226]. Moreover, operant assays for accurate evaluation of neuropathic pain in response to mechanical stimulus have not been described so far. One of the research aims of my disseration project is to develop an orofacial operant assay for behavioral characterization of trigeminal neuropathic pain in response to tactile mechanical stimuli. In the specific aim 1 (SA1), I will demonstrate that rats with neuropathic orofacial pain symptoms exhibit orofacial dysfunction compared to sham treated rats.

2.2 Methods:

The surgical and behavioral methods described here are approved for this study by the Chancellor's Animal Research Committee (ARC) of UCLA.

2.2.1 Bilateral infraorbital nerve constriction (IoNC):

Twelve adult male Sprague-Dawley rats (250 g) are used for this study. Rats are anesthetized with isoflurane (5% for induction and 1% for maintenance) and placed in a metal stereotactic frame. Entire surgery is performed under Carl-Zeiss operation microscope at 20x magnification. Neuropathy is induced bilaterally using the method described previously by Vos et al and Kernisant et al [167,227] (Fig. 2.1 A). A rostro-caudal incision of approximately 7 mm long is made following the curvature of the frontal bone and the adherent muscle is gently teased from the bone to retract the orbital contents. Once the infraorbital nerve is identified and exposed, the nerve is freed from its surrounding connective tissue with the help of a straight forceps (Dumont #4, Fine Science Tools, Foster City, CA). Two 4-0 silk sutures are placed 2 mm apart so that the nerve is barely constricted without compromising the blood flow through the epineurium. The incision above the eye is sutured with 5-0 nylon and rats are allowed to recover for two weeks before testing sessions have been resumed. The surgical procedure for sham treatment is similar to that described for IoNC except the induction of constriction injury. All rats are treated with postoperative analgesics and antibiotics as per the guidelines recommended by the Division of Laboratory Animal Medicine (DLAM) at UCLA.

2.2.2 Operant box design and testing:

An operant box for rats is made following the instructions provided in the Neubert et al manuscript [190]. A drinking box (20.3 cm W x 20.3 cm D x 16.2 cm H) with acrylic walls is constructed with a small drinking alcove in one of the walls (4x6 cm). The floor of the box is lined with an aluminum sheet to serve as ground for 10 V DC circuit (Fig. 2.1 B). The drinking alcove is fitted with 0.009 inch nitinol wires (1 cm long) on either side and a metal spigot attached to 50 ml falcon tube is positioned between the free ends of nitinol wires (Fig 2.1 C). Two independent but similar circuits are designed to record licks and facial contact events of a rat. For the circuit that is used to record licks, positive and negative terminals are connected to a metal spigot and an aluminum sheet lining the floor of the box. For the recording of facial contact events, nitinol wires on both sides of the alcove are connected to the positive and negative terminals of a DC circuit.

Rats are trained for 1 week to access reward (sweetened milk) from the metal spigot while maintaining bilateral vibrissal pad contacts with nitinol wires. The DC circuit to record licks and facial contact events is in turn connected to a digitizer (DIGIDATA 1322A, Axon Instruments) and a personal computer, for event monitoring and offline analysis (Fig. 2.1 D). For a ten minute test session, total number of licks, reward consumed and total facial contact duration is obtained. During offline analysis a threshold for detection of a licking or facial contact event is set 1V above the background noise to eliminate false positive traces. Rewarded licks and drinking efficiency of an individual rat are determined by using the formulas below.

Rewarded licks = $\left[{}^{Mean\ Licks}/_{Gram\ of\ Reward}\right]_{Baseline}$ *x Reward (g) consumed*

Drinking efficiency = $\left[\frac{Total\ Rewarded\ Licks}{Total\ Facial\ Contact\ Duration\ (s)}\right]$

2.2.3 Statistical analysis:

Data is expressed as mean ± standard error of the mean (s.e.m). Difference of means between groups distributed across different time points is compared with two-way repeated measures ANOVA. Friedman repeated measures ANOVA is applied if data distribution is non-parametric. A p value of < 0.05 is considered statistically significant. Results for statistics are generated using Sigmastat 3.5 software.

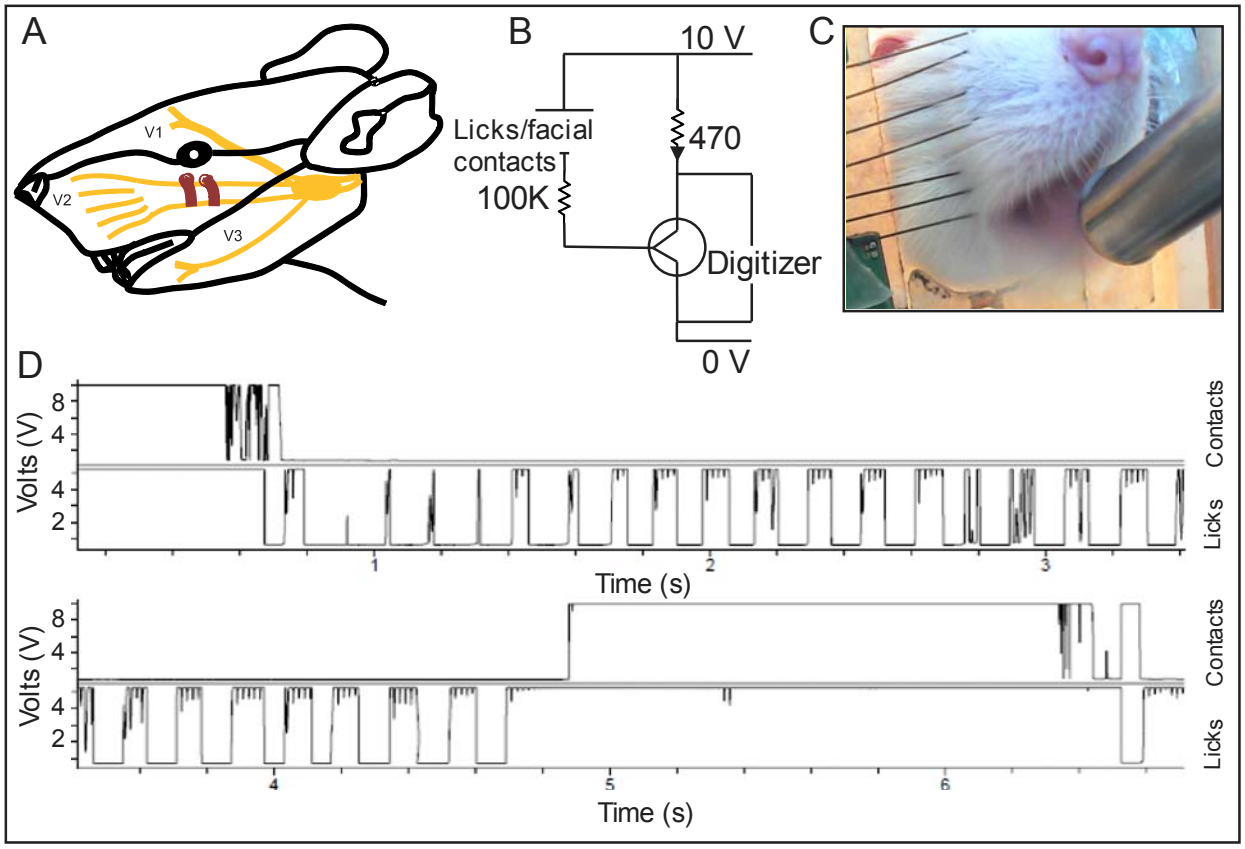


Figure 2.1: Rat trigeminal neuropathy model and operant testing design.
(A) Schematic view of a rat model of trigeminal neuropathic pain induced by chronic constriction injury of infraorbital nerve. V1, V2 and V3 represent three major subdivisions of trigeminal nerve-ophthalmic, maxillary and mandibular respectively. (B) Schematic view of an operant DC circuit used to record total licks and total facial contact duration from an adult rat engaged in operant drinking behavior. A digitizer is connected in parallel to the circuit for event monitoring and offline analysis. (C) Photograph of a rat licking the metal spigot through the drinking alcove of an operant chamber for reward consumption. Either side of drinking alcove was fitted with 0.009 inch Nitinol wires to serve as mechanical stimulators. (D) Sample image of recorded licks and facial contacts acquired by the digitizer from the orofacial operant assay. A drop in potential from 5 or 10 volts to 0 volts indicates a single licking event or facial contact event respectively.

2.3 Results:

2.3.1 Raw operant measures are not significantly different between IoNC and sham groups:

The original manuscript that described operant assay for orofacial pain assessment in rats proposed two indices for evaluation of pain: 1) ratio of licks and facial contact events; 2) ratio of facial contact duration and facial contact events. In our experiments, we did not take facial contact events into consideration because mere sniffing or licking of the metal wires by rats produced an event trace, generating too many false positive contact events. Instead, plotting of raw operant measures (Fig 2.2: A-C) showed no significant difference between IoNC and sham groups at baseline (BL) and 15,18 & 21 days post surgery, suggesting that raw operant measures alone cannot accurately predict painful vs non-painful states. This is in agreement with the previous study that evaluation of licking events alone showed no distinction between responses in the innocuous to noxious stimulus range [190]. The transient decrease in operant measures of sham rats is possibly due to two weeks of abstinence post-surgery [228].

2.3.2 Drinking efficiency is significantly lowered in the IoNC group:

Studies aimed at understanding the drinking behavior of rat demonstrated that licking frequency remain stable at 6-7 Hz, independent of level of water deprivation [229]. Additionally, when rats or mice are subjected to altered environmental conditions either by decreasing or increasing the size of the sipper tube, the size of the licking burst is dramatically changed without any effect on licking frequency [230,231]. Therefore, to determine if IoNC or sham treatment had any effect on orofacial function in rats, we estimated the number of licks required for consumption of a gram of reward, licking frequency, and drinking efficiency. Compared to baseline measurements, the number of licks required for consumption of one gram of reward significantly increased after IoNC (Fig. 2.2 D), suggesting that the amount of reward received

per each lick significantly decreased in the IoNC group. Moreover, comparison of licking frequency without adjusting to the actual reward consumed, showed no significant difference between IoNC and sham groups at all time points tested (Fig 2.2 E). Calculation of drinking efficiency for both IoNC and sham groups showed a significant decrease after IoNC treatment with no deviation from the standard licking frequency in the sham rats (Fig 2.2 F). Our data suggests that modification of licking behavior is attributed to chronic neuropathic pain as a result of IoNC.

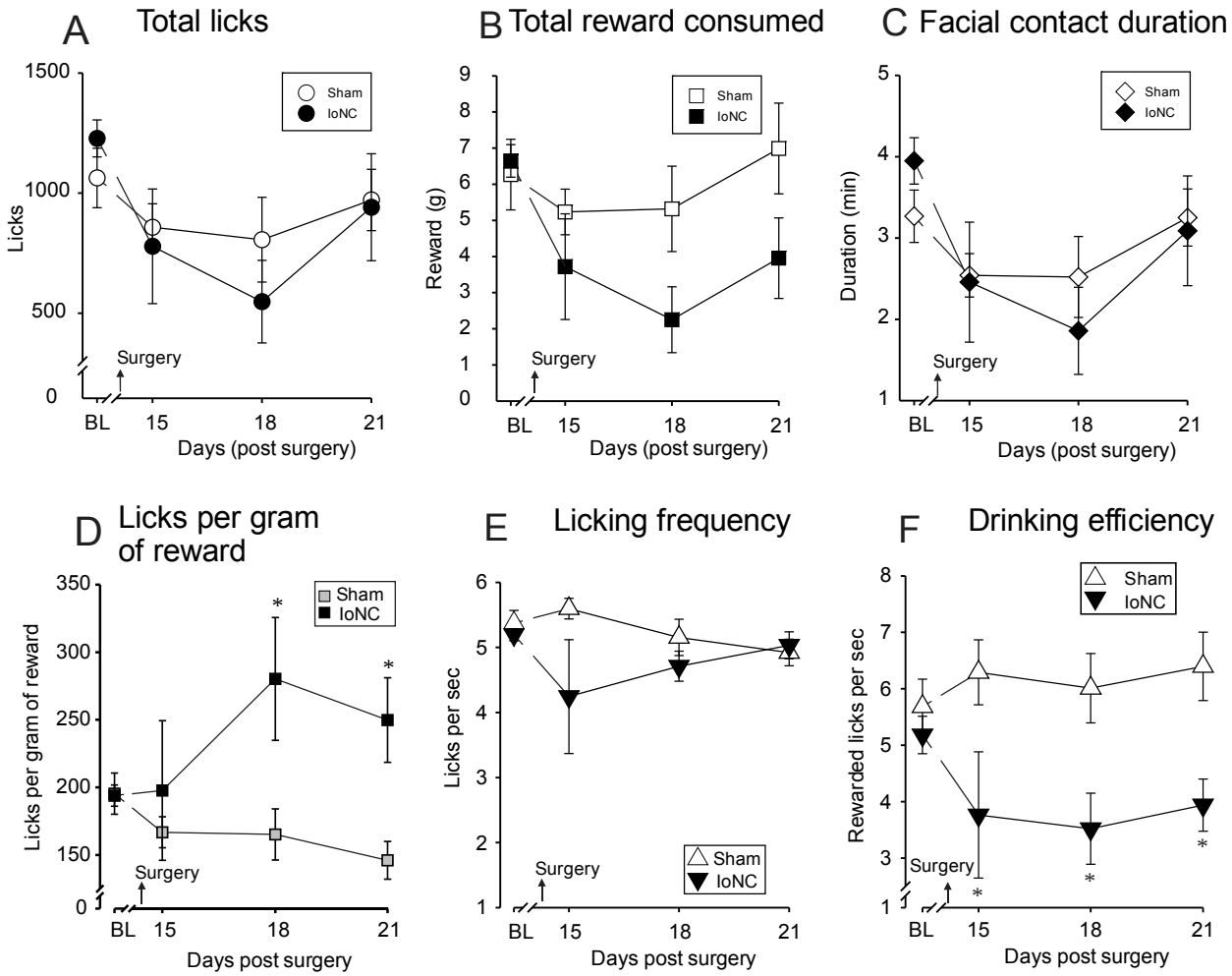


Figure 2.2: Orofacial operant measures.
(A-C) Total licks, total reward consumed and total facial contact duration are recorded for a 10 minute test session before surgery and 15,18 & 21 days after surgery for sham (n=6) and IoNC (n=6) groups. Baseline (BL) measurements represent the mean of two test trials before the surgery. Two-way RM ANOVA showed no significant difference between IoNC and sham groups for total licks, total reward and total facial contact duration. (D) Total number of licks required to consume one gram of reward significantly increased after IoNC (* $p<0.05$ from sham values; two-way RM ANOVA, Holm-Sidak Post-Hoc method). (E) Standard licking frequency is not significantly different between IoNC and sham rats. (F) Drinking efficiency is significantly lowered in the IoNC group at all time points tested after surgery. (*, $p<0.05$; two-way RM ANOVA, Holm-Sidak Post-Hoc method).

2.4 Discussion:

2.4.1 Operant analysis of chronic orofacial pain:

Our current method to evaluate licking function in rats is in good agreement with previous studies that described the microstructure of licking behavior [230,231]. Even though previous observations showed that the amplitude of the licking burst is modified by external stimuli, the role of pain in the modulation of licking function has not been explored. There is some evidence that suggests either electrical activation of infraorbital nerve or mechanical stimulation of tooth pulp suppress tongue EMG activity [232], whereas perioral stimulations of awake rats with food or water enhance jaw opening and tongue protrusion [233]. In our experiment, the nitinol wires fitted on the either side of the drinking alcove served as tactile stimulators for a rat perioral and vibrissal pad region. Our current estimation of licks per gram of reward showed that the amount of reward ingested per each lick is significantly reduced in the IoNC group with no detectable change in the licking behavior of sham rats. A parsimonious explanation for this change in the licking efficiency of IoNC rats is that the orofacial neuropathic pain significantly lowered the amplitude of the tongue licking burst. In our current experimental situation, neuropathic pain can be evidenced by the presence of ongoing or spontaneous pain and dynamic mechanical allodynia. Since the arrangement of nitinol wires had no effect on the drinking efficiency of sham rats, the nitinol wires arranged on either side deliver only innocuous tactile stimuli in sham rats and evoke dynamic mechanical allodynia in IoNC rats.

2.4.2 Neural basis of orofacial dysfunction after IoNC:

Orofacial muscular activity in mammals is regulated by tight coordination of several important brainstem nuclei with continuous feedback from primary afferent pathways and gating by higher-

order brain regions including cortex, cerebellum, basal ganglia and superior colliculus [234]. The sensory information from orofacial region is carried mostly by the primary afferents of all three sub-divisions (V1, V2 & V3) of trigeminal nerve (Fig 2.3). The central fibers of the trigeminal nerve travel in the rostro-caudal extent in the brainstem and anastomose with the second order neurons of the trigeminal brainstem nuclear complex (TBSNC), which is composed of principal sensory nucleus and spinal trigeminal nucleus (subdivided into S. oralis, S.interpolaris and S. caudalis). The principal sensory and S. oralis nuclei receive majority of low threshold tactile input from the orofacial regions. Temperature and pain sensations are carried over to S. caudalis mostly and S. interpolaris to some extent [235]. The projection neurons from the TBSNC carry afferent information to higher order centers such as thalamus and cortex with minor portion of neurons forming local synaptic circuits with motor nuclei of the brainstem [236]. The extent to which the motor output is excitatory or inhibitory is governed by the nature of afferent sensation and descending input from cortical masticatory area [232,233,237]. Central pattern generators (CPGs) of brainstem generate rhythmic and coordinated movements of tongue and jaw muscles to perform orofacial functions such as licking and chewing [238]. Licking frequency of rats is highly stable and is not influenced by change in external stimuli, indicating that CPGs operate independent of feedback from afferent sensation [239]. Moreover, the generation of licking rhythm is independent of the amplitude of tongue-muscle contraction [240] suggesting that the magnitude of tongue licking burst is influenced by descending pathways from higher-order motor areas of the brain. It has been shown that frontal cortical areas regulate tongue protrusions during rhythmic licking in a sensory detection task for mice trained to lick for a reward [241]. Even though the exact pathway involved in the integration of sensory-motor cortices is currently unknown, our data suggests that the orofacial behavior adaptation as a result of trigeminal neuropathic pain is mainly under higher-order motor control. This is evidenced by two of our important observations: 1. No significant difference in the rhythmic licking frequency

between IoNC and sham rats; 2. Significant increase in the number of licks required for consumption of one gram of reward, suggestive of decreased magnitude of jaw opening or tongue licking burst. The methodology described here for the evaluation of orofacial neuropathic pain represents pain processing through an entire neuraxis.

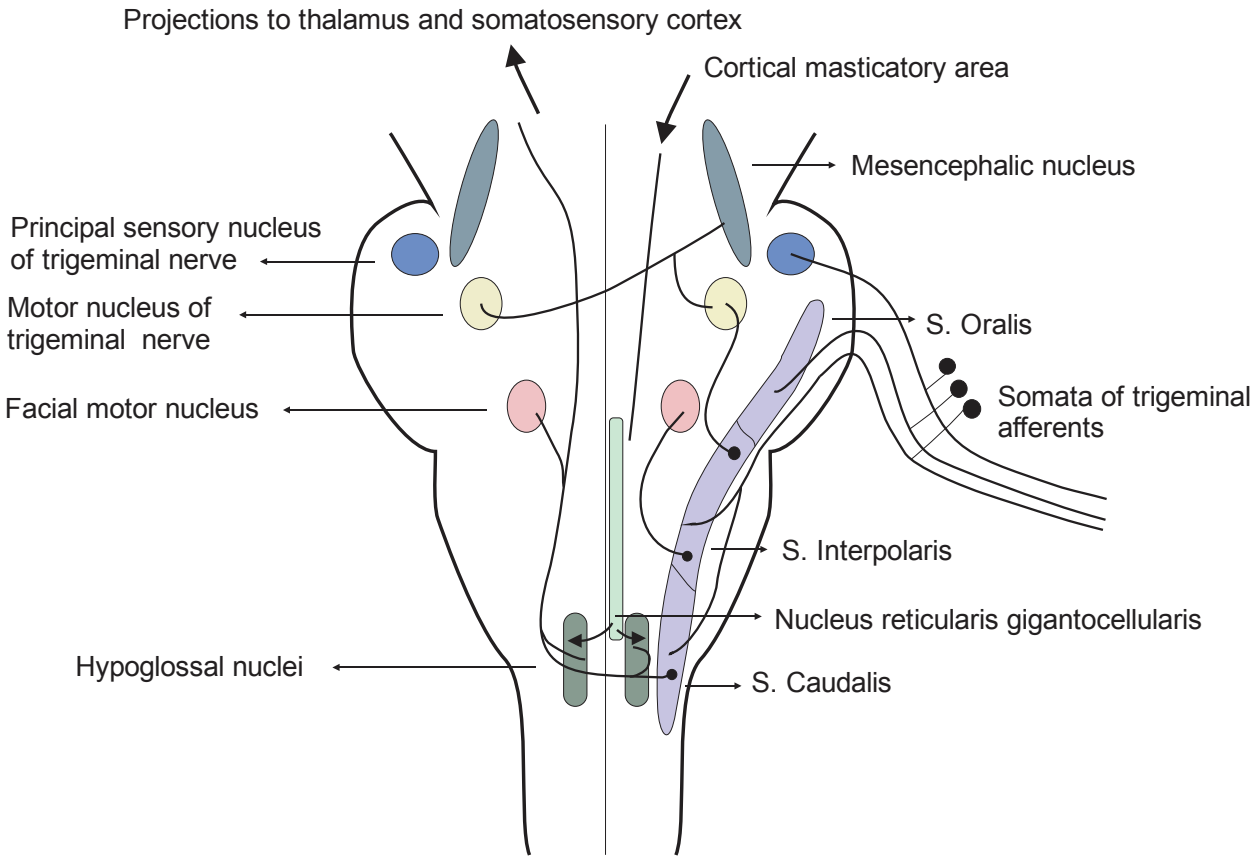


Figure 2.3: Ascending and descending pathways of brainstem
Schematic view of coronal section through mammalian brainstem. For ease of reference, trigeminal afferent connections to the brainstem are depicted unilaterally. (Adapted from Haines D.E, Neuroanatomy)

Chapter III – Specific Aim (SA) 2

SA2: To investigate peripherally restricted cannabinoids for the potential treatment of neuropathic pain

3.1 Introduction:

Perception of pain is induced by stimulation of nociceptors either through direct activation of cell surface receptors capable of detecting harmful mechanical, thermal and chemical stimuli or through receptors specialized in sensing proalgesic factors such as nucleotides, peptides and lipid mediators [242]. Endocannabinoids comprise a subset of lipid derived mediators and are described as potent regulators for the peripheral gating of pain [243]. Endocannabinoids along with their metabolizing enzymes and cognate receptors constitute the endocannabinoid system [244]. Physiological effects of both exogenous and endogenous cannabinoids are largely mediated via cannabinoid receptor subtypes 1 (CB1R) and 2 (CB2R). CBRs are members of the superfamily of G-protein coupled receptors (GPCRs) and are coupled to $G_{i/o}$ proteins [245]. Like other GPCRs, rat CB1R harbors seven transmembrane spanning α-helices with an extracellular N-terminus and an intracellular C-terminus, and is partially homologous to rCB2R. The rCB1R shares more than 97% sequence homology with human CB1R [246]. Neurobiological effects of CB1R activation include motor dysfunction and hypoactivity, immobility, hypothermia, antinociception and deficits in memory and cognition.

3.1.1 CBR distribution and function:

The expression of CB1R is primarily restricted to nervous system with abundant expression in regions involved in pain transmission, similar to opioid receptors [247]. Autoradiographic studies with tritiated CB1R agonist, CP55,940 identified extraordinarily high levels of CB1R in substantia nigra, globus pallidus, hippocampus, cerebellum and cortex in rodents and humans [248,249]. Moreover, immunohistochemistry of rat brain identified CB1R expression in regions

involved in nociception – periaqueductal gray (PAG), rostral ventromedial medulla (RVM) and thalamus [250]. At the neuronal level, CB1Rs are expressed on presynaptic terminals of GABAergic and glutamatergic neurons, modulating neurotransmission [250–252]. CB1R expression was also identified in the regions of spinal cord involved in pain transmission, dorsolateral funiculus, superficial dorsal horn and lamina X, and central terminals of peptidergic class of nociceptive neurons [253]. In the PNS, CB1R expression was detected in all types of DRG neurons with predominant expression in the isolectin B4 positive (IB4+) class of nociceptive neurons [116]. The expression of CB2R is restricted mostly to immune cells and it was originally identified in splenic macrophages [254]. In addition, the CB2R expression has also been detected in B cells, NK cells and microglia [255,256].

The activation of CB1 and CB2 receptors initiates multiple intracellular signaling pathways by engaging the C-terminal domain of CBRs. Intracellular activation of $G_{i/o}$ protein via CB1R inhibits pathway downstream of adenylate cyclase, subsequently inactivating pre-synaptic voltage dependent Ca^{2+} channels (particularly L, N and P/Q types), and activating inwardly rectifying K^{+} channels, with net result in the augmentation of membrane hyperpolarization [257–259]. CB1R activation also induces activation of mitogen activated protein kinase (MAPK) and immediate early gene signaling pathways [245]. Activation of CB2R results in the modulation of extracellular signal regulated kinase 1/2 pathway, inhibiting the release of proinflammatory cytokines [260].

3.1.2 Endocannabinoids and their metabolism:

Among several endocannabinoids discovered, those with high relevance to pain physiology are anandamide and 2-AG [247]. Anandamide binds to both cannabinoid receptors with high affinity at CB1R. Activation of CB1R with anandamide results in the initiation of classic $G_{i/o}$ pathway, with net result of hyperpolarized membrane state. Anandamide also exhibits agonist

activity at TRPV1 with weak binding affinity [261]. Anandamide is synthesized by the intracellular breakdown of N-arachidonylphosphatidylethanolamine (NAPE) by phospholipase D (PLD) [262,263]. The exact mechanism involved in the synthesis of anandamide is still unclear as the basal levels of anandamide remain stable in NAPE-PLD $^{-/-}$ mice [264]. The degradation of anandamide involves intracellular uptake followed by hydrolysis with fatty acid amide hydrolase (FAAH) [265]. 2-AG exhibits similar cannabimimetic effects as anandamide but it has a more prominent role in retrograde signaling at the synapse and is much abundantly expressed than anandamide [266]. It is synthesized and degraded by diacyglycerol lipase (DAGL) and monoacyl glycerol lipase (MAGL), respectively [267].

3.1.3 Cannabinoids and neuropathic pain:

Many synthetic and naturally occurring cannabinoids have been widely tested in pre-clinical studies of acute, chronic inflammatory and neuropathic pain. Major pharmacologically active cannabinoids from the herbal cannabis are Δ^9- and Δ^8-THC, cannabidiol and cannabinol [268]. Synthetic cannabinoids include classical (HU-210), non-classical (CP55,940), aminoalkylindoles (WIN55,212-2), endocannabinoid analogues (arachidonyl-2-chloroethylamide and arvanil) and CBR antagonists (SR141716A and SR144528) [269]. Cannabinoids are effective in suppressing signs of neuropathic pain such as allodynia and hyperalgesia in a wide variety of rodent models of neuropathic pain induced by traumatic nerve injury, chemotherapy induced peripheral neuropathy, streptozotocin induced peripheral neuropathy, and neuropathy as a result of HIV or herpes zoster infection [270]. Moreover, increase in the expression of CB1 and CB2 receptors has been reported in DRG and spinal cord/brain stem after chronic constriction injury of sciatic nerve/infraorbital nerve and spinal nerve ligation [116,271–273]. Acute administration of WIN55,212-2, a mixed agonist at CB1R/CB2R, suppressed all signs of neuropathic pain associated with spinal nerve ligation. In addition, chronic administration of WIN55,212-2 attenuated the development of

mechanical allodynia and glial activation in the spinal cord [274]. Unlike morphine, chronic administration of WIN55,212-2 reduced mechanical allodynia developed after spinal cord injury with no decrease in effectiveness overtime, suggesting no tolerance [275]. When mice subjected to SNL were treated with CP-55,940, a full agonist at CB1R, potent suppression of mechanical allodynia was observed. This anti-allodynic effect was completely diminished in $CB1^{-/-}$ mice but not in $CB2^{-/-}$ mice, suggesting that the CB1R activation mediates majority of antinociceptive actions of mixed CB1/CB2 agonists when administered systemically [276]. The efficacy of CB2 agonists has also been demonstrated in the suppression of neuropathic pain after SNL. The CB2 agonist AM1241 suppressed both thermal hyperalgesia and mechanical allodynia following SNL [272]. Following L5 spinal nerve transection, CB2 receptor immunoreactivity is increased in the ipsilateral dorsal horn and colocalizes with markers of microglia and perivascular cells [277], suggesting that microglial activation plays an important role in the maintenance of neuropathic pain. The efficacy of the inhibitors of endocannabinoid degrading enzymes such as FAAH has also been reported. Oral administration of a reversible FAAH inhibitor, ST4070 suppressed mechanical allodynia observed in vincristine, streptozotocin and chronic constriction nerve injury induced neuropathic pain and correlated with enhanced brain levels of endocannabinoids in these animal models [278]. Clinical studies also suggest that cannabinoids exhibit greater efficacy when used for the management of neuropathic pain compared to acute and postoperative pain [279]. Derivatives of naturally occurring cannabinoids such as Δ^9-THC, Marinol and Sativex have shown some efficacy in the management of HIV induced sensory neuropathy and multiple sclerosis induced neuropathic pain [214,280,281], although CNS mediated side-effects have been reported.

3.1.4 Peripheral targeting of CB1R for the treatment of neuropathic pain:

Site specific administration of cannabinoid analogues in rodent models of inflammatory and neuropathic pain showed marked difference in the potency of analgesia. Intraplantar administration of WIN55,212-2 in a rat model of partial sciatic nerve ligation resulted in more than 70% reduction in mechanical hyperalgesia. This effect is completely blocked by subcutaneous injection of CB1R antagonist SR141716A but not after intrathecal administration [117]. Findings from this study provided preliminary evidence that CB1R expressed on the primary sensory neurons alone can mediate most of the anti-nociceptive effects after cannabinoid administration. More conclusive evidence came from the study that described anti-nociceptive properties of synthetic cannabinoids in a mouse model of neuropathic pain where CB1Rs are deleted in majority of nociceptive primary sensory neurons ($Na_V1.8^+$/SNS-$CB1^-$). These mice exhibited exaggerated responses to noxious mechanical and thermal stimuli, indicating the existence of a basal anti-nociceptive tone mediated by continuous activation of CB1Rs in the periphery. In a spared nerve injury (SNI) model of neuropathic pain, the anti-allodynic effect of WIN55,212-2 is diminished in SNS-$CB1^-$ mice after systemic administration. This effect is fully preserved in control littermates at doses where no signs of CNS side-effects are observed [282].

Evidence from these studies prompted us to consider the development of novel peripherally restricted cannabinoids (PRCBs) for the effective treatment of neuropathic pain without any CNS mediated side effects. These compounds have been developed in collaboration with Dr. Herbert H. Seltzman (Research Triangle Institute, Research Triangle Park of North Carolina). A significant portion of my dissertation work has been to investigate the therapeutic efficacy of PRCBs in rat models of chronic pain with primary emphasis on neuropathic pain. In the current research aim, I will demonstrate that a peripherally acting

cannabinoid is effective in alleviating chronic symptoms of neuropathic pain without causing CNS side effects.

3.2 Preliminary data:

3.2.1 Ligand design and synthesis:

Unique compounds were designed as a series with an indene core and substituted about these rings [283] (Figure 3.1).

3.2.2 In vitro screening:

Novel compounds were first screened for: a) CB1R and CB2R binding, b) Ca^{2+} flux assay for agonist activity in CHO cells stably expressing $G_{\alpha 16}$ and human CB1R (hCB1R) and hCB2R, c) blood-brain barrier permeability using the Madin-Darby canine kidney (MDCK-MDR1) cell line assay. After identifying suitable ligands with high affinity and agonist activity at CB1R/CB2R and low MDCK permeability, ligands were tested for stability in rat plasma and in the rat S9 fraction (Table 1). Subsequently, the most promising compounds were tested in rat models of chronic pain.

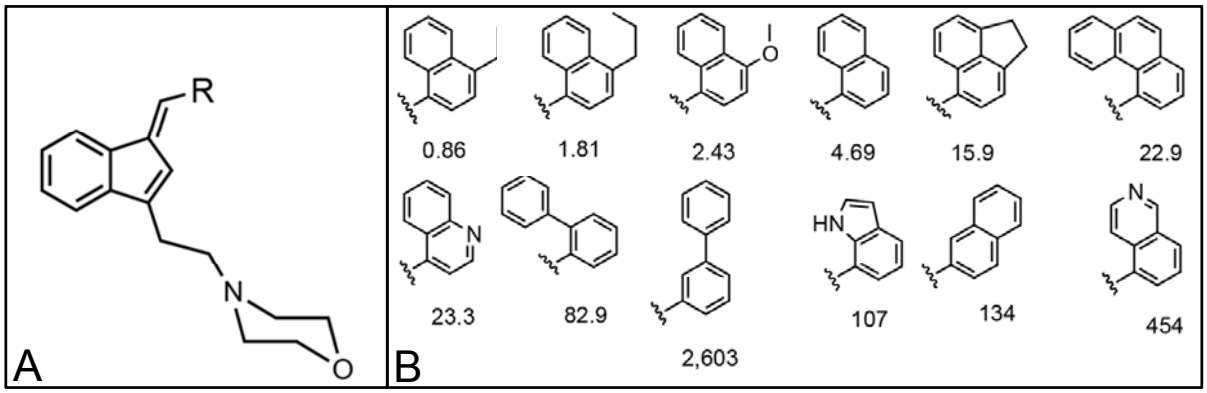


Figure 3.1: Indene series
An indene core is substituted in the C-3 position with an ethyl-4-morpholino group and with a 1-naphthalenylmethylene at the C-1 position (Fig. 1A). The 1-naphthalenylmethylene is further substituted at the 4-position with different alkyl moieties (R group) (Fig. 1B).

E-arylidene morpholinoethyl indene R-group					
Abbreviated name	EtNMI	PrNMI	MoNMI	ENMI	AceNMI
CB1R Ki (nM)	0.86	1.81	2.43	4.69	15.9
CB2R Ki (nM)	0.79	1.00	4.07	5.62	4.22
MDCK B/A ratio	0.000	0.000	0.000	0.001	0.0014
hCB1R EC_{50} (nM)	141	182	196	257	---
hCB1R % E_{Max}	121	108	114	103	---
hCB2R EC_{50} (nM)	163	94	194	138	594
hCB2R % E_{Max}	59	60	52	27	20
% remaining in plasma at 1 hr	---	92	100	98.5	---
% remaining in S9 fraction at 1 hr	---	37	43	22.5	---

Table 3.1: E-Arylidene morpholinoethyl indenes
Structures, hCB1R/hCB2R binding, permeability in the MDCK assay (indicator of blood-brain barrier permeability), Ca^{2+} flux (hCB1R and hCB2R agonist) activity, and metabolic stability.

Note that while the indenes exhibit full agonist activity at hCB1R (% E_{max} versus CP55,940), they exhibit only partial agonist activity at hCB2R, with PrNMI having the highest partial agonist activity at hCB2R (69% E_{max}).

3.3 Methods:

Rat studies described here are approved for this experiment by the Chancellor's Animal Research Committee (ARC) of UCLA

3.3.1 Rat model of chemotherapy induced peripheral neuropathy:

The rat model was developed following instructions provided in the Guindon et al manuscript [284]. Cisplatin is injected intraperitoneally once a week at a dose of 3 mg/kg for 4 weeks. All animals received 4% sodium bicarbonate dissolved in 0.9% saline subcutaneously before each cisplatin injection to help buffer effects of cisplatin.

3.3.2 Sciatic nerve entrapment (SNE) neuropathy model:

Surgical procedure for the SNE model was performed as described previously [168]. Briefly, in rats anesthetized with isoflurane, left sciatic nerve is surgically exposed and three polyethylene cuffs (1 mm long, 2.28 mm outer diameter and 0.76 mm inner diameter) are placed around the sciatic nerve proximal to the trifurcation. Muscle and skin layers are separately closed.

3.3.3 Cerebrospinal fluid (CSF) sampling from rat:

The CSF sampling procedure was adapted from the method described previously by Liu et al for mice [285]. Rats are anesthetized with isoflurane and placed in a stereotactic frame with head position angled approximately 135° to the body axis. A sagittal incision of 10 mm long is made on the back of the neck and subcutaneous tissue and muscles are separated by blunt dissection

with forceps. A micro-retractor is used to hold the muscles apart. Overlying connective tissue is removed carefully to expose the duramater covering cisterna magna. A 30 gauge needle which is connected to polyethylene tubing and 1 cc syringe is advanced carefully through the shiny duramater and the plunger is aspirated slowly to collect 50 µl of CSF.

3.3.4 Behavioral testing:

a. Mechanical sensitivity:

Rats were placed in a plastic-walled cage (10×20×13 cm) with a metal mesh floor (0.6×0.6 cm holes) and allowed to acclimate for 10 min. The amount of pressure (g) needed to evoke a hindpaw withdrawal response was measured 4 times on each paw for SNE model and 2 times on each paw for CIPN model, separated by 30-s intervals using a von Frey-type digital meter (Model 1601C, IITC Instr.). Results of 4 tests per session were averaged for each paw.

b. Cold allodynia:

Cold allodynia was assessed by applying drops of acetone to the plantar surface of hindpaw. Rats were allowed to habituate in a plastic walled cage, as described for examination of mechanical sensitivity. One drop of acetone (~20 µl) was applied through the mesh platform to the plantar surface of hindpaw. Brisk withdrawal or flick of the hindpaw was counted as a positive response and the data are expressed as percent withdrawal.

c. Tail flick test:

A modified Hargreaves apparatus (Model 390, IITC Instr.) is used to measure tail-flick latency (TFL). Radiant heat is directed to a point 3 cm from the tail tip and the TFL observed and timed with a photo cell counter. The intensity of the radiant heat is adjusted for a baseline TFL of approximately 5-7 seconds for naïve rats, with a 25 s cutoff set to avoid tissue damage.

d. Rotarod:

Rats are trained 72 h before the test (3 sessions 24 h apart) to remain for at least 180 s on a rotarod revolving at an acceleration of 4-40 revs over 5 min). Rats are tested immediately before vehicle or drug injections and again at 2, 6, 24 and 48 hrs after injection. The time for which the rats are able to remain on the rotarod is recorded up to a cut-off of 5 min.

e. Hypothermia:

Rats are acclimated to a plastic restrainer apparatus (Model RTV-180 Braintree Scientific Inc.) on the day of testing by placing them in the restrainer twice for 5 min separated by 20 min. Baseline core temperature is taken before treatment, and again at 2, 6, and 24 and 48 hrs after test drug injection.

f. Catalepsy (ring) test:

Rats are placed with their forepaws on a horizontal metal ring (12 cm diameter) at a height that allows their hindpaws to just touch the bench surface. Immobility is recorded as the time for the rat to move off the ring with a 100 s cutoff. Rats are tested before vehicle/drug injections and again at 2, 6, 24 and 48 hrs after injection.

3.3.5 Drugs:

Drugs are prepared as stock solutions in dimethyl sulfoxide (DMSO) or DMSO+Cremaphor EL and administered in bacteriostatic saline vehicle in a volume of ~0.5 ml systemically (i.p.) at doses dictated by their CB1R activity in the in vitro assay. For oral administration, drug stock was dissolved in 20% sweetened condensed milk, titrated to a final volume of ~5 ml and given by gavage.

3.3.6 Liquid chromatography/Mass spectrometry:

Analytes were determined in samples of plasma, brain, and cerebrospinal fluid (CSF) by multiple reaction monitoring of reaction 410.3 → 100.2.

3.3.7 Western blotting:

Total protein from all tissue samples is extracted using PARIS kit (Invitrogen) and concentration is determined by DC protein assay (Biorad). Samples are mixed with a loading dye in 1:1 ratio prior to running on 10 % poly-acrylamide stain free gels (Mini-protean TGX Stain free gels, Biorad). Proteins are segregated under denatured running conditions and transferred to 0.2 µ PVDF (Immun-blot, Biorad) using a wet transfer method. Blots are incubated with anti-L15 CB1R 1° antibody (gift of Prof. Ken Mackie, Indiana University) (2% ECL prime blocking reagent, 1:1000) over night (16 hrs) at 4°C and HRP-conjugated 2° anti-body (2.5% NFDM, 1:2000) at room temperature for 1 hour. Stain-free images are taken prior to application of substrate on the blot using ChemiDoc MP imaging system (Biorad). CB1R bands are visualized by chemiluminescence detection by ChemiDoc MP imager (Biorad). Acquired images are analyzed by ImageLab software (BioRad). Band volume intensity of each lane is normalized to total lane protein.

3.3.8 Quantitative PCR:

Total RNA from all tissue samples is collected using the PARIS kit (Invitrogen) and concentration is determined by spectrophotometer (Nanopearl, IMPLEN). 50 ng of total RNA is used for each qPCR reaction well and reaction is carried out using one-step RNA-Ct kit (Invitrogen) on a 7900 HT real time fast PCR system. Primers and probes used for qPCR reaction are: CB1R (Rn00562880_m1), CB2R (Rn01637601_m1) and GAPDH

(Rn01775763_g1). mRNA fold change is calculated using comparative Ct method (Please refer to SA3 methods for detailed description).

3.3.9 Statistical methods:

Data is expressed as mean ± s.e.m. Before performing the actual test procedures, entire numerical data is verified for normality and equal variance. Difference of means between two groups is compared with unpaired t-test. Before and after treatments are compared with paired t-test. For a multiple group comparison, one-way or two-way ANOVA is applied, as applicable. Repeated treatments are compared with repeated measures ANOVA. Holm-Sidak post hoc method is used to compare individual group differences (pairwise or vs. control). A p value of < 0.05 is considered statistically significant.

3.4 Results:

3.4.1 In vivo screening of E-arylidene morpholinoethyl indenes:

We examined the effectiveness of indene PRCBs in alleviating painful symptoms of SNE induced neuropathic pain. SNE was demonstrated to produce consistent pain behaviors and models the most common complaints of human neuropathic pain patients of mechanical allodynia [69,168]. Fig.3.2 illustrates that systemic injection of PrNMI or MoNMI (0.3 mg/kg) results in large, reversible decreases in mechanical allodynia. By contrast, ENMI has a much smaller effect, consistent with its lower CBR affinity and faster metabolism (Table 3.1).

Pharmacokinetic profiling of novel compounds is prerequisite to their clinical development [286]. Analysis of plasma samples after PrNMI injections yielded its initial pharmacokinetic profile (Fig. 3.2E) which is in good agreement with the time course of its anti-allodynic effects (Fig.

3.2B). Measurements of brain levels are a must for putative peripherally-restricted drugs. Total brain levels include drug partitioned into brain lipids + unbound drug in equilibrium with extracellular fluid. Therefore, cerebrospinal fluid (CSF)/plasma ratios are considered to be more precise estimates of a drug's brain penetration because of the continuity of CSF with extracellular space [287]. However, both measures are needed to confirm minimal CNS access and to compare with other reported peripherally-restricted CB1R ligands [288]. Analysis of plasma, brain and cerebrospinal fluid (CSF) samples confirmed the minimal penetration of PrNMI into the CNS after systemic administration (Fig. 3.2E).

In subsequent experiments we demonstrated that PrNMI was also effective in suppressing neuropathy symptoms after oral administration, which is more representative of future therapeutic uses. The high oral dose of PrNMI (3 mg/kg) likely accounts for its continued anti-allodynic effectiveness at the 24 hr time point (Fig. 3.2F).

3.4.2 CB1R mediated CNS side effects – HU-210 vs. PRCBs:

The CNS-mediated psychotropic actions of CB1R ligands represent their most troubling side effects. The catalepsy, motor performance, hypothermia, and analgesia tests are classically predictive of CNS CB1R activation [289]. Effects in all 4 tests have been thought to be mediated by the activation of central CB1Rs, but it is now well established that peripheral CBRs make a major contribution to the analgesic effects of CBs [282,117]. Therefore, we used the "tetrad" to determine whether the novel ligands have antinociceptive effects and side-effect profile consistent with central CB1R activation. We also studied the potent CB1R agonist, HU-210, to allow comparisons of this positive control with the putatively brain-impermeant analogs which we developed. We used systemic doses of HU-210 [117] and of novel indenes (e.g., Fig. 3.2) consistent with their demonstrated effectiveness in alleviating painful neuropathy symptoms. The experiments were performed using the "tetrad" tests modified for rats, with rotarod

substituting for the spontaneous activity test. Unlike HU-210, PrNMI and other indenes lack effects in the catalepsy, rotarod or hypothermia assays, although a small effect in the tail-flick assay is observed, as expected for analgesic effect of peripherally-acting CB1R ligands (Fig. 3.3).

3.4.3 PRCB alleviates neuropathic pain symptoms via periphery:

Operant assays enable evaluation of cortical processing of pain and are able to appraise the behavior as a result of modified central output [224,290]. CB1Rs are abundantly expressed in basal ganglia and cerebellum which are involved in the gating of rhythmic actions of brain stem such as licking and chewing [234]. The operant assay method (please refer to SA1) that we developed is suitable for simultaneous evaluation of antinociception as well as CNS mediated side-effects due to CB1R activation by cannabinoids. Therefore, we tested the therapeutic efficacy of one of our novel class of PRCBs, PrNMI in suppressing symptoms of neuropathic pain in a rat model of IoNC. Intraperitoneal injection of PrNMI (0.3 mg/kg) resulted in significant improvement in the drinking efficiency of IoNC rats compared to vehicle administration alone (Fig. 3.4B). Similar administration of PrNMI in sham rats had no effect on drinking efficiency, suggesting that the antinociceptive effects of PrNMI are mostly mediated at the periphery (Fig. 3.4A). Analgesic effects of cannabinoids are primarily mediated by CB1Rs expressed on peripheral terminals of nociceptive neurons [282] and brain-permeant CBs were shown to ameliorate the pain symptoms of chemotherapy induced peripheral neuropathy, a dose limiting side-effect of cancer treatment [291,292]. Hence, we studied PrNMI's ability to alleviate CIPN symptoms after local and systemic administrations. Intraplantar injection of PrNMI (0.25 mg/kg) induced complete suppression of CIPN symptoms compared to partial alleviation after intraperitoneal administration (0.25 mg/kg), suggesting that peripheral nerve terminals are potential targets of PrNMI's anti-allodynic effects (Fig. 3.4C-D).

3.4.4 Anti-allodynic effects of PrNMI are largely mediated via CB1R without appreciable tolerance:

Our in vitro studies showed that PRNMI is a full agonist at hCB1R, but only a partial agonist at hCB2R (Table 3.1). To determine, which CBR subtype is responsible for the anti-allodynic actions, we measured the ability of PrNMI to suppress mechanical and cold allodynia in CIPN rats in the presence of CB1R or CB2R selective antagonists. PrNMI was administered alone or in combination with CBR antagonists at 3 day intervals. Co-administration of a CB2R selective inverse agonist, SR144528 had no effect on suppression of mechanical or cold allodynia by PrNMI, whereas co-administration of the CB1R antagonist, SR141716A, blocked the anti-allodynic effects of PrNMI, suggestive of CB1R activation by PrNMI (Fig. 3.5A-F). Chronic administration of centrally acting CBs results in receptor desensitization and development of tolerance, although this is region specific [293,294]. Since tolerance is of major concern for chronic pain medications; we tested the anti-allodynic efficacy of PrNMI in CIPN rats during repeated oral administrations and found no appreciable tolerance after two weeks of testing (Fig. 3.5G-H).

3.4.5 CB1R levels remain stable in PrNMI treated rats:

Internalization of cell surface receptors after ligand binding is observed in some of the GPCRs. While few GPCRs recycle back to cell surface, others are subjected to liposome mediated degradation, constituting tolerance [295,296]. Repeated oral dosing of PrNMI for two weeks showed no considerable tolerance on tests for allodynia in CIPN rats (Fig. 3.5G-H). Therefore, we examined CB1R protein levels in L4-L5 DRG at the end of rat behavioral measurements. Our Western blotting data showed no significant difference in the expression levels of CB1R between PrNMI treated CIPN rats and age matched naïve controls (Fig. 3.6A-B). Moreover, qPCR analysis of steady-state mRNA levels showed 2-5 fold increase in the PrNMI

treated SNE and sham rats, compared to age matched naïve controls (Fig. 3.6C). CB2R mRNA levels are not detected in DRG samples and is in agreement with previous studies that neuronal expression of CB2R is extremely low [272,297].

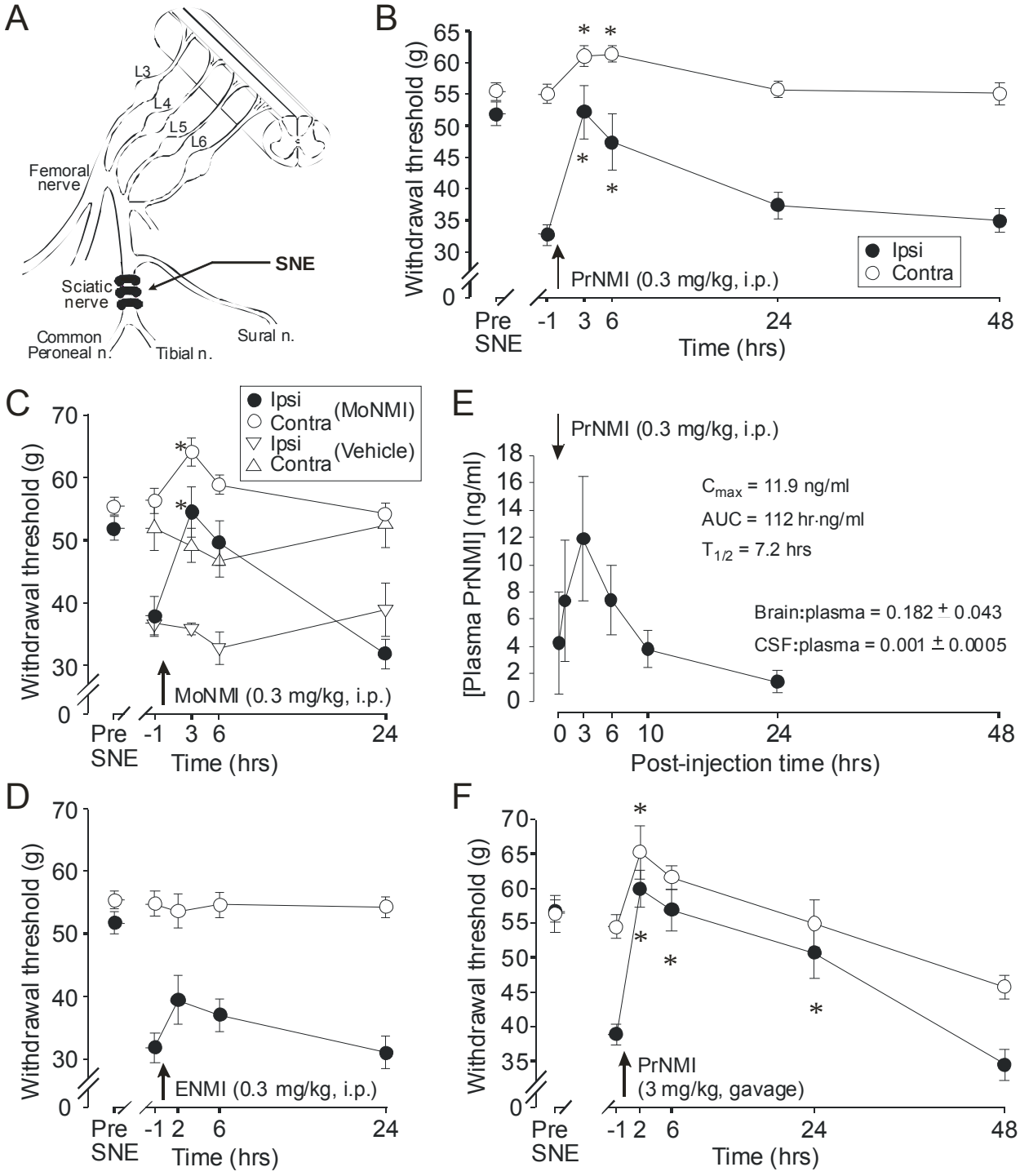


Figure 3.2: Reversible suppression of SNE-induced mechanical allodynia by representative indene PRCBs.

(a) Schematic of sciatic nerve entrapment and relevant peripheral nerve and spinal ganglia. (b) Graph of withdrawal thresholds to mechanical stimulation of hindpaws ipsilateral and contralateral to SNE at 1 hr before and 3, 6, 24, and 48 hrs after PrNMI (0.3 mg/kg, i.p.) injection. At 3 hrs post-injection, ipsilateral

thresholds are increased to levels of pre-drug contralateral thresholds and are indistinguishable from thresholds measured prior to neuropathy development (pre-SNE). Also note the drug-induced small, but significant increases in contralateral thresholds (mean ±SEM, n=8 rats). (c) In the same rats, administration of MoNMI (0.3 mg/kg), but not vehicle alone, results in similar increases in ipsilateral thresholds to pre-SNE values. (d) ENMI (0.3 mg/kg) produces considerably smaller increases in thresholds than PrNMI or MoNMI. *, $p<0.05$ vs. pre-drug (-1 hr) values (one-way ANOVA). (e) Changes in plasma [PrNMI] and calculated pharmacokinetic parameters after injection (0.3 mg/kg, i.p.) in naïve rats (n=3) are consistent with the time course of its effects on SNE neuropathy symptoms. Brain and CSF: plasma ratios of PrNMI obtained from samples collected from 3 other rats at ~75 min after PrNMI (0.3 mg/kg, i.p.) suggest minimal CNS penetration. (f) Oral administration of PrNMI (3 mg/kg) reversibly suppresses SNE neuropathy symptoms (n=8 rats). *, $p<0.05$ vs pre-drug (-1 hr) values (one-way ANOVA, Holm-Sidak post-hoc method).

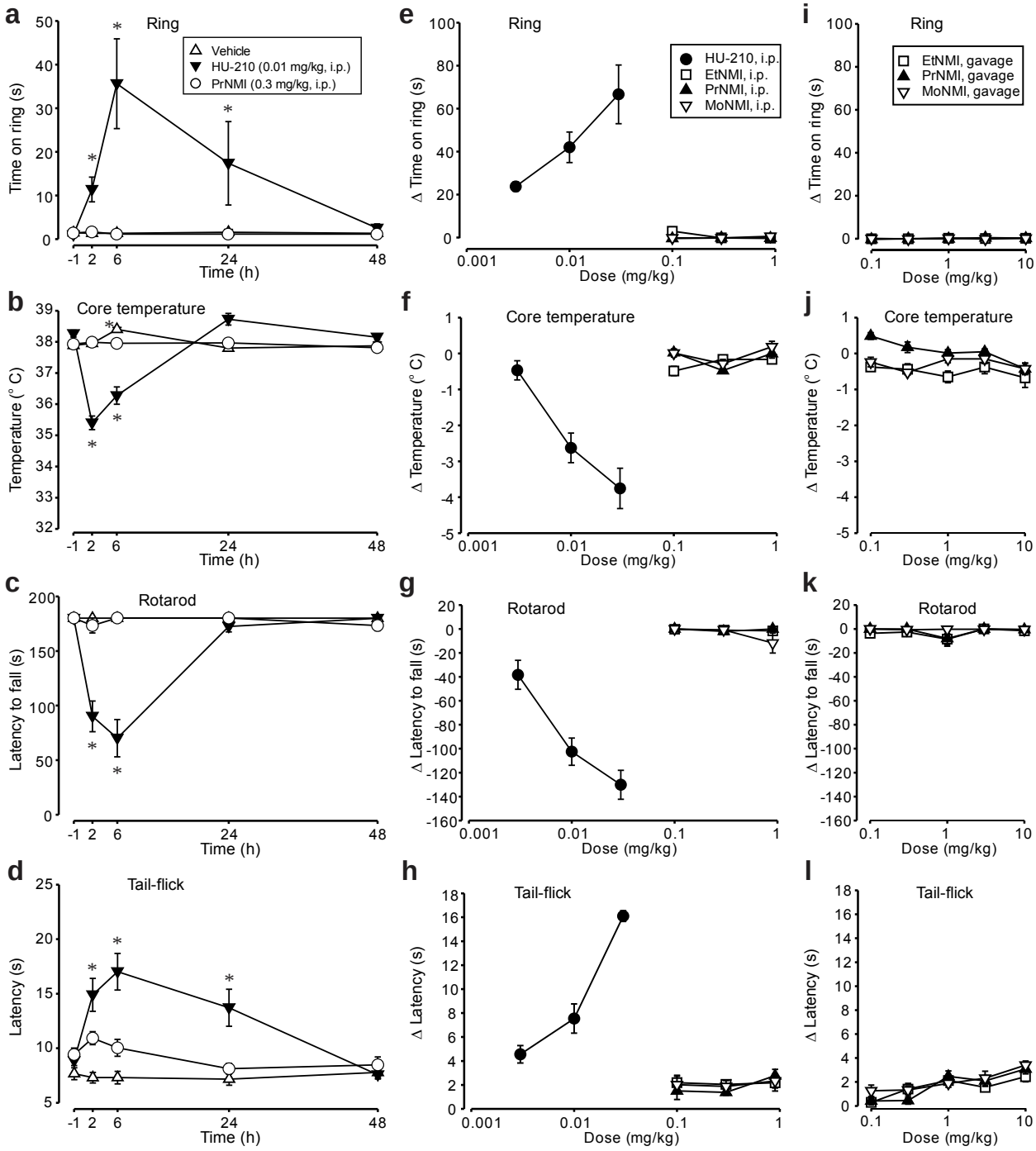


Figure 3.3: Activity of HU-210, PrNMI, EtNMI and MoNMI in the "tetrad" assay.
(a-d) Rats (n=8) were tested in each assay 1 h prior and up to 48 hrs following intraperitoneal injection of vehicle, HU-210 (0.01 mg/kg) or PrNMI (0.3 mg/kg). Note the profound CNS side effects of HU-210 vs. PrNMI in the ring, core temperature, and rotarod tests. The small analgesic effect of PrNMI in the tail-flick assay is consistent with peripheral activation of CBRs. *, $p<0.05$ vs. pre-drug (-1 hr) values (one-way

ANOVA). (e-h) Dose-dependence of brain-permeant HU-210 and peripherally-restricted EtNMI, PrNMI, or MoNMI in the tetrad assays after intraperitoneal administration. Each point represents mean peak effect ± S.E.M. of HU-210 (n=6 rats), EtNMI (n=8 rats), PrNMI (n=8 rats), and MoNMI (n=8 rats), each subtracted from its vehicle control. (i-l) Dose-dependence of EtNMI, PrNMI, and MoNMI in the tetrad assays after oral administration. Each point represents mean peak effect ± S.E.M. of EtNMI (n=8 rats), PrNMI (n=8 rats), and MoNMI (n=8 rats), each subtracted from its vehicle control. Note the relative lack of side effects in the catalepsy, motor incoordination, and hypothermia assays. The small effects in the tail-flick assay are consistent with antinociceptive effects due to activation of peripheral CBRs in naïve rats.

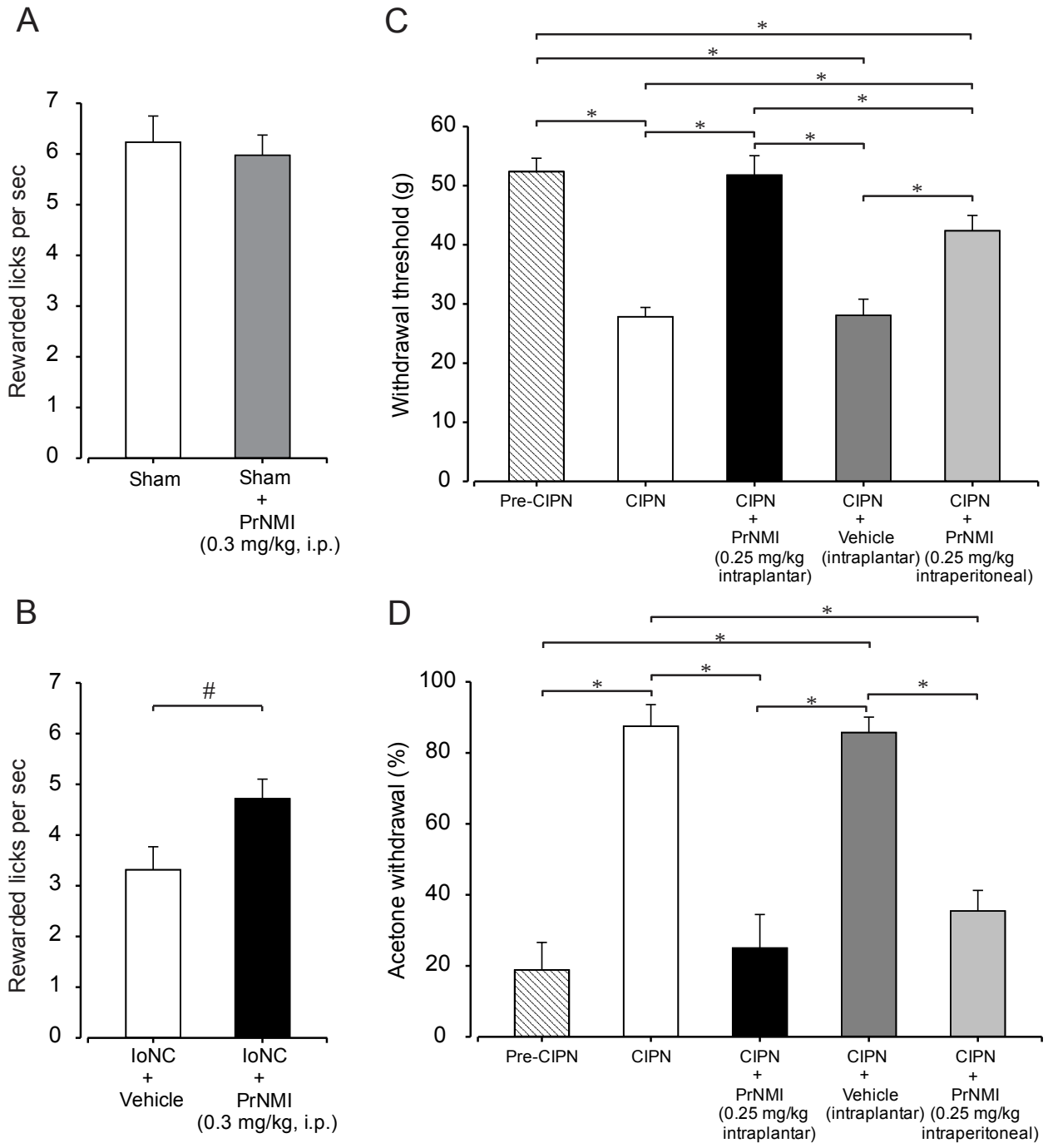


Figure 3.4: Demonstration of peripheral anti-allodynic activity of PrNMI in rat models of IoNC and CIPN.

(A) Evaluation of drinking efficiency by orofacial operant assay, 2 hrs after intraperitoneal administration of PrNMI (0.3 mg/kg) in sham rats. No significant change in the drinking efficiency was observed after PrNMI administration (Paired t-test). (B) Similar administration of PrNMI in IoNC rats resulted in the significant improvement of drinking efficiency in comparison with vehicle alone administration (# $p<0.05$;

Un-paired t-test). (C) Intraplantar administration of PrNMI (0.25 mg/kg) resulted in complete suppression of mechanical allodynia in CIPN rats (n=8). Note the reversal of mechanical withdrawal thresholds to pre-CIPN values after local but not systemic (0.25 mg/kg) or vehicle administrations (* $p<0.05$; one-way ANOVA, Holm-Sidak post-hoc method). (D) In the same rats, cold allodynia suppression is not significantly different between local and systemic administrations.

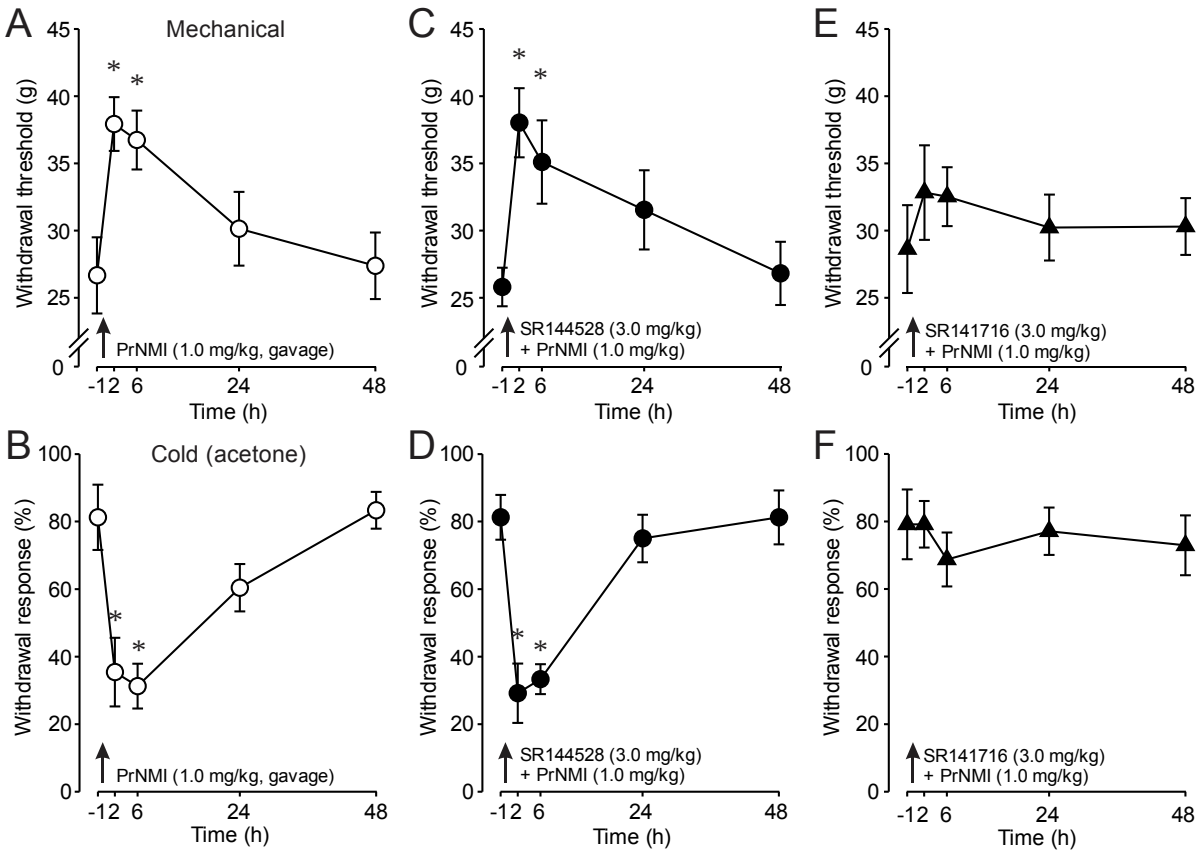

A
Mechanical
Withdrawal threshold (g)
PrNMI (1.0 mg/kg, gavage)
Time (h)
C
SR144528 (3.0 mg/kg)
+ PrNMI (1.0 mg/kg)
E
SR141716 (3.0 mg/kg)
+ PrNMI (1.0 mg/kg)
B
Cold (acetone)
Withdrawal response (%)
D
F
G
Mechanical
Threshold (g)
Time (days)
H
Cold (acetone)

Figure 3.5: Anti-allodynic effects of PrNMI in the CIPN are mediated by CB1Rs without considerable tolerance.

Suppression of cisplatin-induced mechanical (A) and cold (B) allodynia by PrNMI (1.0 mg/kg, gavage). C, D: In the same rats (n=8), co-administration of the selective CB2R inhibitor SR144528 (3 mg/kg, gavage) does not affect the response to PrNMI. E, F: However, PrNMI co-administration with the CB1R inhibitor SR141716A (3 mg/kg, gavage) blocks the response to PrNMI. (*, $p<0.05$ vs. pre-drug; one-way RM ANOVA, Holm-Sidak post hoc method). (G-H) Repeated daily oral administration of PrNMI (↑, 1 mg/kg) for 2 weeks suppresses mechanical (G) and cold (H) allodynia CIPN symptoms. *, $p<0.05$ from baseline values (one-way RM ANOVA, Holm-Sidak post hoc method). Note the significant anti-allodynic effects on all dosing days except day 8. After a 5-day break, PrNMI administration again has a significant effect.

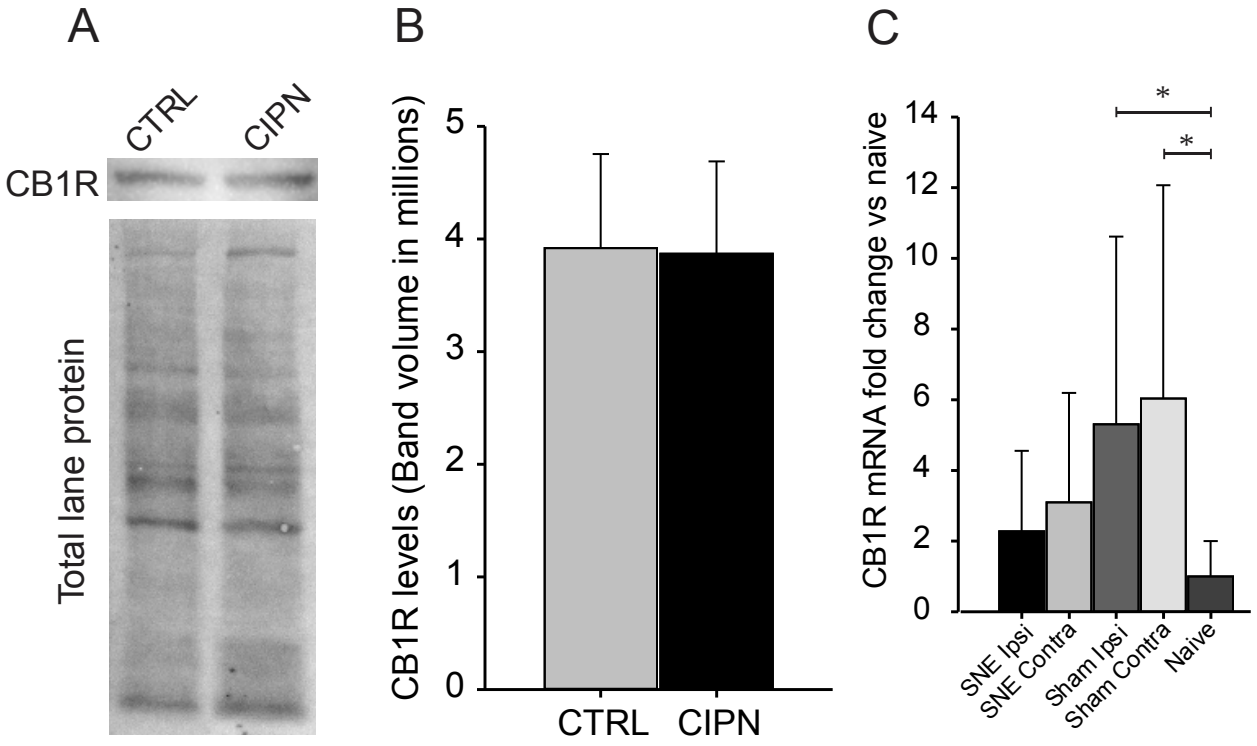


Figure 3.6: Estimation of CB1R mRNA and protein levels in L4-L5 DRG after repeated administrations of PrNMI in CIPN and SNE rats.

(A) Sample image of Western blot with control and CIPN samples loaded on the same gel. (B) Comparison of CB1R levels after normalization to total lane protein between untreated naïve control (n=8) and PrNMI treated CIPN (n=8) rats. No significant difference in CB1R expression levels between control and CIPN samples (t=0.0427; p=0.967) is noted. (C) CB1R mRNA fold change vs. naive in SNE-Ipsi, SNE-Contra, Sham-Ipsi and Sham-Contra samples after normalization to internal GAPDH. Note the higher steady state CB1R mRNA levels in PrNMI treated vs. untreated age matched naïve controls. Statistics were run comparing 2^{-dCt} values and error bars represent S.E.M. of PrNMI treated samples. (* $p<0.05$, sham-ipsi & sham-contra vs. naïve; one-way ANOVA, Holm-Sidak post-hoc method).

3.5 Discussion:

3.5.1 Peripherally restricted cannabinoids alleviate peripheral neuropathy symptoms without CNS side-effects:

A major impediment to the widespread use of cannabinoid based analgesics for the treatment of neuropathic and other chronic pain syndromes is CNS mediated side effects. Evidence from previous studies suggests that local administration of low doses of brain-permeant CBs often produce anti-nociceptive effects without central side-effects [117,298]. However, these doses induce only partial suppression of symptoms associated with chronic pain, as evidenced in our current tests for PrNMI's site of action (Fig. 3.4C-D). Previous pre-clinical studies demonstrated the utility of peripherally acting cannabinoids for the treatment of chronic pain in but failed to show complete segregation of analgesic effects from CNS mediated side-effects [288,299]. The PRCBs described here represent a novel class of cannabinoid based analgesics with high anti-allodynic efficacy and negligible central side-effects. The current thesis work based on pharmacological and behavioral studies demonstrated minimal CNS penetration, negligible central side effects and potent anti-allodynic effects of one of the PRCBs in three different rat models of neuropathic pain. Our comparisons of local vs. systemic administrations delineated the peripheral site as the primary target for anti-allodynic actions of PrNMI (Fig. 3.4 C-D). A significant proportion of human neuropathic pain population complain of ongoing or spontaneous pain and mechanical allodynia and pre-clinical screening of novel pain drugs for the suppression of these neuropathy symptoms is essential for future success at the clinical trial stage [300]. Pharmacological and behavioral studies focused on the development of novel PRCBs failed to characterize the ability of these drugs in the alleviation of spontaneous pain. Our current method of orofacial operant analysis in IoNC rats allowed us for evaluation of ongoing or spontaneous pain and mechanical allodynia after PrNMI administration. However, the magnitude of pain suppression that we observed is lower in operant assays as compared to

reflex withdrawal measures. Differences in treatment efficacy are possibly due to different types of neuropathy models we used for testing. Bilateral IoNC is a more severe type of neuropathy in comparison with the SNE model, suggesting that higher doses may be required for complete suppression of pain symptoms. Moreover, mechanisms underlying CB1R expression changes might differ between IoNC and SNE models. Our future studies will address the potential differences between these models.

3.5.2 Potential mechanisms underlying PRCBs' anti-allodynic actions in neuropathic pain:

Our current methods of in vivo testing showed potent anti-allodynic effects of PRCBs in neuropathic rats yet minimal or negligible effects in healthy controls. Neuropathic state is characterized by sensitization of afferent sensory neurons [301] and is partly attributed to changes in the endocannabinoid system [116,284]. CB1Rs on the peripheral nerve terminals mediate majority of anti-nociceptive effects [282] and it was postulated that CB1R activation induces inhibition of neurotransmitter release by afferent sensory neurons, and efferent sympathetic nerve terminals [302,303]. Results from the current study are in agreement with previous findings that the ability of PrNMI to suppress allodynia is greater after local administration (Fig. 3.4C-D). Moreover, our co-administration studies demonstrated that the PrNMI's ability to alleviate neuropathic pain is primarily mediated via CB1Rs (Fig. 3.5A-F). Other potential factors such as disruption of blood-nerve barrier (BNB) and alterations in the expression of BNB efflux transporters have been reported under certain chronic pain states [304,305]. These changes might enhance the penetration and availability of PRCBs to primary sensory neurons; therefore, anti-allodynic effects are observed only in neuropathic states.

CB1Rs undergo agonist induced internalization and some of them are subjected to lysosomal degradation [306]. Our Western blot analysis showed no significant change in the total CB1R protein levels after repeated treatments with PrNMI (Fig. 3.6A-B). In contrast, an increase

in the CB1R gene expression was noted (Fig. 3.6C). Chronic administration of Taxol (anti-cancer agent) induced neuropathy rats with CB2R agonists enhanced the expression of both CBR mRNAs in the spinal cord sections of L4-L5 DRG and this effect was blocked by co-administration with CB2R antagonist [307]. Although underlying mechanisms are unknown, we expect that the increased gene expression is a compensatory mechanism for the maintenance of normal CBR protein levels. Our study showed only preliminary evidence of PRCBs' effect on CB1R gene expression. Further characterization is necessary to fully understand the molecular effects of PRCBs.

3.5.3 Development of PRCBs as future therapeutics:

Previous studies pursuing the development of peripherally restricted mixed CB1R/CB2R agonists and CB2R agonists showed limited success [288,298,299,308]. In addition, the success of peripherally restricted endocannabinoid breakdown inhibitors is limited by off target effects at TRPV1 [309]. Implementation of PRCBs at the clinical level requires further screening for safety/toxicity and off-target effects. Our in vitro and in vivo screening studies showed CB1R as the primary target for PRCBs anti-allodynic action. However reliance on pharmacological studies alone is not sufficient to determine the specificity of novel drug entities. Previous study on transgenic mice with conditional deletion of CB1R in majority of nociceptive neurons demonstrated the specificity of peripheral CB1R mediated anti-nociceptive effects. Also, the role of CB1R activation-mediated anti-allodynia in large diameter fibers has not been ruled out. A future study based on transgenic mice with conditional deletion of CB1R in nociceptive as well as sensory and sympathetic neurons is of paramount importance for the further characterization of PRCBs off target effects. In addition, preclinical screening for safety pharmacology and toxicology such as monitoring of blood pressure, heart rate, and body temperature and cage

activity in freely moving animals is necessary for the development of PRCBs as future therapeutics for neuropathic pain treatment.

Chapter IV - Specific Aim (SA) 3

SA3: To investigate axonal accumulation of $Na_V1.8$ in a rat model of trigeminal neuropathic pain

4.1 Introduction:

Augmented depolarized state of primary afferent sensory neurons under neuropathic pain conditions is mainly attributed to altered expression levels of ion channels and receptors. Genetic deletion of cnr1 gene encoding CB1R in all of $Na_V1.8$ positive nociceptive neurons resulted in exaggerated responses to noxious mechanical and thermal stimuli in transgenic mice [282]. Sensory transduction of external stimuli into electrochemical gradient in primary afferent neurons is propagated along the nerve in the form of action potentials whose activity is regulated by voltage gated sodium channels. Most commonly expressed voltage gated sodium channels in trigeminal and dorsal root ganglia are $Na_V1.6$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ [310].

4.1.1 Voltage gated sodium channel structure and function:

Voltage gated sodium channels are heteromeric transmembrane protein complexes consisting of pore forming α-subunit and auxiliary β-subunits. The α-subunit consists of four homologous domains (D1-DIV) with each domain having six transmembrane helices (S1-S6) (Fig. 4.1). The S4 helix of each domain acts as a voltage sensor. P loops in each domain forms the selectivity filter for the permeation of Na^+ ions. The S6 segment of each domain lines the pore and serves as binding site for local anesthetics and anticonvulsants. Inactivation gate formed by the connecting loop of DIII and DIV domains regulates fast inactivation of the sodium channel.

Under resting conditions, Na^+ channels remain in a closed state. Upon depolarization, the voltage sensor formation of the S4 segment moves in an outward direction, allowing the pore to permit Na^+ ions to flow through. The outward movement of the voltage sensor activates the

inactivation gate, allowing for fast inactivation of Na^+ channels. Fast inactivation of Na^+ channels is responsible for action potential termination.

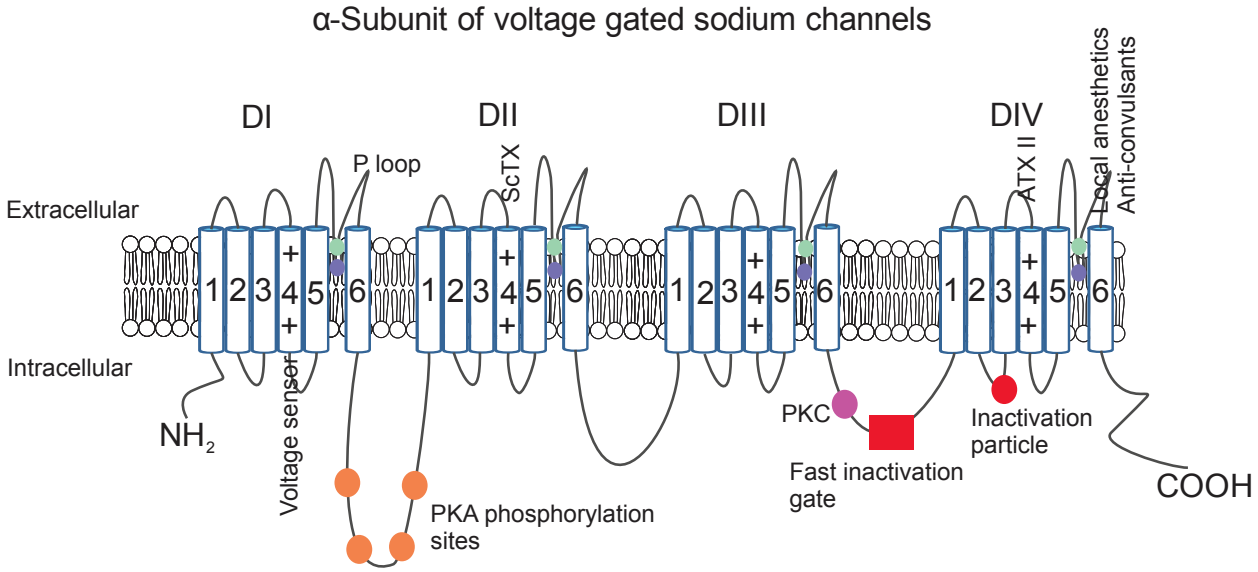


Figure 4.1: Structure of voltage gated sodium channel (Na_V)
Schematic diagram showing the secondary structure of voltage gated sodium channel α-subunit with important regulatory sites. DI-DIV represents four transmembrane domains of α-subunit. Each domain is formed of six transmembrane helices, with voltage sensor in the 4th helix. Tetrodotoxin (TTX) binding site is located on the pore of outer vestibule formed by four domain tertiary structure (Not shown in the diagram)

4.1.2 Voltage gated sodium channels and neuropathic pain:

Paroxysmal and spontaneous pain like sensations in neuropathic pain occurs as a result of changes in the excitability of peripheral sensory neurons. Voltage gated sodium channels are key determinants of neuronal hyperexcitability and altered expression of these ion channels can be related to abnormal neuronal firing. Voltage gated sodium channels with a mechanistic role in the pathophysiology of neuropathic pain are $Na_V1.3$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$.

$Na_V1.3$:

Neuronal expression of $Na_V1.3$ in adult DRG is very low, but it is abundantly expressed during the neonatal period [311]. However, the expression of $Na_V1.3$ dramatically increases in the DRG and spinal dorsal horn neurons after peripheral nerve injury [59,312]. $Na_V1.3$ is a TTX sensitive ion channel and accumulates at the axonal tips of injured neurons, causing ectopic firing [313]. Co-localization studies identified that $Na_V1.3$ upregulation after nerve injury is confined to medium to large diameter DRG neurons [314]. It has been shown that ventral root transection resulted in the pro-inflammatory cytokine mediated upregulation of $Na_V1.3$ in L4-L5 DRG. Despite increased expression of $Na_V1.3$ in injured DRG neurons, its role in neuropathic pain is still unclear. Intrathecal administration of antisense oligonucleotides targeting $Na_V1.3$ mRNA did not alleviate mechanical and cold allodynia developed after spared nerve injury, although its expression was decreased [315]. In contrast, shRNA virus mediated knockdown of $Na_V1.3$ mRNA by direct injection into injured DRG did partially alleviate the mechanical allodynia developed after spared nerve injury [59].

$Na_V1.6$:

At the periphery, this particular sodium channel is abundantly expressed in myelinated large diameter neurons and accumulates at the nodes of Ranvier. $Na_V1.6$ can cause repetitive firing of sensory neurons and exhibit excitatory and persistent resurgent currents [316]. Downregulation of $Na_V1.6$ mRNA has been reported in the DRG neurons after peripheral nerve injury [317]. In contrast, accumulation of $Na_V1.6$ protein proximal to the site of injury in the infraorbital nerve has been noticed [318]. shRNA mediated knockdown of $Na_V1.6$ mRNA after direct injection into inflamed DRG completely blocked pain behaviors, and spontaneous activity in medium and large diameter neurons [319]. In addition to amelioration of pain behaviors and abnormal

spontaneous activity, local knockdown of $Na_V1.6$ mRNA prevented sympathetic sprouting around myelinated neurons in the spinal nerve ligation model of neuropathic pain [320]. Symptoms of cold allodynia after treatment with anti-neoplastic agents like oxaliplatin are shown to be due to persistent and resurgent currents produced by $Na_V1.6$ sodium channel [321]. These findings provide satisfactory evidence for the emerging role of $Na_V1.6$ in the maintenance of allodynic states under various neuropathy conditions.

$Na_V1.7$:

$Na_V1.7$ is a TTX-sensitive sodium channel and is selectively expressed in sensory, sympathetic, and myentric neurons [322]. $Na_V1.7$ produces fast activating, fast inactivating and slowly repriming currents [323]. $Na_V1.7$ is primarily implicated in human inherited neuropathy disorders [56]. Mutations in the coding sequence of $Na_V1.7$ gene cause altered firing properties of sensory neurons and reduced threshold for activation in nociceptive neurons [324]. In contrast, mutations with inherited loss of $Na_V1.7$ function results in congenital insensitivity to pain in humans [325]. $Na_V1.7$ acts as a threshold channel for initiation of action potentials in nociceptive neurons and sets the gain in nociceptors [326]. Reduced levels of $Na_V1.7$ mRNA and protein in DRG neurons after peripheral nerve injury has been reported in animal models and in human studies [317,327]. However, an increase in the expression of $Na_V1.7$ in the axons of painful human neuromas has been reported [328]. Genetic knockout of $Na_V1.7$ in sensory neurons alone did not prevent the development of neuropathic pain after peripheral nerve injury whereas elimination in both sensory and sympathetic neurons completely suppressed neuropathic pain symptoms [329]. This suggests a role for $Na_V1.7$ in sympathetically maintained neuropathic pain.

$Na_V1.8$:

$Na_V1.8$ is a TTX-resistant sodium channel, expressed in majority of nociceptive neurons (A-δ and C). $Na_V1.8$ contributes to most of the Na^+ current during action potential initiation and propagation, and is expressed along the peripheral axon shafts and free nerve terminals in the skin, in addition to its expression in cell bodies [330,331]. Because of its delayed inactivation and repetitive firing properties, $Na_V1.8$ ion channel is described as an important target for the regulation of ectopic firing in injured nerves under neuropathic pain conditions [332]. In addition, abnormal expression of $Na_V1.8$ mRNA and protein has been reported proximal to the site of sciatic nerve injury with no change or decrease in the cell bodies of injured neurons [57,333]. Histological examination of lingual neuroma specimens of symptomatic trigeminal neuropathic pain patients identified abnormal axonal accumulation of $Na_V1.8$ protein [67]. Strengthening previous findings, studies aimed at antisense and shRNA mediated knockdown of $Na_V1.8$ mRNA, demonstrated alleviation of mechanical allodynia and thermal hyperalgesia in rat models of neuropathic pain [68,69].

$Na_V1.9$:

$Na_V1.9$ is a TTX-resistant sodium channel, selectively expressed in small diameter C-fiber neurons. The voltage dependency of activation for $Na_V1.9$ is close to resting membrane potential and has ultraslow inactivation, leading to persistent currents [334]. Previous studies suggest a role of $Na_V1.9$ in inflammatory and diabetic neuropathic pain. $Na_V1.9$ expression in lumbar DRG neurons increases under chronic inflammatory conditions [335]. In painful diabetic neuropathy, $Na_V1.9$ expression is increased in large diameter neurons rather than in small diameter nociceptive neurons [336]. However, $Na_V1.9$ role in neuropathic pain is still unclear because $Na_V1.9$ mRNA and protein levels are downregulated in several other animal models of

neuropathic pain [317,337], and antisense knockdown of $Na_V1.9$ did not ameliorate signs of neuropathic pain [338].

4.1.3 Targeting $Na_V1.8$ mRNA 3' UTR for the potential treatment of neuropathic pain:

Despite significant advances in the knowledge of $Na_V1.8$ role in neuropathic pain pathogenesis, progress in the development of new therapeutics has been poor. A potent $Na_V1.8$ inhibitor, A-803467 was shown to be highly effective in the suppression of chronic inflammatory and neuropathic pain in rat models, yet it failed to progress to clinical trials [339,340]. Development of $Na_V1.8$ specific small molecule drugs is still challenging because of high structural similarity with other voltage gated sodium ion channels, suggesting alternate routes for targeted therapy. A feasible approach is to modulate post-transcriptional and translational mechanisms for specific knockdown and long-term pain alleviation [341]. However, mechanisms underlying co/post-transcriptional regulation of $Na_V1.8$ are still unknown. Studies on the membrane trafficking of $Na_V1.8$ by annexin II light chain or contactin and post-translational modifications did not elucidate mechanisms involved in the abnormal axonal accumulation in neuropathic pain [330,342–344]. Previous studies focused on axonal transport of certain mRNAs after peripheral nerve injury described the potential role of untranslated regions (UTR) in the sub-cellular redistribution of mature mRNAs [345]. In addition to protein coding sequence, mature mRNAs harbor 5' and 3' untranslated regions that determine stability, translational efficacy and sub-cellular localization [346]. Even though the role of 5'UTR in the sub-cellular localization of mRNA has been proposed, the vast majority of studies suggest 3' UTR as the potential region of importance for the axonal transport of mRNAs [347,348]. 3' UTRs retain multiple cis-acting elements including, stabilization elements, destabilization elements and motifs for the coupling of RNA transport binding proteins [349]. Moreover, 3'UTRs also possess miRNA binding sites, cytoplasmic polyadenylation elements

(CPEB), conserved 5' and 3' splice sites and tandem polyA signals (pA) [350]. Tandem arrangement of polyA signals facilitates alternative polyadenylation with a net result in the relative gain or loss of cis-acting elements, miRNA binding sites and introns/exons [351]. Previous work from Thakor et al demonstrated that the increased abundance of $Na_V1.8$ mRNA in the injured nerve is not due to enhanced stability as a result of polyA tail elongation [57]. Hence, we focused our work on identifying changes in the 3' UTR of $Na_V1.8$ mRNA anticipating that these changes might occur as a result of alternative splicing or alternative polyadenylation or a combination of both. Careful examination of rat genomic DNA sequence downstream of $Na_V1.8$ stop codon revealed four canonical polyA signals, potential sites for alternative polyadenylation.

In this specific aim of my thesis, I focused on determining the steady state mRNA levels of voltage gated sodium channels in the ganglia and nerve, and obtaining the sequence information of the $Na_V1.8$ mRNA 3' alternate UTR in a rat trigeminal neuropathic pain model.

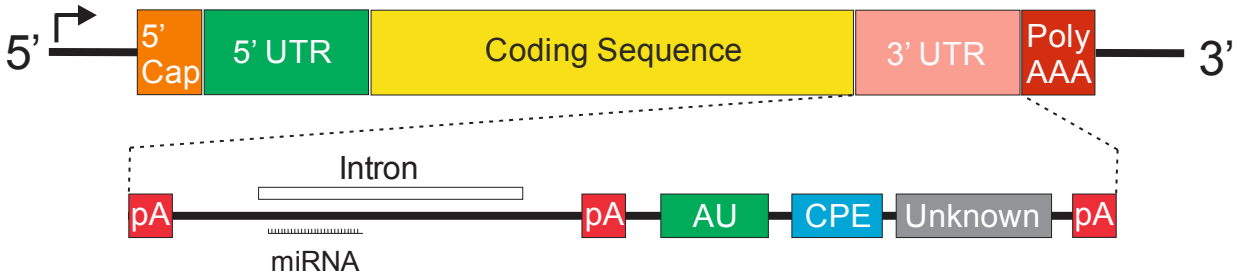


Figure 4.1: mRNA schematic showing 3' UTR regulatory elements.
5'-3' end of mRNA is shown from left to right. 5' Cap: Methyl-guanosine cap; 5' UTR: 5' Untranslated region; Coding sequence: mature mRNA sequence for the translation of final protein; 3' UTR: 3' Untranslated region; PolyAAA: PolyA tail (50-250 nucleotides); pA: polyA signal; AU: Adenine/Uridine rich elements; CPE: Cytoplasmic polyadenylation elements; miRNA: microRNA binding sites.

4.2 Preliminary data:

4.2.1 RNA-Seq read alignment of rat $Na_V1.8$ 3' UTR:

RNA-Seq is a high throughput sequencing technology that allows simultaneous evaluation of all genes expressed in a given sample. Fragmented mRNA pieces are sequenced and reads are aligned to a reference genome, aiding us in the visualization of unknown regions of genome expressed, such as novel exons, non coding RNAs and untranslated regions. Hirai et al extracted RNA from axons & soma of cultured neurons of DRG isolated from SNE rats and processed them for RNA-Seq. Our preliminary findings [362] showed that $Na_V1.8$ mRNA is expressed beyond the conventional size of UTR that has been described previously [352].

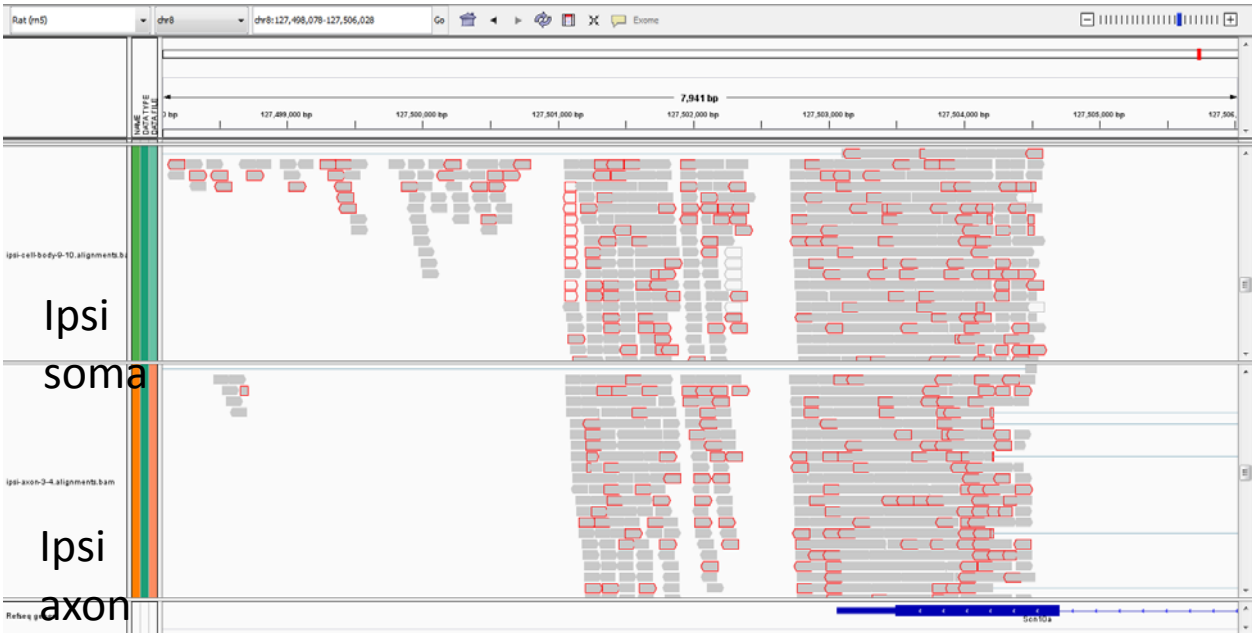


Figure 4.2: RNA-Seq read alignment
Sequenced mRNA reads are aligned to rat reference genome (rn5) using TopHat short read aligner and visualized on Broad's Integrated Genome Viewer (IGV). Last exon and conventional UTR length is indicated by the blue bar at the bottom. Coordinates of the browser window are adjusted to view rat $Na_V1.8$ last exon & 3'UTR and ~ 5 kb beyond. Browser genomic coordinates: 127,499,000-127,506,000

(3'-5'). Read pair with missing read is highlighted in red color. Top read alignment – ipsi cell body; bottom read alignment – ipsi axon.

4.3 Methods:

4.3.1 RNA extraction:

At the end of behavioral testing, all rats are euthanized by decapitation with a metal guillotine after being anesthetized with isoflurane (5%). Trigeminal ganglia and infraorbital nerve are extracted in ice cold artificial cerebro-spinal fluid (ACSF) bubbling with carbogen (95% O_2 and 5% CO_2) and stored in RNAlater solution (Ambion) at -20°C until further use. On the day of RNA extraction, tissue samples are placed in a Dounce Homogenizer loaded with 1 ml of Qiazol lysis reagent (Qiagen) and disrupted with a glass pestle until the solution has become homogenous. Total RNA is extracted using Qiagen RNeasy lipid tissue mini kit and concentration is measured with IMPLEN Nanopearl spectrophotometer (Genotyping Core, UCLA).

4.3.2 cDNA synthesis and qPCR:

0.2 µg of total RNA is reverse transcribed using Advantage RT for PCR kit (Clontech). For a 20 µl reaction volume, 12.5 µl of RNA is mixed with 1 µl of random hexamer primer and heated to 70°C for 2 minutes followed by snap cooling on ice for a minute. The RNA primer mix along with other contents of the reaction is incubated for 1 hr at 42°C and the cDNA reaction is terminated by heating at 94°C for 5 min. The reaction mix is further diluted to 100 µl with DEPC treated water prior to use in qPCR.

qPCR is performed with Taqman based hydrolysis probes and Gene Expression master mix (Life Technologies) on a 384 well plate loaded with triplicates of each cDNA sample. The

final volume of qPCR reaction for a single well is set to 10 µl. The following probes are purchased from Invitrogen to determine steady state mRNA levels of all tissue samples.

mRNA	Taqman GE ID
$Na_V1.3$	Rn00565438_m1
$Na_V1.5$	Rn00565502_m1
$Na_V1.6$	Rn00570506_m1
$Na_V1.7$	Rn00591020_m1
$Na_V1.8$	Rn00568393_m1
$Na_V1.9$	Rn00570487_m1
GAPDH	Rn01775763_g1

qPCR reaction was run on an Applied Biosystem 7900HT Fast real time PCR system. The following thermal cycling conditions have been applied for the qPCR reaction.

Time and Temp.			
Initial setup		Each of 40 cycles	
		Denature	Anneal/Extend
Hold	Hold	Cycle	
2 min 50˚C	10 min 95˚C	15 sec 95˚C	1 min 60˚C

Fold change in gene expression is calculated using the formula $log_{10}2^{-ddCt}$ where *ddCt* represents $[Ct_{Nav}\text{-}Ct_{GAPDH}]_{Experimental}\text{-}[Ct_{Nav}\text{-}Ct_{GAPDH}]_{Control}$. Ct is the threshold cycle for fluorescence detection of sample reaction on a thermal cycler.

4.3.3 3' RACE (Rapid amplification of cDNA ends):

1 µg of total RNA is reverse transcribed by Superscript III reverse transcriptase (Invitrogen) using cDNA cloning primer and PCR amplified by $Na_V1.8$ specific forward primer and 3' RACE PCR primer.

4.3.4 Digoxigenin (DIG) labeled DNA probe synthesis & Southern blot:

A 340 bp of $Na_V1.8$ 3' UTR immediately after stop codon is PCR amplified using forward (F) and reverse (R) primers followed by electrophoretic separation on 1.5% agarose gel. Band size corresponding to 340 bp is excised from the gel and DNA is purified by QIAquick (Qiagen) gel extraction kit. Purified DNA is cloned and sequenced. A 340 bp DIG labeled DNA is prepared by PCR amplification of $Na_V1.8$ 3' UTR cloned plasmid (Please refer to section 4.3.5) using PCR DIG-probe synthesis kit (Roche). 3' RACE products are electrphoretically separated on 1.5% agarose gel and transferred to positively charged nylon membrane (Roche) by wet capillary transfer (Southern transfer) overnight. DNA is UV cross linked (Stratagene) to nylon membrane and incubated in a rolling 50 mL Falcon tube with DIG labeled DNA probe at 42°C for 16 hours. After incubation, membrane is washed and blocked (DIG wash and block buffer set, Roche), and incubated with anti-DIG AP antibody (Roche). Bands are visualized under chemiluminescence after application of ready to use CSPD (Roche).

4.3.5 Cloning and sequencing:

Taq DNA polymerase amplified PCR products are gel purified and cloned into pCR4-TOPO vector (Invitrogen) and transformed to one shot TOP10 chemically competent *E.coli* (Invitrogen). Colonies are grown overnight on Amp-agar plates (Invitrogen) and further processed for propagation and plasmid isolation next day. Size of the cloned insert is confirmed by EcoRI digestion (Thermo scientific) and sequenced by ABI 3730xl bioanalyzer (Genoseq Core, UCLA).

4.3.6 Primers:

GTTTATGGCGACCAATCTCTCCAAAG (F1); GAGGTCGCTGCTAAGG (F2); CCATGACCCTCAGTCTCTCC (F3); GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTV (cDNA cloning primer); GGCCACGCGTCGACTAGTAC (3' RACE Primer); GTGAGCATGCAGTCATGC (R1); CATAGCAGGAAGATCTGG (R2); GGCTATAATATTTTATTCAC (R3); CCGCTTACAGATTATCGTCC (R4)

4.4 Results:

4.4.1 Selective Na_V mRNA subtype upregulation in infraorbital nerve after IoNC:

Histological examination of lingual nerve neuromas of human patients identified abnormal expression of $Na_V1.7$, $Na_V1.8$, $Na_V1.9$, TRPV1 and P2X7 [67,328,353,354]. Among them, only $Na_V1.8$ and $Na_V1.9$ expression correlated with painful symptoms of neuropathic pain. Moreover, abnormal expression of $Na_V1.6$ protein in injured infraorbital nerve has been reported after IoNC in a rat model of trigeminal neuropathic pain [318]. To determine if Na_V mRNA steady state levels are altered after IoNC we harvested trigeminal ganglia and infraorbital nerves of IoNC and sham rats 6 weeks after surgery and estimated their mRNA expression levels. Our qPCR data showed approximately ten-fold increases in the expression levels of $Na_V1.6$, $Na_V1.8$ and $Na_V1.9$ mRNA in injured infraorbital nerve compared to sham nerve with no significant change in the mRNA levels of these three sodium channels in the injured trigeminal ganglia (Fig. 4.3 A-B). In contrast, we did not observe any significant change in the expression of $Na_V1.3$, $Na_V1.5$ and $Na_V1.7$ mRNAs in the injured trigeminal ganglia or infraorbital nerves. Increased expression of $Na_V1.3$ mRNA and protein has been reported in the DRG of injured nerves [59]. However, we only

observed a trend of $Na_V1.3$ mRNA upregulation in the trigeminal ganglion of the injured nerves, which did not reach statistically significance (unadjusted p, 0.526). This suggests that IoNC induces selective axonal transport of Na_V mRNA subtypes.

4.4.2 3' RACE and Southern blot identify an alternative 3' UTR of $Na_V1.8$ mRNA:

3' RACE enables amplification of unknown 3' ends of mature mRNA ending with a polyA tail [355]. To determine potential 3' UTR alternative variants, we used an anchored forward primer 356 bp upstream of the stop codon and RACE-PCR amplified the total RNA collected from trigeminal ganglia and infraorbital nerves of IoNC rats. Southern probing with the 340 bp DIG labeled DNA probe matching the $Na_V1.8$ cDNA sequence downstream of stop codon identified an 835 bp band corresponding to the size of the previously published 3' UTR sequence and two additional bands of approximately 1500 bp and 1900 bp in both trigeminal ganglion and infraorbital nerve. This supports our previous findings that distally mapped RNA-Seq reads indeed belong to the extended 3' UTR region of $Na_V1.8$ mRNA rather than non-coding RNAs or some unknown intergenic expression.

4.4.3 $Na_V1.8$ mRNA alternative 3' UTR:

Cloning and sequencing of the 1900 bp band revealed that the alternate 3'UTR of $Na_V1.8$ mRNA is produced as a result of alternative splicing coupled with alternative polyadenylation. The locations of donor and acceptor sites of 3' UTR intron are 400 bp and 1251 bp from the stop codon respectively, and the 3' end is formed by selective utilization of the 4^{th} canonical polyA signal (~2.4 kb from stop codon). Multiz alignment of genomic sequences downstream of $Na_V1.8$ mRNA stop codon revealed that the intron boundaries are conserved in most of the mammals (Fig. 4.4D). The fate of mRNA is determined by the type of RNA binding proteins

(RNA-BPs) bound to the cis-acting elements of mRNA [347]. Since the alternative 3' UTR has an extended sequence, we scanned for potential RNA-BP sites for both conventional and alternative UTRs. Our scanning results identified additional RNA-BP sites for proteins involved in the regulation of alternative splicing, mRNA transport, translational efficiency and mRNA stability confined to the alternative 3' UTR (Table 4.1). This suggests that the cellular fate of mRNAs encoding a given protein is determined by the UTR variants expressed.

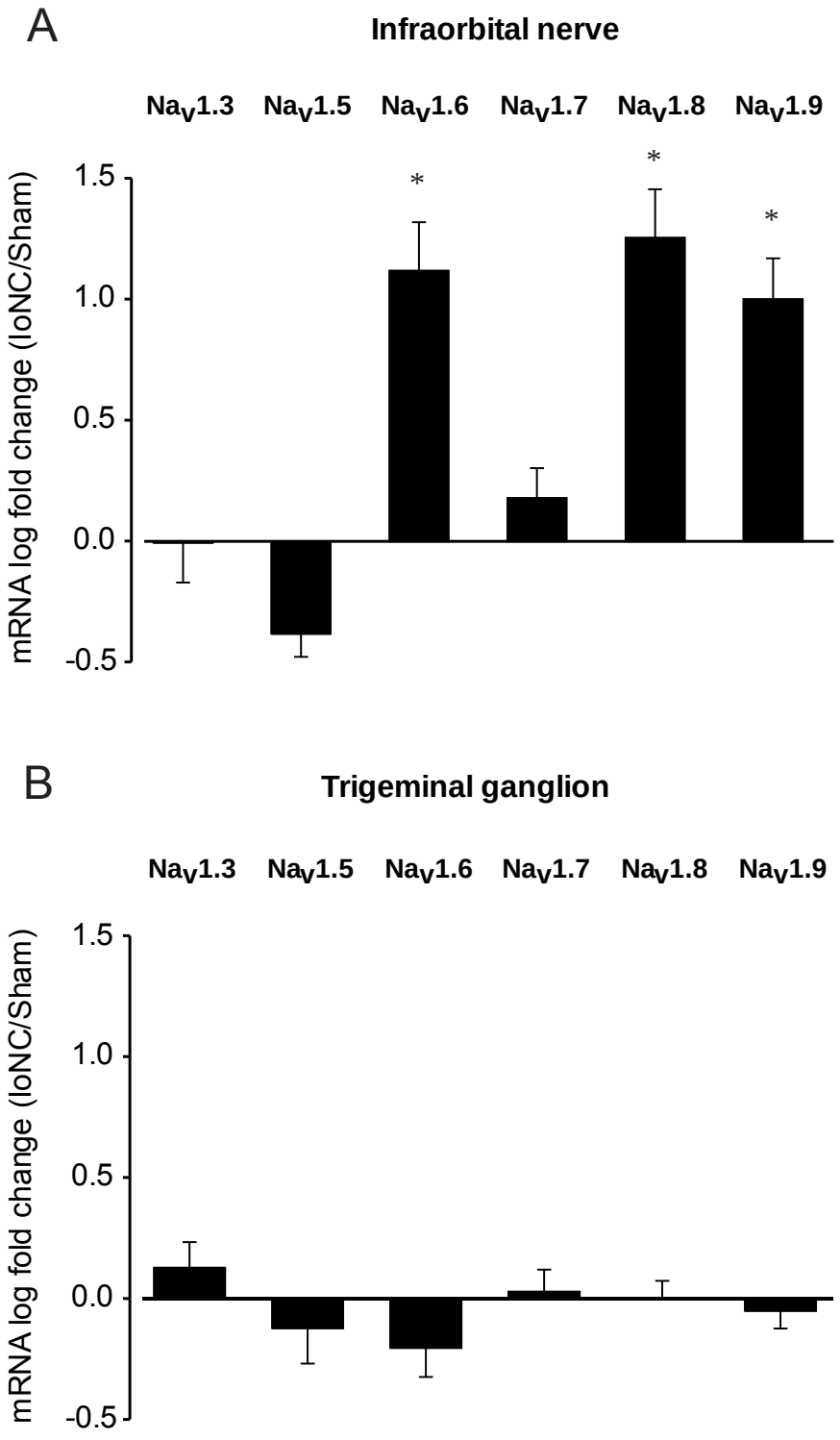
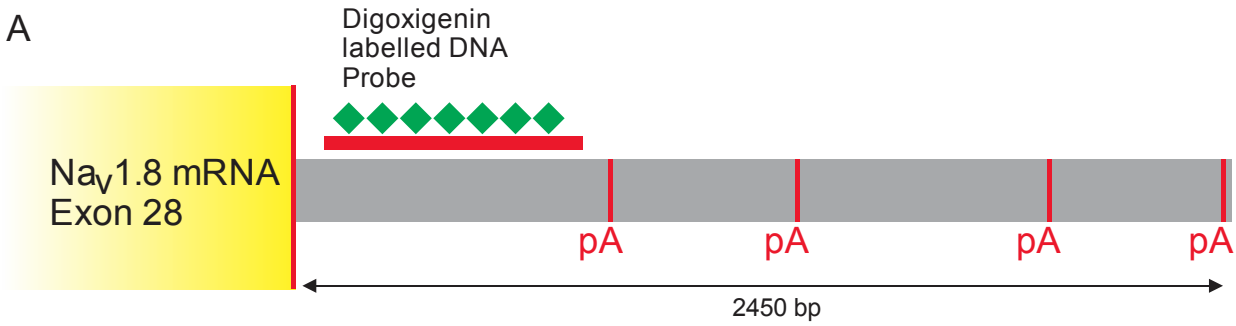
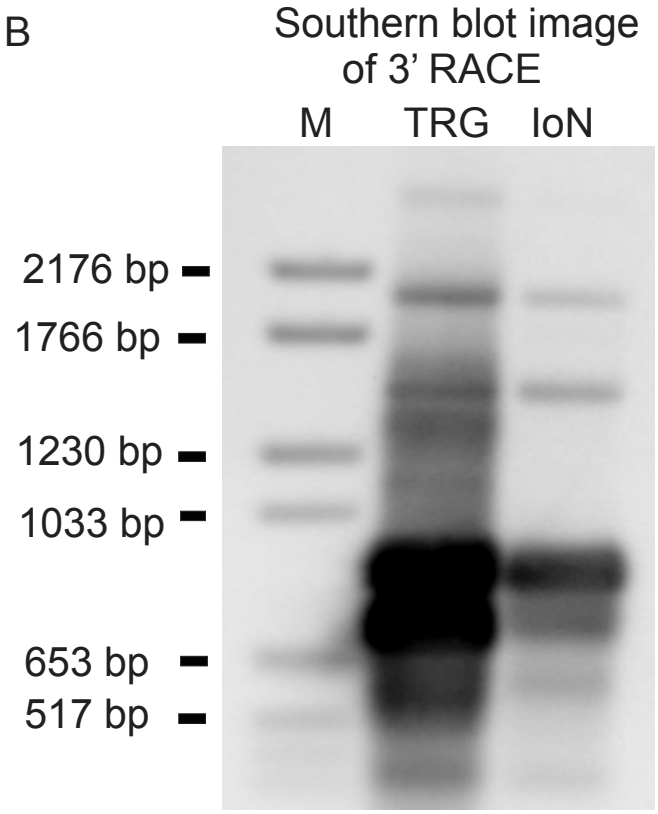


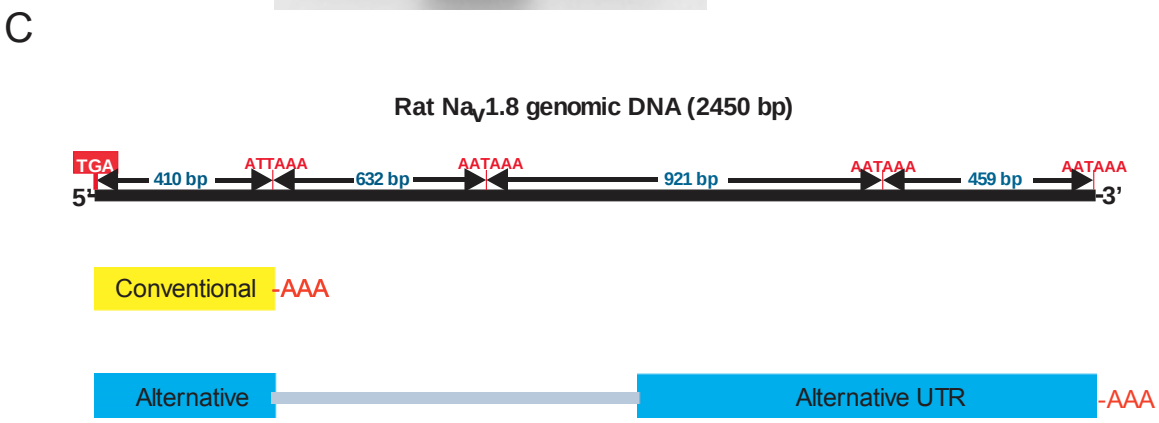
Figure 4.3: qPCR data of IoNC and sham rats

Steady state Na_v mRNA levels in infraorbital nerve (A) and trigeminal ganglion (B) expressed as log fold change (IoNC/Sham). Two-way ANOVA with Holm-Sidak post-hoc method revealed significant increase

in the expression of $Na_V1.6$, $Na_V1.8$ and $Na_V1.9$ mRNAs in the infraorbital nerve after IoNC *(* $p<0.05$)*. Statistics were run comparing $\log_{10}2^{-dCt}$ values of IoNC (n=4) and sham (n=3) samples. Error bars represent standard error of mean of IoNC samples


A
Digoxigenin
labelled DNA
Probe
Na$_V$1.8 mRNA
Exon 28
pA
pA
pA
pA
2450 bp


B
Southern blot image
of 3' RACE
M
TRG
IoN
2176 bp
1766 bp
1230 bp
1033 bp
653 bp
517 bp


C
Rat Na$_V$1.8 genomic DNA (2450 bp)
TGA
410 bp
ATTAAA
632 bp
AATAAA
921 bp
AATAAA
459 bp
AATAAA
5'
3'
Conventional
-AAA
Alternative
Alternative UTR
-AAA

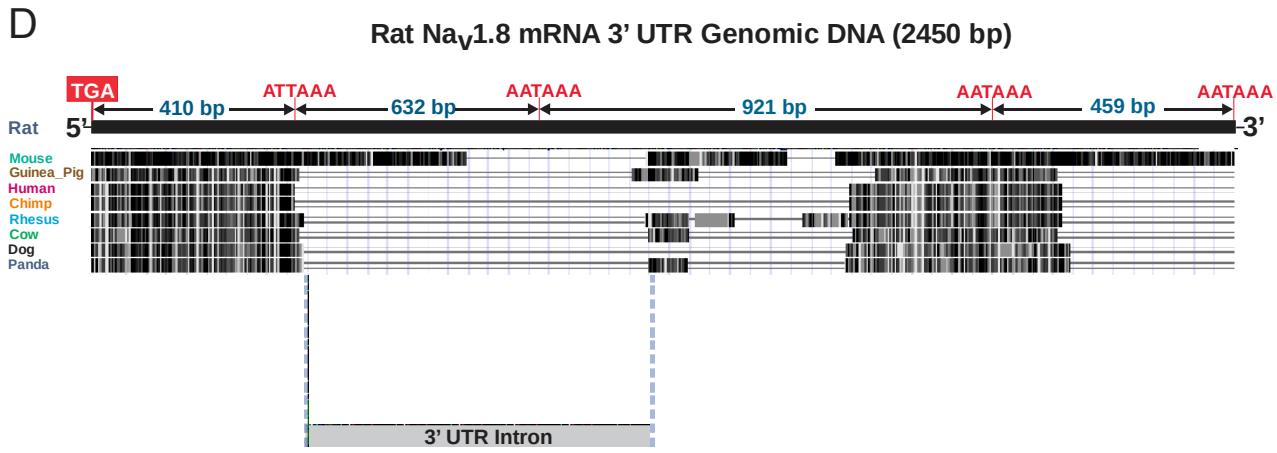


Figure 4.4: Na$_V$1.8 mRNA 3' UTR description

(A) Schematic view of Na$_V$1.8 mRNA last exon, annotated 3 'UTR (1st pA) and genomic DNA sequence (~2.5 kb) showing tandem arrangement of polyA (pA) signals. A DIG labeled DNA probe matching the sequence of proximal end of 3' UTR is indicated in red. (B) Southern blot image of 3' RACE. DIG labeled DNA ladder is shown in the first lane (M), and TRG and IoN of IoNC samples on the second and third lanes respectively. Two faint bands at 1500 bp and 1900 bp correspond to alternative 3' UTR. (C) Schematic view of rat Na$_V$1.8 mRNA conventional and alternative 3' UTRs in comparison with rat genomic DNA sequence. Intron is indicated by thin grey line connecting UTR exons (Blue). (D) Multiz sequence alignment of Na$_V$1.8 mRNA alternative 3' UTR for rat and other mammalian species. Highly conserved regions are highlighted in black color.

4.4.4 Rat Na$_V$1.8 mRNA Conventional and Alternative 3' UTR sequences:

Na$_V$1.8 mRNA conventional 3'UTR

TGAAGGCACTCAGGCATGCACAGGGCAGGTTCCAATGTCTTTCTCTGCTGTGCTAACTCCTT
CCCTCTGGAGGTGGCACCAACCTCCAGCCTCCACCAATGCATGTCACTGGTCATGGTGTCAG
AGCTGAATGGGGACATCCTTGAGAAAGCCCCCACCCCAATAGGAATCAAAAGCCAAGGATAC
TCCTCCATTCTGACGTCCCTTCCGAGTTCCCAGAAGATGTCATTGCTCCCTTCTGTTTGTGA
CCAGAGACGTGATTCACCAACTTCTCGGAGCCAGAGACACATAGCAAAGACTTTTCTGCTGG
TGTCGGGCAGTCTTAGAGAAGTCACGTAGGGGTTGGCACTGAGAATTAGGGTTTGCATGACT
GCATGCTCACAGCTGCCGGACAATACCTGTGAGTCGGCCATTAAAATTAATATTTTTAAAGT
TAAAAAAAAAAAAAAAAA

$Na_V1.8$ mRNA alternative 3'UTR

TGAAGGCACTCAGGCATGCACAGGGCAGGTTCCAATGTCTTTCTCTGCTGTGCTAACTCCTTCC
CTCTGGAGGTGGCACCAACCTCCAGCCTCCACCAATGCATGTCACTGGTCATGGTGTCAGAGCT
GAATGGGGACATCCTTGAGAAAGCCCCCACCCCAATAGGAATCAAAAGCCAAGGATACTCCTCC
ATTCTGACGTCCCTTCCGAGTTCCCAGAAGATGTCATTGCTCCCTTCTGTTTGTGACCAGAGAC
GTGATTCACCAACTTCTCGGAGCCAGAGACACATAGCAAAGACTTTTCTGCTGGTGTCGGGCAG
TCTTAGAGAAGTCACGTAGGGGTTGGCACTGAGAATTAGGGTTTGCATGACTGCATGCTCACAG
CTGCCGGACAATACCT-gtgagtcggcc-----[851 nt intron]-----cactctcag-
GGCTCCAGATGCTCTGCGGTCAGAACTTGAAGATGCTGACCTAGGAGCTGGAAAGCCGAGTCCT
AAATTTTTAAAGTTTTTAAAAATATACATGTATGTGGGAACATGTATGTGTGAGTACGAGTGCA
GCAGCCAGAGATGGCATTGGGGCCCCTGCAGCTGGAGTTTACAGATGTTCGTGAGCCCCCTGAC
GTAGGTGTTGAGAACCAAATTCAAGTCTTCTGCAAGACCAGAAAGTGTTCTTAAGGGGTTGGGG
ATTTAGCTCAGTGGTAGAGCGCTTGCCTAGGAAGCGAAAGGCCCTGGGTTCTGTCCCCAGCTCC
GAAAAAAAAAGAACCAAAAAAAAAAAAAAAAGAAAAAAAGAAAGTGTTCTTAACCACCCAGCCC
TAACTGGCTCTATTTTAATTCTGTACTGATTGCCTGACTAGCTGGGGCTAAGATCTTTCTCCCC
CATGACCCTCAGTCTCTCCGCTTTTAAAGTTTGTAAGAATAGGAGAGATGAGACAGCTCTCAGT
CACATGCTTAGATGTCAGCGGAACTGGGATGGGGGGATAACATTGCTGTGTCCAGATCTTCCCG
CTATGATTGGCCAAGGCAGATTTGGACTATGTTCTAGGTTCAGTTTTATATCTCATAACAGAAC
TTTAAAAAGGCCATTATAGTCCCATGGAACTAGGACTCCATAATTAGTATAATTAAACGCAAAC
TGGATATTTCAAGAAAACTCTGGTGAATAAAATATTATAGCCAGAAGGCTTTTTTATCTATCCA
ATGAATATTTGTAAGTACTTGTTATATAACAGACATTGCCCTGAGAACGAGAAAGGAAGCAGGA
GCTAAGCTCCGTAGATGAATTGATGTTTATTTGGTGTATATCCTGGTATCAACTTGACACAACC
AAGAGTGTCTGGGAAGAGGAAGCCTCAGTTGAGGGATTGCCCAGATCGAGTTGGCCTGCTGCCA
TGTCTGTGAGGGATTGTATTGACAGACAAGTAGTGTGTAAGAGCCCACCCAGCCCACTGTGGAC
AGTGCTGTCGCTGGCAGGTGGCCCTGGGTTATAAGAAAGCTGGCTTAGCATGAGCCAGAGAAAG
CAACCCAGTAAAAAGCATCCCTCCATGGTCTCTGCTTCAGCTCCTGACTCCTGGTCCCTTCGTT
GGCTTCTCTCAATAGTGGACGATAATCTGTAAGCGGAATAAAACTTTTCATCCCTAAAAAAAAA
AAAAAAAA

Stop codon (dark blue); Alternative intron (yellow); Poly A signal sequence (red)

RNA-BP Uniprot Gene ID	Function	Sequence motif
zfp 36	mRNA stability	AAAAAAGAAAG
a2bp1	Alternative splicing, Neuronal excitability[356]	UGCAUG
sfrs2	Pre-mRNA splicing	AGGAGAGAU
hnrnpa1	mRNA metabolism and transport	UAGGGU
nono	Pre-mRNA splicing	AGGGA
eif4b	Translation initiation	GCGGAAC
sap49	Pre-mRNA splicing	GUGUGA
pabpc1	mRNA translation turnover	AAAAA
rbmy1a1	Pre-mRNA splicing	CUCAA
ybx2-a	Stability and translation of mRNAs	AAGAUC
fus	Pre-mRNA splicing and nuclear export	GGUG
aco1	mRNA stability and translation	CAGUGC
pum2	Translation control	UGUA
sfrs9	Alternative splicing	AGGAG
ptbp1	Pre-mRNA processing, transport and metabolism	AGAUCUUUCUCCCCC
mbnl1	Regulates pre-mRNA alternative splicing	UGCU
elavl1	mRNA stability	GUUU
khsrp	Alternative splicing and mRNA localization	GUCC
ybx1	Translational control	UCUGCG
ythdc1	Efficiency of mRNA splicing, processing and stability	GAGUAC
vts1	mRNA stability	GCUGGUG
rbmx	Alternative splicing	CCAG
khdrbs3	Alternative splicing and splice site selection	UAUAAU/ CAUAAU
sfrs13a	Alternative splicing	AGAGAAG
sfrs1	Prevents exon skipping and regulates alternative splicing	AGGA

Table 4.1: RNA binding proteins
Sequences of $Na_V1.8$ mRNA conventional and alternative 3' UTRs are scanned for potential RNA binding proteins (RBPs) using human RBP database developed by UToronto. RBPs common to both the 3' UTRs are highlighted in red color. RBPs exclusive to the alternative 3' UTR are highlighted in blue. Sequence recognition motif on the mRNA sequence for each RBP is shown in the last column.

4.5 Discussion:

4.5.1 $Na_V1.8$ mRNA axonal accumulation and 3' UTR alternative splicing:

Structural and functional plasticity of neurons occurs as a result of increases or decreases in the expression of certain proteins. This type of altered protein expression in neurons is based on the regulation of their spatial and temporal availability, which is in part mediated by subcellular localization of mRNAs [357]. Our qPCR data showed that the $Na_V1.8$ mRNA is increased several fold in the injured nerve along with other subtypes ($Na_V1.6$ and $Na_V1.9$). mRNAs with similar protein coding sequence retain multiple 3' UTRs which determine their stability, transport and translation [345]. Our current research work demonstrates that $Na_V1.8$ mRNA exists with an alternative 3' UTR. A 1500 bp band that appeared on Southern blot may be as a result of alternate choice of splice acceptor site. Our future work will address the sequence information of this band. Akopian et al reported a $Na_V1.8$ mRNA clone from rat naïve DRG with a 3' UTR size of ~2 kb [352]. Even though the sequence information of this long UTR has not been published, it appeared to be a result of using an alternative polyadenylation site (3rd polyA site) rather than alternative splicing in the 3' UTR. This difference in the size of 3' UTRs observed may be due to sample tissue used for cloning and sequencing of $Na_V1.8$ mRNA. Most of our current work is carried out on RNA samples isolated from ganglia and nerves of neuropathy rats. Our extensive search for $Na_V1.8$ mRNA alternative 3' UTR sequences did not result in the identification of previously defined 2 kb variant in the nerve injured samples [352]. Additional experiments involving direct mRNA quantitation such as Northern blot or RNAse protection assay are required to detect if 3' UTR alternative splicing occurs as a result of peripheral nerve injury.

4.5.2 $Na_V1.8$ mRNA alternative 3' UTR as potential therapeutic target:

More than one 3' UTR for a given mRNA facilitates activity dependent regulation of translation. This has been demonstrated in the case of BDNF and GluR2 mRNAs. Normally, long 3' UTRs are maintained in a dormant stage, while short 3' UTR mRNAs continue to translate in order to maintain basal physiological levels of protein. Drug or electrical stimulation induced neuronal activity induces the inhibition of translational suppression of long 3' UTRs, assisting in the development of neuronal plasticity [358–360]. In addition, mRNAs localized to specific cellular compartments serve distinct functions. For example, the long BDNF 3' UTR in dendrites promotes spine maturation and pruning, as knock down of this variant blocked spine head enlargement [361]. Alternative splicing in the 3' UTR facilitates gain of novel secondary structures, aggregation of cis-acting elements and escape from miRNA mediated mRNA degradation [350] (Fig. 4.5). Based on the sequence information of $Na_V1.8$ mRNA alternative 3' UTR, and the results of scanning for RBP sequence recognition motifs (Table 4.1), we predict that newly acquired cis-acting elements of alternative 3' UTR may serve as potential sites for the binding of transacting factors involved in the abnormal axonal accumulation of $Na_V1.8$ mRNA. In addition, cis-elements required for RBPs involved in the axonal regulation of translation such as ribosome recruitment and translation initiation are mapped exclusively to alternative 3' UTR (e.g. Pabpc1), indicating that injury caused axonal upregulation of $Na_V1.8$ protein could be influenced by local translation. Escape from miRNA mediated decay through alternative splicing also enhances the stability of mRNA, thus increasing half life.

To the best of our knowledge, this is the first study that described an alternative 3' UTR for the mRNA of $Na_V1.8$ protein, a key mediator in the physiological and pathological transmission of pain signals. We expect that the discovery of this novel variant may advance the knowledge

of $Na_V1.8$ role in the pathogenesis of neuropathic pain. Based on the acquisition of novel regulatory sequences for enhanced transport and translation, we predict that the alternative 3' UTR variant is a promising target for the specific knockdown of injury-enhanced $Na_V1.8$ mRNA and protein without detrimental effects on basal expression levels. The current information for cis-acting elements and RBPs is largely based on *in silico* screening. Further experimental validation is required to identify the potential role of this variant under normal and pathological conditions. Future studies involving shRNA or anti-sense mediated knockdown and UV-cross linking-immunoprecipitation (IP) experiments will address the potential role of this alternative variant and associated RBPs in the maintenance of neuropathic pain.

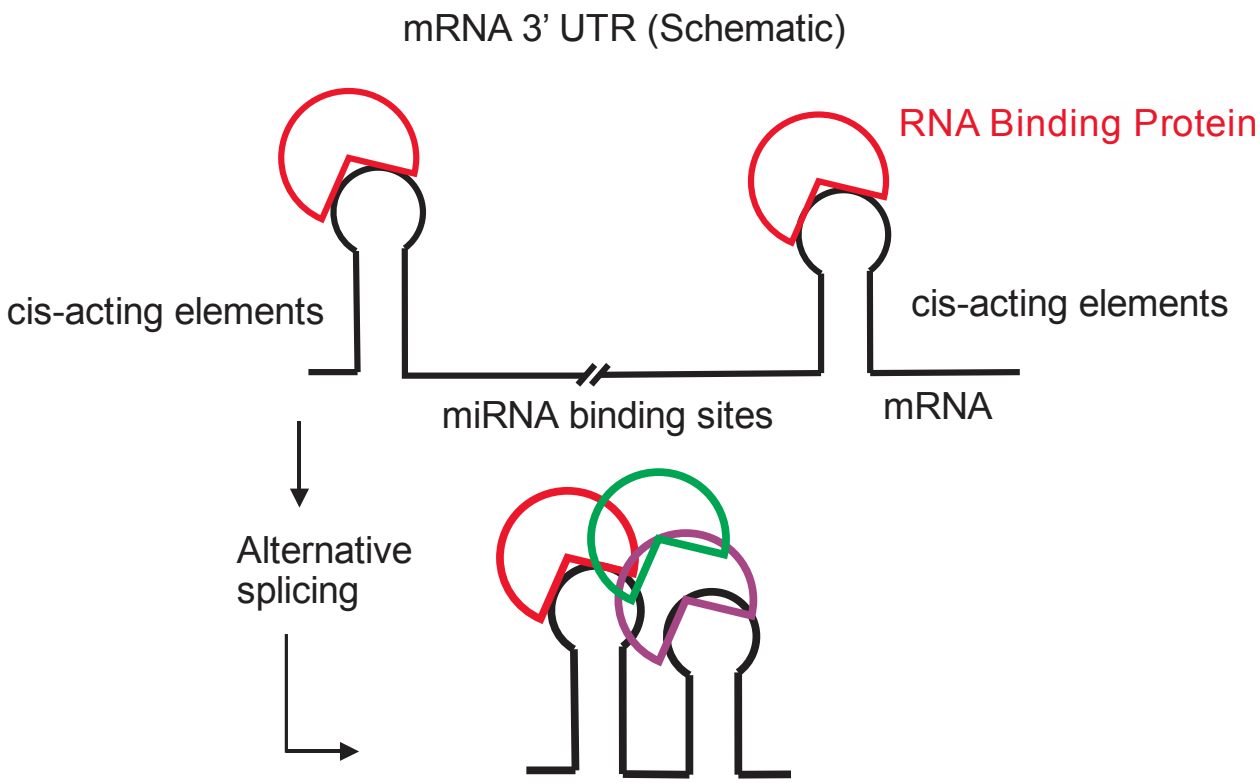


Figure 4.5: mRNA 3' UTR alternative splicing
mRNA 3' UTR schematic showing alternative splicing, loss of miRNA binding sites, novel secondary structure formation and aggregation of RNA binding proteins.

FINAL WORD

Chronic pain is difficult to treat because it is difficult to diagnose. Unlike predictors of cardiac health condition such as heart rate and blood pressure, there is no objective indicator of chronic pain condition. Given our understanding of disease pathogenesis, and technological advancements, I hope that chronic pain can be diagnosed and treated successfully in the future.

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