EFFECTS OF ELECTRICAL STIMULATION ON NEUROPATHIC PAIN AND NEURONAL SPROUTING IN SPINAL DORSAL HORN AFTER SPARED NERVE INJURY IN RATS

KANYARAT BAMRUNGSUK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (ANATOMY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007

Thesis

Entitled

EFFECTS OF ELECTRICAL STIMULATION ON NEUROPATHIC PAIN AND NEURONAL SPROUTING IN SPINAL DORSAL HORN AFTER SPARED NERVE INJURY IN RATS

Miss Kanyarat Bamrungsuk
Candidate

Lt.Col. Supin Chompoopong,
Ph.D. (Anatomy)
Major-Advisor

Asst. Prof. Kanokwan Tilokskulchai,
Ph.D. (Physiology)
Co-Advisor

Assoc. Prof. Arraya Sa-ngiampong,
M.Sc. (Anatomy)
Co-Advisor

Assoc. Prof. Wandee Apinhasmit,
D.D.S., Ph.D. (Anatomy)
Co-Advisor

Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies

Assoc. Prof. Jantima Roongruangchai,
D.D.S., Ph.D. (Anatomy)
Chair
Master of Science Programme in
Anatomy
Faculty of Medicine Siriraj Hospital

Thesis
Entitled

EFFECTS OF ELECTRICAL STIMULATION ON NEUROPATHIC PAIN AND NEURONAL SPROUTING IN SPINAL DORSAL HORN AFTER SPARED NERVE INJURY IN RATS

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Anatomy) on 19 October, 2007

Miss Kanyarat Bamrungsuk
Candidate

Assoc. Prof. Sukumal Chongthammakun,
Ph.D. (Anatomy)
Chair

Lt.Col. Supin Chompoopong,
Ph.D. (Anatomy)
Member

Asst. Prof. Kanokwan Tilokskulchai,
Ph.D. (Physiology)
Member

Assoc. Prof. Pansiri Phansuwan,
Ph.D. (Anatomy)
Member

Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

Clin. Prof. Piyasakol Sakolsatayadorn,
M.D., F.R.C.S.T.
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENTS

The success of this thesis can be attributed to the extensive support and assistance from my major advisor, Lt.Col. Supin Chompoopong and my co-advisors, Asst. Prof. Kanokwan Tilokskulchai, Assoc. Prof. Arraya Sa-ngiampong, and Assoc. Prof. Wandee Apinhasmit. In addition, I deeply thank them for their valuable advice and guidance in the research. Specially thanks to the chair of defense thesis committee, Assoc. Prof. Sukumal Chongthammakun, and the external examiner, Assoc. Prof. Pansiri Phansuwan.

I am grateful to all lecturers and staff of Department of Anatomy, Faculty of Medicine Siriraj Hospital for their valuable advice, kindness and generosity in supporting some materials used in this research.

I would like to thank Department of Microbiology and Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University and my friends in our laboratory for their valuable advice, kindness support, and helpfulness in laboratory technique.

Finally, I am grateful to my family for their entirely care encouragement, counseling and love. The usefulness of this thesis, I dedicate to my parents, my brother and the entire teachers who have taught me since my childhood.

Kanyarat Bamrungsuk

EFFECTS OF ELECTRICAL STIMULATION ON NEUROPATHIC PAIN AND NEURONAL SPROUTING IN SPINAL DORSAL HORN AFTER SPARED NERVE INJURY IN RATS

KANYARAT BAMRUNGSUK 4736154 SIAN/M

M.Sc. (ANATOMY)

THESIS ADVISORS: SUPIN CHOMPOOPHONG, Ph.D. (ANATOMY), KANOKWAN TILOKSKULCHAI, Ph.D. (PHYSIOLOGY), ARRAYA SA-NGIAMPONG, M.Sc. (ANATOMY), WANDEE APINHASMIT, Ph.D. (ANATOMY).

ABSTRACT

The aim of this study was to determine whether low frequency electrical stimulation (ES) had an antinociceptive effect on the neuropathic pain after spared nerve injury (SNI) in rats, assessed by pain behavioral response and substance P immunoreactivity (SPir) in dorsal root ganglion (DRG) and spinal dorsal horn. Fifty two adult male rats weighing 250-300 grams were randomly assigned into four groups (n=13 each). Two groups underwent SNI with or without ES treatment. The other two groups were subjected to sham operation with or without ES treatment. The SNI was performed by transecting the tibial and common peroneal nerves, leaving the sural nerve intact. The ES of 2 Hz was applied intramuscularly for 30 minutes daily for five consecutive days in the first week, followed by twice a week for another two consecutive weeks. The ES was started on day 15 after surgery when behavioral pain was developed markedly. The development of mechanical allodynia was determined by the paw withdrawal threshold in response to von Frey filament stimulation at the sural skin area of hind paw, on days-1, 8, 15, 23 and 32 after surgery. The myelinated primary afferents were labeled with cholera toxin B subunit (CTBir) and the distribution of SPir was determined in L4-L6 DRG and spinal dorsal horn. The results revealed that 1) after SNI, the paw withdrawal threshold was significantly decreased from a baseline (17.8±0.2 g) in all behavioral tests on day 8: 1.2±0.2 g, day 15: 2.4±0.6 g, day 23: 1.6±0.5 g and day 32: 1.6±0.4 g. 2) The ES could attenuate the mechanical allodynia with an increase in paw withdrawal threshold after two weeks: baseline: 18.0±0.0 g, day 8: 3.3±0.9 g, day 15: 2.9±0.8 g, day 23: 4.6±0.8 g and day 32: 8.7±2.0 g. 3) The SNI could increase SPir in the ipsilateral L4-L6 DRG and spinal dorsal horn significantly and these effects could be attenuated by the ES. 4) The neuronal sprouting that was stained by CTBir distributed into laminae I&II following SNI and could be decreased after ES treatment.

These results indicate that the ES treatment applied intramuscularly has antinociceptive effects on both pain behavioral response and neuronal activation of the DRG and spinal dorsal horn via substance P in SNI rats. Therefore, low frequency ES might be used as a supplement therapy for relieving neuropathic pain.

KEY WORDS: LOW FREQUENCY ELECTRICAL STIMULATION, SPARED NERVE INJURY, NEUROPATHIC PAIN

114 pp.

ผลของการกระตุ้นไฟฟ้าต่อความเจ็บปวดและการงอกของเส้นใยประสาทบริเวณไขสันหลังส่วนหลังในหนูที่ได้รับการบาดเจ็บของเส้นประสาทบางส่วน (EFFECTS OF ELECTRICAL STIMULATION ON NEUROPATHIC PAIN AND NEURONAL SPROUTING IN SPINAL DORSAL HORN AFTER SPARED NERVE INJURY IN RATS)

กันยารัตน์ บำรุงสุข 4736154 SIAN/M

วท.ม. (กายวิภาคศาสตร์)

คณะกรรมการควบคุมวิทยานิพนธ์: สุพิน ชมภูพงษ์ ปรด. (กายวิภาคศาสตร์) กนกวรรณ ติลกสกุลชัย Ph.D. (สรีรวิทยา) อารยา เสงี่ยมพงษ์ วท.ม. (กายวิภาคศาสตร์) วันดี อภิณหสมิต ปรด. (กายวิภาคศาสตร์)

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการกระตุ้นไฟฟ้าความถี่ต่ำ ในการลดความเจ็บปวดต่อภาวะบาดเจ็บของเส้นประสาทบางส่วนในหนูแรท ด้วยการวัดพฤติกรรมตอบสนองต่อความเจ็บปวดและติดตามการปรากฏของ substance P ในปมประสาทไขสันหลังและไขสันหลังส่วนหลัง หนูแรทเพศผู้จำนวน 52 ตัว น้ำหนัก 250-300 กรัม แบ่งออกเป็น 4 กลุ่ม ๆ ละ 13 ตัว โดยสองกลุ่มแรกจะถูกทำให้มีการบาดเจ็บของเส้นประสาท โดยได้รับและไม่ได้รับการกระตุ้นไฟฟ้า อีกสองกลุ่มเป็นกลุ่มปกติที่ได้รับและไม่ได้รับการกระตุ้นไฟฟ้า การบาดเจ็บของเส้นประสาทบางส่วนกระทำด้วยการตัดเส้นประสาท tibial และ common peroneal โดยคงเหลือเส้นประสาท sural ไว้ ทำการกระตุ้นไฟฟ้าด้วยความถี่ต่ำ 2 Hz ผ่านกล้ามเนื้อ ครั้งละ 30 นาทีต่อวัน เป็นเวลา 5 วันติดต่อกันในสัปดาห์แรก และ 2 ครั้งต่อสัปดาห์ในอีก 2 สัปดาห์ถัดมา โดยเริ่มกระตุ้นไฟฟ้าวันที่ 15 หลังการบาดเจ็บ ซึ่งเป็นวันที่พฤติกรรมตอบสนองต่อความเจ็บปวด แสดงออกมาอย่างเด่นชัด การทดสอบพฤติกรรมตอบสนองต่อความเจ็บปวด โดยวัดการชักเท้าหนี หลังกระตุ้นการสัมผัสด้วย von Frey filaments ที่บริเวณด้านนอกของฝ่าเท้าผ่านทางเส้นประสาท sural ใน 1 วันก่อนการบาดเจ็บ (baseline), วันที่ 8, 15, 23 และ 32 หลังการบาดเจ็บ ศึกษาการปรากฏของเส้นประสาทนำความรู้สึกที่มีเยื่อหุ้มไมอีลิน ด้วยการย้อม cholera toxin B subunit (CTBir) และการกระจายของ substance P (SP) ในปมประสาทไขสันหลังและไขสันหลังส่วนหลัง ระดับ L4-L6 ผลการศึกษาพบว่า 1) หลังจากได้รับการบาดเจ็บเส้นประสาทบางส่วน การตอบสนองต่อความเจ็บปวด มีค่า withdrawal threshold ลดลง จาก baseline (17.8±0.2 กรัม) ในตลอดช่วงการทดลอง (วันที่ 8: 1.2±0.2 กรัม, วันที่ 15: 2.4±0.6 กรัม, วันที่ 23: 1.6±0.5 กรัม และวันที่ 32: 1.6±0.4 กรัม) 2) การกระตุ้นไฟฟ้ามีผลลดความไวต่อการรู้สึกเจ็บ โดยการเพิ่มขึ้นของค่า withdrawal threshold ในสัปดาห์ที่ 2 อย่างมีนัยสำคัญ; baseline: 18.0±0.0 กรัม, วันที่ 8: 3.3±0.9 กรัม, วันที่ 15: 2.9±0.8 กรัม, วันที่ 23: 4.6±0.8 กรัม และวันที่ 32: 8.7±2.0 กรัม 3) การบาดเจ็บของเส้นประสาทบางส่วนนี้ มีผลต่อการเพิ่มขึ้นของ SP ในปมประสาทและไขสันหลังในข้างเดียวกับที่มีการบาดเจ็บของเส้นประสาท และ SP ลดลงเมื่อได้รับการกระตุ้นไฟฟ้า 4) มีการงอกของเส้นใยประสาทที่ย้อมด้วย CTBir กระจายเพิ่มขึ้นสู่ laminae I&II ของไขสันหลังส่วนหลังในหนูภาวะมีการบาดเจ็บของเส้นประสาทบางส่วน และลดลงเมื่อได้รับการกระตุ้นไฟฟ้า

การศึกษาครั้งนี้สามารถระบุได้ว่า หลังเกิดการบาดเจ็บของเส้นประสาท จะเกิดการเปลี่ยนแปลงของการกระจายของ SP ในไขสันหลังและปมประสาทระดับเอว (L4-L6) ซึ่งน่าจะมีบทบาทในการเปลี่ยนแปลงกระบวนการรับรู้ความรู้สึกเจ็บปวดในวิถีประสาท มีผลสอดคล้องกับการเกิด neuropathic pain และการกระตุ้นไฟฟ้าความถี่ต่ำ มีผลต่อการลดภาวะ neuropathic pain ภายหลังได้รับบาดเจ็บของเส้นประสาท ซึ่งน่าจะเป็นทางเลือกหนึ่งในการรักษาเพื่อช่วยบรรเทาความเจ็บปวดเรื้อรังเนื่องจากการบาดเจ็บของระบบประสาทส่วนปลายในผู้ป่วยต่อไป

114 หน้า

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

%	Percent
°	Degree
Ach	Acetylcholine
BSA	Bovine serum albumin
C	Celsius
CCI	Chronic constriction injury model
cm	Centimetre
CNS	Central nervous system
CTB	Cholera toxin B subunit
CTBir	CTB immunoreactivity
DRG	Dorsal root ganglion
ed.	Edition
EMG	Electromyography
ES	Electrical stimulation
ETOIMS	Electrical twitch obtaining electrical stimulation
et al.	And colleagues
FITC	Fluorescein isothiocyanate
g	Gram
h	Hour
Hz	Hertz
IgG	Immunoglobulin G
IMS	Intramuscular stimulation
kg	Kilogram
mA	Milliamperes
MEPZs	Motor end-plate zone
mg	Milligram
ml	Millilitre
mm	Millimetre

LIST OF ABBREVIATIONS (Continued)

ms	Millisecond
O.C.T.	Optimal Cutting Temperature compound
PBS	Phosphate buffer saline
PNS	Peripheral nervous system
PSL	Partial sciatic nerve ligation injury model
p-value	Probability of obtaining a result at least as "impressive" as that obtained
s	Second
SCS	Spinal cord stimulation
SD	Standard deviation
SNI	Spared nerve injury model
SNI-ES	Spared nerve injury model with electrical stimulation treatment
SNL	Spinal nerve ligation injury model
SNT	Sciatic nerve transection injury model
SP	Substance P
SPir	Substance P immunoreactivity
SPSS	Statistical package for social science
TCM	Traditional Chinese Medicine
TENS	Transcutaneous electrical nerve stimulation
WDR	Wide dynamic range neuron

CHAPTER I
INTRODUCTION

Background and rationale

Humans or animals with a peripheral nerve injury also have abnormal pain that called neuropathic pain. Neuropathic pain is a complex syndrome resulting from diverse lesions or disease to the peripheral and central nervous system such as trigeminal neuralgia, polyneuropathy, postherapeutic neuralgia and painful diabetic neuropath (1, 2). The common etiology of peripheral nerve injury is due to trauma, metabolic disease, toxin, compression and chronic musculoskeletal pain.

Clinical symptoms of neuropathic pain include spontaneous ongoing pain that is stimulus-independent, noxious stimuli induces more pain response (hyperalgesia) and a pain that is elicited by innocuous stimuli (allodynia). This pathology persists for a long time after the damages tissue has healed and is often resistant to common therapeutic interventions (3-5). Patients are usually treated with tricyclic or serotonin and norepinephrine uptake inhibitors, antidepressants, anticonvulsants, topical agents, local anaesthetics, antiarrhythmics, non-narcotic and narcotic analgesics that all have limited efficacy and undesirable side effects (6-8). The alternative therapies commonly used to relieve pain are the needling technique such as spinal cord stimulation (SCS), traditional acupuncture and transcutaneous electrical nerve stimulation (TENS).

As described by Melzack (9) and Simon (10), traditional acupuncture uses fine acupuncture needles at acupuncture points on meridians to relieve pain. They suggest that acupuncture point, muscle trigger points, and motor end-plate zones (MEPZs) may be identical. In 1996, Gunn (11) introduced intramuscular stimulation (IMS) to relieve myofascial pain by dry needling muscles, with acupuncture needles, at tender motor points. Based on the fact that neuropathic pain is a supersensitivity phenomenon, and its treatment requires desensitization. Lomo (12) has shown in animal experiments that supersensitivity and other features of denervated muscle can be reversed by electrical stimulation. IMS uses some of the tools of acupuncture, but it differs fundamentally in that it is based on current Western research in physiology,

rather than Chinese medicine. IMS is applied directly to the site of the pain called trigger point, rather than to remote points based on maps of energy flow (11). Recently, Chu et al. (13) have shown that musculoskeletal pain relief can be achieved by additional changes to classical acupuncture and IMS methods. They suggested that needle insertion or manipulation induces local muscle twitches, which may mediate musculoskeletal pain relief (14). They demonstrated that significantly greater pain relief occurred with electrical twitch-obtaining intramuscular stimulation (ETOIMS) than dry needling of the same muscle or overlying skin (13). Neuropathic pain occurred following neuropathy responds well to IMS treatments by desensitizing the affected nerve and muscles (15). Furthermore, there is a supporting evidence of choosing points for being stimulated by electrical stimulation, based on neuro-anatomical principle. This clinical model suggested strong analgesic effect of needling by application on damaged tissue or points anywhere that share on innervations via the same spinal segment as the injured tissue (16).

During the past decade, the mechanisms of neuropathic pain are still incompletely understood and treatment is often unsatisfactory. Therefore, the different animal models for studying the neuropathic pain have been intensively developed such as chronic constriction model (CCI), partial sciatic nerve model (PSL) and spinal nerve ligation (SNL) models. Most of these models have been developed in the rat, including sciatic nerve constriction (17), spinal nerve ligation (18), and tight ligation of 1/3 to 1/2 of the sciatic nerve (19). Although these models have all been extensively used to study a board range of behavior, pharmacological responses and induction of alterations in the somatosensory system and will continue to be important tools. However, the degree of damage in these models is inherently difficult to reproduce leading to some variability in the number of responders and their behaviors. Therefore, Decosterd and Woolf (20) developed a spared nerve injury (SNI) model, which is produced by complete transection of the common peroneal and tibial distal branches of the sciatic nerve leaving the sural branch intact. This procedure was reported that it is both reliable and easy to perform.

Because there is great interest in defining the molecular mechanisms that contribute to the development of neuropathic pain, there is particular value to study the alterations in central neuronal processing which play an important role in the

development of such pathological pain. A key mechanism, of how injured primary afferents contribute to abnormality in the sensory pathway, is not fully understood (21). One possible mechanism whereby primary afferents might influence spinal or supraspinal sites following peripheral nerve injury is a change in expression and distribution of neuropeptides by axon sprouting (22). In the spinal cord, substance P (SP) appears to be involved in the mechanism of hyperexcitability of dorsal horn neurons via a direct action on the postsynaptic cells or via potentiation of the excitatory effects of glutamate (23, 24). Neurons containing SP as well as other neuropeptides exhibit changes in gene expression and peptide levels in the dorsal root ganglia (DRG) and the spinal dorsal horn after axotomy (25). Noguchi et al (24) reported that SP was synthesized in injured DRG neurons and contributed to hyperexcitability of neurons and changes in gene expression in gracile nucleus neurons, including those that project to the thalamus. The changes in excitability of gracile nucleus neurons is very likely to affect the activity of neurons in the thalamus and somatosensory cortex (26), so that SP in damaged primary afferent neurons may contribute to abnormal neuropathic sensations, such as dysesthesias and mechanical allodynia (27).

Therefore, the present study chose the spared nerve injury model to develop the peripheral neuropathy and is aimed to test the efficacy of electrical stimulation treatment similar to IMS and modified ETOIMS on neuropathic pain behavioral responses, mechanical allodynia in SNI. In addition to evaluating the behavioral consequences of sparing sural nerve of the distal branches of sciatic nerve, the DRG neurons and central termination of these branches were examined by studying injury and treatment-induced changes in the substance P immunoreactivity (SPir) in DRG and spinal dorsal horn.

CHAPTER II
OBJECTIVES

The main aim of the present study was:

1. To demonstrate the development of mechanical allodynia after nerve injury in SNI rat model.

2. To investigate the relieving effect of low frequency (2Hz) electrical stimulation (ES) applied intramuscularly on the mechanical allodynia in SNI rats.

To achieve these objectives, two main studies were included:

1. In the pain behavioral tests, the paw withdrawal threshold to non nociceptive mechanical stimulation was evaluated to indicate the mechanical allodynia.

2. In the immunofluorescent histochemistry, the SP immunoreactivity was examined in the spinal dorsal horn and dorsal root ganglia.

CHAPTER III
LITERATURE REVIEW

Pain is an unpleasant sensation and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Damage of tissues whether induced by disease, inflammation, or accident (or provoked by surgical operation or other therapeutic measures) constitutes a noxious stimulus and causes cellular breakdown with liberation of biochemical substances. Pain is separate and distinct from nociception, the system which carries information, about inflammation, damage or near-damage in tissue, to the central nervous system. These activate special receptors of nociceptors that can be sensitive to heat, cold, mechanical stimuli, or chemical mediators. Although nociceptive impulses are often called pain impulses, pain is not experienced until information reaches the brain in normally conscious persons, in which the sensory and emotional experience takes place (28).

Pain can be adaptive sensation, an early warning to protect the body from tissue injury. Pain can also be maladaptive, reflecting pathological function of the nervous system. Multiple molecular and cellular mechanisms operate alone and in combination within the peripheral and central nervous systems to produce the difference forms of pain (29).

Classification of pain

Pain can be classified to consider it along a continuum of duration as acute and chronic pain. Acute pain and chronic pain differ in their etiology, pathophysiology, diagnosis and treatment.

Acute pain: pain of peripheral origin is usually cause by tissue damage, inflammation or a disease process of the skin, subcutaneous tissue, or deep somatic structures, spasm of muscles. It is a symptom of a disease process experienced in or around the injured or diseased tissue. Acute pain is nociceptive in nature, and occurs secondary to chemical, mechanical and thermal stimulation of A-delta and C-

polymodal pain receptors (30). The pathophysiology of acute pain is fairly well understood: its diagnosis is usually not difficult, hence the therapy is effective. As a result of effective treatment, the self-limiting nature of the disease or injury, or both, the pain and associated responses usually disappear within days or weeks (31, 32).

Chronic pain: was originally defined as pain for extended periods of time that accompanies a disease process (e.g., Rheumatoid arthritis) or that is associated a with an injury that has not resolved within an expected period of time (e.g., low back pain, phantom limb pain) are all referred to as chronic (32). Chronic pain syndrome consists of a wide range of symptoms likely to be mediated by multiple mechanisms. Therefore, blockade of just one of these mechanisms may not provide full pain relief in every patient. A better approach may be a combination therapy involving two compounds with independent mechanism of action. Mechanical hypersensitivity can be the most debilitating symptom in some patients suffering from neuropathic pain (33). Chronic pain can be refractory to multiple treatment modalities. If chronic pain is inadequately treated, associated symptoms can include chronic anxiety, fear, depression, sleeplessness and impairment of social interaction. Chronic, non–malignant pain is predominately neuropathic in nature and involves damage either to the peripheral or central nervous systems (30).

The pain sensation can be further split into distinct categories. Pain normally serves as a warning device, an alarm system activated in response to damage to the organism. This nociceptive is an activated only by noxious stimuli acting on a specialized high-threshold sensory apparatus (figure 3.1). The threshold for eliciting pain has to be high enough that it does not interfere with normal activities but low enough that it can be evoked before tissue damage occurs. This threshold is not fixed and can be shifted either up or down, which may be either adaptive or maladaptive. Shifts in pain threshold and responsiveness are an expression of neural plasticity, the neurobiological means by which changes in the nervous system can modulate the response to any stimulus. Such plasticity or modifiability of the sensory system essentially characterized clinical pain syndrome (34).

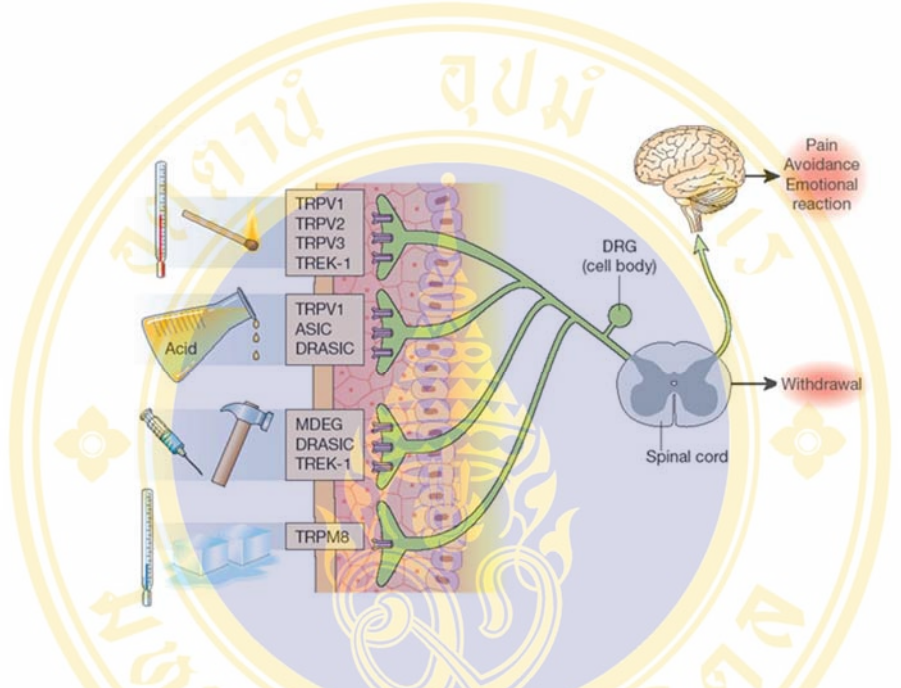


Figure 3.1 Nociceptive pain: noxious stimuli, such as heat, cold, acid, pin prick, are transduced into electrical activity at the peripheral terminals of nociceptors by specific receptors or ion channels sensitive to heat, mechanical stimuli, protons and cold. This activity is conducted to the spinal cord and, after transmission in central pathways, to the cortex, where the sensation of pain is experienced (29).

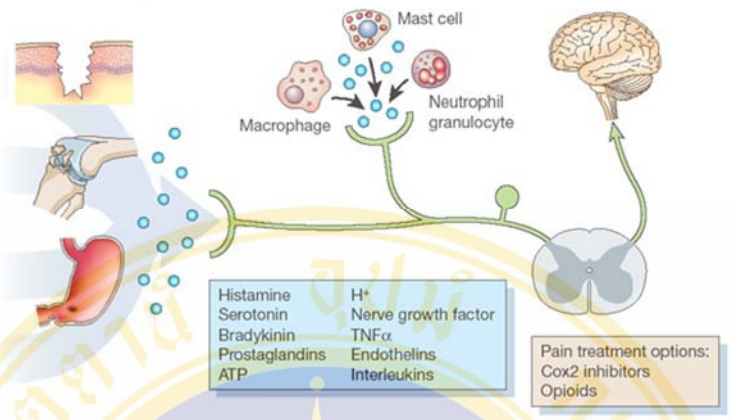


Figure 3.2 Inflammatory pain: damaged tissues, inflammatory and tumor cells release chemical mediators that activate or modify the stimulus response properties of nociceptors afferents. This sets up changes in the responsiveness of neurons in the central nervous system (29).

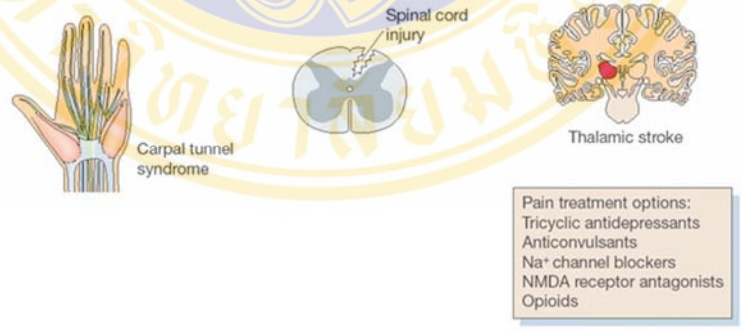


Figure 3.3 Neuropathic pain arises from lesions or disease or dysfunction of the nervous system. Conditions affecting the peripheral nervous, as in carpal tunnel syndrome, the spinal cord after traumatic injuries or the brain after stroke, diabetic disease can all cause neuropathic pain, which is characterized by a combination of neurological deficits and pain (29).

Once tissue has been damaged, the multiple chemical mediators are released from damaged and inflammatory cells. The chemical mediators are rich in cytokines, growth factors, kinins, purines, amines, prostanoids and ions, including protons (35). Some inflammatory mediators directly activate nociceptors, evoke pain others act together to produce a sensitization of the somatosensory nervous system, which is characteristic for inflammatory pain, enabling easier activation of the pain pathway until the tissue heals (29) (figure 3.2).

Maladaptive plasticity represents those changes that generate spontaneous and exaggerated pain with no discernable protective or reparative role. The pain becomes the pathology, typically via damage to or disease or dysfunction of the peripheral or central nervous system, termed ‘neuropathic pain’ (figure 3.3).

Perception of pain

The highly individual and subjective nature of pain is one of the factors that made it difficult to define and to treat clinically. Nociceptive, inflammatory and neuropathic pain results from diverse mechanism. In some patients, a single mechanism may produce their pain; in others, multiple mechanisms may contribute. The some symptom, pain in response to light touch of the skin, may be generated by a number of mechanisms. Moreover, a single mechanism, upregulation of a voltage-gated sodium channel, may potentially produce different symptoms like spontaneous burning pain or paresthesias (pins and needles) (36).

Nociception is the role sole mechanisms that produce pain. Nociceptors A-delta and C-somatosensory afferent terminals converse external noxious stimuli such as noxious thermal, mechanical, or chemical stimulus into electrical activity in the peripheral terminals of nociceptor sensory fibers. Nociceptor responses directly to some noxious stimuli and indirectly to others by means of one or more chemical released from cells in the traumatized tissue include bradykinin, histamine, prostaglandins, substance P and other related peptides, acidity, ATP, serotonin and acetylcholine. Each originates from a different population of cells, but all act to decrease the threshold for activation of nociceptors. Nociceptor function is substantially modified in response to tissue damage, inflammation or injury of the nervous system. Post-translational and transcriptional changes can profoundly alter the

threshold, excitability and transmission properties of nociceptors, contributing to pain hypersensitivity and spontaneous pain (29).

Nociceptors have three classes of nociceptors can be distinguished on the basis of the type of stimulus. 1) The mechanical nociceptors require strong, often painful tactile stimuli, such as a pinch, in order to respond. They have small-diameter, thinly myelinated A-delta fibers that conduct signals at about 2–14 m/s. 2) The thermal nociceptors are excited by extremes of temperature, >45^{o}C or <5^{o}C, as noxious heat or cold. They also have small diameter, thinly myelinated A-delta fibers that conduct signals. 3) The polymodal nociceptors respond to a variety of destructive mechanical, thermal and chemical stimuli. They are activated by noxious mechanical stimuli, such as pinch or puncture, by noxious heat and cold, and by irritant chemical applied to the skin. These nociceptors have small-diameter, nonmyelinated C-fibers that conduct slowly, generally at velocities of less than 2 m/s. Stimulation of these receptors in human evokes sensations of slow, burning pain (37).

Central transmission of pain impulse

In morphological, nociceptors are free nerve ending. As with all neurons involved in somatic sensation, the cell bodies of the primary afferent pain neurons are located in the dorsal root ganglia (38). The free nerve endings connected to small myelinated axons and unmyelinated C-fibers. The myelinated fibers, when excited, are responsible for a transient sensation of fast pain, the C-fibers for slow, burning pain. The fibers terminate in the dorsal horns of the spinal cord, where they make synapses on second-order cells that send their axons to the contralateral side to ascend in the spinothalamic pathway (figure 3.4). These pathways are shared with fibers carrying temperature information. Activity in the pain pathway can be modulated at all levels, beginning at the segment of entry into the descending influences (39).

Nociceptive and polymodal impulses from tissues are transmitted via fibers in spinal nerves. This process is the synaptic transfer and modulation of input from one neuron to another. Nerve impulses in primary pain afferent fibers propagate to dorsal horn mediated direct monosynaptic contact or through multiple interneurons, some of

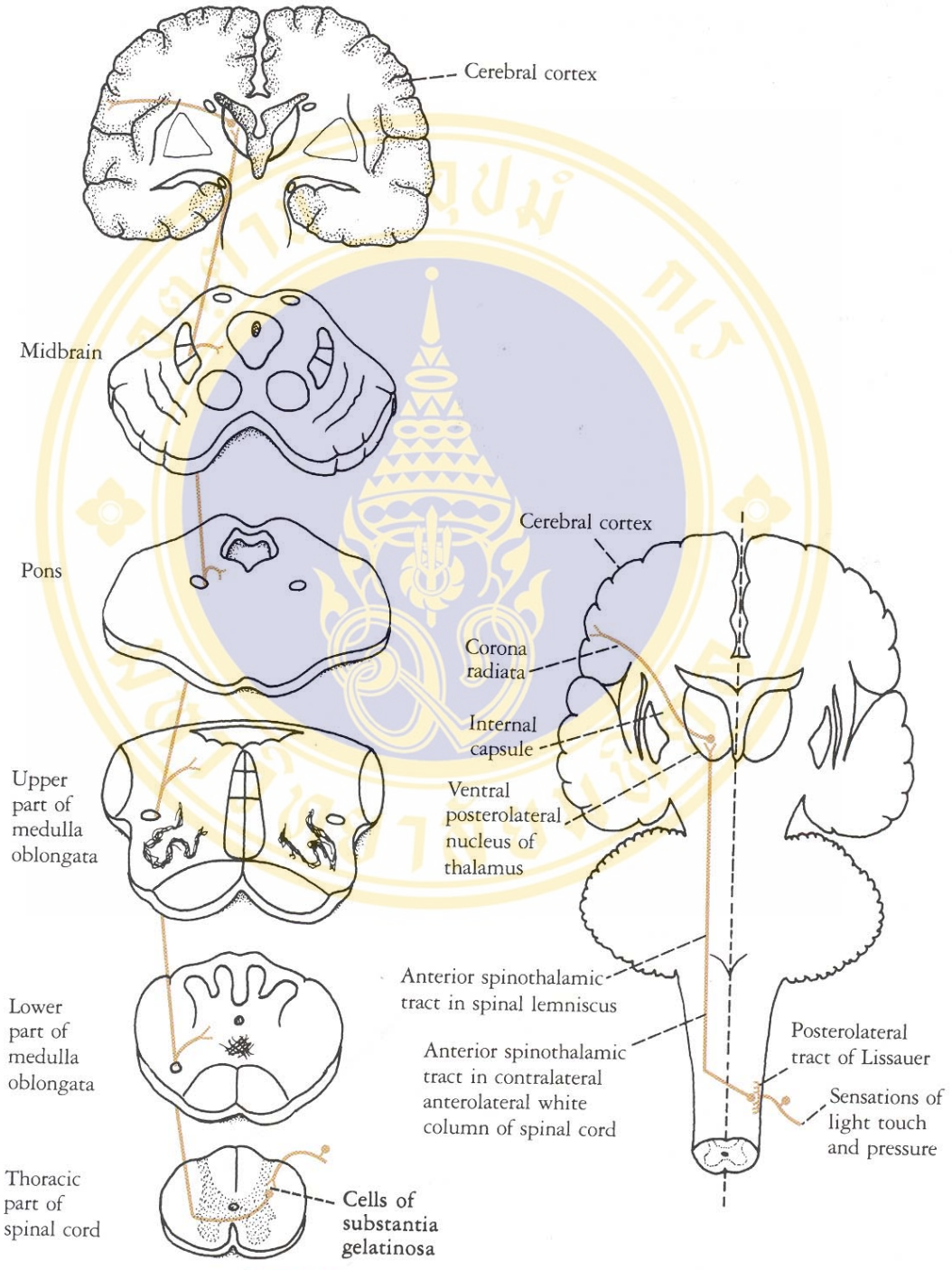


Figure 3.4 Schematic diagram of the spinothalamic pathway.

which are excitatory and some inhibitory. Synaptic transmission between nociceptors and interneuron or second order neurons is mediated by chemical neurotransmitter released from central terminal of pain afferent fibers. The principle excitatory neurotransmitter released by A-delta and C-fibers as well as nonnociceptive afferent is the glutamate, which evokes excitatory synaptic potentials in dorsal horn interneurons or spinothalamic neuron. In addition, the primary pain afferent fibers also elicit slow excitatory postsynaptic potentials in dorsal horn neurons by releasing other peptide transmitter. Excitatory neuropeptides, substance P (SP), are released together at synapses which act coordinately on ligand ion channels receptors on the postsynaptic membrane of the spinothalamic neuron. Neuropeptides, including SP, appear to enhance and prolong the actions of glutamate. The release of neuropeptides from a single afferent fiber is likely to influence many postsynaptic dorsal horn neurons. This feature, together with the result of significant increase of neuropeptide levels in persistent pain conditions, are indicated that peptide actions contribute both to excitatory of dorsal horn neurons and to the unlocalized character of many pain conditions.

The primary nociceptive afferent fibers terminate predominantly in the spinal dorsal horn. The dorsal horn can be subdivided into 6 distinct laminae on the basis of the cytological features of its resident neurons (figure 3.5). Classes of primary afferent neurons that convey distinct modalities terminate in distinct of the spinal dorsal horn. Nociceptive neurons are located in the superficial dorsal horn, in the lamina I and lamina II (substantia gelatinosa). Most A-delta nociceptive fibers of high threshold and heat nociceptive and C-polymodal nociceptive fibers give direct synaptic input to these dorsal horn neurons. Many of the neuron are located in lamina I responds exclusively to noxious stimulation and nociceptive-specific neuron which project to higher brain centers. Some neurons in this layer, called wide dynamic range neurons (WDR), respond in a graded fashion to both non-noxious and noxious mechanical stimulation. The lamina II is made up almost exclusively of interneurons (both excitatory and inhibitory), some of which respond only to nociceptive inputs while others also respond to non-noxious stimuli.

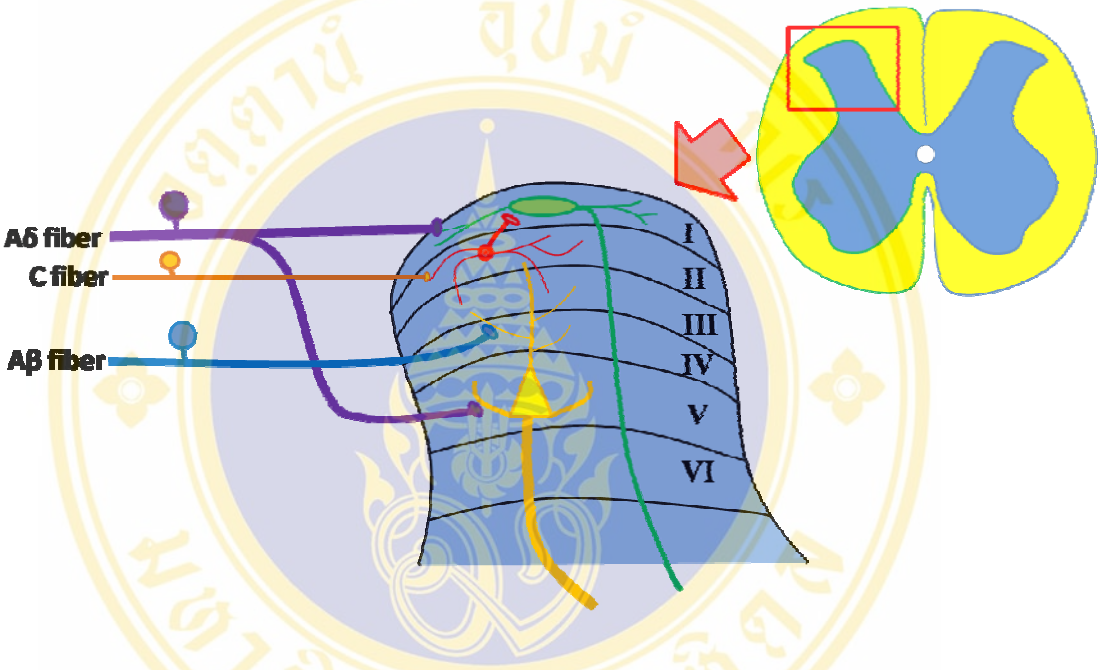


Figure 3.5 Nociceptive afferent fibers terminate on projection neurons in the spinal dorsal horn. Lamina I have wide dynamic range neurons that respond to noxious stimulation. The interneuron on lamina II, some of which respond to nociceptive inputs and others also respond to non-noxious stimuli. In laminae III and IV respond to non-nociceptive input (A-beta fibers). Laminae V and VI have wide dynamic range neurons receive input from A-beta fibers as well as indirect and direct input from A-delta and C-fibers.

The laminae III and IV are located ventral to the lamina II and contain neurons that receive monosynaptic input from A-beta fibers. These neurons respond predominantly to non-noxious stimuli and have quite restricted receptive fields that are organized topographically. Lamina V contains primarily WDR neurons that project to the brain stem and to regions of the thalamus. These neurons receive input from A-beta and A-delta fibers. They also receive input from C-fibers, either directly on their dendrites, which extend dorsally into the superficial dorsal horn, or indirectly via excitatory interneurons that themselves receive input directly from C-fibers. Many neurons in lamina V also receive nociceptive input from visceral structure.

Neurons in lamina VI receive inputs from large diameter afferents from muscles and joints and respond to non-noxious manipulation of joints. These neurons are thought not to contribute to the transmission of nociceptive messages.

Pain has 2 difference types: fast and slow pain. *Fast pain* is an abrupt and sharp sensation that is carried by A-delta fibers. *Slow pain*, carried by C-fibers, is a sickening burning sensation.

These are two principle theories concerning pain: (1) the theory that considers pain a specific sensory modality; (2) the pattern theory that maintain that the impulse pattern for pain is produced by intense stimulation of nonspecific receptors.

Neuropathic pain

In adult animals, most neurons are postmitotic; only in a few instances do neuroepithelial stem cells persist. Thus neurons lost to injury or disease cannot be replaced. Nerve cells can, regenerate severed axonal and dendritic processes to reestablish synaptic connections.

The ability of regenerating axons to locate and innervate appropriate targets varies widely among species, from neuron to neuron, and with development age. Neurons in fetal or neonatal mammals also are able to reestablish appropriate synaptic connections with targets in the periphery after injury. Axons in the adult mammalian peripheral nervous system can regenerate; buy show less specificity in contacting peripheral targets. In the adult mammalian CNS, successful regeneration is often blocked by proteins on the surfaces of astrocytes and oligodindroglia that inhibit axon growth.

Damage to a peripheral nerve causes changes within the cell body that promote neuronal survival, axonal regeneration and functional recovery. The distal portion of the axon degenerates together with a short length of axon proximal to the site of the lesion. The glial cells that had formed the myelin sheath of the distal segment of the nerve dedifferentiate, proliferate, and together with invading microglia and macrophages, phagocytize the axonal and myelin remnants. Within a few hours, new axonal sprouts emerge from near the tip of the proximal stump and begin regenerating (39) (figure 3.6).

Reactive changes associated with peripheral nerve injury are not limited to the cell body and nerve stumps it could induce changes to the central targets of primary afferent neurons. Peripheral nerve injury of neurons with C-fiber exhibit transganglionic degeneration of their terminations within Rexed's lamina II of dorsal horn, while peripheral nerve injury of medium to large neurons induces collateral sprouting of myelinated A-fibers from laminae I and III/IV into lamina II in rats, cats and primates (29, 37).

Neuropathic pain is described as "burning", "electric", "tingling", and "shooting" in nature. It can be continuous or paroxysmal in presentation (30). It originates from pathology of the nervous system. Innumerable diseases may be the culprits. Examples include autoimmune disease (e.g. multiple sclerosis), metabolic diseases (e.g. diabetic neuropathy), infection (e.g. shingles and the sequel, postherpetic neuralgia), vascular disease (stroke), trauma, and cancer. A rule without apparent exception is that the lesion leading to pain must directly involve the nociceptive pathways (40). Namely, not all lesions of nociceptive pathways induce pain. A lesion of the peripheral nerve may induce pain, but simply severing dorsal roots seems to have little chance of creating lasting pain (41).

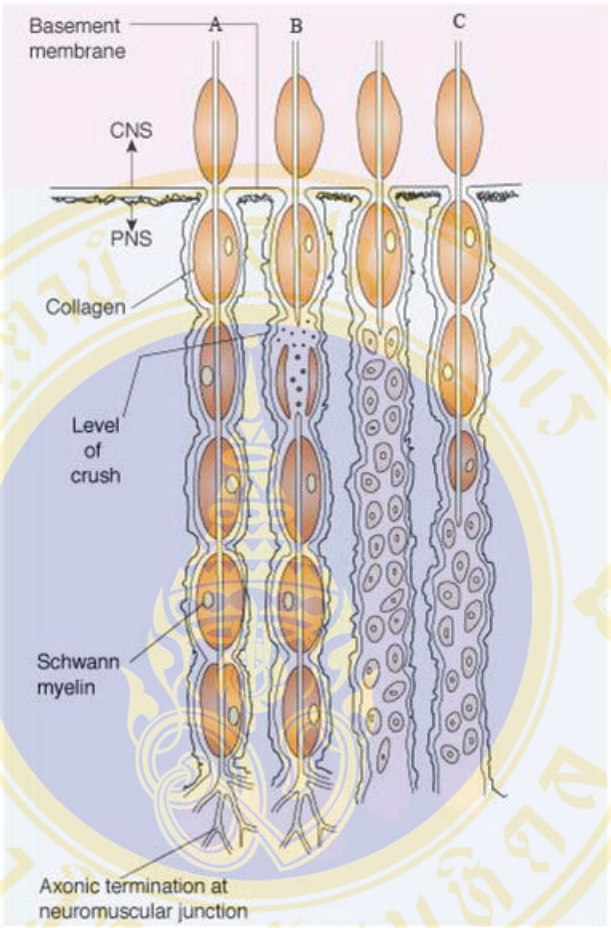


Figure 3.6 Degenerative changes after axotomy:

(A) A typical neuron.

(B) After axotomy the nerve terminal, the distal segment of the axon, and a short length of the proximal segment of the axon degenerate. Schwann cells dedifferentiate, proliferate and together with invading microglia and macrophages, phagocytize the axonal and myelin remnants. The axotomized neuron undergoes chromatolysis, presynaptic terminals retract and degenerative changes may occur in pre- and postsynaptic cells.

(C) The axon regenerates along the column of Schwann cells within the endoneurial tube and sheath of basal lamina that had surrounded the original axon.

Symptom of neuropathic pain

Abnormal pain stated that results from a peripheral nerve injury are commonly referred to as neuropathic pain. The liability for pain appears to vary from person to person, from nerve to nerve, between males and females, and even with age. What appears to be the same lesion may induce no pain in one person but severe pain in another (42). Many patients with neuropathic pain exhibit persistent or paroxysmal pain that is independent of a stimulus. This stimulus-independent pain can be shooting, lancinating, or burning and may depend on activity in the sympathetic nervous system. These pathological phenomena persist for a long time after the damaged tissue has healed (5, 20). Spontaneous activity in nociceptor C-fibers is thought to be responsible for persistent burning pain and the sensitization of dorsal horn neurons. Similarly, spontaneous activity in large myelinated A-fibers (which normally signal innocuous sensations) is related to stimulus-independent paraesthesias and, after central sensitization, to dysaesthesias and pain (43).

Neuropathic pain is often present as a combination of several heterogeneous symptoms. Selective nerve damage in animals also leads to induction of multiple signs that appear to be indicative of pain. For example, rats with chronic construction injury show hyperalgesia and allodynia provoked by thermal and mechanical stimuli. These animals also show what has been termed spontaneous pain.

Stimulus-evoke pain is a common component of peripheral nerve injury or damage and has two key features: hyperalgesia and allodynia (43, 44).

Hyperalgesia: is defined as an increased sensitivity to a normally painful stimuli and is the result of abnormal processing of nociceptor (44, 45). Primary hyperalgesia, caused by sensitization of C–fibers, occurs immediately within the area of the injury. Secondary hyperalgesia, caused by sensitization of dorsal horn neurons, occurs in the undamaged area surrounding the injury. Stimulus-evoked hyperalgesia are commonly classified into subgroups on the basis of modality; mechanical, thermal, or chemical. Abnormal sensitivity is not confined to the periphery. The neuromas, a swelling at the proximal end of the injured nerve which contains regenerative axon sprouts, commonly exhibits exquisite mechanical sensitivity because of altered membrane properties of both C- and A-axons (46, 47).

Allodynia: is defined as pain resulting from a stimulus that ordinarily does not elicit a painful response (e.g. light touch) and can be produced in two ways: by the action of low threshold myelinated A-fibers on an altered central nervous system; and by a reduction in the threshold of nociceptor terminals in the periphery (48, 49). Because allodynia as a clinical diagnosis does not implicate a particular mechanism, it may be more useful to subclassify it under the umbrella of hyperalgesia, so that the diagnosis of symptoms can be more suitably aligned with mechanisms (43, 46).

Mechanical allodynia is a prominent feature of inflammatory, neuropathic, and postoperative pain syndromes. The results of this study support previous reports that two distinct types of mechanical allodynia can be detected in animal models of neuropathic pain (50). They have been termed static and dynamic allodynia after the type of stimulus that leads to their induction. Both types of allodynia are also present in patients suffering from neuropathic pain (51). It has been suggested that static allodynia is pressure-evoked pain signaled by un-myelinated afferent fibers, whereas dynamic allodynia is brush-evoked pain, signaled by large myelinated afferent fibers (51, 52). While the static component is only found in the injured area, the dynamic component also extends into a halo of undamaged tissue surrounding the injury. The A-fibers that appear to signal dynamic allodynia (52) do not contain SP then after chronic inflammation can induce synthesis of SP in A-beta fibers (53).

Neuropathic pain reflects both peripheral and central sensitization mechanisms. Abnormal signals arise not only from injured axons but also from the intact nociceptors that share the innervations territory of the injured nerve (54).

Mechanisms of Neuropathic pain

A variety of pain-related phenomenon of both central and peripheral mechanisms, have been associated with peripheral nerve injury (table 3.1). These are generally not mutually exclusive and it is entirely possible that any one of these (or more likely a combination) contribute to symptomatology in individual patients suffering from neuropathic pain.

The peripheral mechanism of neuropathic pain occurring after peripheral nerve damage has been characterized in great (although still incomplete) detail. Injured peripheral nerve fibers give rise to an intense and prolonged input of ectopic activity to

the central nervous system and in some cases also secondary changes of the excitability of dorsal horn neurons.

At the cellular level formation of new channels, upregulation of certain receptors and down regulation of others, altered local or descending inhibition are some of the biological features that can contribute to a hyperexcitability, which is assume to be a chronic pain (55, 56).

The neuronal hyperexcitability has a wide spectrum of manifestations including increase in cellular excitability, expansion of neuronal receptive fields, change of modality to which neurons respond, recruitment of silent neurons or circuits and a neuronal reorganization in dorsal horn (47, 56).

At this point of time, we do not know the exact sequence of changes in mechanisms and how they may influence each other. After lesion of central pathways, neurons in the spinal cord and brain which lost normal input may also change their response characteristics and exhibit signs of hyperexcitability in a fashion mimicking that seen after peripheral nerve injury (43).

1. Peripheral mechanisms

Following a peripheral nerve injury (e.g. crush, stretch, or axotomy) sensitization occurs which is characterized by spontaneous activity by the neuron, a lowered threshold for activation and increased response to a given stimulus. Should the injured nerve be a nociceptor then increased nervous discharge will equate to increased pain. Following nerve injury C–fiber nociceptors can develop new adrenergic receptors and sensitivity, which may help to explain the mechanism of sympathetically maintained pain.

Table 3.1 Summary of the phenomena observed after experiment peripheral nerve injury in peripheral and central nervous system, may be contributed to neuropathic pain (55, 57)

Peripheral mechanisms	Central mechanisms
- Nociceptor sensitization - Ectopic and spontaneous discharge - Ephaptic conduction - Alterations on ion channels expression - Collateral sprouting of primary afferent neuron - Sprouting of sympathetic neurons into the DRG - Phenotype switch of A-beta fibers	- Central sensitization - Spinal reorganization - Changes in inhibitory pathways

Sensitization of primary afferent nociceptor terminals

Injury and inflammation of tissue result in profound changes to the chemical environment of the peripheral terminal of nociceptors. Damaged cells and inflammatory cells release intracellular content, such as cytokines, histamine, serotonin, bradykinin, prostaglandins, adenosine triphophatase, interleukins, cytokines, and growth factors, are recruited to the site of damage (35, 43). With these inflammatory components such as bradykinin or prostaglandins bind to G-protein-coupled receptors and induce activation of protein kinase A and C in nociceptor peripheral terminals, which then phosphorylate ion channels and receptors. As a result, the threshold of activation of transducer receptors and the excitability of the peripheral terminal membrane increases, producing a state of heightened sensitivity (58).

Some of these factors act directly on the nociceptor terminal to activate it and produce pain (nociceptor activators), and others sensitize the terminal so that it become hypersensitive to subsequent stimuli (nociceptor sensitizers).

Following injury, peripheral nociceptors are prolonging sensitized by ectopic discharge leading to release of neuropeptides (Substance P and CGRP) contributed to sensitization of the peripheral terminal of injured and uninjured fibers. As a result, sensitization of C- and A-delta fibers, is characterized by ongoing discharges, a lowered threshold for activation and increased response to a given stimulus (56, 59).

Ectopic discharges and ephaptic conduction

Generally, in normal primary afferent neurons, it is rare for firing threshold to be reached without the input of a stimulus. Two types of sodium channel are found in sensory neurons; the first type is sensitive to tetrodotoxin, a potent puffer-fish toxin, is responsible for the initiation of the action potential and exists in all sensory neurons. The second type are insensitive to tetrodotoxin, found only on nociceptor sensory neurons, have much slower activation and inactivation kinetics than the tetrodotoxin sensitive channels, and are implicated in pathological pain states (60).

Following nerve injury, however, it has been demonstrated that there is a large increase in the level of spontaneous firing in the afferent neurons linked to the injury site. The sodium channels in damaged nerves differ pharmacologically and demonstrate different depolarization characteristics (61). They begin to accumulate in

the axon at the neuroma site and along the length of the axon (62). It has increase in transcription or altered trafficking of sodium channels as well as a reduction in potassium channels increases membrane excitability sufficiently so that action potential is generated spontaneously (figure 3.7). This has been termed ectopic discharge and has also been demonstrated in humans, suffering from neuropathic pain.

Ochoa and colleagues (63) reported spontaneous firing of C-fiber nociceptors and low threshold A-fibers mechanoreceptors after nerve injury in human beings. Ectopic discharges were originally arising in the neuromas itself. The spontaneous activity and ectopic sensitivity to mechanical, thermal, and chemical stimuli that originate from the traumatic neuromas have been well documented (64). Neuromas are composed of abnormal sprouting axons and have a significant degree of sympathetic innervations (65).

However, further studies revealed that some ectopic discharges could also originate from the DRG and other points along the nerve (55). Having demonstrate that presence of ectopic firing in peripheral nerve injury animal models (66), as well as in human, it is important to investigate the cause of these discharges. A small number of A-fibers (10%) exhibit sub-threshold membrane oscillations in their resting state or under depolarization condition (67). This increased oscillatory behavior leads to an increase in ectopic firing as the oscillations more frequently reach threshold and subsequent “cross-excitation” of other neurons serve to amplify this effect.

Further animal investigations suggest that abnormal electrical connections can occur between adjacent demyelinated axons. These are referred to as ephapses. "Ephaptic cross talk" may result in the transfer of nerve impulses from one axon to another. This evidence has been demonstrated the cross-exhibition or ephaptic between A- and C-fibers that developed in dorsal root ganglion (68).

These observations suggest that the development of ectopic activity may be particularly important for the development of hyperalgesia, allodynia and ongoing pain associated with nerve injury (55). In addition, it is now recognized that two populations of afferent fibers develop ectopic activity following nerve injury, the injured sensory neuron themselves and their uninjured neighbors (69, 70). Moreover, more recent study has supported a direct correlation in the time course of behavioral changes following spinal nerve injury with that of ectopic activity in A-fibers that

gives only occur 3-4 weeks following axotomy and may persist for many weeks following injury. Thus, pain arising as a result of peripheral nerve damage may reflect activity in both damaged as well as intact sensory neurons (55).

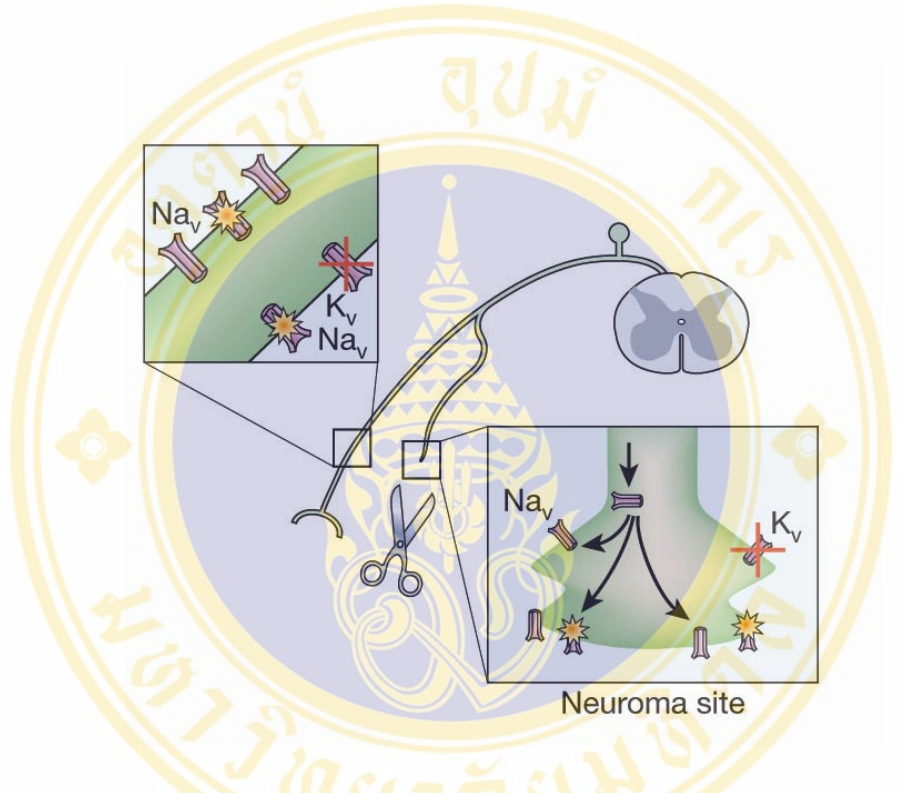


Figure 3.7 Ectopic discharge: it has increase in transcription or altered trafficking of sodium channels as well as a reduction in potassium channels increases membrane excitability sufficiently so that action potential is generated spontaneously (29).

Coupling between the sympathetic and sensory nervous system

There has been know that sympathetic nervous system can affect the severity of neuropathic pain (56). After peripheral nerve injury, it often initiates regional changes in circulation and temperature, which probably to aid the inflammatory response (71). Nerve injury also induces the sprouting of sympathetic axons into the dorsal root ganglion where they form baskets around the cell bodies of sensory neurons (72) and may constitute a mechanism in which sympathetic activity initiates activity in sensory fibers. Sympathetically maintained pain theoretically requires specific treatment, such as sympathetic blocks, guanethidine, or α1 antagonists that would not be appropriate for patients with sympathetically independent pain. Clinicians have observed for many years that in a small subset of patients suffering from neuropathic pain, the pain is somewhat dependent on activity in the sympathetic nervous system. In certain of these patients, selective anesthetic blockade of the sympathetic nervous system leads to dramatic relief of pain (73). This is often referred to as sympathetic maintained pain (56, 74). This phenomenon is due to the formation of an abnormal connection between the sensory and the sympathetic nervous system. Thus, sympathetically maintained pain is a specific example of the importance of the intact nociceptors in the pathogenesis of a particular neuropathic pain disorder (54).

2. Central mechanisms

Anatomical re-organization at central levels

Under normal physiological conditions, different classes of primary afferent neuronal fibers terminate in specific laminae of the dorsal horn. As a generalization, A-beta fibers (large myelinated afferents) penetrate the dorsal horn, travel ventrally, and terminate in lamina III and deeper. C-fibers (small unmyelinated afferents) penetrate directly and generally terminate no deeper than lamina II. After peripheral tissue injury or damage to the nervous system, the low-threshold sensory fibers, which normally only produce innocuous sensations like light touch, can begin to produce pain, a very substantial change in the normal functional specificity of the sensory system. The central terminals of myelinated non-nociceptive A-beta afferents sprout in the dorsal horn and form new connections with nociceptive neurons in laminae I&II. This re-wiring of the circuitry of the spinal cord may contribute to persistent pain

hypersensitivity (29). Woolf and colleagues demonstrated that after sciatic nerve axotomy, the central terminals of the large myelinated primary afferent neurons sprouted into lamina II of the dorsal horn (75). Moreover, Woolf and co-worker then demonstrated that this sprouting occurred within 1 week, was at its highest 2 weeks post injury and persisted over 6 months post axotomy (76). In addition, Lekan and colleagues (77) investigated the A-beta fibers sprouting in the spinal nerve injury model of neuropathic pain and established a robust sprouting of A-beta fibers within 2-4 weeks post-surgery (77). It should be noted that optimal sprouting does not occur until 2 weeks post-surgery and so cannot be solely responsible for the allodynia observed in neuropathy models (55). One consequence of this synaptic rearrangement is that second-order neurons within the laminae I and II of spinal dorsal horn, that normally receive predominantly high threshold sensory input, begin to receive inputs from low threshold mechanoreceptors. This misinterpretation of information within the spinal cord may result in low threshold sensory information being interpreted as nociceptive, thus, providing another explanation for the emergence of allodynia after peripheral nerve injury (55). This rewiring of synaptic connections could explain why tactile allodynia occurs in patients with postherpetic neuralgia who have absent nociceptor innervation of the skin (78).

After peripheral tissue injury or damage to the nervous system, low-threshold sensory fibers, which normally only produce innocuous sensations like light touch, can begin to produce pain, a very substantial change in the normal functional specificity of the sensory system (figure 3.8). Increase in synaptic transmission in the dorsal horn (central sensitization) can begin almost immediately as a result of activity-dependent phosphorylation and trafficking of receptors or ion channels. Central sensitization can be sustained for some time by transcriptional changes which alter the excitability of neurons. Structural alterations in the synaptic contacts of low threshold afferents with pain transmission neurons, or a reduction of inhibitory mechanisms due to a loss of interneurons, represent persistent changes in the CNS that eventually result in a fixed state of sensitization (29).

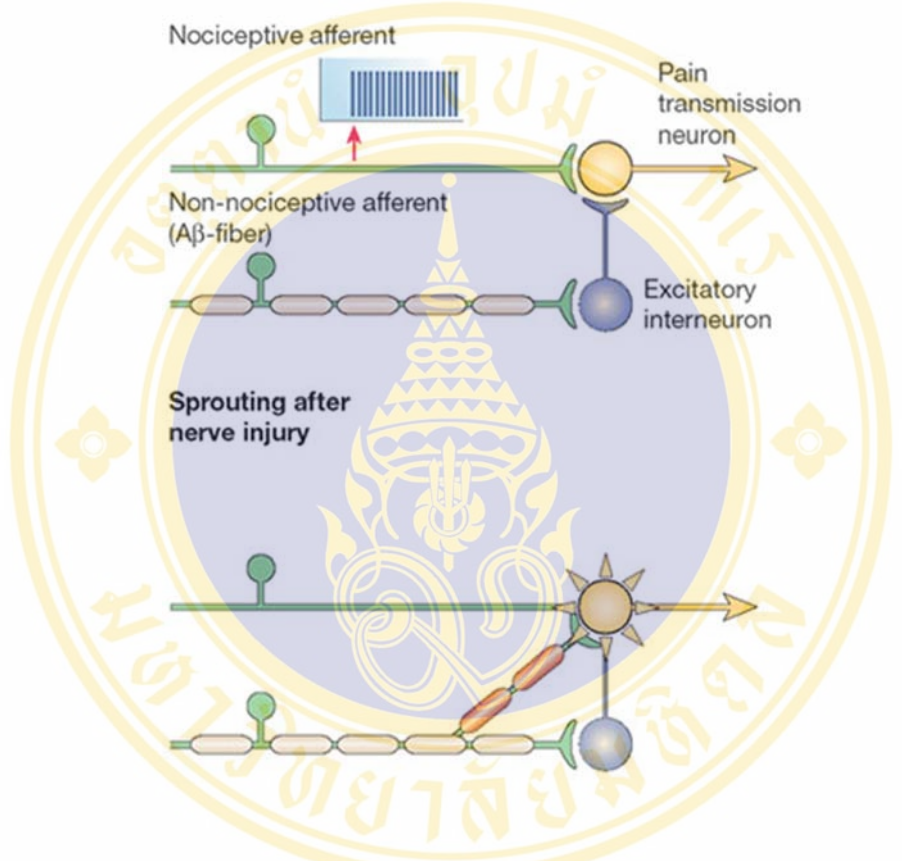


Figure 3.8 After peripheral nerve injury, the central terminals of myelinated non-nociceptive A-beta afferents sprout in the dorsal horn and form new connections with nociceptive neurons in laminae I and II. This re-wiring of the circuitry of the spinal cord may contribute to persistent pain hypersensitivity.

Spinal cord hyperexcitability

The afferent barrage associated with peripheral nerve injury is associated with the development of a sustained state of hyperexcitability of dorsal horn neurons, a process dubbed ‘central sensitization’. In dorsal horn nociceptive neurons, this is evident in a phenomenon referred to as ‘‘windup,’’ where continual low-frequency stimulation of C-fiber afferents leads to an increasing response in the dorsal horn cell (79). Repetitive episodes of "wind–up" may precipitate long–term potentiation, which involves a long lasting increase in the efficacy of synaptic transmission. Both "wind–up" and long–term potentiation are believed to be part of the sensitization process involved in many chronic pain states (30) and may contribute to hyperalgesia. However, the exact relationship of the relatively short lived phenomenon of wind-up and the persistent stated of central sensitization remain to be fully elucidated (55).

Loss of inhibitory from supraspinal pathways

In fact, not all stimulus-independent pain is mediated by spontaneous activity in primary afferent. A continuing spontaneous pain from damaged C-fiber nociceptors caused hyperexcitability of spinal cord neurons in the dorsal horn, is also important in central sensitization (56, 80). Neurons in the dorsal horn of the spinal cord not only receive excitatory input from primary afferent but also by inhibitory inputs that can be segmental or descend from the brain. Thus, an increase in the inhibition would decrease activity of the dorsal horn neuron (43). However, after peripheral nerve injury may reduce the amount of inhibitory control. There are several proposed mechanisms can account for the loss of inhibition. For example, loss of intrinsic modulatory systems such as inhibitory interneurons can cause excessive nociceptive input to the dorsal horn cell (56). The expression of cholycystokinin, an endogenous inhibitor of opiate receptors, is upregulated in injured sensory neurons. Furthermore, interneurons in lamina II, many of which are inhibitory (81), are thought to die after peripheral nerve injury. The degeneration of inhibitory interneurons in lamina II following peripheral nerve injury is thought to be an excitotoxic mechanism (43, 56). Consequently, the disinhibition occurred as a result of these process increases the likelihood that a dorsal horn neuron will fire spontaneously or in an exaggerated way in response to primary input (43).

In addition the basis of transcutaneous electrical nerve stimulation, which activate segmental inhibitory pathways, or brain stimulators, which activate descending inhibitory pathways (43).

The opioid system fails in neuropathic animals

It is generally accepted that opioids are less effective in relieving neuropathic pain than inflammatory pain. Although, the exact extent of this is controversial, the balance of evidence supports the view of an unfavorable shift in the dose response function for opioids in neuropathy. There are a number of plausible explanations for this observation, including a loss of peripheral opioid effects, loss of spinal opioid receptors and increased activity in physiological opioid antagonists systems (82).

In the spinal cord, opioid-receptors are localized predominantly on the pre-synaptic terminals of primary afferents in the superficial dorsal horn. However, after peripheral axotomy, a decrease in immunocytochemical receptor staining has been reported and also after dorsal rhizotomy, neonatal C-fiber degradation and chronic construction injury (83) a decrease on opioid receptor binding is observed, presumably reflecting the degeneration of primary afferent neurons.

In addition, immune cells may be involved in inflammatory pain, cancer pain and pain after nerve injury. They are activated both in the periphery and within the central nervous system in response to tissue damage, inflammatory or mechanical nerve lesions (84). The immune reaction may increase nociception through the release of cytokines, but granulocytes and monocytes can also promote analgesia by secreting beta-endorphin and enkephalin (85).

The allodynia and hyperalgesia associated with neuropathic pain may be best explained by: (1) the development of spontaneous activity of afferent input (2) the sprouting of large primary efferents (e.g. A-beta fibers from lamina III into laminae I&II), (3) sprouting of sympathetic efferents into neuromas and dorsal root and ganglion cells, (4) elimination of intrinsic modulatory systems and (5) up regulation of receptors in the dorsal horn which mediate excitatory processes (30).

Substance P

SP is a well-established neurotransmitter of nociception in both the peripheral and the central nervous system (86-88). Its role in nociceptive and inflammatory pain has been studied extensively (89-91). In primary afferent nerves, SP is located over synaptic vesicles in the small unmyelinated neurons concerned with nociception or pain and their endings in the dorsal layers of the spinal cord (92-94). The substantia gelatinosa of the dorsal horn contains a high concentration of SP (95-98). After peripheral nervy injury, the expression of these neuropeptides by these cells is downregulated. However, large myelinated A-beta fibers begin to express these neuropeptides, a phenotypic switch (99). Thus, low threshold stimuli, activating A-beta fibers, may cause release of SP in the dorsal horn and thereby generate a state of central hyperexcitability that is normally produced only by nociceptor drive. Release of these substances may lead to the sensitization of peripheral sensory terminals of injured and uninjured fibers.

SP would become more prominent in neuropathic pain states where myelinated fibers are primarily affected, resulting in an increase in the relative proportion of the remaining small and unmyelinated fibers (91, 100). Complex changes in SP levels in dorsal root ganglion (DRG) neurons have been described after nerve injury (101, 102) (figure 3.9).

Peripheral nerve injury results in the degeneration of primary sensory neurons which contain and release SP as neurotransmitter (103). Most of the studies concerning SP in connection with peripheral nerve injury have been conducted using the axotomy model and numerous studies have established that SP is downregulated in sensory neurons and that the SP content decreases in the dorsal horn (22, 103, 104).

Sciatic nerve section produced a 75-80% depletion of SP in the dorsal horn but not change the SP content of the ventral horn. The onset of SP depletion occurred within 7 days and was maintained for 2 months (103). Decreased levels of SP have been reported to occur at 60 days following chronic constriction injury of the sciatic nerve (105). The SP content of the dorsal root ganglia and both the peripheral and central branches of primary sensory neurons was also reduced after sciatic nerve section. Dorsal rhizotomy produced an 80% depletion of SP in the dorsal horn (103).

In partial sciatic nerve ligation models (PNL), 60 days after injury, the release of SP was increased and significantly higher than the release in the corresponding group of axotomized rats. The PNL rats displayed mechanical hypersensitivity which unrelated to late change in SP release but it may contribute to the maintenance of neuropathic pain (106).

SP would become more prominent in neuropathic pain stated where myelinated fibers are primarily affected, resulting in an increase in the relative proportion of the remaining small and unmyelinated fibers (87). A de novo expression of SP in large myelinated A-beta fiber, which normally do not synthesize SP (24), has been reported to occur up to 14 days both after chronic constriction injury (107) and after axotomy (101). A-beta fiber stimulation induces significant SP release after proximal spinal nerve lesion but not after distal sciatic axotomy (108).

Anatomical evidence of large myelinated fiber participation in nociception following nerve injury is well documented in the description of A-beta fiber terminal rearrangement in the dorsal horn to project to superficial laminae, which normally receive principally nociceptive information and are devoid of large fiber input (75). The expression of SP in large myelinated A-beta fiber leading to the notion that stimulation of low-threshold A-beta fiber may lead to SP mediated tactile allodynia (108).

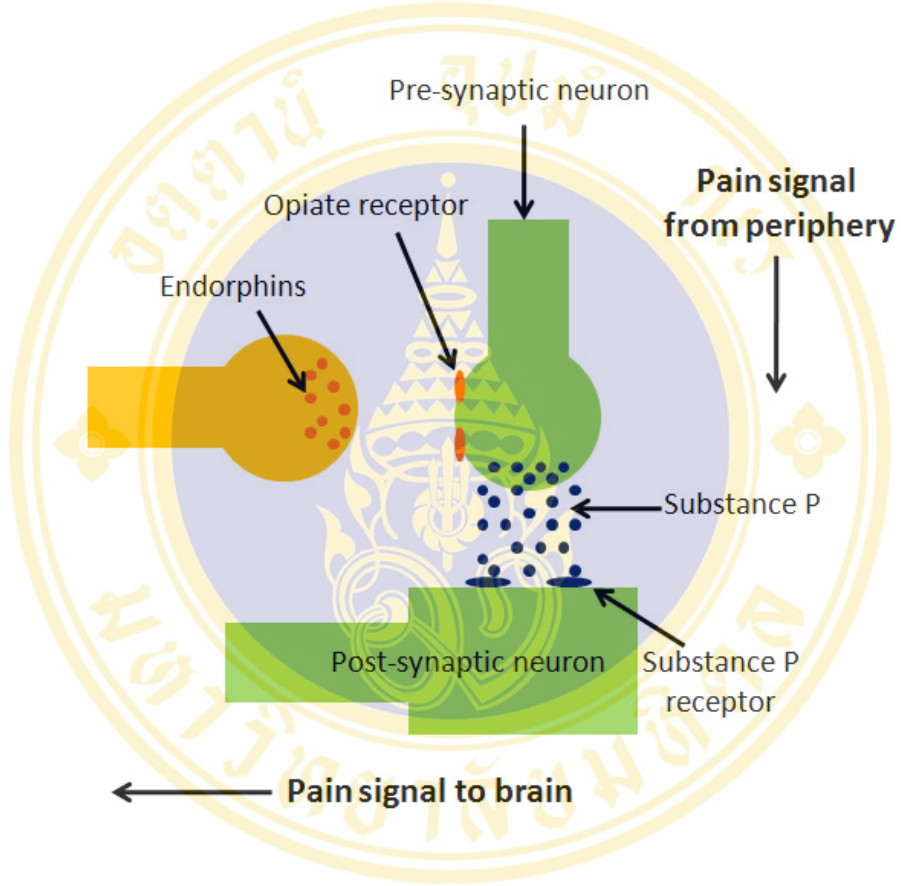


Figure 3.9 Substance P travels across the synapse between neurons to transmit a pain signal.

Animal models of nerve injury

Neuropathic pain is caused by peripheral nerve injury (table 3.2). Peripheral nerve injuries cause sensory loss which usually improves as regeneration occurs, but the protective function of the nociceptive system can become disordered as a consequence of injury. This can present clinically as the phenomena of hyperalgesia and allodynia that called neuropathic pain (109). At present, the mechanisms of neuropathic pain are still incomplete understood and treatment is often unsatisfactory. In order to evaluate the mechanisms of neuropathic pain and to identify novel approaches to therapy, different animal models for neuropathic pain have been intensively studied for years (110). Unlike inflammatory pain, human volunteer models of neuropathic pain have not yet to be developed. Also, as most patients do not developed neuropathic pain following nerve injuries. Therefore, animal models have been developed to result in highly reproducible and frequent development of allodynia and hyperalgesia (55). The advantage of these models is that allow analysis of pain-related behavior by determination of mechanical and thermal withdrawal thresholds because of the partial nerve injury whereas after complete nerve transection only spontaneous pain and autotomy was observable (111). The majority of currently used neuropathic pain models share alterations in hind limbs cutaneous sensory thresholds following injury of a peripheral, usually sciatic, nerve as a common stimuli (figure 3.10) and allodynia to cold and mechanical stimuli are used as outcome measures.

The sciatic nerve transection (SNT) consists of the section of the sciatic nerve and encapsulation of its cut end in a polythene tube. The animal have anaesthesized affected limb and autotomy, the animal to attack the anaesthetic limb, in the result (112).

The chronic constriction injury (CCI) is placement of loose constrictive ligatures around the common sciatic nerve at mid-thigh level in rat brought about behavior that appeared analogous to human neuropathic pain conditions. An immune response to the sutures leads to nerve swelling and nerve constriction. The rats engaged in protective behavior and had lowered thresholds to heat, cold, mechanical stimuli, and possibly spontaneous pain (or dysesthesia) (17). The association of this

behavior is an inflammatory reaction develops in response to the consequentially a loss of most A- and some C-fibers. It has been demonstrated that anti-inflammatory treatments of CCI rats decreases the associated thermal hyperalgesia (113) and so it is speculated that there is a significant inflammatory component in the development of the painful neuropathy. There is a degree of operator variability with this model, particularly in relation to the difference in the tightness of the ligatures.

The partial sciatic nerve ligation (PNL) also consists of injury to the sciatic nerve at mid-thigh level. In this model, a tight ligation is created around 33-50% of the sciatic nerve, leaving the rest of the nerve uninjured (19). This is associated with the development of spontaneous pain-like behavior, allodynia and hyperalgesia. Although this model is regarded as having less of an inflammatory component than the CCI model, there is still likely to be variability in the actual number of ligated neurons per animal.

The spinal nerve ligation (SNL) Chung and colleagues devised a now frequently used model in rat whereby one or more spinal nerves that innervates the foot is cut. It consists of injury to the L5, L6 and spared L4 spinal nerve which contribute to the sciatic nerve (18). Once again, this is associated with the development of spontaneous pain-like behavior as well as long-lasting hyperalgesia to noxious heat (at least 5 weeks) and mechanical allodynia (at least 10 weeks) of the affected foot.

The spared nerve injury (SNI) is produced by complete transection of common peroneal and tibial distal branches of the sciatic nerve, leaving the sural branch intact (20) results in early (<24 h), prolonged (>6 months), robust (all animals are responders) behavioral modifications. The mechanical (von Frey and pinprick) sensitivity and thermal (hot and cold) responsiveness is increased in the ipsilateral sural and to a lesser extent saphenous territories, without any change in heat thermal thresholds. Another SNI model, spared the tibial branch of the sciatic nerve, cut the common peroneal and sural branches (114) produced robust mechanical allodynia while leaving heat sensibility intact.

Spared nerve injury develops a marked hypersensitivity to innocuous mechanical von Frey hair stimulation of the hindpaw (sural nerve skin area) shortly after the surgery. This is present in every animal (20).

In figure 3.11, the different zones of the dorsal and plantar surfaces of the rat hind paw innervated by the sciatic terminal branches and the saphenous nerve are shown. Therefore, the lateral side of the dorsal and plantar surface which innervated by the sural branch (figure 3.12) is the zone for applying von Frey filament testing (sparing sural branch will be used in this study).

The spared nerve injury model differ from the spinal segmental nerve model (18), the chronic constriction injury model (17) and the partial sciatic nerve injury model (19) in that the effect of distal intact axons with degenerating axons is restricted, and it permits behavioral testing of the non-injured skin territories adjacent to the denervated areas. The mechanical (von Frey and pin prick) sensitivity and thermal (hot and cold) responsiveness is increased in the ipsilateral sural territories (20).

Although these models of CCI, PSL, SNL and SNI have all been extensively used to study a broad range of behavior, pharmacological response and induction of alterations in the somatosensory system and will continue to be important tools. Interestingly, the degree of damage in these models is inherently difficult to reproducible leading to some variability in the number of responders and their behaviors.

Table 3.2 Reviews of nerve injury

Type of injury	Site of injury	Pain behavioral response	Reference
Sciatic nerve transection (SNT)	Complete transection of a sciatic nerve	Pain referred to area in the absence of any sensory input	Wall et al.,1979 (112)
Chronic constriction injury (CCI)	Loosely constrictive ligatures around the sciatic nerve	Hyperalgesia, allodynia and, possibly, spontaneous pain	Bennett and Xie, 1988 (17)
Partial sciatic nerve ligation (PNL)	Unilaterally ligated of the sciatic nerve	The plantar surface of affected foot was hyperesthetic to non-noxious and noxious stimuli	Seltzer et al., 1990 (19)
Spinal nerve ligation (SNL)	Tight ligation of two spinal segmental nerves, L5 and L6, close to the dorsal root ganglion, leaving the L4 intact	Long-lasting hyperalgesia to noxious heat, mechanical allodynia and spontaneous pain in affected foot	Kim and Chung, 1992 (18)
Spared nerve injury (spared sural branch)	Axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact	Increase the mechanical sensitivity and thermal responsiveness in ipsilateral sural territories	Decosterd and Woolf, 2000 (20)
Spared nerve injury (spared tibial branch)	Axotomy and ligation of the common peroneal and sural nerves leaving the tibial nerve intact	Robust mechanical allodynia while leaving heat sensibility intact	Shannon et al., 2003 (114)

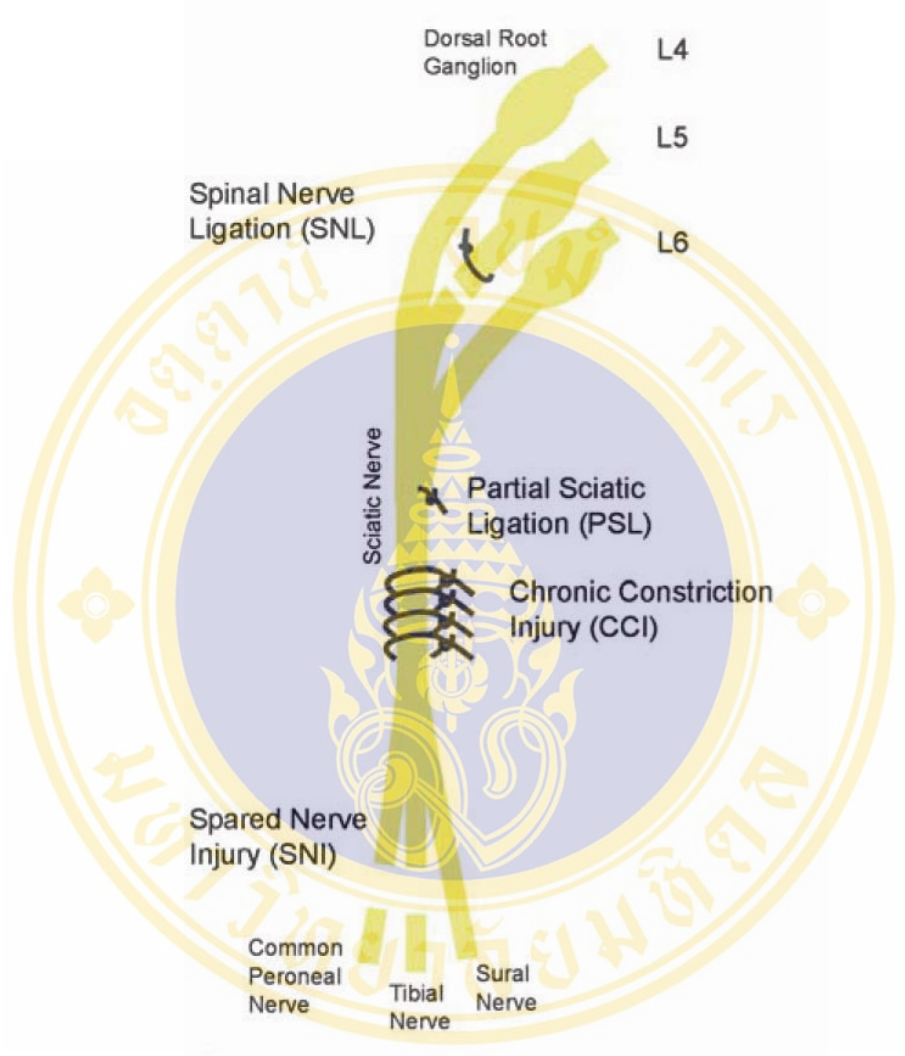


Figure 3.10 Four different nerve injury models are shown. The chronic constriction injury (CCI) model involves placement of four loose chromic-gut ligatures on the sciatic nerve (17). In the partial sciatic ligation (PSL) model, a portion of the sciatic nerve is tightly ligated (19). In the spinal nerve ligation (SNL) model, one or more spinal nerves going to the foot are ligated and cut (18). In the spared nerve injury (SNI) model, the common peroneal and tibial nerves are cut, sparing the sural nerve (20). In each model, only a portion of the afferents going to the foot is lesioned (54).

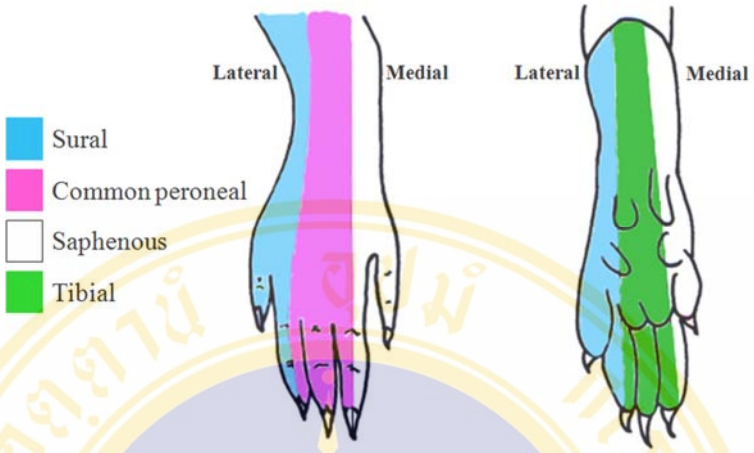


Figure 3.11 Different zones of the dorsal and plantar surfaces of the rat paw innervated by the sciatic terminal branches and the saphenous nerve.

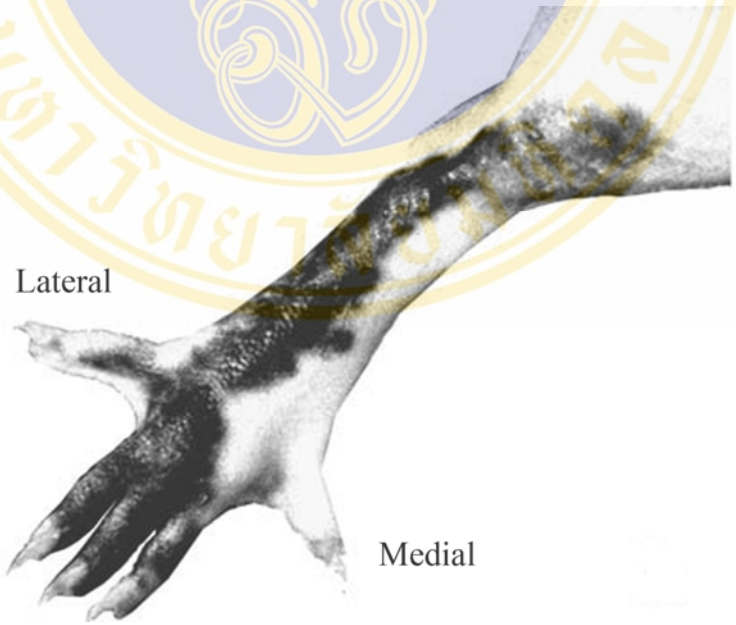


Figure 3.12 Noxious stimulation of the cutaneous nerves reveals the innervated territories as indicated by Evans blue. Each of the colored area following electrical stimulation of the sural nerve (115).

Electrical stimulation

The use of electrical stimulation for the relief of chronic pain (table 3.3) is an age-old technique, for example, the use of electrical shocks for the relief of chronic pain when suffering from severe headache the patient was recommended 'electrotherapy' using electric eels (116). Electrical stimulation has been extensively used by applying directly into spinal cord, peripheral nerve (117) in an effort to improve the management of acute and chronic pain.

Spinal cord stimulation (SCS)

The SCS in a well-established method for managing a variety of chronic neuropathic pains and the use of an implantable device for direct spinal cord stimulation (118). The proposed mechanism of SCS induced analgesia is based on Melzack and Wall's gate control theory described to recruitment of large diameter A-beta fibers in the dorsal column and preventing the transmission of pain impulse via small diameter C-fibers (119). The antidromic conduction and levels of endogenous analgesic substances and other amino acid, e.g. substance P, within the central nervous system also have been studied and found after using SCS treatment. Although, spinal cord stimulation is successful in relieving inoperable and intractable neuropathic pain (120), they have main complication related to infection, bleeding, equipment failure and the development of tolerance. Therefore, other less invasive electro-analgesic modalities have been increasingly wide-used for a variety treatment of neuropathic pain instead of invasive and expensive analgesic intervention with potentially serious side effects demonstrated in SCS (120).

Transcutaneous electrical nerve stimulation (TENS)

The TENS involves the transmission of electrical energy from an external stimulator to the peripheral nervous system via cutaneously placed conductive gel pads (120). TENS is a non-drug therapy clinically used to relieve acute and chronic pain (121). TENS can be subclassified as high frequency (>50 Hz) and low frequency (<10 Hz) (122) and is used for pain relief patient populations, including post-operative pain, acute and chronic pain, musculoskeletal and neuropathic pain conditions (123). The proposed mechanism of TENS are thought to be involved both spinal; gate

control, frequency-dependent blockage (124) and supraspinal theories (i.e. release of endogenous neuromediators) (120, 123). From a systemic review has demonstrated that TENS provided moderate analgesia but contradictory evidence whether or not they would benefit patients with chronic pain. Moreover, several studies showed no significant difference between placebo TENS and TENS for pain relief in-patients with acute and chronic low back pain, temporomandibular joint disorders, or arthritis (125). By contrast, animal model of neuropathic pain showed that TENS provided pain relief via a depressive action on the enhanced mechanical responsiveness of the spinal neurons in this model (126). Also, the literature of randomized placebo-controlled trials indicated that TENS could significantly reduce analgesic consumption for postoperative pain. Therefore, the current data are still conflicting and limited but contradictory evidence that TENS is beneficial in the management of chronic pain in the short-term to long-term (124).

Electroacupuncture

There are numerous electrostimulation units available today recommended for electrocupuncture and TENS. Acupuncture, part of Traditional Chinese Medicine (TCM), has been widely practiced and used in the treatment of pain for centuries. Eventually, acupuncture has been further studied and increasing acceptance for pain relief in Western medicine. Their approach emphasized using acupuncture points on Western understanding of myofascial trigger points, the nervous system, or recent scientific discoveries about the likely mechanism of acupuncture (127). The stimulus, acupuncture points is believed to be effective only at certain points. However, comparison with anatomical atlas showed that many of these points correspond with small nerve bundles either cutaneous, vascular and muscular or motor points (128).

Electroacupuncture was developed methods of acupuncture since it has been found to be more effective to stimulate the needles electrically at acupuncture points from a pulse generator. Electroacupuncture units are designed to deliver either variable amplitudes or frequencies, or various trains of pulse at different frequencies and intensities, high intensity treatment currents appear superior to TENS because it produces prolonged analgesia and has very few side effects (129). More recently, current data-analysis of randomized-controlled acupuncture trials provided some

evidences for the efficacy of acupuncture in chronic low back pain, has found a significant improvement by traditional acupuncture in chronic low back pain compared to routine care at the end of treatment (130).

Intramuscular stimulation (IMS)

Recently, a new treatment for neuropathic pain called intramuscular stimulation (IMS) (11). Based on the fact that neuropathic pain is a supersensitivity phenomenon, and its treatment requires desensitization. Lomo has shown that in animal experiments that supersensitivity and other features of denervated muscle can be reversed by electric stimulation (131). IMS uses some of the tools of acupuncture, but it differs fundamentally in that it is based on current Western research in physiology. IMS is applied directly to the site of the pain called trigger point, rather than to remote points based on maps of energy flow. Acupuncture in often useful to neutralize these trigger points, as well as medical procedures such as local anesthesia and corticosteroids (132).

Neuropathic pains occur following neuropathy responds well to IMS treatments by desensitizing the affected nerve and muscles. Furthermore, there is a based on neuro-anatomical principle. This clinical model suggested strong analgesic effect of needling by application on damaged tissue or points anywhere that share innervations via the same spinal segment as the injured tissue (16).

Unlike external forms of stimulation, stimulation from a needle lasts for several days until the miniature wounds heal. Needling has another unique benefit unavailable to other forms of local therapy (133).

Electrical twitch obtaining intramuscular stimulation (ETOIMS)

The basis of pain relief after electromyography (EMG) is that intramuscular needle movements lead to insertional activity and microtwitches (14). Occasionally, acupuncture needle penetration or manipulation in classical or electrical acupuncture, intramuscular stimulation, and trigger-point localization also evoke small local twitches. ETOIMS applies electricity using; pulse duration of 0.5 msecs, frequency of 2 Hz, and a current strength of 2 mA; through a monopolar EMG needle electrode to deep motor end-plate zones (MEPZs) for stimulated to twitch (13). These

microtwitches can cause immediate muscle fiber contraction and then relaxation, which may be the basis of pain relief with needling tender MEPZs (14, 134). Muscle contractions improve skin and muscle circulation. Electrical stimulation–induced contractions improve circulation of the lower leg by the physiologic pumping action of muscle, reducing venous stasis/pooling and edema (135). Immediately after muscle contraction, muscle microvessels exhibit increased convective (flow of red blood cells) and diffusive (perfused capillary surface area) transport (136). The use of low-frequency transcutaneous nerve stimulation (2 Hz), producing moderate muscle contractions, leads to a transient, local increase in blood flow in muscle and skin (137, 138). A recent report with blood flow measurements in the common femoral artery showed that surface-twitch contractions at 3 Hz increase perfusion in human leg muscles (139). Therefore, ETOIMS-mediated muscle contractions may produce pain relief through the following mechanisms of action: spinal cord reflex closure of the pain gate, intramuscular exercise, and enhanced tissue perfusion (13).

Table 3.3 Review of electrical stimulation treatment

Type of stimulation	Manifestation	Activation	Reference
Spinal cord stimulation (SCS)	Stimulus parameters similar to the used on clinical practice	Activate dorsal column pathway	Meyerson et al., 1995 (118)
Acupuncture-like transcutaneous electrical nerve stimulation	Low frequency (2 Hz), high intensity (4-5 mA)	Activate endogenous opiate mechanism	Leem et al., 1995 (126)
Lo-TENS	Low frequency (2 Hz), high intensity (4-5 mA)	Activate endogenous opiate mechanism	Nam et al., 2001(140)
Hi-TENS	High frequency (100 Hz), continuous mode of 200 microsecond	Activate large diameter afferent fiber in mechanism of Gate control theory	Radihakrishnan and Sluka, 2005 (122)
Electrical Twitch Obtaining Intramuscular Stimulation (ETOIMS)	Intramuscular stimulation by low frequency (2 Hz), 2 mA and 0.5 msec	Activate local muscle twitches, desensitize and relax muscle fibers, and local increase in blood flow in muscle and skin	Chu et al., 2004 (13)

Denervation supersensitivity

The function and integrity of all innervated structures are dependent on the flow of nerve impulses in the intact nerve to provide a regulatory or trophic effect. When this flow (probably a combination of axoplasmic flow and electrical input) is blocked, innervated structures are deprived of the trophic factor, which is necessary for the control and maintenance of cellular function (141).

When a unit is destroyed in a series of efferent neurons, an increased irritability to chemical agents develops in the isolated structure, the effect being maximal in the part directly denervated. All denervated structures develop supersensitivity, but it is now know that physical interruption and total denervation are not necessary any circumstance that impedes the flow of motor impulses for a period of time can rob the effector organ of its excitatory input and cause disuse supersensitivity in that organ and in associate spinal reflexes (142).

After the nerve in the mammalian muscle fiber is cut, the muscle becomes supersensitive to a variety of chemicals. In example, the concentration of acetylcholine (ACh) required to produce depolarization, or shortening of muscle, is reduced by a factor of several hundred to a thousand. The increase in chemosensitivity is not restricted to the physiological transmitter-Ach but occurs for a wide variety of chemical substances and even makes the muscle more sensitive to stretch and pressure (143). Other changes occur in denervated muscle, such as a gradual atrophy of wasting of muscle fibers (144).

The role of muscle activity itself as an important factor in controlling supersensitivity was further shown in other experiment in which supersensitive denervated muscles in the rat were stimulated directly through electrodes permanently implanted around the muscle. Repetitive direct stimulation of muscles over several days caused the sensitive area to become restricted, so that once again only the synaptic region was sensitive to Ach (131). The frequency of stimulation and the length of the quiescent intervals were important variables in the development or reversal of supersensitivity. This explains why denervated mammalian muscle fibers develop supersensitivity in spite of the ongoing contractions associated with fibrillation. Sampling the activity of individual fibers showed that fibrillation is cyclical, periods of activity alternating with inactivity. The level of spontaneous

activity is, however, below that required to reverse the effects of denervation on the distribution of Ach receptors (145). Like the development of supersensitivity, the turnover of junctional receptors is regulated by muscle activity (146). Direct electrical stimulation of denervated muscle also restores normal stability to Ach receptors at synaptic sites.

When part of their synaptic input is destroyed, neurons, like muscles, undergo changes. The skeletal muscle fibers, innervated neurons are highly sensitive to the transmitter Ach at selected spots on their surfaces, immediately under the presynaptic terminals. The restriction of receptors to synaptic site in these neurons is not as complete as in skeletal muscle, however approximately 20% of the receptors may be extrasynaptic (147). In addition, the mechanisms that regulate Ach receptor distribution appear to be different in nerve and muscle cells, and to vary among neuronal cell types as well.

The importance of disuse supersensitivity cannot be overemphasized. When a nerve malfunction, the structures it supplied become supersensitive and will behave abnormally. These structures over-react with many forms of input, not only chemical, but physical as well, including stretch and pressure. Supersensitive muscle cells can generate spontaneous electrical impulses that trigger false pain signals of provoke involuntary muscle activity and supersensitive nerve fibers can become receptive to chemical transmitters at every point along their length instead of at their terminals only. Sprouting may occur, and denervated nerves are prone to accept contacts from other types of nerves including autonomic and sensory nerve fibers.

Not only are denervated muscles amenable to innervations, but they actively induce undamaged nerves to spout new terminal branches. For example, if a muscle is partially denervated, the remaining axon terminals will sprout and innervate the denervated fibers (148). As with regulation of Ach receptors synthesis and degradation, muscle inactivity appears to play a key role in this inductive process. Sprouting and hyperinnervation occur if muscle activity is prevents by blocking action potential propagation in the nerve (149). Denervation-induced sprout has also been observed for sensory axons innervating skin and spinal cord, for preganglionic axons in autonomic ganglia, and for several axonal projections in the brain (145).

In fact, disuse supersensitivity is basic and universal, yet not at all well known or credited. The important role of supersensitive structures following neuropathy of denervation has been, until recently neglected. The diverse pain syndrome of apparently unknown causation can be attributed to development of hypersensitive receptor organs and supersensitivity in pain sensory pathway (132).

CHAPTER IV
MATERIALS AND METHODS

All experiments (figure 4.1) were performed on adult Sprague Dawley male rats (average weight 250-300 g). During the experimentation period, the rats were housed in individual cages with free access to food and water and at a controlled room temperature (22±2°C) with a twelve-h light/dark cycle. All procedures complied with the ethical guidelines for pain experimentation on awake animals (150) and were approved by the Ethical Committee on Animal Experiments of Mahidol University.

Experimental design and groups

This study was based on results obtained from 52 rats randomly distributed into four groups (n= 13 each). Two groups underwent spared nerve injury (SNI) and SNI with the electrical stimulation treatment (SNI-ES). The other two groups were subjected to sham operation (sham) and sham with the electrical stimulation treatment (sham-ES).

sham-operation:	After anesthesia, the sciatic nerve and its branches were exposed without lesion.
sham-ES:	After anesthesia, the sciatic nerve and its branches were exposed without lesion and given ES of 2 Hz.
SNI:	The right tibial and common peroneal nerves were transected and spared the sural nerve intact without ES treatment.
SNI-ES:	The right tibial and common peroneal nerves were transected and spared the sural nerve intact and given ES of 2 Hz.

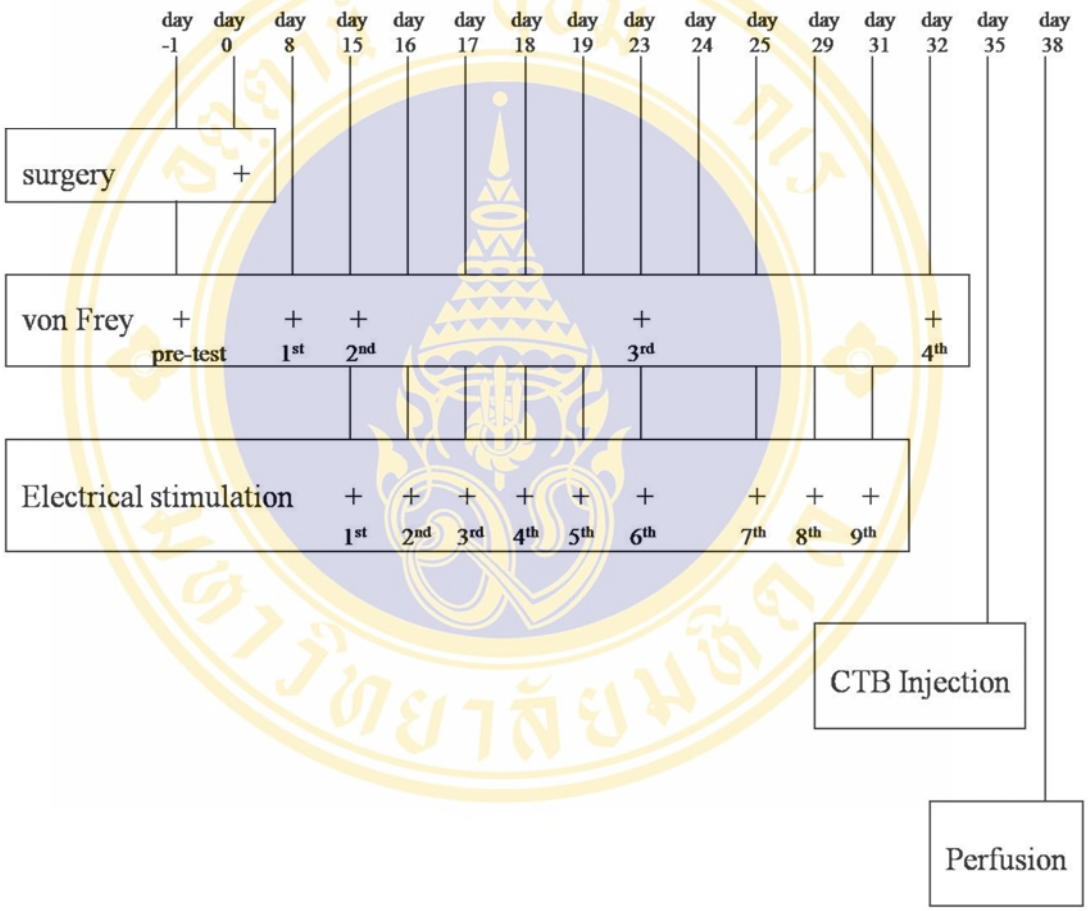


Figure 4.1 Experimental designs.

Nerve injury surgical procedure

Peripheral neuropathy following Decosterd and Woolf model (20) was induced in the SNI and SNI-ES groups. This model was selected for the behavioral test, since the SNI procedure comprised an axotomy and ligation of two of three terminal distal branches of the sciatic nerve and almost all animals in this model showed early-onset and prolonged mechanical hypersensitivity (20). Briefly, under pentobarbital sodium anesthesia (40 mg/kg, intraperitoneally), the skin on the lateral surface of the right thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves (figure 4.2). The common peroneal and the tibial nerves were transected close to the position that nerves enter the muscles and leaving the sural branch intact. Great care was taken to avoid any contact with or stretching of the intact sural nerve. Muscle and skin were closed with 3-0 silk in two layers. A single dose of antibiotics was administered (Ampicillin 8000 u/rat, Sigma, St Louis, MO). Each animal was allowed to recover for 24 h before further testing. In sham controls the right sciatic nerve and its branches were identically exposed without any lesion. In all groups, the contralateral thigh remained unoperated.

Electrical stimulation treatment

After 14 days of surgery, sham-ES and SNI-ES rats were placed loosely in a plastic holder, with their hindlimbs protruding. On the ipsilateral side to surgery, the electrical stimulation treatment by two pairs of electrode was applied to the anterior tibial muscle and the gastrocnemius muscle (151, 152). Briefly, each pair of electrode composed of two stainless-steel needles of 0.25 mm in diameter and 5 mm in length were inserted to a depth of 5 mm, one needle at the motor point of each muscle about 10 mm below the knee joint and another one below that point on fleshy muscle fiber close to the insertion of that muscle. Then, an electrical impulse generator (figure 4.3) was attached to the needles, the cathode was connected on the first needle, and the anode was connected to another one. The low frequency (2 Hz) with pulse duration of 0.3 ms pulse width and a current strength of 0.2-0.3 mA were applied to the inserted needle for 30 minutes daily for five consecutive days in the first week, followed by

twice a week for another 2 consecutive weeks (figures 4.4, 4.5). For the control, needles were inserted at the same location without electrical stimulation.

Behavioral pain test: Mechanical allodynia

The animals were tested for behavioral responsiveness only after a period of at least one week of habituation to the testing environment and observer. To avoid the effect of the circadian cycle, all behavioral assessments were performed during the same period (7:30-11:00 AM). The experimenter was blinded to the group of the animals. As a routine protocol, the animals received behavioral testing without anesthesia. The baseline measurements for each tested parameter were established on one separate day preceded the surgery. The animals were then tested on the days 8, 15, 23 and 32 after the surgery for an average period of 5 weeks to establish the level of mechanical allodynia. All animals were placed in a small plastic cage (on 11×13×24 cm) with an open wire mesh bottom 1 h before starting the behavioral test (figure 4.7). So that their grooming and exploratory behaviors cease and all four paws were placed on the bottom. Mechanical allodynia was assayed by using a series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) (figure 4.6) (153). The hairless plantar surface of the hind paw was probed by a set of 18 filaments, marked from 1.65 to 6.5. The respective bending forces were in the range of 0.005 to 125.892 g (154). All tests were performed on the lateral plantar surface of the right hind paw. The von Frey filaments were applied perpendicularly to the planter surface of the paw with an upward force just sufficient to bend the microfilament for 1-2 s (figure 4.8). In the absence of response (indicated by paw withdrawal), the filament of next greater force was used. In the presence of response, the filament of next lower force was used. Each von Frey hair was used five times on each hind paw at 30 s intervals. The response threshold was defined as the lowest force that caused at least one paw withdrawals out of the five consecutive applications (155). Special care was taken to stimulate the lateral plantar surface, which is the area of the skin innervated by the sural nerve. In sham operated animals, larger diameter filaments (more than 18 g) could not be used because they caused the paw to be lifted by the filament.

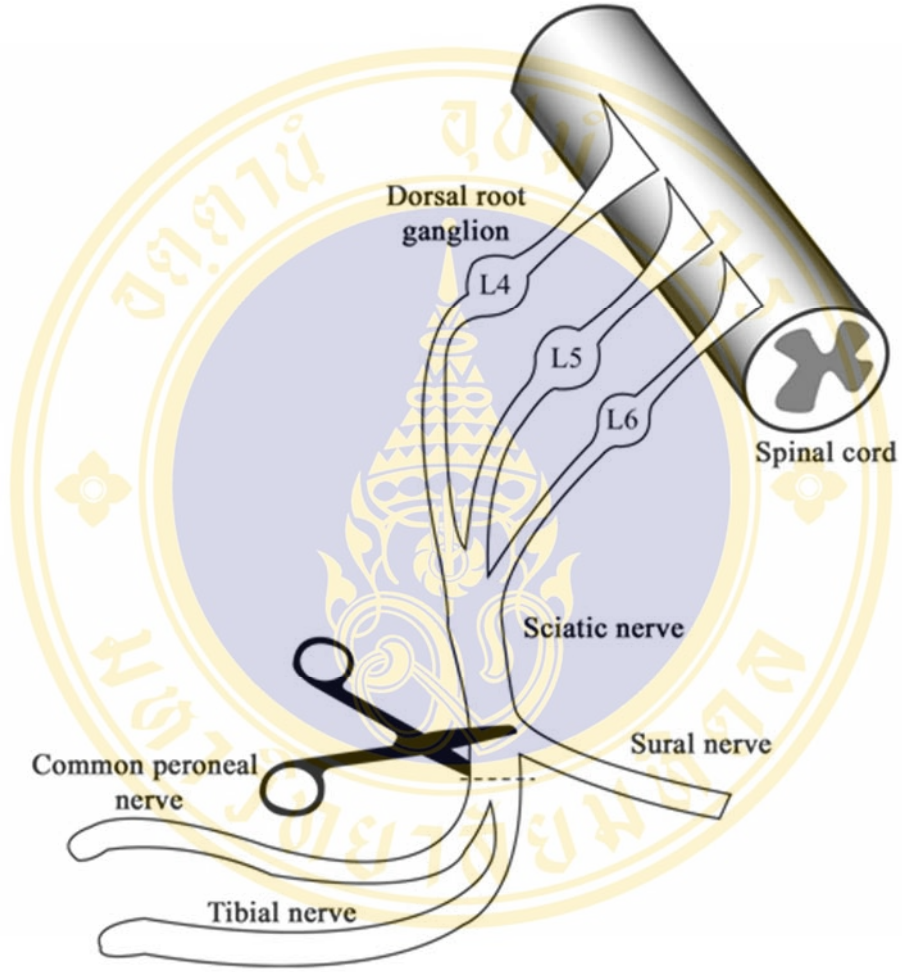


Figure 4.2 The sciatic nerve with three terminal branches: tibial, common peroneal and sural. The surgery was exposed the three terminal branches then cut the tibial and common peroneal nerve and spared the sural nerve intact.

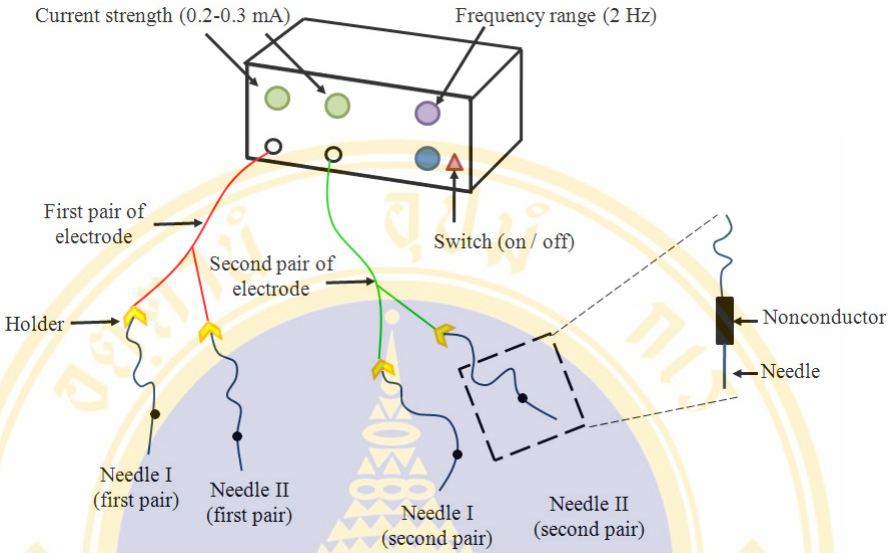


Figure 4.3 The electrical impulse generator.

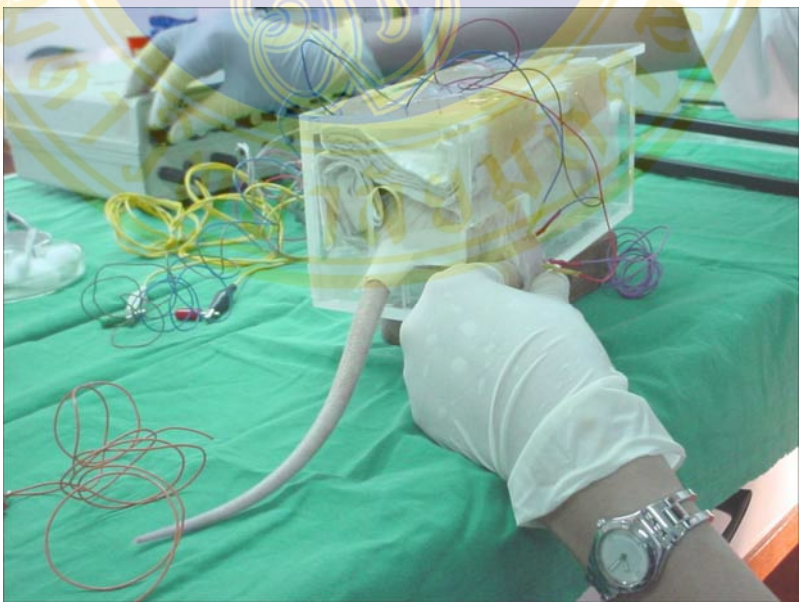


Figure 4.4 The electrical stimulation treatment with low frequency (2 Hz).

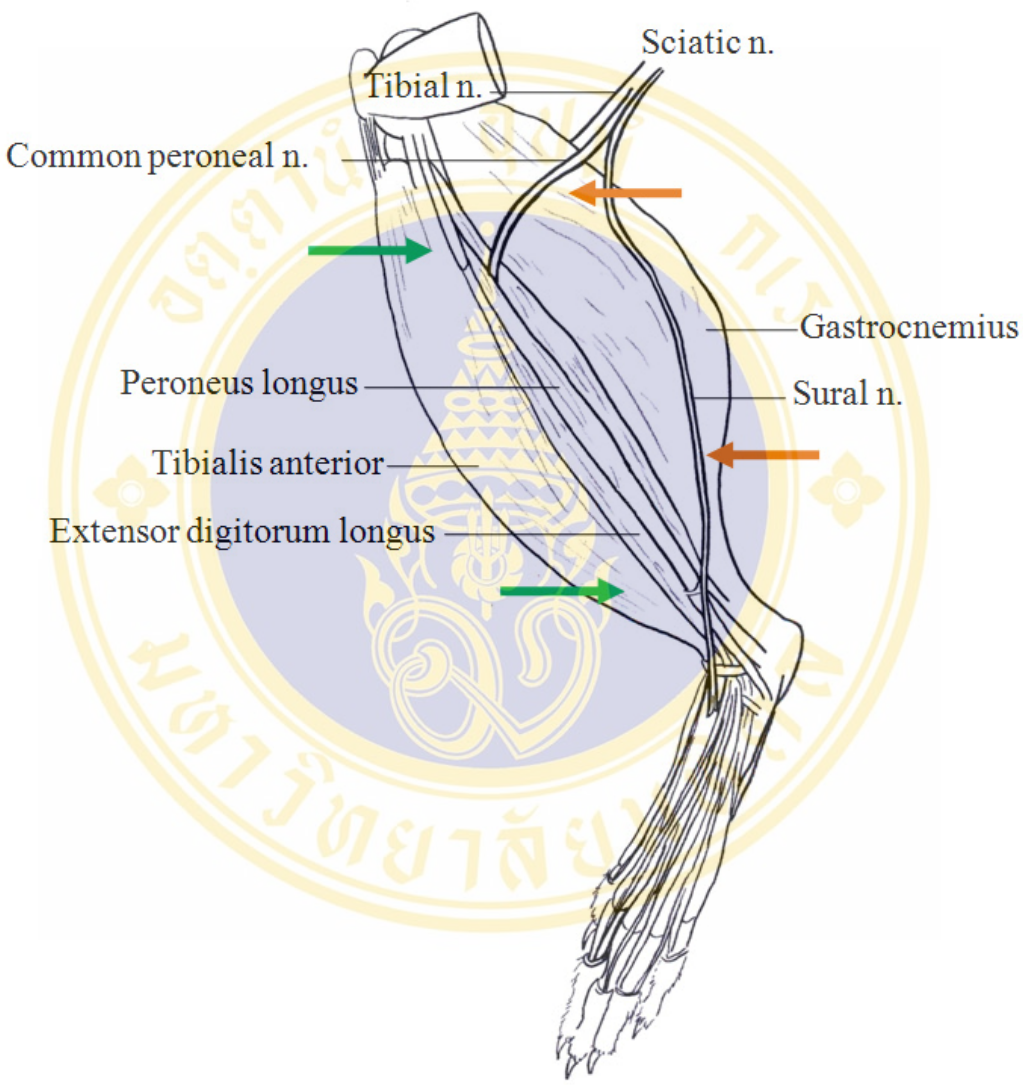


Figure 4.5 The two pairs of electrode, each pair composed of one needle at the motor point of each muscle about 10 mm below the knee joint and another one below that point on fleshy muscle fiber close to the insertion of that muscle.

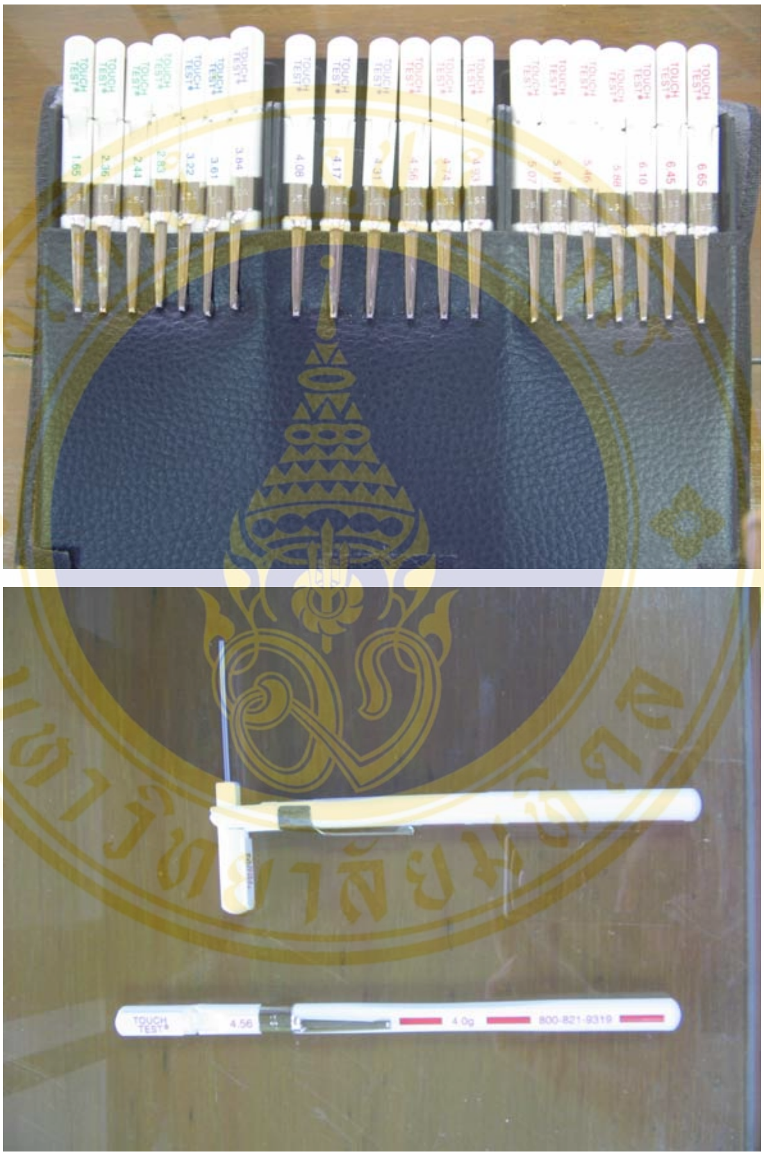


Figure 4.6 The touch-test sensory evaluators: von Frey filaments.

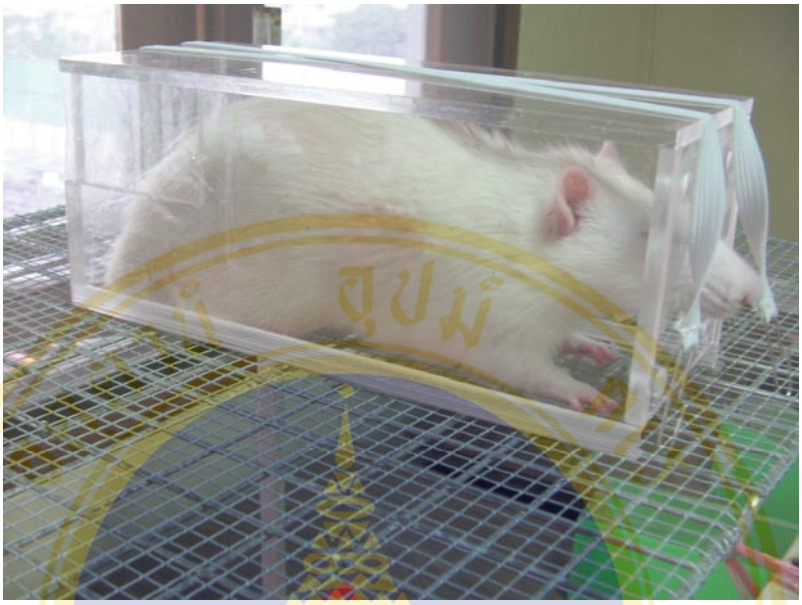


Figure 4.7 Rat was placed in plastic cage with an open wire mesh bottom 1 h before starting the behavioral test.

Figure 4.8 A series of touch-test sensory were applied perpendicularly to the lateral side of plantar surface of right hind paw with sufficient forced to bend the filament for 2-3 sec.

Tracing of afferent projections to the dorsal horn

Cholera toxin B-subunit (CTB) have been used to map the central projections of cutaneous myelinated primary afferents in the spinal dorsal horn of rats (156). At the end of behavioral testing (32 days after surgery), all animals were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). Right sciatic nerves were reexposed and injected proximal to the injury site with small volumes (2.5–3 microlitres) of CTB (sigma, 1% dissolved in distilled water) by using Hamilton microsyringes. Three days later at Day 35, the animals were terminally anesthetized, perfused and prepared for CTB immunofluorescent staining as described below.

Tissue preparation and immunofluorescent staining

SP and CTB were determined in SNI and sham rats with or without electrical stimulation treatment by immunofluorescent histochemistry. All animals were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) at 35 days after surgery and perfused transcardially with 100 ml saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) at pH 7.4. The L4-L6 spinal cord and DRG (figure 4.9) were removed, placed in the same fixative overnight at 4°C, and transferred to 30% sucrose (w/v) in 0.1 M PBS for three days for cryoprotection. Serial coronal section of spinal cord and DRG at a thickness of 40 micrometers and 20 micrometers, respectively were prepared in a Leitz cryostat and mounted on slides. The sections were treated with phosphate-buffered saline (PBS, 0.1M, pH 7.2) containing 1% Bovine serum albumin (BSA) and 0.05% Tween-20 for 30 min at room temperature. The primary antiserum, goat polyclonal antiserum directed against SP (1:50; Santa Crus Biotechnology) and rabbit anti-cholera toxin B (1:1000, Sigma) in 0.1M PBS containing 1% BSA and 0.05% Tween-20 was used for the incubation with the sections for overnight at 4°C. The sections were washed in 0.1 M PBS and then incubated in the secondary antiserum, FITC-conjugated donkey anti-goat IgG (1:500; Santa Crus Biotechnology) and 1.5% Texas Red anti-rabbit IgG (Vector laboratories) for 3 h at room temperature. After several rinses in 0.1 M PBS, sections were cover slipped with Vectashield (Vector) and observed under a Carl Zeiss laser confocal microscope (figure 4.10).

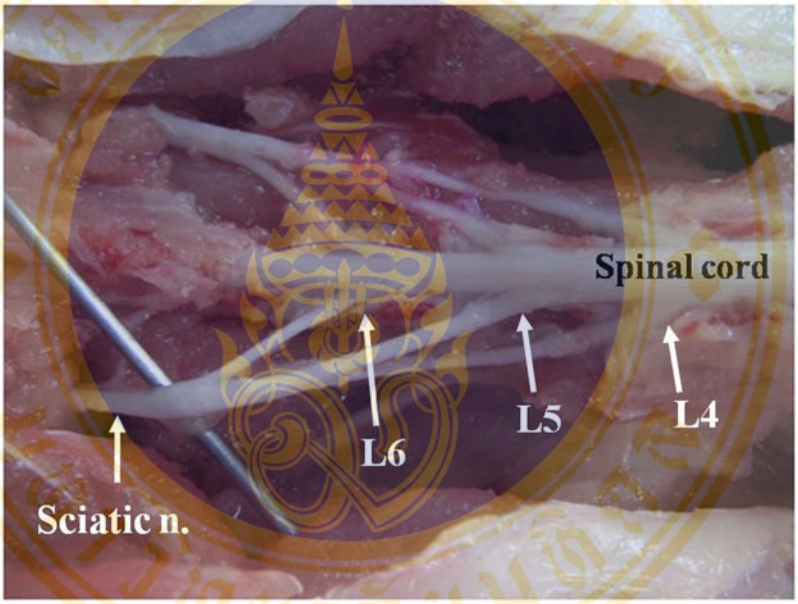


Figure 4.9 The L4-L6 level of dorsal root ganglia.

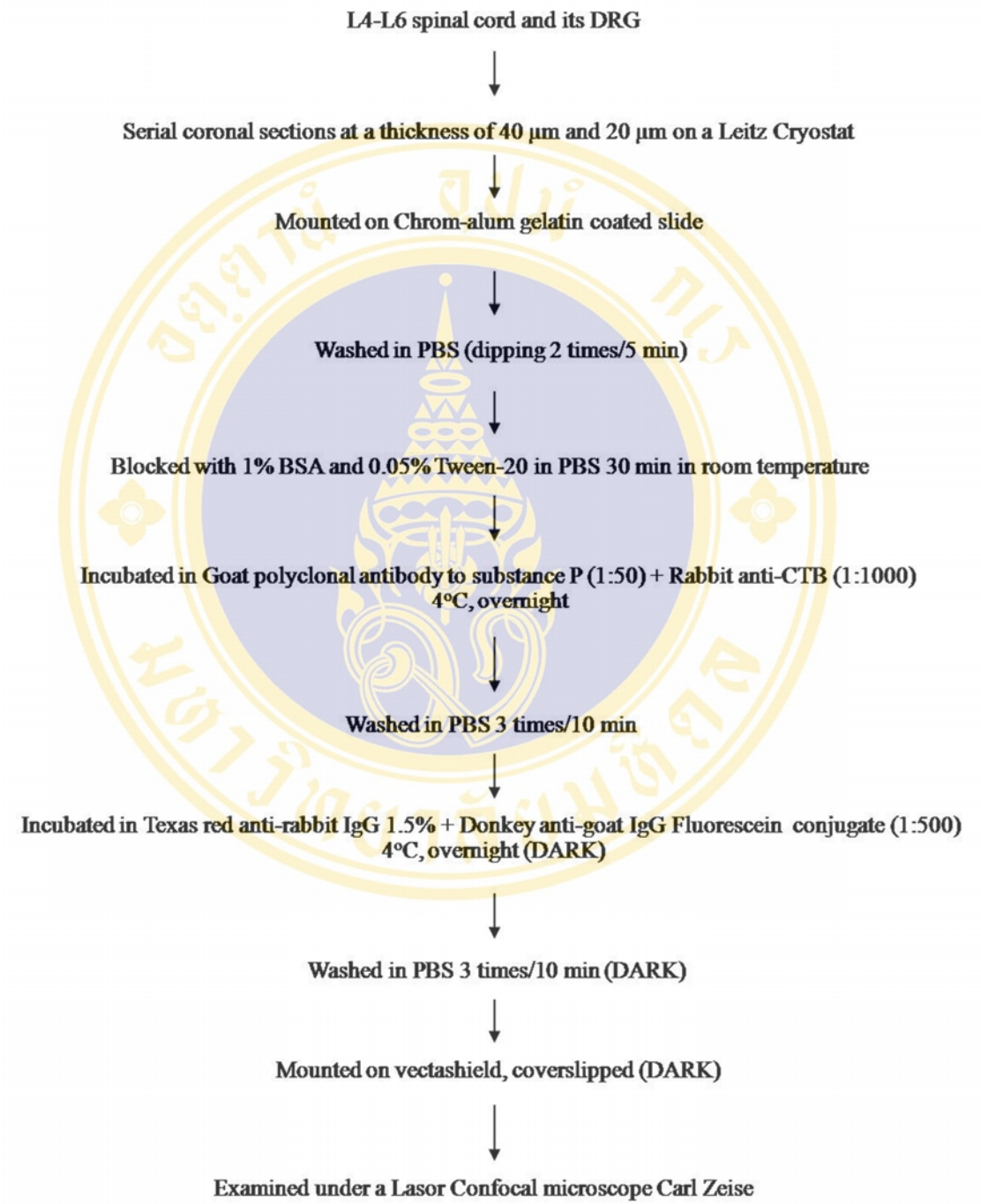


Figure 4.10 Immunofluorescence staining.

Image analysis

Image analysis of SP immunostaining was performed on five nonadjacent sections from each animal in the SNI, SNI-ES and sham groups. To determine changes in SP immunostaining in the spinal dorsal horn and DRG, the density of SP immunoreactivity (SPir) was quantified using a computer-based image analysis system (the free UTHSCSA Image Tool program, 3.00) (157). While viewing the monitor, upper and lower thresholds of grey level density were set such that only specific SP immunoreaction product was accurately discriminated from the background in the outlined laminae I–IV of the dorsal horn and DRG. The pixel-by-pixel was read by the computer (158). Subsequently, the total area of discriminated pixels was divided by the area of the outlined dorsal horn. Since the staining on the contralateral side was equivalent to that seen in unoperated animals, the percentage in the subdivisions within the dorsal horn was calculated as ipsilateral/ contralateral X 100. The values from the five random sections were averaged for each animal.

Data analysis

Animals were randomly assigned to each group. To determine the paw withdrawal thresholds response to von Frey filaments stimulation, the significance of difference among experimental groups were compared using one-way analysis of variance (ANOVA). The Bonferroni post hoc test was used to identify specific mean differences. The changes in SP staining in spinal dorsal horn and DRG were compared by using independent sample t-test if only two groups were applied. The data were represented as mean ± standard error mean (mean ± S.E.M.). Differences were considered significant if $P < 0.05$ and 0.001.

CHAPTER V
RESULTS

The main aims of the present study were, first, to demonstrate that the development of neuropathic pain was observed in SNI rat model and second, to investigate whether the low frequency (2Hz) electrical stimulation can modulate the mechanical allodynia following SNI. In this study, the result was divided into two main parts: the results of pain sensitivity assessment and immunofluorescent histochemistry. In the pain behavioral tests, the paw withdrawal threshold to non-nociceptive mechanical stimulation of the sural skin territory was evaluated to indicate the mechanical allodynia. In the immunofluorescent histochemistry, the SP immunoreactivity, and the CTB immunoreactivity was examined in the spinal dorsal horn and dorsal root ganglia.

I. Pain sensitivity assessment

1. General observations

After surgery, the majority of rats showed normal weight gain and normal level of general activity. Autotomy was never observed. All SNI rats developed signs indicative of a marked sensory hypersensitivity of the hindpaw ipsilateral to the nerve injury. The SNI procedure induced sensory-motor alterations due to the injury to the tibial and common peroneal nerves and the hamstring muscles. The SNI rats held the affected paw in a guarding behavior, no weight bearing on the affected side, using medial surface to walk. When the rats lifted the injured limb, it appeared that the movement was an action at the hip than at the knee or ankle.

2. The effects of SNI on pain behavioral signs of neuropathic pain

The SNI rats (n=13) developed a marked withdrawal response to innocuous mechanical von Frey filament stimulation of the lateral surface of hindpaw (sural nerve skin area), which was sign of mechanical allodynia. This was present in every animal and developed shortly after the surgery.

Before surgery, the sham and SNI groups had withdrawal threshold baseline (day-1) at 17.4±0.6 g and 17.8±0.2 g respectively (table 5.1).

After surgery, the sham group had no changed in withdrawal threshold, closed to baseline (day 8: 17.0±0.8 g, day 15: 17.0±0.8 g, day 23: 17.2±0.6 g, day 32: 17.2±0.6 g).

In SNI group, the withdrawal threshold was decreased extensively when tested at day 8, day 15, day 23 and day 32 (1.2±0.2 g, 2.4±0.6 g, 1.6±0.5 g and 1.6±0.4 g respectively). Spared sural nerve injury resulted in a significant decrease in mechanical threshold compared to sham-operated rat or baseline day-1, 7 days after surgery till the end of observation (figure 5.1, $p<0.001$). By contrast, no significant mechanical allodynia was in the sham (figure 5.1) or on the contralateral paw (not shown) at any time during 5 weeks of observation.

3. The effects of low frequency (2Hz) electrical stimulation on behavioral signs of mechanical allodynia following SNI

Before surgery, sham-ES and SNI-ES groups have withdrawal threshold baseline (day-1) at 15.9±0.9 g and 18.0±0.0 g respectively (table 5.2).

The sham-ES group has slightly decreased of withdrawal threshold after surgery (day 8: 12.9±1.8 g), when we treated the animals with electrical stimulation at 2nd, 3rd and 4th weeks of the experiment have resulted in increased of withdrawal threshold in day 15, 23 and 32 (15.0±1.2 g, 16.0±1.1 g and 16.9±0.9 g respectively).

The SNI-ES group has decreased of withdrawal threshold after surgery (day 8: 3.3±0.9 g, day 15: 2.9±0.8 g), when we treated the animals with electrical stimulation at 2nd, 3rd and 4th weeks of the experiment have resulted in increased of withdrawal threshold in day 23 and day 32 (4.6±0.8 g, 8.7±2.0 g respectively).

In figure 5.2, the withdrawal threshold of the SNI-ES rats at day 8, 15, 23 and 32 were significantly decreased when compared with the sham-ES group on the corresponding day (day 8: 3.3±0.9 g, day 15: 2.9±0.8 g, day 23: 4.6±0.8 g, day 32: 8.7±2.0 g vs. day 8: 12.9±1.8 g, day 15: 15.0±1.2 g, day 23: 16.0±1.1 g, day 32: 16.9±0.9 g respectively, $p<0.001$).

Table 5.1 Comparison of mean paw withdrawal threshold (g) of sham and SNI groups at day-1 (before surgery), day 8, 15, 23 and 32

Note: values are presented as mean ± SEM, n=13/group

* Statistically significant (at p-value < 0.001) compared to day-1

Day	Paw withdrawal threshold (grams)		p-value
	sham	SNI	
Day-1	17.4±0.6	17.8±0.2	0.770
Day 8	17.0±0.8	1.2±0.2*	0.000
Day 15	17.0±0.8	2.4±0.6*	0.000
Day 23	17.2±0.6	1.6±0.5*	0.000
Day 32	17.2±0.6	1.6±0.4*	0.000

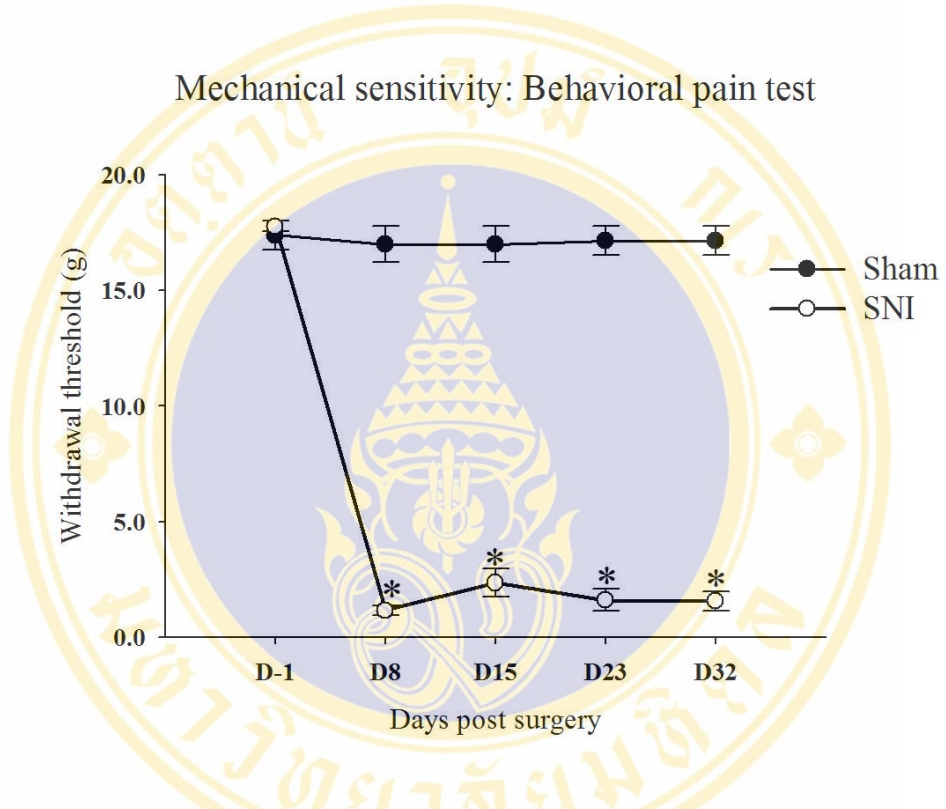


Figure 5.1 Response to mechanical stimulation with von Frey filaments in the sural skin territory for sham and SNI. Statistically significant difference was observed 7 days after surgery until day 32, * p-value < 0.001. Each point in each curve represents the mean ± SEM. Measurements of a neuropathic manifestation made at the indicated day in one group of rats (n=13). The values of significance of differences were measured with reference to the level measured at day-1 each curve.

Table 5.2 Comparison of mean paw withdrawal threshold (g) of sham-ES, SNI and SNI-ES groups at day-1 (before surgery), day 8, 15, 23 and 32

Note: values are presented as mean ± SEM, n=13/group

* Statistically significant (at p-value < 0.05) compared to day-1

**Statistically significant (at p-value < 0.001) compared to day-1

Day	Paw withdrawal threshold (grams)			p-value
	sham-ES	SNI	SNI-ES	
Day-1	15.9±0.9	17.8±0.2	18.0±0.0	0.861
Day 8	12.9±1.8	1.2±0.2	3.3±0.9	0.105
Day 15	15.0±1.2	2.4±0.6	2.9±0.8	0.648
Day 23	16.0±1.1	1.6±0.5	4.6±0.8*	0.026
Day 32	16.9±0.9	1.6±0.4	8.7±2.0**	0.000

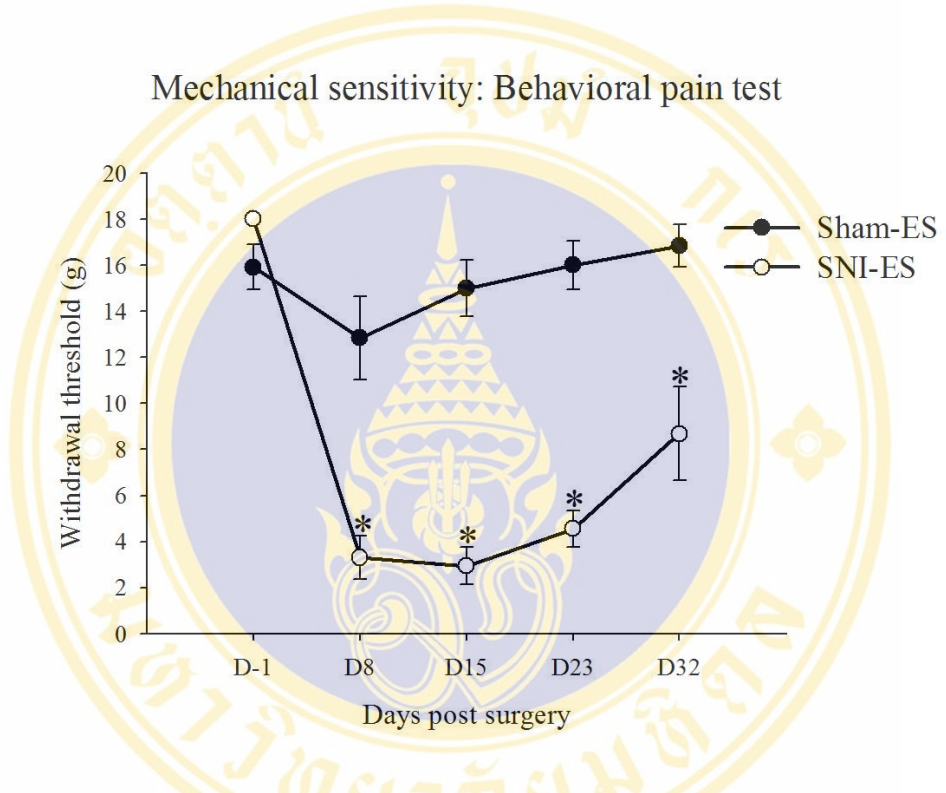


Figure 5.2 Response to mechanical stimulation with von Frey filaments in the sural skin territory for sham-ES and SNI-ES. Statistically significant difference was observed 7 days after surgery until day 32, * p-value < 0.001. Each point in each curve represents the mean ± SEM. Measurements of a neuropathic manifestation made at the indicated day in one group of rats (n=13). The values of significance of differences were measured with reference to the level measured at day-1 each curve.

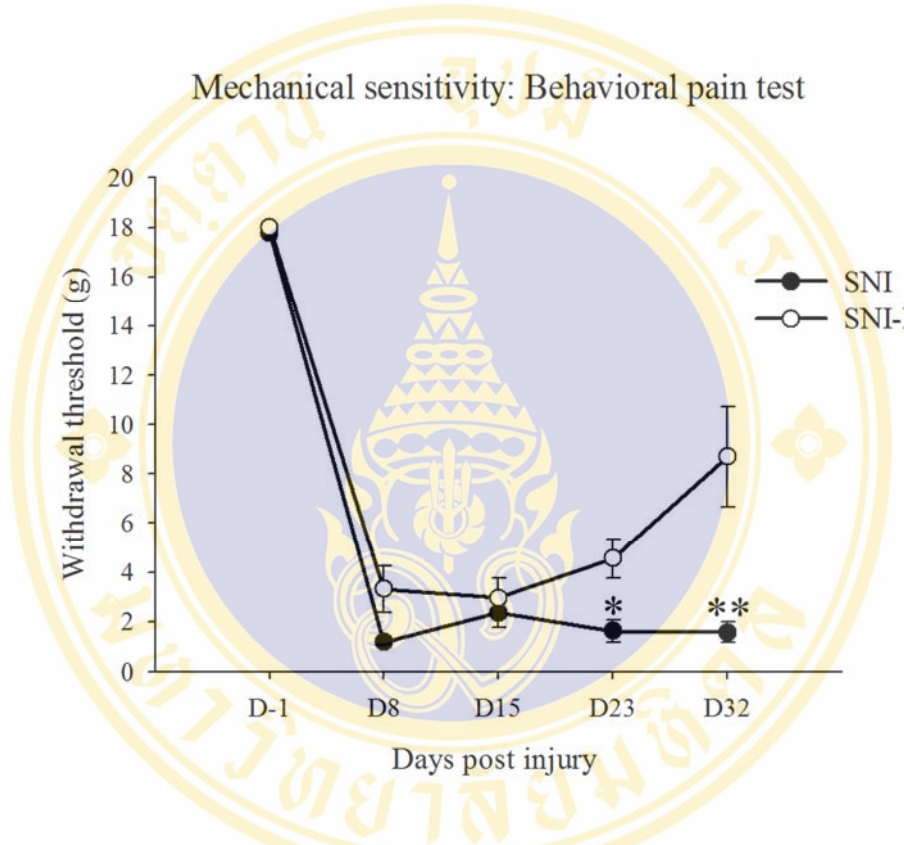


Figure 5.3 Response to mechanical stimulation with von Frey filaments in the sural skin territory for SNI and SNI-ES. Statistically significant difference was observed 7 days after surgery until day 32, * p-value < 0.05 and ** p-value < 0.001. Each point in each curve represents the mean ± SEM. Measurements of a neuropathic manifestation made at the indicated day in one group of rats (n=13). The values of significance of differences were measured with reference to the level measured at day-1 each curve.

Moreover, in table 5.2 and figure 5.3, the withdrawal threshold of SNI-ES rats were increased in day 23 and 32 when compared with SNI group on the corresponding day (day 23: 4.6±0.8*g, day 32: 8.7±2.0** g vs. day 23: 1.6±0.5 g, day 32: 1.6±0.4 g respectively, * $p<0.05$ and ** $p<0.001$).

After nerve injury, mechanical allodynia developed from the first postinjury day to the end of the observation (5 weeks). There were decreased response thresholds on the von Frey test on the injured side (figure 5.3). After low frequency electrical stimulation treatment, the mechanical allodynia in SNI rats were attenuated significantly after day 7 till the end of observation ($p<0.001$). But after low frequency electrical stimulation treatment, the sham showed slightly decrease in pain behavioral threshold after surgery till day 8 of observation and turned to baseline till the end of observation (figure 5.2).

II. Immunofluorescent histochemistry

The SPir was localized in DRG and spinal dorsal horn by using immunofluorescent histochemistry at 5 weeks after surgery. In addition, the SPir was measured as mean gray level by using image analysis.

SPir expression in DRG induced by sham and SNI was shown in figure 5.4 whereas that induced by sham-ES and SNI-ES was shown in figure 5.5 and by SNI and SNI-ES was shown in figure 5.6.

SPir expression in spinal dorsal horn induced by sham, SNI and SNI-ES was shown in figures 5.8.

Mean gray level of SPir of DRG and spinal dorsal horn expressed as mean±SEM was shown in table 5.3 and 5.5 respectively. The percentage change of SPir in L4-L6 DRG (table 5.4) and spinal dorsal horn (table 5.6) were also shown.

In addition, the percentage changes of SPir in DRG (figure 5.7) were compared among groups by bar graph.

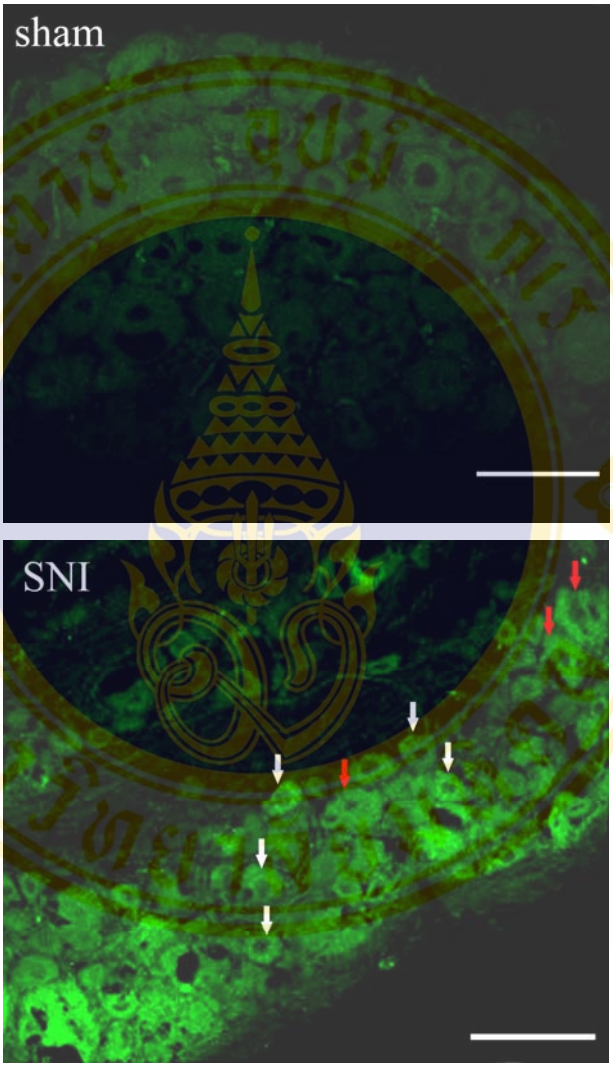


Figure 5.4 Photomicrographs of DRG showing SPir induced by sham and SNI operation. Note that the SNI group demonstrated the different increase in SPir in medium (white arrows) and large neuron (red arrows). Scale bar; 100 micrometers.

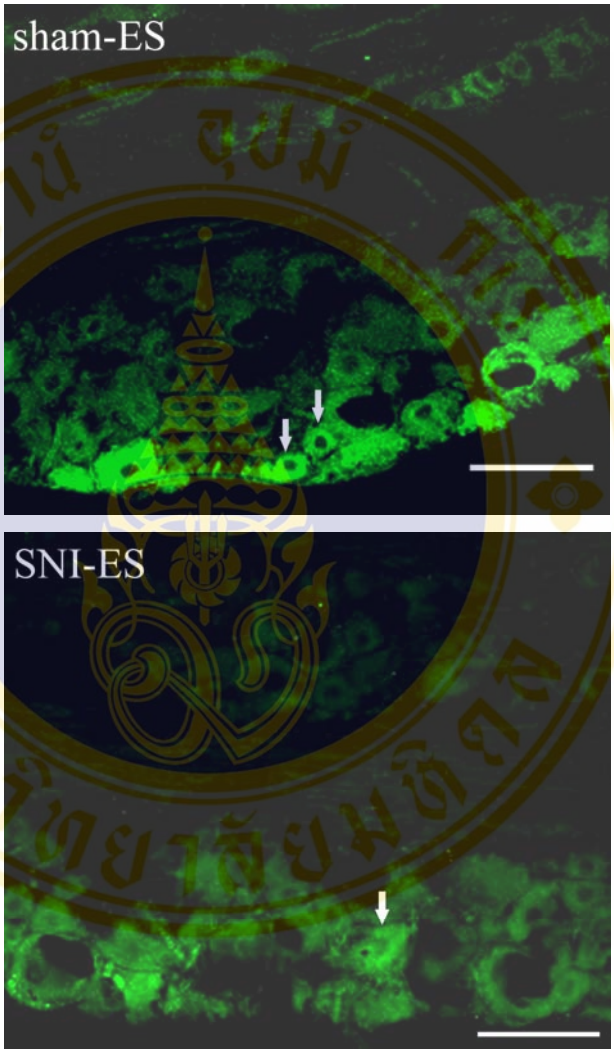


Figure 5.5 Photomicrographs of DRG showing SPir induced by sham-operation with electrical stimulation treatment (sham-ES) and SNI with electrical stimulation treatment (SNI-ES). Note that electrical stimulation treatment could attenuate the increase of SPir after SNI but increase SPir in some cells in sham-ES. Scale bar; 100 micrometers. Arrows indicated SPir in DRG neurons.

Figure 5.6 Photomicrographs of DRG showing SPir induced by SNI and SNI-ES. Note that electrical stimulation treatment could attenuate the increase of SPir after SNI. Scale bar; 100 micrometers.

Table 5.3 Mean gray level of substance P expressed as mean ± SEM from 5 random sections of DRG per rat was measured by Image Tool Analysis program 3.0

Rats	Mean grey level of SP of Dorsal root ganglion (Mean ±SEM)			
	sham	**sham-ES**	**SNI**	**SNI-ES**
1	11±21.45	11±23.85	27±39.86	18±37.69
2	8±17.24	13±11.17	27±28.31	18±34.77
3	10±16.34	11±23.44	28±39.86	20±36.59
4	10±16.56	10±25.55	32±28.39	16±20.09
5	11±18.04	12±10.08	27±21.22	18±21.22
Mean±SEM	10±2.08	11.4±7.53	28.2±8.14	18±8.67

Table 5.4 The percentage change of SPir in L4-L6 DRG of sham, sham-ES, SNI and SNI-ES group (*Statistical significant at p<0.001)

	SPir				p-value
	sham	**Sham-ES**	**SNI**	**SNI-ES**	
DRG	100.0±5.5 (10±2.1)	114.0±5.5 (11.4±7.3)	282.0±6.0* (28.2±8.1)	180.0±5.7* (18.0±8.7)	0.000

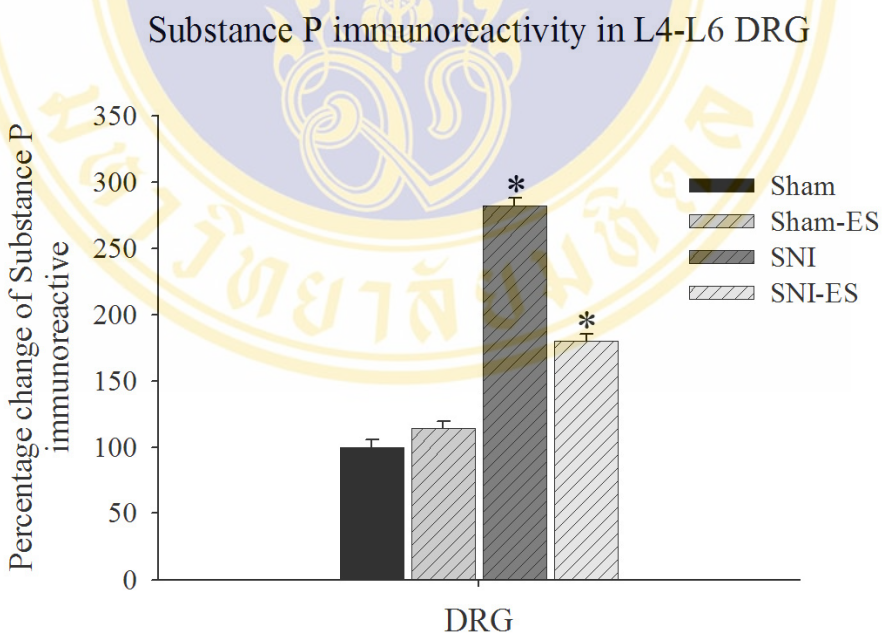


Figure 5.7 The graph shows percentage change of SPir in DRG in all groups. Statistically significant difference was observed at day 35, * p-value < 0.001. Each point in each graph represents the mean ± SEM.

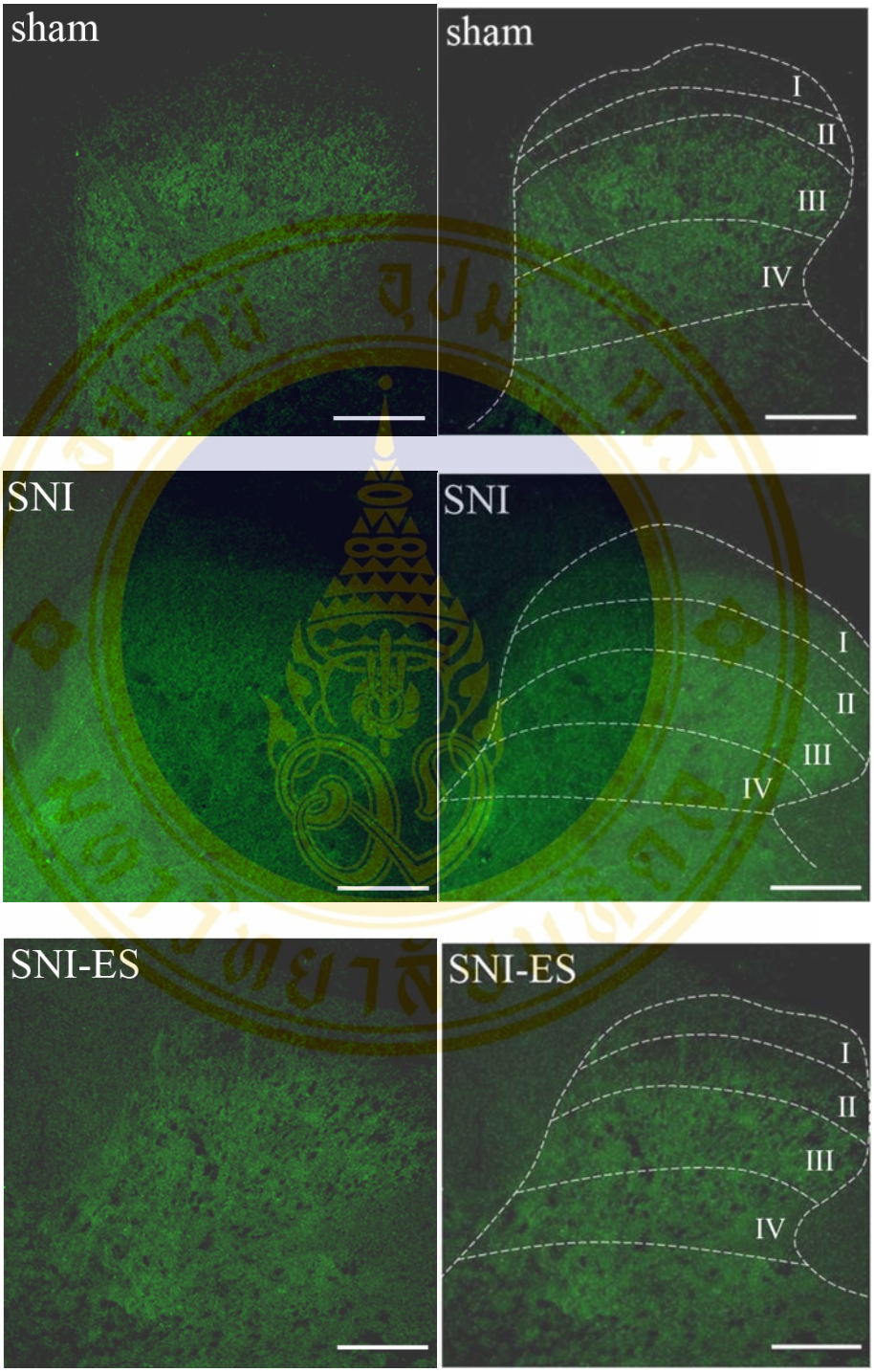


Figure 5.8 Photomicrographs of spinal dorsal horn showing SPir induced by sham, SNI and SNI-ES operation. Note that the SNI group has increase in SPir compared with sham group and has decrease in SNI-ES group. Scale bars; 200 micrometer micrometers.

Table 5.5 Mean gray level of substance P expressed as mean±SEM from 5 random sections of spinal dorsal horn per rat was measured by Image Tool Analysis program 3.0

Rats	Mean grey level of SP of Spinal dorsal horn (Mean ±SEM)			
	sham	**sham-ES**	**SNI**	**SNI-ES**
1	60±42.47	81±44.58	142±31.81	80±47.32
2	63±42.93	79±44.63	142±31.62	76±47.43
3	60±42.54	74±44.56	122±36.86	80±47.75
4	62±42.79	76±44.81	142±31.60	73±47.04
5	62±42.84	77±44.91	142±31.49	78±47.16
Mean±SEM	61.4±0.20	77.4±0.15	138±2.34	77.4±0.27

Table 5.6 The percentage change of SPir in L4-L6 spinal dorsal horn of sham, sham-ES, SNI and SNI-ES group (*Statistical significant at p<0.001)

	SPir				p-value
	sham	**sham-ES**	**SNI**	**SNI-ES**	
Spinal dorsal horn	100.0±5.6 (61.4±0.2)	126.1±6.2 (77.4±0.6)	224.8±9.0 (138±2.3)	126.1±6.3 (77.4±0.3)	1.000

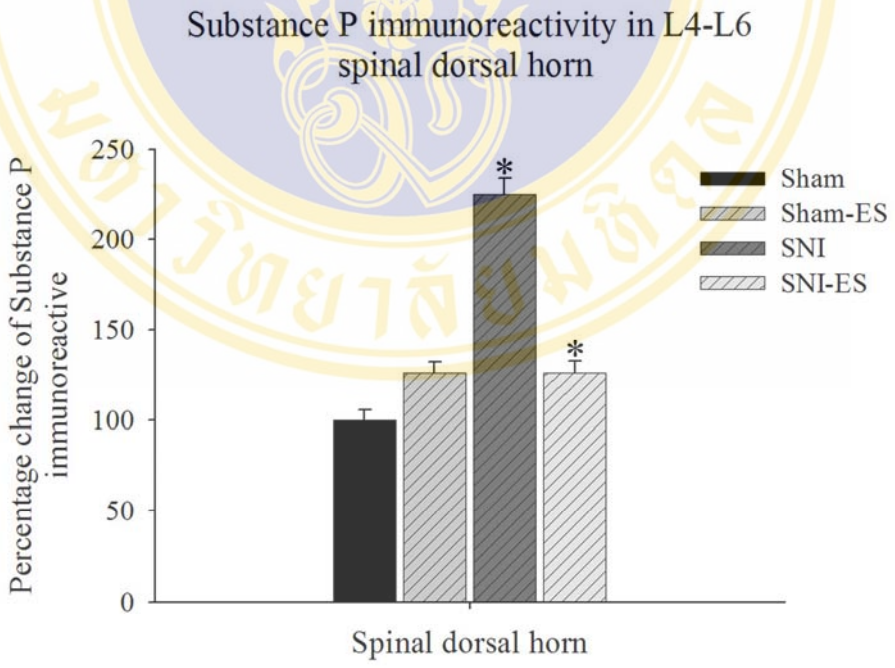


Figure 5.9 The graph shows percentage change of SPir in spinal dorsal horn in all groups. Statistically significant difference was observed at day 35, * p-value < 0.001. Each point in each graph represents the mean ± SEM.

1. The expression of SPir

SPir in DRG

In figure 5.4, the SPir in DRG of sham group was compared with SNI group. In sham, SPir was distributed throughout the DRG but less intense than SNI.

In figure 5.5, the SPir in DRG of sham-ES was compared with SNI-ES group. In sham-ES, SPir was distributed the throughout the DRG and intense in some cells (white arrows). In SNI-ES, the SPir was distributed in some DRG neurons (white arrow) but more intense than sham-ES.

In figure 5.6, the SPir in DRG of SNI was compared with SNI-ES group. In SNI, SPir was increased in the small, medium (white arrows) and large (red arrows) DRG neurons. In SNI-ES, the distribution of SPir was decreased and showed less than SNI but also intense in some cells.

SPir in spinal dorsal horn

In figure 5.8, the SPir in spinal dorsal horn of sham, SNI and SNI-ES groups were compared. In SNI, SPir was increased when compared with sham. In SNI-ES, SPir was decreased and distributed in lamina III&IV and dispersed to lamina II similar to sham.

2. Quantitative image analysis

Image analysis of mean gray level of SPir was performed on five non adjacent sections from each animal in all groups by using Image Tool program 3.0.

2.1 Increase of SPir in DRG and spinal dorsal horn after SNI

In the immunofluorescent histochemistry, the SPir was examined in the DRG and spinal dorsal horn. The data were shown in tables 5.3 and 5.7.

Table 5.4 showed the percentage change of SPir in L4-L6 DRG and spinal dorsal horn of the sham and SNI group. In figure 5.7, in the sham group, there was the significant difference in the percentage change of SPir in L4-L5 DRG (100.0±5.5) when compared with SNI group (282.0±6.0).

In figure 5.7, in the sham group, there was decrease in percentage change of SP in L4-L5 of spinal dorsal horn (100.0±5.6) when compared with SNI group (224.8±9.0).

Note that, after SNI, both in DRG and spinal dorsal horn showed the increase of SPir (282.0±6.0, 224.8±9.0 respectively, $p<0.001$).

2.2 The effects of low frequency electrical stimulation treatment on SPir in DRG and spinal dorsal horn after SNI

Table 5.5 showed the percentage change of SPir in L4-L6 DRG and spinal dorsal horn of sham-ES and SNI-ES group. In figure 5.7, in the sham-ES group, there was the significant difference in the percentage change of SPir in L4-L5 DRG (114.0±5.5) when compared with SNI-ES group (180.0±5.7).

But in figure 5.9, in the sham-ES group, there was no difference in L4-L5 of spinal dorsal horn (126.1±6.2) when compared with SNI-ES group (126.1±6.3).

In table 5.6 shown the percentage change of SPir in L4-L5 DRG of the SNI-ES was increased but less than SNI group (180.0±5.7 vs. 282.0±6 respectively, $p<0.001$ in figure 5.7) similar in spinal dorsal horn (126.1±6.3 vs. 224.8±9.0 respectively, $p<0.001$ in figure 5.9).

III The expression of CTB immunoreactivity

Three days before sacrifice of rat, the injured nerve was injected with cholera toxin B subunit (CTB) that effectively labels both peripheral and central A-beta axons. The effect of SNI was evaluated by analyzing the extent of CTB (CTBir) and substance P (SPir) immunoreactivities in L4-L6 dorsal root ganglia (DRG) and spinal dorsal horn at day 35 after surgery.

On the basis of the use of CTB as a selective tracer for A-beta fibers, neuronal sprouting was studied in the spinal dorsal horn. In sham operation group, CTBir was present in laminae III and IV. In SNI group, CTBir distributed in laminae III and IV and dispersed into laminae I and II, a region that normally receives only C-fiber input. In addition, CTBir was shown co-localization with SPir in medium- and large-size DRG neurons.

In DRG after SNI without any stimulation (figure 5.10B), the CTBir labeled neurons (red color) were distributed throughout the DRG in medium- and large-size neuron. In addition, there were co-localizing expressions with SPir only in medium- and large-sized neurons (figure 5.10C: white arrows).

In spinal dorsal horn, the labeling of CTBir were shown when study in sham (figure 5.11B), SNI (figure 5.12B) and SNI-ES (figure 5.13B). In sham, CTBir central terminals were present intensely in laminae III-IV (figure 5.11B and C). After SNI, CTBir central terminals distributed mainly in laminae III-IV and dispersed through laminae I-II (figures 5.12B and C: white arrows). In addition, the electrical stimulation treatment may affect changes of the CTBir in laminae I-IV (figures 5.13B and C). It was shown less intensely than in SNI and less dispersely into laminae I-II.

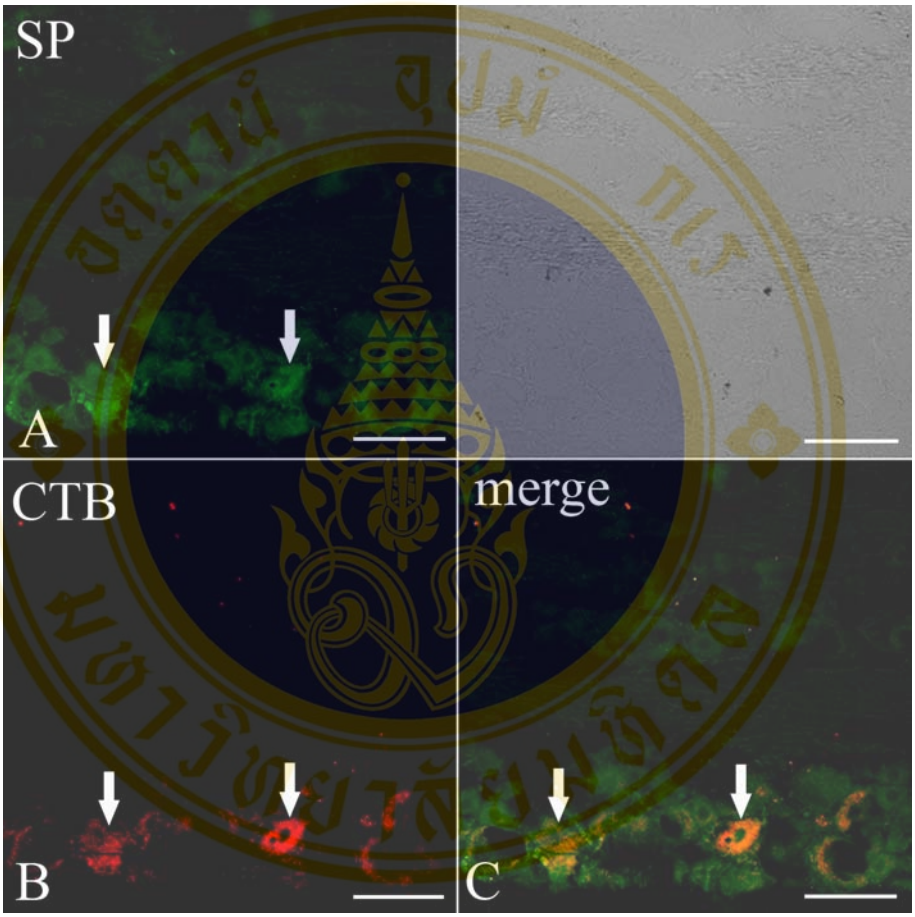


Figure 5.10 Photomicrographs of DRG showing SPir (A), CTBir (B), and the co-localization (C) of SPir (green) and CTBir (red) in both medium and large DRG neurons (white arrows) but not in small DRG neuron following SNI, scale bars; 100 micrometers.

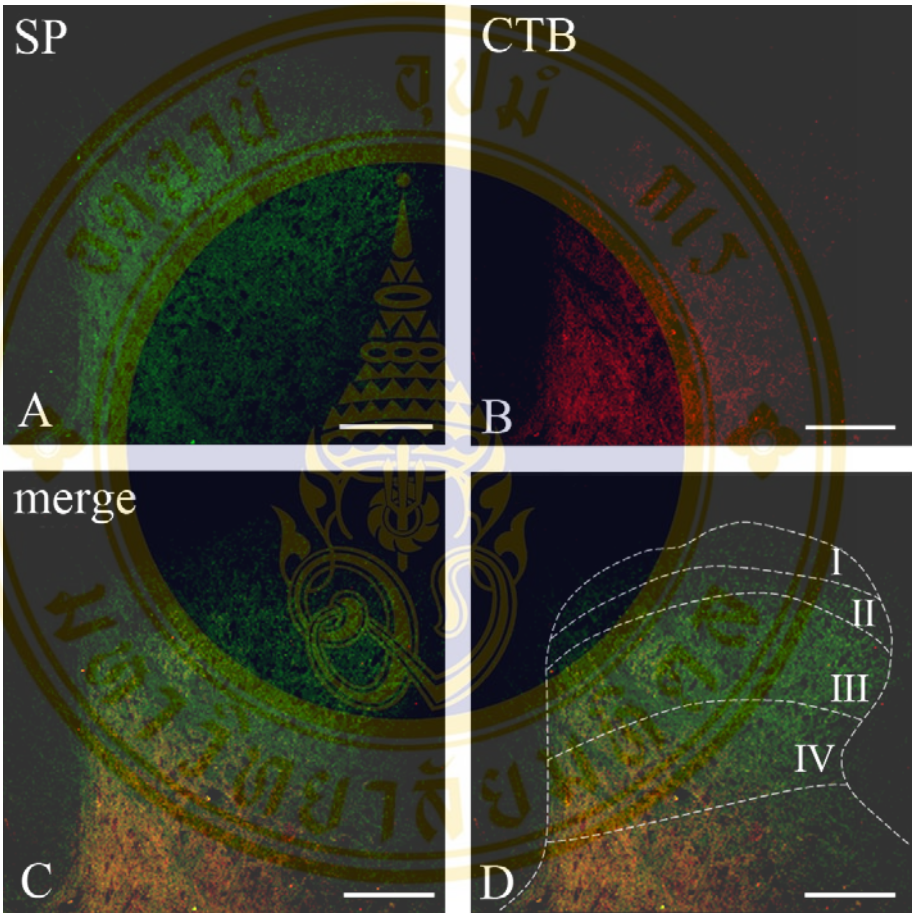


Figure 5.11 Photomicrographs of spinal dorsal horn showing SPir (A) and CTBir (B) of normal primary afferent fibers induced by sham. SP primary afferent central terminations (green) were concentrated mainly in laminae III&IV and dispersed in I&II (A). CTB-labeled terminals (red) were present in laminae III-IV (B). Double labeling showing SPir co-localized with CTBir were shown in C and D. Laminae I-IV were indicated in D. Scale bars; 200 micrometers.

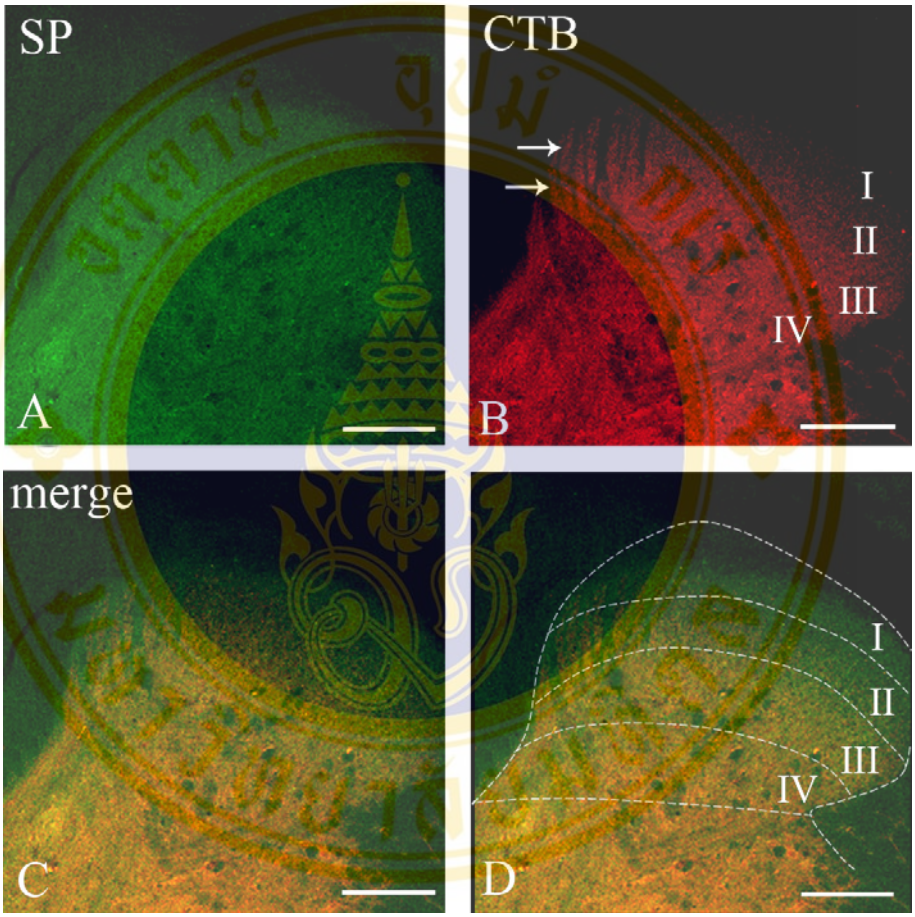


Figure 5.12 Photomicrographs of spinal dorsal horn showing SPir (A) and CTBir (B) of abnormal primary afferent fibers induced by SNI. SP primary afferent central terminations (green) were concentrated more intensely in laminae I-IV and II (A). CTB-labeled terminals (red) were present mainly in laminae III-IV and some axon sprouting into lamina II (B) (white arrows). Double labeling showing SPir co-localized with CTBir were shown in C and D. Laminae I-IV were indicated in D. Scale bars; 200 micrometers.

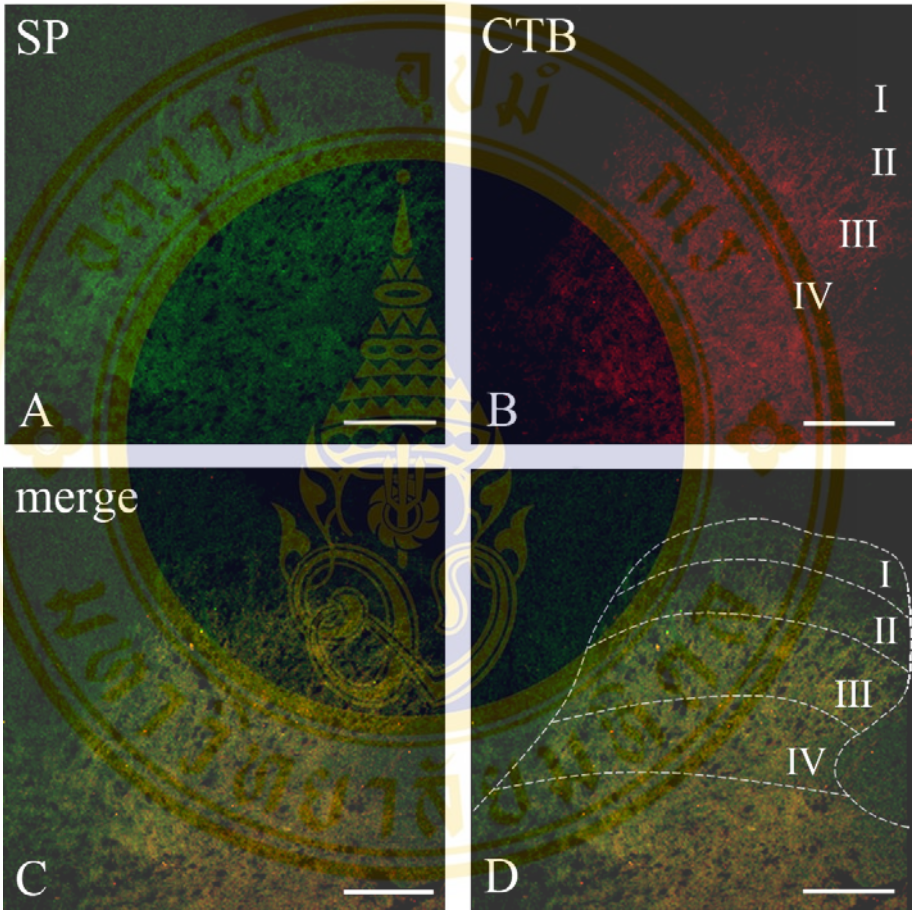


Figure 5.13 Photomicrographs of spinal dorsal horn showing SPir (A) and CTBir (B) of primary afferent fibers induced by SNI-ES. SP primary afferent central terminations (green) distributed less intensely in laminae I&II (A). CTB-labeled terminals (red) were present mainly in laminae III-IV (B). Double labeling showing SPir co-localized with CTBir were shown in C and D. Laminae I-IV were indicated in D. Scale bars; 200 micrometers.

CHAPTER VI
DISCUSSION

Neuropathic pain is an impair condition that often results from partial injury to a peripheral nerve and is often resistant to common therapeutic interventions (43, 44, 159). The present study showed that the SNI model (20) of neuropathic pain, employing a complete transection of common peroneal and tibial distal branches of the sciatic nerve leaving the sural branch intact, consistently resulted in mechanical allodynia.

The results also demonstrate that low frequency (2 Hz) electrical stimulation can reduce the signs of mechanical allodynia, produced by SNI of the nerve innervating the rat hind limb. Consistent with the decreased mechanical allodynia, SPir in DRG and spinal dorsal horn induced by SNI was decreased following electrical stimulation. In addition, CTBir in SNI rat was co-localized with SPir in medium- and large-sized DRG neurons and also distributed more intensely in laminae III-IV and dispersed up to laminae I-II. Electrical stimulation affected on CTBir distribution in spinal dorsal horn. It showed decrease CTBir in laminae I-II, concentrated mainly in laminae III-IV.

Spared nerve injury model

According to different types of nerve injury shown in table 3.2, SNI procedure was decided to use in this study as a partial denervation model which was both technically easy to perform and subjected to minimal variability in the degree of damage. This is a model that enabled a direct way of investigating changes in both injured primary sensory neurons and in neighboring intact sensory neurons. In addition, the essential idea was to investigate their relative contributions to the pathophysiology of pain that was attenuated following the electrical stimulation. Therefore, the activation of the spared intact sural nerve which arises from the cells of origin in the same level of DRG and spinal dorsal horn (L4-L6) of the injured tibia and

common peroneal nerves was suitable analyze the central mechanism involved the effects of electrical stimulation.

The present study demonstrates that all rats subjected to SNI displayed evident signs of neuropathic in the operated leg. A pronounced mechanical allodynia, a sign neuropathic pain, was observed. The current data confirmed the previous reports, (3, 20, 154) that SNI in rats produced consistent reduction of mechanical nociceptive threshold by using von Frey filaments. A significant decreased allodynia was onset in first postinjury day till the end of the behavioral observation (5 weeks). The neuropathic pain-like symptom has been described before in peripheral nerve injury models. This symptom was observed during the period of regeneration (within the first three weeks) (8, 160), that is before reinnervation of the paw has been established. In addition, a previous reports (161) demonstrated that the maximum changes were observed more than 8 weeks in the SNI model.

Substance P immunoreactivity

The previous attempts have been made to elucidate the potential neurobiological mechanisms which contribute to the pathogenesis of neuropathic pain (20, 162, 163). Some of them are almost immediate (nerve injury discharge-evoked central sensitization), intermediate (development of ectopic excitability and alteration in phenotype in injured and intact afferents), delayed (structural changes). In the line with these idea, the nerve injury is followed by the upregulation and downregulation of various substances in DRG (22, 162). Some of these changes are related to the degenerative and regenerative changes occurring in the peripheral and central branches of the sensory neurons (164, 165).

SP is the main excitatory sensory neuropeptide of small-diameter primary afferents and is released into the spinal dorsal horn following noxious stimulation (166). In addition, some damaged large A-fibers appear to undergo a phenotypic shift and begin to express SP (24, 34). It was known from the earlier evidence that peripheral nerve injury triggers hyperexcitability in neurons in the DRG-dorsal horn-thalamic pathway (26). This pathway transmits information about noxious stimuli.

In the present study by immunofluorescent localization of SPir, it was found that the increased SPir was localized in ipsilateral medium- to large-sized DRG

neurons following SNI. This finding is in line with previous demonstration of Noguchi, et al (24, 101) that SP is synthesized in injured DRG neurons and contributes to hyperexcitability of neurons and changes in gene expression in gracile nucleus neurons, including those that project to the thalamus (24). This led to the suggestion that the effects seen in the gracile nucleus may be directly related to activity in large myelinated primary afferents that express SPir after nerve injury and ascend the dorsal columns (24). More evidence indicated by in situ hybridization that peripheral axotomy of lumbar DRG neurons resulted in the appearance of preprotachykinin mRNA that encoded the neuropeptides SP and neurokinin A (167). In addition, the immunohistochemistry revealed that large-sized myelinated fibers, in L5 dorsal root contained SPir only on the side ipsilateral to the L5 spinal nerve transection. This showed that newly synthesized SP in large DRG cells is transported, via large myelinated fibers, toward the CNS (24, 101).

DRG neuronal size is correlated with the caliber of primary afferent axons. Normally tachykinin peptides, SP in particular, are localized to small DRG neurons, which are usually associated with unmyelinated afferent axons (168). These axons terminate mainly in superficial laminae I and II of the spinal dorsal horn and carry information from peripheral nociceptors. Medium and large cells in the DRG have myelinated axons that carry information from low threshold mechanoreceptors and terminate mainly in laminae III and IV of the spinal dorsal horn and dorsal column nuclei of the medulla oblongata (168).

In this study, an increased SPir was demonstrated intensely in laminae III and IV and some disperse of SPir in laminae I and II of the ipsilateral spinal dorsal horn following SNI when compared to sham-operated group. These data are consistent with the suggestion that the peripheral neuropathy induces phenotypic changes predominantly in myelinated afferents, the sensory neurons that normally response to mechanical stimulation. Therefore, these SPir axons terminated in laminae III and IV. There may be some sprouting of these SPir axons in laminae I and II that normally transmitted the signal through a pain pathway (169).

Several lines of evidence demonstrate clear differences in SP release between partial and complete nerve injury. Numerous studies have been conducted using the axotomy model and established that SP is down regulated in sensory neurons and that

the SP content decreases in the dorsal horn (22, 103, 104, 170) . It has also been suggested that a decrease in spinal SP levels may be involved in the induction of neuropathic signs following sciatic nerve transection or chronic constriction injury (105). In contrast to the axotomy case, it has been observed that there is a proportionate increase of SP in uninjured neurons following partial sciatic nerve ligation, the most frequently used partial nerve injury models (171). Partial nerve injury models are often regarded as more relevant for the understanding of neuropathic pain in patients (172).

In our experiment, the SNI model recently developed by Decosterd and Woolf (2000) as a variant of partial denervation was used (20). This model differs from other earlier models that it permits behavioral testing of the non injured skin territories (innervated by spared intact axon) adjacent to the denervated areas. And it was suggested to be used as the additional resource for unraveling the mechanisms responsible for the production of neuropathic pain. The present results demonstrated the increased SPir both in DRG and spinal dorsal horn at five weeks after SNI in comparison to sham-operated groups. Furthermore, there was the correlation between decreased mechanical withdrawal threshold in the behavioral pain test and increased SP synthesis.

The mechanisms of transmission of neuropathic allodynia have been discussed in several studies (20, 169, 173). Nociceptive transmission through the larger mechanosensory neurons during neuropathic conditions has been attributed to one or more of the following mechanisms: phenotypic changes affecting the injured and the intact A-beta afferent (53), sympathetic sprouting primarily around large neurons (174), abnormal sprouting of A-beta afferents into laminae I and II (169, 173) and expression of SP instead of glutamate, either in A-beta fibers (24, 53) or in laminae III and IV neurons known to contribute pain signaling of larger mechanosensory neurons in response to normally nonpainful mechanical stimuli (24, 175). Accordingly, these phenotypic changes have been reported to occur earlier after nerve injury.

These findings raise the possibility that the increased SP release as observed in the present study arises from uninjured axons of sciatic nerve. It is also suggested that partially denervated tissues in the nerve, skin, and other locations include spinal glial cell activation may enhance the release of SP or other substances that, in turn, sensitize

the intact nociceptors. Therefore, these abnormalities in the intact nociceptor, which arise in the context of Wallerian degeneration, may also play a role in creating or maintaining the abnormal pain state (175). However, it should also be pointed out that pathophysiological mechanisms and neurochemical changes may be different for different animal models of neuropathic pain.

Electrical stimulation

As found in the present study, the elevated pain threshold (antinociceptive effect) was significantly reversed to the threshold close to normal in the SNI rats after low frequency electrical stimulation intramuscularly on the denervated muscles such as tibialis anterior and lateral head of gastrocnemius muscles and pass through the skin area supplied by the sural nerve. This attenuation of neuropathic allodynia in awaken rats was caused by using the stimulation current in the intensity that can produce a motor contraction. The possible mechanisms were proposed according to the present technique of electrical stimulation which aimed at stimulation the intact somatic afferent fibers in the skin and innervated muscles and also denervated-muscles. Based on the fact that neuropathic pain is a supersensitivity phenomenon and IMS treatments acted by desensitizing the affected nerve, dorsal horn neuron and denervated muscles (15). In addition, as described by Lomo (12) that in animal experiments the supersensitivity and other features of denervated muscle could be reversed by electrical stimulation. Moreover, it was reported that muscle contractions improve skin and muscle circulation (13). Therefore, in the present study, the use of low-frequency electrical stimulation (2 Hz), producing moderate muscle contractions, leads to a transient, local increase in blood flow in muscle and skin. Daily stimulation sessions produced progressive recovery from the neuropathic manifestration that was more evident for improvement of mechanical allodynia by decreasing the ectopic discharge from the muscle and skin after nerve injury.

Previous investigation by Wu et al (176) described that Wallerian degeneration in the peripheral nerve may play an important role in at least some types of neuropathic pain. Schwann cells of such Remak bundles would synthesize and release growth factors, cytokines, and short-acting intermediates that could affect uninjured afferents sharing the same Remak bundle. Therefore, in the present study, the injured

tibial and common peroneal nerve may affect the uninjured sural afferents that connect to the same level of spinal cord.

CTB immunoreactivity

This study found CTBir in medium- and large-DRG neurons and laminae I-IV following SNI. In contrast, SNI-ES, CTBir was found in laminae III-IV after electrical stimulation treatment. The effects of CTB, retrograde tracer to injured medium- and large-DRG neurons, which used to study central termination of injured neurons in spinal dorsal horn.

In SNI, found CTBir in lamina I&II, we suggested that the central termination of medium- and large-DRG neurons were sprouted into laminae I&II and synapse with secondary neuron in laminae I&II to interpreted pain impulse that may correlated with the increase of mechanical allodynia after SNI.

In SNI-ES, CTBir shown in laminae III&IV, we suggested that the low frequency (2 Hz) electrical stimulation may effected in decrease of sprouting that may correlated with the decrease of mechanical allodynia after electrical stimulation in SNI rats.

This study suggest that the possibility considered here is that peripheral neuropathy possibly induced SP synthesis in myelinated afferent fibers and their activation by innocuous mechanical stimulation induced SP release. Thus, the attenuated effects by electrical stimulation on the ectopic activity in injured afferents especially A-beta fibers may affect some changes on central terminal of A-fiber such as decrease in sprouting of A-fiber in lamina II. SP synthesis and release may be one of the neurochemical mechanisms involved and it could be modified for relieve pain.

CHAPTER VII
CONCLUSION

The present study demonstrated that changes in term of behavioral neuropathic pain and SPir in L4-L6 DRG and spinal dorsal horn are occurred after SNI. Furthermore, an antinociceptive effect of low frequency electrical stimulation intramuscularly on the behavioral neuropathic pain and SPir were also evaluated. The results are as followed (table 7.1):

1. The present study demonstrated that the SNI model of neuropathic pain, employing a transection of the tibial and common peroneal nerves leaving the sural nerve intact resulted in marked mechanical allodynia and gradually decreased response thresholds. The SNI rat displayed quick onset (within the first day) and long-lasting (more than 5 weeks) behavioral signs of robust mechanical allodynia.

2. The present results also demonstrated that the upregulation of SPir in L4-L6 DRG and spinal dorsal horn 5 weeks after SNI. This may contribute to the establishment and persistent of neuropathic pain especially mechanical allodynia. In addition, the CTBir belonged to the myelinated primary afferents was shown to concentrate intensely in laminae III&IV and dispersed into laminae I&II after SNI. These changes indicated the sprouting of large A-beta fibers.

3. Interestingly, as the 2 Hz-ES was applied intramuscularly once a day for 5 consecutive days in the first week, followed by twice a week for other 2 consecutive weeks. It was started on day 15 after injury when the animals developed highest peak of pain. The 2 Hz-ES could significantly reduced mechanical allodynia by increasing the response threshold. In addition, it could decrease the SPir in DRG and spinal dorsal horn and affect the changes in CTBir distribution intensely in laminae III&IV.

Therefore, the low frequency ES treatment applied intramuscularly has antinociceptive effects on both pain behavioral response and neuronal activation of the DRG and spinal dorsal horn via SP in SNI rats. The present study suggests that the 2 Hz-ES might be used as an alternative therapy for relieving neuropathic pain.

Table 7.1 The conclusion of this study

	SNI	**SNI-ES**
Mechanical allodynia	increase	decrease
SP immunoreactivity (DRG & spinal dorsal horn)	increase	decrease
CTB immunoreactivity	lamina I-II, III-IV	lamina III-IV

Further investigation

Further studies are needed to elucidate the time course of SPir during the first week after SNI for more clearly understanding in the antinociceptive effects of 2 Hz-ES on neuropathic pain. To achieve this study, the Western blot analysis and immunohistochemistry staining will be performed. It is still possible that other factors than SP are also involved in the mechanism of neuropathic pain.

REFERENCES

1. Helle KE, Gordon BM. Pharmacological characterisation of the spared nerve injury model of neuropathic pain. Pain. 2002;98(1-2):151-61.
2. Crisp T, Giles JR, Cruce WL, McBurney DL, Stuesse SL. The effects of aging on thermal hyperalgesia and tactile-evoked allodynia using two models of peripheral mononeuropathy in the rat. Neurosci Lett. 2003 Mar 20;339(2):103-6.
3. Shields S, Eckert Wr, Basbaum A. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. J Pain. 2003 Oct;4(8):465-70.
4. Decosterd I, Allchorne A, Woolf CJ. Progressive tactile hypersensitivity after a peripheral nerve crush: non-noxious mechanical stimulus-induced neuropathic pain. Pain. 2002 Nov;100(1-2):155-62.
5. Won R, Jung S, Park Y, Chung S, Lee B. Crossed-withdrawal reflex in a rat model of neuropathic pain: implications in neural plasticity. Neurosci Lett. 2004 Oct 21;369(3):239-44
6. Besson J. The neurobiology of pain. Lancet. 1999 May 8;353(9164):1610-5.
7. Galer B. Neuropathic pain of peripheral origin: advances in pharmacologic treatment. Neurology 1995 Dec;45(12 Suppl 9):S17-25.
8. Attal N. Chronic neuropathic pain: mechanisms and treatment. Clin J Pain. 2000 Sep;16(3):S118-S30.
9. Melzack R. Acupuncture and related forms of folk medicine, in wall PD Melzack R (eds): Textbook of pain. Edinburgh: Churchill Livingstone; 1984.
10. Simon D, Travell J, Simon L. Myofascial Pain and Dysfunction: The Trigger Point Manual, Vol 1. Upper Half of the Body. Baltimore: Williams and Wilkins; 1999.
11. Gunn C. Treatment of chronic pain: Intramuscular stimulation for myofascial pain of radivulopathic origin. London: Churchill livingston; 1996.
12. Lomo T. What controls the position, number, size, and distribution of neuromuscular junctions on rat muscle fibers? J Neurocytol. 2003 Jun-Sep;32(5-8):835-48.

13. Chu J, Yuen K, Wang B, Chan R, Schwartz I, Neuhauser D. Electrical twitch-obtaining intramuscular stimulation in lower back pain: A pilot study. Am J Phys Med Rehabil. 2004;83:104–11.
14. Chu J, Schwartz I. The muscle twitch in myofascial pain relief: effects of acupuncture and other needling methods. Electromyogr Clin Neurophysiol. 2002 Jul-Aug;42(5):307-11.
15. Gunn C. Re: Electrical twitch-obtaining intramuscular stimulation in lower back pain: a pilot study. Am J Phys Med Rehabil. 2006;85(12):1015-6.
16. Bradnam L. A Proposed Clinical Reasoning Model for Western Acupuncture. NZ Journal of Physiotherapy 2003;31(1):40-5.
17. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain. 1988 April;33(1):87-107.
18. Kim S, Chung J. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain. 1992;50(3):355-63.
19. Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain. 1990;43(2):205-18.
20. Decosterd I, Woolf C. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain. 2000 Aug;87(2):149-58.
21. Woolf C. Evidence for a central component of post-injury pain hypersensitivity. Nature. 1983 Dec 15-21;306(5944):686-8.
22. Hökfelt T, Zhang X, Wiesenfeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. Trends Neurosci. 1994 Jan;17(1):22-30.
23. Levin M, Hui-Chan C. Conventional and acupuncture-like transcutaneous electrical nerve stimulation excite similar afferent fibers. Arch Phys Med Rehabil. 1993 Jan;74(1):54-60.
24. Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J Neurosci. 1995;15:7633-43.
25. Noguchi K, De León M, Nahin R, Senba E, Ruda M. Quantification of axotomy-induced alteration of neuropeptide mRNAs in dorsal root ganglion neurons

with special reference to neuropeptide Y mRNA and the effects of neonatal capsaicin treatment. J Neurosci Res. 1993 May 1;35(1):54-66.

26. Guilbaud G, Benoist J, Jazat F, Gautron M. Neuronal responsiveness in the ventrobasal thalamic complex of rats with an experimental peripheral mononeuropathy. J Neurophysiol. 1990 Nov;64(5):1537-54.
27. Baron R, Maier C. Phantom limb pain: are cutaneous nociceptors and spinothalamic neurons involved in the signaling and maintenance of spontaneous and touch-evoked pain? A case report. Pain. 1995 Feb;60(2):223-8.
28. Woolf C. Pain: moving from symptom control toward mechanism-specific pharmacologic management. Ann Intern Med. 2004 Mar 16;140(6):441-51.
29. Scholz J, Woolf C. Can we conquer pain? Nat Neurosci. 2002 Nov;5 Suppl:1062-7.
30. Steven R. Understanding Neuropathic Pain; USC Pain Management, USC Medical Center. Los Angeles; 2000.
31. Goodman G. Outcomes measurement in pain management: issues of disease complexity and uncertain outcomes. J Nurs Care Qual. 2003 Apr-Jun;18(2):105-11; quiz 12-3.
32. Holden J, Pizzi J. The challenge of chronic pain. Adv Drug Deliv Rev. 2003 Aug 28;55(8):935-48.
33. Gonzalez M, Field M, Hughes J, Singh L. Evaluation of selective NK(1) receptor antagonist CI-1021 in animal models of inflammatory and neuropathic pain. J Pharmacol Exp Ther. 2000 Aug;294(2):444-50.
34. Woolf C, Salter M. Neuronal plasticity: increasing the gain in pain. Science. 2000 Jun 9;288(5472):1765-9.
35. Mantyh P, Clohisy D, Koltzenburg M, Hunt S. Molecular mechanisms of cancer pain. Nat Rev Cancer. 2002 Mar;2(3):201-9.
36. Woolf C, Max M. Mechanism-based pain diagnosis: issues for analgesic drug development. Anesthesiology. 2001 Jul;95(1):241-9.
37. White F, Kocsis J. A-Fiber Sprouting in Spinal Cord Dorsal Horn Is Attenuated by Proximal Nerve Stump Encapsulation. Exp Neurol. 2002 Oct;177(2):385-95.
38. Eric R, James H. Principles of neural science. Elsevier. 1985.

39. John G, Robert M, Bruce G. From neuron to brain: A cellular and molecular approach to the function of the nervous system; 1992.
40. Boivie J, Leijon G, Johansson I. Central post-stroke pain--a study of the mechanisms through analyses of the sensory abnormalities. Pain. 1989 May;37(2):173-85.
41. Li Y, Dorsi M, Meyer R, Belzberg A. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain. 2000 Apr;85(3):493-502.
42. Treede R, Meyer R, Raja S, Campbell J. Peripheral and central mechanisms of cutaneous hyperalgesia. Prog Neurobiol. 1992;38(4):397-421.
43. Woolf C, Mannion R. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet. 1999 Jun 5;353(9168):1959-64.
44. Koltzenburg M, Scadding J. Neuropathic pain. Curr Opin Neurol. 2001 Oct;14(5):641-7.
45. Kolzenburg M. Basic mechanisms of peripheral neuropathic pain. In: Institute of child health and instistute of neurology at University College London. UK; 2004.
46. Jensen T, Gottrup H, Sindrup S, Bach F. The clinical picture of neuropathic pain. Eur J Pharmacol. 2001 Oct 19;429(1-3):1-11.
47. Regan J, Peng P. Neurophysiology of cancer pain. Cancer Control. 2000 Mar-Apr;7(2):111-9.
48. Pitcher G, Henry J. Nociceptive response to innocuous mechanical stimulation is mediated via myelinated afferents and NK-1 receptor activation in a rat model of neuropathic pain. Exp Neurol. 2004;186(2):173-97.
49. Sandkühler J, Liu X. Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. Eur J Neurosci. 1998 Jul;10(7):2476-80.
50. Field M, Bramwell S, Hughes J, Singh L. Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones? Pain. 1999 Nov;83(2):303-11.
51. Ochoa J, Yarnitsky D. Mechanical hyperalgesias in neuropathic pain patients: dynamic and static subtypes. Ann Neurol. 1993 May;33(5):465-72.

52. Koltzenburg M, Lundberg L, Torebjörk H. Dynamic and static components of mechanical hyperalgesia in human hairy skin. Pain. 1992 Nov;51(2):207-19.
53. Neumann S, Doubell T, Leslie T, Woolf C. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature. 1996 Nov 28;384(6607):360-4.
54. Campbell J, Meyer R. Mechanisms of neuropathic pain. Neuron. 2006 Oct 5;52(1):77-92.
55. Bridges D, Thompson S, Rice A. Mechanisms of neuropathic pain. Br J Anaesth. 2001 Jul;87(1):12-26.
56. Teng J, Mekhail N. Neuropathic Pain: Mechanisms and Treatment Options. Pain Pract. 2003;3(1):8-21.
57. Hansson P. Difficulties in stratifying neuropathic pain by mechanisms. Eur J Pain. 2003;7(4):353-7.
58. Svensson C, Yaksh T. The spinal phospholipase-cyclooxygenase-prostanoid cascade in nociceptive processing. Annu Rev Pharmacol Toxicol. 2002;42:553-83.
59. Kandel E, Schwartz J, Jessell T. Principles of Neural Science. New York: McGrew-Hill.
60. Novakovic S, Tzoumaka E, McGivern J, Haraguchi M, Sangameswaran L, Gogas K, et al. Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. J Neurosci. 1998 Mar 15;18(6):2174-87.
61. Yaksh T, Chaplan S. Physiology and pharmacology of neuropathic pain. Anesthesiol Clin North America 1997;15 (2):335-52.
62. Devor M. The pathophysiology of damaged peripheral nerves. In: Wall PD, Melzack R, eds. Textbook of pain. Edinburgh: Churchill Livingstone; 1994.
63. Ochoa J, Torebjörk H, Culp W, Schady W. Abnormal spontaneous activity in single sensory nerve fibers in humans. Muscle Nerve. 1982;5(9S):S74-7.
64. Blumberg H, Jänig W. Discharge pattern of afferent fibers from a neuroma. Pain. 1984 Dec;20(4):335-53.
65. Chung K, Kim H, Na H, Park M, Chung J. Abnormalities of sympathetic innervation in the area of an injured peripheral nerve in a rat model of neuropathic pain. Neurosci Lett. 1993 Nov 12;162(1-2):85-8.

66. Han H, Lee D, Chung J. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain. 2000 Feb;84(2-3):253-61.

67. Liu C, Amir R, Devor M. Effect of age and nerve injury on cross-excitation among sensory neurons in rat dorsal root ganglia. Neurosci Lett. 1999 Jan 8;259(2):95-8.

68. Devor M, Wall P. Cross-excitation in dorsal root ganglia of nerve-injured and intact rats. J Neurophysiol. 1990;64(6):1733-46.

69. Belgrade M. Following the clues to neuropathic pain. Distribution and other leads reveal the cause and the treatment approach. Postgrad Med. 1999 Nov;106(6):127-32, 35-40.

70. Gold M. Spinal nerve ligation: what to blame for the pain and why. Pain. 2000 Feb;84(203):117-20.

71. Dworkin R, Backonja M, Rowbotham M, Allen R, Argoff C, Bennett G, et al. Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. Arch Neurol. 2003 Nov;60(11):1524-34.

72. McLachlan E, Jänig W, Devor M, Michaelis M. Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. Nature. 1993 Jun 10;363(6429):543-6.

73. Raja S, Treede R, Davis K, Campbell J. Systemic alpha-adrenergic blockade with phentolamine: a diagnostic test for sympathetically maintained pain. Anesthesiology. 1991 Apr;74(4):691-8.

74. Wolfe G. Treatment of painful peripheral neuropathy. J Clin Neuromuscul Dis. 2002 Dec;4(2):50-9.

75. Woolf C, Shortland P, Coggeshall R. Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature. 1992 Jan 2;355(6355):75-8.

76. Woolf C, Shortland P, Reynolds M, Ridings J, Doubell T, Coggeshall R. Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. J Comp Neurol. 1995 Sep 11;360(1):121-34.

77. Lekan H, Carlton S, Coggeshall R. Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy. Neurosci Lett. 1996 Apr 26;208(3):147-50.

78. Baron R, Saguer M. Postherpetic neuralgia. Are C-nociceptors involved in signalling and maintenance of tactile allodynia? Brain. 1993 Dec;116(Pt 6):1477-96.

79. Pockett S. Spinal cord synaptic plasticity and chronic pain. Anesth Analg. 1995 Jan;80(1):173-9.

80. Nicholson B. Responsible prescribing of opioids for the management of chronic pain. Drugs. 2003;63(1):17-32.

81. Willis W, Coggeshall R. Sensory mechanisms of the spinal cord. New York: Plenum Press; 1991.

82. Stone L, Vulchanova L, Riedl M, Williams F, Wilcox G, Elde R. Effects of peripheral nerve injury on delta opioid receptor (DOR) immunoreactivity in the rat spinal cord. Neurosci Lett. 2004 May 6;361(1-3):208-11.

83. Truong W, Cheng C, Xu Q, Li X, Zochodne D. Mu opioid receptors and analgesia at the site of a peripheral nerve injury. Ann Neurol. 2003 Mar;53(3):366-75.

84. Watkins L, Milligan E, Maier S. Glial activation: a driving force for pathological pain. Trends Neurosci. 2001 Aug;24(8):450-5.

85. Rittner H, Brack A, Machelska H, Mousa S, Bauer M, Schäfer M, et al. Opioid peptide-expressing leukocytes: identification, recruitment, and simultaneously increasing inhibition of inflammatory pain. Anesthesiology. 2001 Aug;95(2):500-8.

86. De Felipe C, Herrero J, O'Brien J, Palmer J, Doyle C, Smith A, et al. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature. 1998 Mar 26;392(6674):394-7.

87. Meert T, Vissers K, Geenen F, Kontinen V. Functional role of exogenous administration of substance P in chronic constriction injury model of neuropathic pain in gerbils. Pharmacol Biochem Behav. 2003 Aug;76(1):17-25.

88. Skilling S, Smullin D, Larson A. Differential effects of C- and N-terminal substance P metabolites on the release of amino acid neurotransmitters from the spinal cord: potential role in nociception. J Neurosci 1990 Apr;10(4):1309-18.

89. Quartara L, Maggi C. The tachykinin NK1 receptor. Part II: Distribution and pathophysiological roles. Neuropeptides. 1998 Feb;32(1):1-49.

90. Snijdelaar DG, Dirksen R, Slappendel R, Crul BJ. Substance P. Eur J Pain. 2000 121-35;4(2).

91. Klaus L, Yvonne T, Mathias B, Bernd LF. Substance P and Affective Disorders: New Treatment Opportunities by Neurokinin 1 Receptor Antagonists? Neuropsychobiology. 2002;45:2-6.

92. Chan-Palay V, Palay S. Ultrastructural identification of substance P cells and their processes in rat sensory ganglia and their terminals in the spinal cord by immunocytochemistry. Proc Natl Acad Sci U S A. 1977 Sep;74(9):4050-4.

93. Cuello A, Jessell T, Kanazawa I, Iversen L. Substance P: localization in synaptic vesicles in rat central nervous system. J Neurochem. 1977 Oct;29(4):747-51.

94. Pickel V, Reis D, Leeman S. Ultrastructural localization of substance P in neurons of rat spinal cord. Brain Res. 1977 Feb 25;122(3):534-40.

95. Hökfelt T, Kellerth J, Nilsson G, Pernow B. Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. Brain Res. 1975 Dec 19;100(2):235-52.

96. Hökfelt T, Ljungdahl A, Terenius L, Elde R, Nilsson G. Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. Proc Natl Acad Sci U S A. 1977 Jul;74(7):3081-5.

97. Kanazawa I, Jessell T. Post mortem changes and regional distribution of substance P in the rat and mouse nervous system. Brain Res 1976 Nov 26;117(2):362-7.

98. Takahashi T, Otsuka M. Regional distribution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section. Brain Res 1975 Apr 4;87(1):1-11.

99. Miki K, Fukuoka T, Tokunaga A, Noguchi K. Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: up-regulation in a subpopulation of primary afferent sensory neurons. Neuroscience. 1998 Feb;82(4):1243-52.

100. Theo FM, Kris V, Frank G, Vesa KK. Functional role of exogenous administration of substance P in chronic constriction injury model of neuropathic pain in gerbils. Pharmacology Biochemistry and Behavior. 2003 August;76(1):17-25.
101. Noguchi K, Dubner R, Leon D, Senba E, Ruda M. Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons. J Neurosci Res. 1994 April;37(5):596-603.
102. McLachlan E, Hu P. Axonal sprouts containing calcitonin gene-related peptide and substance P form pericellular baskets around large diameter neurons after sciatic nerve transection in the rat. Neuroscience. 1998 June;84(4):961-5.
103. Jessell T, Tsunoo A, Kanazawa I, Otsuka M. Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. Brain Res. 1979 May 25;168(2):247-59.
104. Barbut D, Polak J, Wall P. Substance P in spinal cord dorsal horn decreases following peripheral nerve injury. Brain Res. 1981 Feb 2;205(2):289-98.
105. Kajander K, Xu J. Quantitative evaluation of calcitonin gene-related peptide and substance P levels in rat spinal cord following peripheral nerve injury. Neurosci Lett. 1995 Feb 17;186(2-3):184-8.
106. Wallin J, Schött E. Substance P release in the spinal dorsal horn following peripheral nerve injury. Neuropeptides. 2002 August;36(4):252-6.
107. Marchand J, Wurm W, Kato T, Kream R. Altered tachykinin expression by dorsal root ganglion neurons in a rat model of neuropathic pain. Pain. 1994 Aug;58(2):219-31.
108. Malcangio M, Ramer M, Jones M, McMahon S. Abnormal substance P release from the spinal cord following injury to primary sensory neurons. Eur J Neurosci. 2000;12(1):397-9.
109. Andrew D, Greenspan J. Modality-specific hyper-responsivity of regenerated cat cutaneous nociceptors. J Physiol. 1999 May 1;516(3):897-906.
110. Zimmermann M. Pathobiology of neuropathic pain. Eur J Pharmacol. 2001 Oct 19;429(1-3):23-37.

111. Lindenlaub T, Sommer C. Partial sciatic nerve transection as a model of neuropathic pain: a qualitative and quantitative neuropathological study. Pain. 2000 Dec 15;89(1):97-106.

112. Wall P, Devor M, Inbal, Scadding W, Schonfeld D, Seltzer Z, et al. Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. Pain. 1979 Oct;7(2):103-11.

113. Wagner R, Janjigian M, Myers R. Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-alpha expression. Pain. 1998 Jan;74(1):35-42.

114. Shannon DS, William AE, Allan IB. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. The Journal of Pain. 2003 October;4(8): 465-70.

115. Li A, Zhang J, Xie Y. Human acupuncture points mapped in rats are associated with excitable muscle/skin-nerve complexes with enriched nerve endings. Brain Res. 2004 Jun 25;1012(1-2):154-9.

116. Kane K, Taub A. A history of local electrical analgesia. Pain. 1975 Jun;1(2):125-38.

117. Turk D. Clinical effectiveness and cost-effectiveness of treatments for patients with chronic pain. Clin J Pain. 2002 Nov-Dec;18(6):355-65.

118. Meyerson B, Ren B, Herregodts P, Linderoth B. Spinal cord stimulation in animal models of mononeuropathy: effects on the withdrawal response and the flexor reflex. Pain. 1995 May;61(2):229-43.

119. Melzack R, Wall P. Pain mechanisms: a new theory. Science. 1965 Nov 19;150(699):971-9.

120. White P, Li S, Chiu J. Electroanalgesia: its role in acute and chronic pain management. Anesth Analg. 2001 Feb;92(2):505-13.

121. Johnson M, Ashton C, Thompson J. Long term use of transcutaneous electrical nerve stimulation at Newcastle Pain Relief Clinic. J R Soc Med. 1992 May;85(5):267-8.

122. Radhakrishnan R, Sluka K. Deep tissue afferents, but not cutaneous afferents, mediate transcutaneous electrical nerve stimulation-Induced antihyperalgesia. J Pain. 2005 Oct;6(10):673-80.

123. Wright A, Sluka K. Nonpharmacological treatments for musculoskeletal pain. Clin J Pain. 2001 Mar;17(1):33-46.
124. Bjordal J, Johnson M, Ljunggreen A. Transcutaneous electrical nerve stimulation (TENS) can reduce postoperative analgesic consumption. A meta-analysis with assessment of optimal treatment parameters for postoperative pain. Eur J Pain. 2003;7(2):181-8.
125. Fargas-Babjak A. Acupuncture, transcutaneous electrical nerve stimulation, and laser therapy in chronic pain. Clin J Pain. 2001 Dec;17(4 Suppl):S105-13.
126. Leem J, Park E, Paik K. Electrophysiological evidence for the antinociceptive effect of transcutaneous electrical stimulation on mechanically evoked responsiveness of dorsal horn neurons in neuropathic rats. Neurosci Lett. 1995 Jun 16;192(3):197-200.
127. Kaptchuk T. Acupuncture: theory, efficacy, and practice. Ann Intern Med. 2002 Mar 5;136(5):374-83.
128. Bowsher D. Mechanisms of acupuncture, In: Theory and Basis Science.
129. Lundeberg T, Stener-Victorin E. Is there a physiological basis for the use of acupuncture in pain? Int Congr Ser. 2002;1238:3-10.
130. Leibing E, Leonhardt U, Köster G, Goerlitz A, Rosenfeldt J, Hilgers R, et al. Acupuncture treatment of chronic low-back pain -- a randomized, blinded, placebo-controlled trial with 9-month follow-up. Pain. 2002 Mar;96(1-2):189-96.
131. Lomo T, Westgaard R. Control of ACh sensitivity in rat muscle fibers. Cold Spring Harb Symp Quant Biol. 1976;40:263-74.
132. Gunn C. Intramuscular stimulation (IMS) the technique. In The stitute for the study and treatment of pain (iSTOP). USA: Vancouver; 1996.
133. Ross R, Vogel A. The platelet-derived growth factor. Cell. 1978 Jun;14(2):203-10.
134. Hong C, Simons D. Pathophysiologic and electrophysiologic mechanisms of myofascial trigger points. Arch Phys Med Rehabil. 1998 Jul;79(7):863-72.
135. Faghri P, Van Meerdervort H, Glaser R, Figoni S. Electrical stimulation-induced contraction to reduce blood stasis during arthroplasty. IEEE Trans Rehabil Eng. 1997 Mar;5(1):62-9.

136. Pittman R. Oxygen supply to contracting skeletal muscle at the microcirculatory level: diffusion vs. convection. Acta Physiol Scand. 2000 Apr;168(4):593-602.

137. Sherry J, Oehrlein K, Hegge K, Morgan B. Effect of burst-mode transcutaneous electrical nerve stimulation on peripheral vascular resistance. Phys Ther. 2001 Jun;81(6):1183-91.

138. Wikström S, Svedman P, Svensson H, Tanweer A. Effect of transcutaneous nerve stimulation on microcirculation in intact skin and blister wounds in healthy volunteers. Scand J Plast Reconstr Surg Hand Surg. 1999 Jun;33(3):195-201.

139. Janssen T, Hopman M. Blood flow response to electrically induced twitch and tetanic lower-limb muscle contractions. Arch Phys Med Rehabil. 2003 Jul;84(7):982-7.

140. Nam T, Choi Y, Yeon D, Leem J, Paik K. Differential antinociceptive effect of transcutaneous electrical stimulation on pain behavior sensitive or insensitive to phentolamine in neuropathic rats. Neurosci Lett. 2001 Mar 23;301(1):17-20.

141. Connon W, Rosenblueth A. The supersensitivity of denervated structures, A law of denervation. New York: The MacMillan Company; 1949.

142. Sharpless S. Supersensitivity-like phenomena in the central nervous system. Fed Proc. 1975 Sep;34(10):1990-7.

143. Kuffler S, Dennis M, Harris A. The development of chemosensitivity in extrasynaptic areas of the neuronal surface after denervation of parasympathetic ganglion cells in the heart of the frog. Proc R Soc Lond B Biol Sci. 1971 Apr 27;177(49):555-63.

144. Spector S. Trophic effects on the contractile and histochemical properties of rat soleus muscle. J Neurosci. 1985 Aug;5(8):2189-96.

145. Purves D, Sakmann B. The effect of contractile activity on fibrillation and extrajunctional acetylcholine-sensitivity in rat muscle maintained in organ culture. J Physiol. 1974 Feb;237(1):157-82.

146. Fumagalli G, Balbi S, Cangiano A, Lømo T. Regulation of turnover and number of acetylcholine receptors at neuromuscular junctions. Neuron. 1990 Apr;4(4):563-9.

147. Sargent P, Pang D. Acetylcholine receptor-like molecules are found in both synaptic and extrasynaptic clusters on the surface of neurons in the frog cardiac ganglion. J Neurosci. 1989 Mar;9(3):1062-72.

148. Brown M, Holland R, Hopkins W, Keynes R. An assessment of the spread of the signal for terminal sprouting within and between muscles. Brain Res. 1981 Apr 6;210(1-2):145-51.

149. Brown M, Ironton R. Motor neurone sprouting induced by prolonged tetrodotoxin block of nerve action potentials. Nature. 1977 Feb 3;265(5593):459-61.

150. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983 Jun;16(2):109-10.

151. Kim J, Min B, Schmidt D, Lee H, Park D. The difference between electroacupuncture only and electroacupuncture with manipulation on analgesia in rats. Neurosci Lett. 2000 Feb 4;279(3):149-52.

152. de Medeiros M, Canteras N, Suchecki D, Mello L. Analgesia and c-Fos expression in the periaqueductal gray induced by electroacupuncture at the Zusanli point in rats. Brain Res. 2003 May 30;973(2):196-204.

153. Chaplan S, Bach F, Pogrel J, Chung J, Yaksh T. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994 Jul;53(1):55-63.

154. Baliki M, Calvo O, Chialvo D, Apkarian A. Spared nerve injury rats exhibit thermal hyperalgesia on an automated operant dynamic thermal escape Task. Mol Pain. 2005 May 26;1(1):18.

155. Tal M, Bennett G. Extra-territorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. Pain. 1994 Jun;57(3):375-82.

156. Woodbury C, Ritter A, Koerber H. On the problem of lamination in the superficial dorsal horn of mammals: a reappraisal of the substantia gelatinosa in postnatal life. J Comp Neurol. 2000 Jan 31;417(1):88-102.

157. Wilcox C, Dove S, McDavid W, Greer D. UTHSCSA ImageTool 3.00 ed. San Antonio, Texas: The University of Texas Health Science Center; 2002.

158. Imbe H, Okamoto K, Kadoya T, Horie H, Senba E. Galectin-1 is involved in the potentiation of neuropathic pain in the dorsal horn. Brain Res. 2003 Dec 12;993(1-2):72-83.

159. Sindrup S, Jensen T. Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. Pain. 1999 Dec;83(3):389-400.

160. Kingery W, Lu J, Roffers J, Kell D. The resolution of neuropathic hyperalgesia following motor and sensory functional recovery in sciatic axonotmetic mononeuropathies. Pain. 1994 Aug;58(2):157-68.

161. El K, Hawwa N, Baliki M, Atweh S, Jabbur S, Saade N. Attenuation of neuropathic pain by segmental and supraspinal activation of the dorsal column system in awake rats. Neuroscience. 2002;112(3):541-53.

162. McMahon S. Neuropathic pain mechanisms, in: M.A. Giamberardino (Ed.), Pain 2002-An Updated Review: Refresher Course Syllabus. Seattle: IASP Press; 2002.

163. Ossipov M, Lai J, Malan Jr. T, Porreca F. Spinal and supraspinal mechanisms of neuropathic pain. Ann NY Acad Sci. 2000;909:12-24.

164. Cameron AA, Cliffer KD, Dougherty PM, Willis WD, Carlton SM. Changes in lectin, GAP-43 and neuropeptide staining in the rat superficial dorsal horn following experimental peripheral neuropathy. Neurosci Lett. 1992;131:249–52.

165. Cameron AA, Cliffer KD, Dougherty PM, Garrison CJ, Willis WD, Carlton SM. Time course of degenerative and regenerative changes in the dorsal horn in a rat model of peripheral neuropathy. J Comp Neurol. 1997;379:428– 42.

166. Duggan AW, Hendry IA. Laminar localization of the sites of release of immunoreactive substance P in the dorsal horn with antibodycoated microelectrodes. Neurosci Lett. 1986;68:134– 40.

167. Gautreau A, Kerdelhué B. Simultaneous quantitation of substance P-encoding preprotachykinin alternatively spliced mRNAs and substance P

receptor NK-1 mRNA by an RNase protection assay. Brain Res Brain Res Protoc. 1998 Jan;2(2):133-40.

168. Coggeshall R, La Forte R, Klein C. Calibration of methods for determining numbers of dorsal root ganglion cells. J Neurosci Methods. 1990 Dec;35(3):187-94.

169. Blomqvist A, Craig A. Is neuropathic pain caused by the activation of nociceptive-specific neurons due to anatomic sprouting in the dorsal horn? J Comp Neurol. 2000 Dec 4;428(1):1-4.

170. Jessell T, Tsunoo A, Kanazawa I, Otsuka M. Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. Brain Res. 1979 May 25;168(2):247-59.

171. Ma W, Bisby M. Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. Eur J Neurosci. 1998 Jul;10(7):2388-99.

172. Laird J, Bennett G. An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy. J Neurophysiol. 1993 Jun;69(6):2072-85.

173. Bester H, Beggs S, Woolf C. Changes in tactile stimuli-induced behavior and c-Fos expression in the superficial dorsal horn and in parabrachial nuclei after sciatic nerve crush. J Comp Neurol. 2000 Dec 4;428(1):45-61.

174. Hu P, McLachlan E. Long-term changes in the distribution of galanin in dorsal root ganglia after sciatic or spinal nerve transection in rats Neuroscience 2001 Apr 4;103(4):1059-71

175. Campbell J. Nerve lesions and the generation of pain. Muscle Nerve 2001 ;24(10):. 2001 Oct;24(10):1261-73.

176. Wu G, Ringkamp M, Murinson B, Pogatzki E, Hartke T, Weerahandi H, et al. Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents. J Neurosci. 2002 Sep 1;22(17):7746-53.

APPENDIX

APPENDIX A
RAW DATA

Table A1 Weight of animal models: sham group

Day	Weight of sham group (grams)												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
-1	202	209	212	206	204	209	202	216	211	207	204	214	203
0	205	210	209	207	210	210	209	216	210	208	205	214	204
8	261	269	263	257	262	259	262	258	264	268	268	257	260
15	317	326	313	326	315	316	313	318	313	313	320	315	308
23	344	332	339	332	339	334	332	339	344	338	332	338	341
32	353	358	346	351	347	346	351	358	347	352	354	358	347
35	352	347	351	354	350	354	361	350	354	361	350	352	347
38	351	350	354	354	354	353	347	353	347	352	352	355	362

Table A2 Weight of animal models: sham-ES group

Day	Weight of sham-ES group (grams)												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
-1	210	201	211	207	198	209	202	211	211	209	204	213	206
0	210	203	210	209	199	209	205	210	213	209	207	214	207
8	262	257	260	268	259	264	268	268	267	261	269	263	262
15	313	320	319	315	312	313	318	322	317	326	315	321	325
16	312	320	320	315	314	312	317	320	315	325	315	322	325
17	317	323	322	317	316	313	317	322	315	328	317	322	325
18	318	325	324	318	316	315	318	324	316	328	319	324	325
19	310	327	329	317	320	317	322	325	317	329	321	325	327
23	332	339	344	336	341	335	341	344	332	339	336	340	335
25	338	341	345	338	342	338	343	345	336	342	340	342	337
29	350	343	346	341	343	342	345	346	339	344	342	343	340
31	358	347	351	342	343	346	345	349	343	347	341	345	343
32	358	347	352	342	342	347	346	351	345	349	342	347	345
35	361	350	354	343	344	350	347	352	347	351	344	350	349
38	362	354	353	347	346	353	352	359	349	353	347	352	352

Table A3 Weight of animal models: SNI group

Day	Weight of SNI group (grams)												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
-1	211	211	204	213	206	204	212	206	204	207	201	211	208
0	210	212	210	209	207	203	210	209	203	208	203	210	209
8	262	257	260	267	261	269	257	262	257	260	260	268	253
15	326	313	322	326	326	313	322	315	313	322	326	321	325
23	344	332	332	338	338	332	339	344	338	339	344	338	341
32	352	346	338	347	346	346	351	347	346	351	347	346	354
35	354	349	347	351	351	354	351	354	350	354	350	354	361
38	358	350	350	354	356	355	352	352	352	355	355	353	362

Table A4 Weight of animal models: SNI-ES group

Day	Weight of SNI-ES group (grams)												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
-1	199	202	208	206	206	204	207	209	211	204	202	208	208
0	203	203	210	209	210	203	210	209	209	207	203	210	209
8	260	268	260	268	257	260	268	268	267	261	262	257	260
15	326	314	321	326	312	319	321	322	326	317	318	322	315
16	327	314	319	328	314	312	320	324	325	312	321	327	316
17	330	316	322	327	316	313	323	326	326	318	323	328	315
18	333	318	324	330	318	318	325	327	328	320	325	329	318
19	332	320	329	331	320	317	322	325	332	321	328	325	323
23	336	336	341	321	321	320	329	329	334	329	332	327	328
25	339	337	345	339	337	324	339	334	337	334	335	333	333
29	344	343	349	345	344	328	345	336	340	337	337	336	340
31	349	345	352	348	345	329	346	342	343	339	341	339	345
32	350	347	357	347	348	335	349	346	342	342	342	342	348
35	354	350	357	354	350	338	349	354	345	344	344	348	349
38	357	354	361	353	352	343	352	357	349	347	347	352	352

APPENDIX B

IMMUNOFLUORESCENT PROTOCOL FOR SP AND CTB

A. Solutions and reagents

1. 0.2 M sodium phosphate buffer, pH 7.4

500 ml 0.2 M sodium monophosphate ($Na_2HPO_4.12H_2O$)

Adjust with solution of 0.2 M sodium biphosphate ($NaH_2PO_4.2H_2O$) to pH 7.4.

2. Sodium phosphate buffer saline (PBS), pH 7.4

5.92 g $NaH_2PO_4.2H_2O$

58.00 g $Na_2HPO_4.12H_2O$

18.00 g NaCl

Make to 2 litres with distilled water. Adjust pH to 7.4.

3. 60% sucrose in distilled water

30.00 g Sucrose

Make to 50 ml with distilled water.

4. 30% sucrose in 0.1 M phosphate buffer

50 ml 0.2 M phosphate buffer, pH 7.4

50 ml 60% sucrose in distilled water

Mix and store at 4^oC.

5. 1% BSA and 0.05% Tween-20 in PBS

1.00 g BSA

50 microlitres Tween-20

950 microlitres PBS, pH 7.4

Mix and store at 4^oC.

6. Primary antibody (goat polyclonal antibody to substance P: Santa Crus Biotechnology) at 1:50 dilutions

40 microlitres goat polyclonal antibody to substance P
960 microlitres PBS, pH 7.4

Vortex and store at 4°C.

7. Primary antibody (rabbit anti-cholera toxin B: Sigma) at 1:1000 dilutions

10 microlitres rabbit anti-cholera toxin B
990 microlitres PBS, pH 7.4

Vortex and store at 4°C.

8. Secondary antibody (Donkey anti-goat IgG Fluorescein conjugate: Santa Crus Biotechnology) at 1:500 dilutions

100 microlitres Donkey anti-goat IgG Fluorescein conjugate
100 microlitres PBS, pH 7.4

Vortex and store at 4°C.

9. Secondary antibody (Texas red anti-rabbit IgG: Vector laboratories) at 1.5% dilutions

10 microlitres Texas Red anti-rabbit IgG
990 microlitres PBS, pH 7.4

Vortex and store at 4°C.

B. Immunofluorescent staining process

After perfusion and sample collection.

1. Fixation

- Post-fix in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 4°C, overnight.
- Soak in 30% sucrose, at 4°C, overnight.

2. Cryostat sectioning

- Mount sample in optimal cutting Temperature compound (OCT).
- Cryostat serial coronal sectioning, 40 and 20 micrometres thickness of spinal dorsal horn and DRG, respectively.

- Mount sections on gelatin-coated slides and store at 4^0C until use.

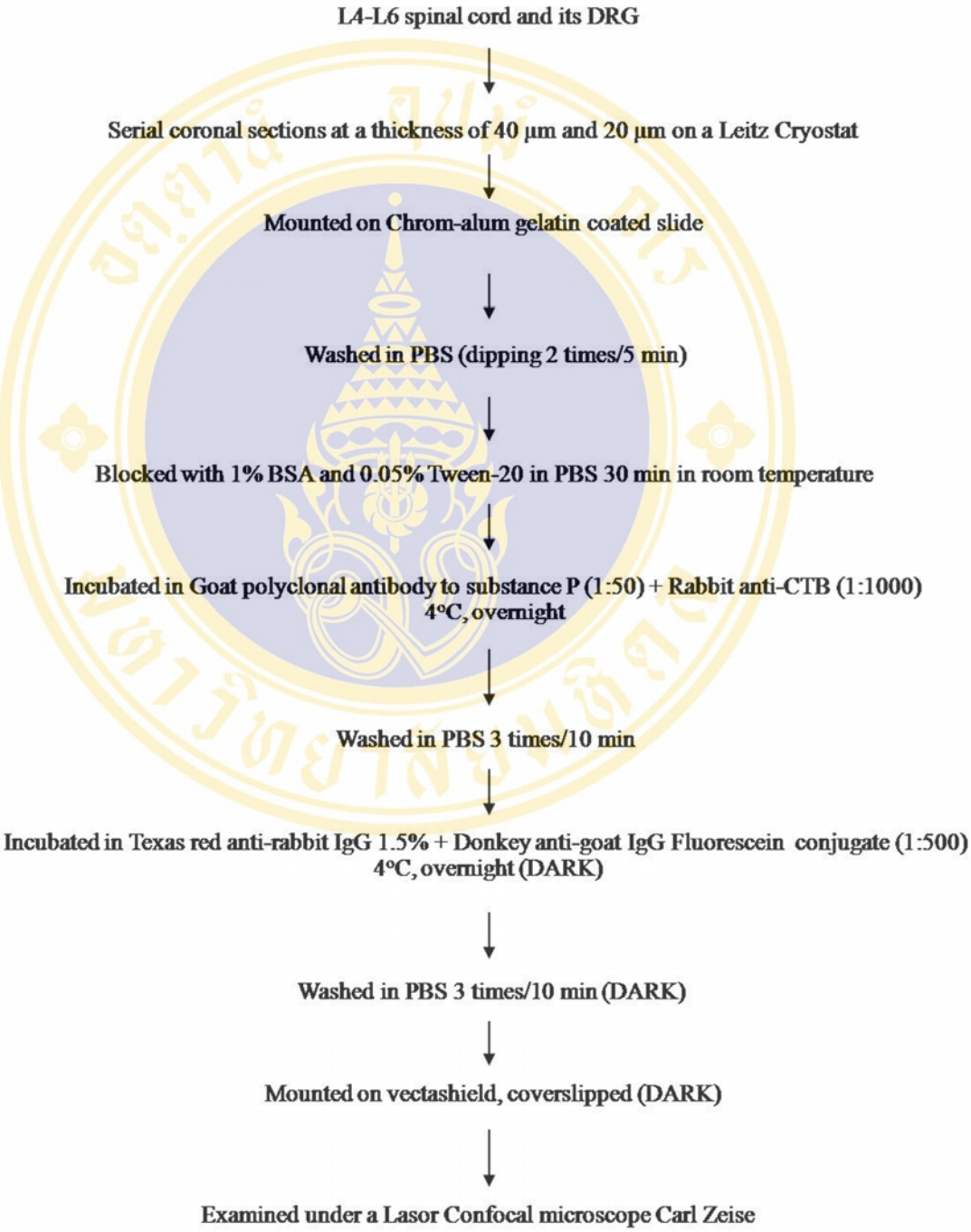
3. Blocking

- Wash slides 2 times, 5 minutes each with PBS at room temperature.
- Block with 1% BSA and 0.05 Tween-20 in PBS for 30 minutes at room temperature.

4. Staining

- Incubate with primary antibody at 1:50 dilution of goat polyclonal antibody to substance P and 1:1000 dilution of rabbit anti-cholera toxin B in PBS in the moist chamber at 4^oC, overnight.
- Wash slides 3 times, 10 minutes each with PBS.
- Incubate with secondary antibody at 1:500 dilution of donkey anti-goat IgG fluorescein conjugate and 1.5% dilution of Texas red-rabbit IgG in PBS in the dark moist chamber at 4^oC, overnight.
- Wash slide 3 times, 10 minutes each with PBS in dark room.
- Air dry, mount and cover slip in dark room.
- Lasor Confocal microscope examination.

Protocol of immunofluorescence



BIOGRAPHY

NAME	Miss Kanyarat Bamrungsuk
DATE OF BIRTH	7 February 1983
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Huachiew Chalermprakiet University, 2004: Bachelor of Physical therapy Mahidol University, 2007: Master of Science (Anatomy)
HOME ADDRESS	108/20 Isaraphab Road, Bangkok Yai, Bangkok, 10600, Thailand E-mail: g4736154@student.mahidol.ac.th
RESEARCH GRANT	Siriraj Graduate Scholarship, 2004