ELECTROPHYSIOLOGICAL, IMMUNOHISTOCHEMICAL AND PHARMACOLOGICAL STUDIES ON AN ANIMAL MODEL OF PERIPHERAL NEUROPATHY INDICATE A PROMINENT ROLE OF Aβ SENSORY NEURONS IN NEUROPATHIC PAIN

By

YONG FANG ZHU

B.Sc, M.Sc

A Thesis

Submitted to the school of Graduate Studies

In Partial Fulfillment of the Requirements

For the degree

Doctor of Philosophy

McMaster University

Doctorate of Philosophy (2011) McMaster University

(Neuroscience and Behavioral Sciences) Hamilton, Ontario

TITLE: ELECTROPHYSIOLOGICAL, IMMUNOHISTOCHEMICAL AND PHARMACOLOGICAL STUDIES ON AN ANIMAL MODEL OF PERIPHERAL NEUROPATHY INDICATE A PROMINENT ROLE OF Aβ SENSORY NEURONS IN NEUROPATHIC PAIN

AUTHOR: Yong Fang Zhu, B.Sc. (Nanjing Normal University)

M.Sc. (East China Normal University)

M.Sc. (McMaster University)

SUPERVISOR: Professor James, L. Henry

NUMBER OF PAGES: xiii, 192

ABSTRACT

Based on the concept that the tactile hypersensitivity and the central sensitization observed in animal models of peripheral neuropathy are maintained by peripheral drive from primary sensory neurons, the present project measured the changes in electrophysiological, immunohistochemical, and pharmacological properties of the dorsal root ganglia (DRG) neurons induced by a peripheral neuropathy. The aim of this study was to make a systematic survey and a unique understanding of changes that occur in primary sensory neurons that can sustain peripheral drive in this model. The data of this study indicate a prominent role of large diameter Aβ-fibers, including low threshold mechanoreceptors in peripheral neuropathy.

ACKNOWLEDGEMENTS

Many sincere thanks go to my supervisor, Dr. James, L. Henry, for his scientific guidance and his spiritual support. I would especially like to express my appreciation for his provision of an environment for growth. I would also like to thank my supervisory committees, including Dr. Alexander K Ball, Dr. Jan Huizinga and Dr. Wolfgang Kunze. I am grateful to Dr. Ball for supervising the immunohistochemical part of this project. I call myself a lucky person because I had a committee that allowed me to work freely and always stimulated intellectual thinking throughout my project.

My work would not have been as enjoyable without the friendship and help of my labmates, both past and present. Dr. Kiran Yashpal, who created a warm atmosphere in working place and Qi Wu, whom I consider to be a brilliant person, taught me a lot of lab skills and tricks. Liliane Dableh, whom I shared the behavior test experience and Yu Fang Wang, who helped me collecting immunohistochemical data, gave me a lot of helps for my project. Those good times we shared will be remembered forever.

Finally, I would like to thank enormously my dear husband Zhi Jun, who has been with me all along. He has been there in my failures and triumphs, always backing me up, giving me comfort, love and support. Special thanks for his effort in helping me to set up my system bench. I also want to thank my two lovely sons Alan and Peter, who make me laugh and enjoy life together with my husband. They gave me the strength to continue my study.

I dedicate this thesis work to all the above people, for you gave me the opportunity to be the best of me. This thesis is also dedicated HIM. Without you this could not have been possible.

PREFACE

This thesis is a “sandwich” style thesis. Chapter 1 is the general introduction and objective, and Chapter 7 is the general discussion and conclusion. Chapters 2, 3, 4, 5, 6, 7 have been or will be submitted for publication as peer-reviewed research papers, and are currently in the review process.

Preface of each chapter describes the details of the submitted articles, as well as my contribution to the multiple-authored work.

All chapters have been reproduced with permission of all co-authors. Irrevocable, non-exclusive licenses have been granted to McMaster University and to the National Library of Canada from all publishers. Copies of permission and licenses have been submitted to the School of Graduate Studies.

TABLE OF CONTENTS

DESCRIPTIVE NOTE……...ii

ABSTRACT……...iii

ACKNOWLEDGEMENT……....iv

PREFACE ……..……..vi

TABLE OF CONTENTS……...……....vii

LIST OF TABLES……..……ix

LIST OF FIGURES……..……...x

LIST OF ABBREVIATION……..……....xii

CHAPTER 1. General introduction and objective……..……1
Reference list……..……..……9

CHAPTER 2. Changes in functional properties of A-type but not C-type sensory DRG neurons in vivo in a rat model of peripheral neuropathy…….....14
Preface……..……15
Abstract……..……..16
2.1. Introduction……..….16
2.2. Methods……....…18
2.3. Results……..….24
2.4. Discussion…….....37
2.5. Conclusions…….……..…45
2.6. Tables and Figures……..…...45
Reference list……...…….....55

CHAPTER 3. Excitability of Aβ sensory neurons is altered in an animal model of peripheral neuropathy ……..….....….61
Preface……..……62
Abstract……..……..…63
3.1. Introduction……..…….63
3.2. Methods……..……...65
3.3. Results……..……72
3.4. Discussion……..……...78
3.5. Conclusions…….....83
3.6. Tables and figures…….....84
Reference list…….…….....……....96

CHAPTER 4. Substance P is expressed in Aβ low-threshold mechanoreceptor neurons in an animal model of peripheral neuropathy……..……...….100
Preface……..……..101
Abstract……..……102
4.1. Introduction……..……...102
4.2. Methods..……..……...105
4.3. Results……...……..112
4.4. Discussion……..……114
4.5. Conclusion...……..……..118
4.6. Tables and Figures…….....118
Reference list...…..……..……129

CHAPTER 5. Pregabalin reduces action potential amplitude in sensory neruons in control and neuropathic rats……..……133
Preface……..……..134
Abstract……..……...…….135
5.1. Introduction……..……...135
5.2. Methods..……..……..137
5.3. Results……..…….……..142
5.4. Discussion……..…….....145
5.5. Conclusion……...……148
5.6. Tables and Figures……...……...…….149
References…….…….……159

CHAPTER 6. Pregabalin: early daily treatment induces persisting antinociception in a rat model of peripheral neuropathic pain …….....162
Preface……...…….163
Abstract……...…..…….164
6.1. Introduction…….....……..164
6.2. Methods..……...……166
6.3. Results……....…….168
6.4. Discussion……..……....172
6.5. Conclusions..……...……175
6.6. Tables and Figures……...…175
Reference list…….……..…….....180

CHAPTER 7. General discussion and conclusion……....183
Reference list……..……....190

LIST OF TABLES

CHAPTER 2

Table 2.1 Comparison of properties of high-threshold mechanoreceptive and low-threshold mechanoreceptive DRG neurons between control and neuropathic rats.....46

Table 2.2 Locations of receptive fields of high-threshold mechanoreceptors and low-threshold mechanoreceptors in both the control and neuropathic rats…….....…..48

CHAPTER 4

Table 4.1. Substance P and identified DRG neurons……..…….....119

Table 4.2. Distribution of SP expression in different size neurons……....120

CHAPTER 5

Table 5.1 Effects of pregabalin on electrophysiological parameters in control and neuropathic DRG sensory neurons…….....150

CHAPTER 6

Table 6.1 Weekly comparison of mean tactile threshold and P value of each group176

Table 6.2 Specific comparisons of antinociceptive effects of i.p. administered pregabalin with early treatment vs. late treatment; with single phase vs. multiple phase treatment; and also with acute vs. long-term treatment.....177

LIST OF FIGURES

CHAPTER 2

Figure 2.1 Examples of APs recorded from mechanoreceptive neurons…….....49

Figure 2.2 Comparison of AP resting membrane potential and amplitude of DRG neurons between control and neuropathic rats…….....50

Figure 2.3 Comparison of AP dynamic parameters of DRG neurons between control and neuropathic rats…….....51

Figure 2.4 Comparison of AHP variables of DRG neurons between control and neuropathic rats…….....53

Figure 2.5 Comparison of dorsal root conduction velocity of DRG neurons between control and neuropathic rats…….....54

CHAPTER 3

Figure 3.1 Comparison of 50% withdrawal threshold between control and neuropathic rats…….....…..85

Figure 3.2 Comparison of the mechanical response threshold of the Aβ-fiber sensory neurons in control and neuropathic rats.....86

Figure 3.3 Ongoing discharge in neuropathic rats…….....88

Figure 3.4 Comparison of the activation threshold of Aβ-fiber neurons in response to intracellular current injection, between control and neuropathic rats……..…….....89

Figure 3.5 A comparison the repetitive discharge characteristics of DRG cells produced by intracellular current injection……..90

Figure 3.6 Comparison of current activation threshold of Aβ-fiber neurons in response to stimulation of the dorsal roots, between control and neuropathic rats…….....92

Figure 3.7 Comparison of the repetitive discharge characteristics of DRG cells evoked by dorsal root stimulation between control and neuropathic rats…….....93

Figure 3.8 Comparison of APs evoked by dorsal root paired-pulse stimulation and by soma current injection 95

CHAPTER 4

Figure 4.1 Localization of substance P in identified DRG neurons. …….121

Figure 4.2 Control and neuropathic rats differ in the expression of SP by muscle spindle neurons..... …….....…….....……..123

Figure 4.3 Quantification of SP in identified control and neuropathic DRG neurons.....124

Figure 4.4 Cell sizes and SP in control and neuropathic DRG neurons..... 126

Figure 4.5 Localization of SP expression in serial DRG sections..... 128

CHAPTER 5

Figure 5.1 Mechanical withdrawal thresholds in the von Frey test after i.v. administration of 5, 1 and 0 mg/kg of pregabalin, at various times following administration 152

Figure 5.2 Effects of i.v. administration of pregabalin on electrophysiological properties of Aβ-fiber LTM cutaneous neurons…….....……...153

Figure 5.3 Effects of i.v. administration of pregabalin on electrophysiological properties of Aβ-fiber low muscle spindle neurons…….....……154

Figure 5.4 Example to show pregabalin effect ongoing discharge Aβ-fiber muscle spindle neurons.....155

Figure 5.5 Examples of intracellular recordings illustrating the somatic APs by different peripheral site stimulations from an Aβ-fiber muscle spindle neuron before and 15 min after pregabalin administration in a neuroapthic rat …….....156

Figure 5.6 Examples of intracellular recordings illustrating effects pregabalin administration on different types of HTM neurons …….....…157

CHAPTER 6

Figure 6.1 Comparison of the antinociceptive effects of i.p. administered pregabalin in the Mosconi and Kruger animal model of peripheral neuropathic pain, comparing early treatment vs. late treatment …….....……178

LIST OF ABBREVIATIONS

AHP: afterhyperpolarization

AHPA: afterhyperpolarization amplitude

AHP50: afterhyperpolarization duration to 50% recovery

AP: action potential

APA: action potential amplitude

APdB: action potential duration at base

APDF: action potential fall time

APDR: action potential rise time

CCD: chronic compression of DRG

CCI: chronic constriction injury of sciatic nerve

CGRP: calcitonin gene related peptide

CNS: central nerve system

CUT: cutaneous mechanoreceptor;

CV: conduction velocity

DRG: dorsal root ganglia

GDNF: glial cell derived neurotrophic factor

GF: guard/field neuron

HTM: high-threshold mechanoreceptor

i.p. intraperitoneally

i.v. intravenously

LTM: low-threshold mechanoreceptor

MFR: maximum AP falling rate

MRR: maximum AP rising rate

MS: muscle spindle neuron

Nav: voltage-gated sodium channel

NeP: neuropathy model

NT-3: neurotrophin-3

NGF: nerve growth factor

PDR: Partial dorsal rhizotomy

PE: polyethylene

PB: phosphate buffer

RA: rapidly adapting neuron

RI: refractory interval

SA: slowly adapting neuron

SG: substantia gelatinosa

SP: substance P

TNF: tumor necrosis factor

TTX: Tetrodotoxin

VGCC: voltage-gated calcium channel

Vm: resting membrane potential

WDR: wide dynamic range

CHAPTER 1

General introduction and objective

The International Association for the Study of Pain (IASP) defines neuropathic pain as "Pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey and Bogduk, 1994). In contrast to nociceptive pain, neuropathic pain may be exaggerated (hyperalgesia), provoked by innocuous stimulation (allodynia), and/or occurs spontaneously (Devor, 1983; Richards, 1967). Currently, the mechanism and treatment of neuropathic pain are not fully understood (Finnerup et al., 2007; Hawksley, 2006). The mechanisms underlying neuropathic pain are complex and may involve several independent pathophysiologic mechanisms in both the peripheral and central nervous systems (Campbell and Meyer, 2006) and may include a large variety of changes in components such as transduction, conduction, transmission, modulation and perception in sensory processing in pain pathways.

An overview of the present mechanisms of neuropathic pain was described under the headings of ectopic discharge, sensitization of sensory terminals, cross excitation, neurotransmitter change, coupling between the sympathetic and sensory nervous systems, spinal cord reorganization, spinal cord increased excitability, spinal cord decreased inhibition, supraspinal influences (Saxby Pridmore, 2002). These mechanisms increase our understanding of neuropathic pain and open the door for therapeutic advances in this field. However, one of the essential questions regarding neuropathic pain mechanisms is the determination of the relative contributions by peripheral vs. central nervous systems. Several hypotheses have been proposed in neuropathy. One view suggests that changes within the central nervous system specifically in the spinal dorsal horn (Moore et al., 2002; Coull et al., 2003; Sotgiu and Biella, 2000) alone mediate neuropathic pain (Sandkuhler and Liu, 1998).

Another view suggests that mechanisms at the spinal dorsal horn level that maintain neuropathic pain require only facilitatory input from supraspinal structures (Bian et al., 1998; Pertovaara et al., 1997; Porreca et al., 2001; Carlson et al., 2007; Kauppila et al., 2002; Kovelowski et al., 2000; Saade et al., 2006b; Saade et al., 2006a; Suzuki et al., 2004; Gardell et al., 2003; Ossipov et al., 2000); Contrary to the notion that predominantly central mechanisms are the basis of neuropathic pain, studies of afferent sensory pathways in models of peripheral neuropathy have revealed several different possibilities. Evidence exists that pain following peripheral nerve injury is maintained by ectopic activity in primary sensory neurons and this activity of neurons is thought to arise from the dorsal root ganglion (DRG), along the axons or in the peripheral nerve terminals (Devor, 2006; Liu et al., 2000; Amaya et al., 2006; Yang et al., 2005; Katz and Gold, 2006; Schaible, 2007). Constant barrage of synaptic activity from primary afferents is thought to induce or maintain central sensitization observed in animal models of neuropathic pain (Lang et al., 2006; Campbell and Meyer, 2006). Therefore, it is important in understanding neuropathic pain to understand the properties of primary sensory neurons and how they adapt to or change in response to nerve injury.

Our earlier studies on the Mosconi and Kruger animal model (Mosconi and Kruger, 1996) of neuropathic pain show that activation of Aβ-fiber sensory neurons including Aβ-fiber low-threshold mechanoreceptor (LTM) neurons by low-threshold, innocuous mechanical stimulation of the peripheral receptive fields would elicit a nociceptive type of response of dorsal horn neurons, specifically a response with an afterdischarge, which normally is observed only in response to noxious stimulation. These data implicated Aβ-fiber sensory neurons input in contributing to central

hyperexcitability and ultimately neuropathic pain. To our knowledge, there is presently no direct evidence regarding the effects of experimental peripheral neuropathy on functionally identified Aβ-fiber associated neurons at the level of dorsal root ganglion (DRG). Therefore, the present study was done with this objective.

Based on our hypothesis that induction of the model by inserting a polyethylene cuff around the sciatic nerve induces changes in functional and structural properties of DRG neurons, this study focused on these primary sensory neurons using intracellular recording *in vivo* on the intracellular electrophysiological, immunohistochemical, pharmacological properties of DRG neurons in the Mosconi and Kruger model.

Aim 1: To measure effects of sciatic nerve cuff on DRG neuron electrophysiological properties

DRG neurons convey somato sensory information as action potentials (APs) to the central nerve system (CNS) (Fang et al., 2005). In order to compare the configuration of the APs of fully characterized DRG neurons in the neuropathic rats vs. control rats, intracellular electrophysiological recording experiments were done in vivo. This recording method and approach enables unambiguous identification of the functional types of sensory neuron recorded. So far the few intracellular studies in neuropathic rat DRGs that have studied the relationship between somatic AP variables, including sensory properties in vivo, were limited to C-fiber sensory neurons with no study to our knowledge on functional subtypes of any high-threshold mechanoreceptor (HTM) or low-threshold mechanoreceptor (LTM) neurons within any conduct velocity (CV) groups.

We therefore used intracellular recordings from the somata of DRG neurons in vivo in control rats and in neuropathic pain model to examine and compare mechanosensitive neuron types to determine whether there are: (1) differences in somatic AP configuration, which will reflect differences in different membrane channel function, expression and distribution; (2) changes in the CV, which would be expected if there are changes in axon function, possibly due to changes in ion channel distribution or in myelination (Djouhri and Lawson, 2001); (3) relationship between AP configuration and response to receptive field stimulation, which uniquely reflects the threshold of activation in defined neuron; (4) differences in spike shape and frequency to varied single pulse stimulation, paired-pulse stimulation, which are used to measure neuron excitability, as well as relative and absolute refractory periods; (5) correlation between different sites of stimulation and APs, which reveal the site of abnormal ectopic action potential generation.

Such knowledge of the normal electrophysiological properties of DRG neurons is important as a basis for understanding any changes in their properties in neuropathic pain. Finally, selected neurons revealed to have significant changes have been injected intracellularly with neurobiotin. Thus, functionally-identified neurons *in vivo* received further immunohistochemical analysis (the second aim) *in vitro*.

Aim 2: To determine expression of substance P in functionally-identified DRG neuron types

Substance P (SP) is an important peptide responsible for nociceptive transmission from the peripheral to the central nervous system (Henry, 1976). It is

released from nociceptive primary sensory neurons in response to intense and persistent stimulation (Lawson et al., 1997).

SP and tachykinin NK-1 receptors have been implicated in animal models of neuropathic pain (Pitcher and Henry, 2004). Other evidence has shown that SP mRNA in DRG following nerve injury is unregulated (Noguchi et al., 1995). SP binding in dorsal horn after nerve injury is increased (Aanonsen et al., 1992). An NK-1 receptor antagonist attenuates allodynia and hyperalgesia associated with sciatic nerve cuff (Cahill and Coderre, 2002). Although some studies have reported that NK-1 receptor antagonists have failed to relieve clinical pain (Hill, 2000; Urban and Fox, 2000; Cahill and Coderre, 2002; Sindrup et al., 2006), given changes in SP levels in DRGs in humans with peripheral neuropathy (Batbayar et al., 2004) and in animal models of neuropathic pain (Hofmann et al., 2003; Uehara et al., 2004; Swamydas et al., 2004), SP may play other roles, e.g. in changes in spinal processing of nociceptive information (Wallin and Schott, 2002) in peripheral tissue health or in sensitivity of peripheral terminals (Jang et al., 2004; Gibran et al., 2002).

Following a spinal nerve lesion, increased SP immunoreactivity was identified in the lager myelinated fibers in the dorsal root (Noguchi et al., 1995). More recently, Malcangio showed that a low-intensity electrical stimulus (only able to activate the Aβ-fiber sensory neurons) applied to the dorsal root induced the aberrant release of SP in the spinal cord weeks after the spinal nerve lesion. This event was considered the consequence of the *de novo* expression of SP in Aβ-fiber sensory neurons (Malcangio et al., 2000). To date, though, there has been no study of SP distribution in functionally-classified DGR neurons in vivo. Therefore, neurobiotin-filled,

functionally-identified neurons were counterstained for SP immunoreactive material to gain information on a cross-section of neuron types but also to focus on large-diameter neurons that normally do not express SP in order to determine the percentage of the population that begins to express SP after sciatic nerve injury.

Aim 3: To determine the effects of pregabalin on the changes in response properties of DRG neurons

Clinically, neuropathic pain is resistant to conventional treatment. However, pregabalin (Lyrica) has a broad analgesic effect on mechanical and thermal hypersensitivity associated with nerve injury (Field et al., 1999; Field et al., 1997). The upregulation of the spinal dorsal horn calcium channel α2δ subunit contributes to peripheral nerve injury-induced tactile allodynia. Basal expression of this subunit may occur presynaptically and postsynaptically in the spinal cords and nerve injury induces mainly presynaptic expression (Han et al., 2007). Pregabalin is thought to express its effect via an action on the auxiliary α2δ subunit of the voltage-gated calcium channel (VGCC) (Dooley et al., 2007; Field et al., 1997; Han et al., 2007; Taylor et al., 2007) and although there seems to be reasonable evidence implicate other possible mechanisms as well (Lynch, et al., 2004), it seems to be even more reasonable to assume that inhibition of the subunit of the VGCC is a paramount action. Pregabalin has been proposed to reduce the depolarization-induced calcium influx at nerve terminals, resulting in a reduction of the presynaptic release of excitatory neurotransmitters, including glutamate, noradrenalin, SP and calcitonin gene-related peptide (CGRP) (Fink et al., 2000; Han et al., 2007). Therefore, pregabalin binding to

the α2δ subunit might be effective at blocking hyperalgesia and allodynia in a rat model of peripheral neuropathy (Han et al., 2007).

While it is inherent in theories regarding the mechanisms of the analgesic action of pregabalin that its site of the action is on the central terminals of sensory neurons in the spinal cord and brain stem (Dooley et al., 2007; Taylor et al., 2007), this may not be exclusively the case. The present project focused on the possibility that there is a peripheral site of action of pregabalin. Thus, it is hypothesized that pregabalin acting in the periphery might affect the activity of peripheral neurons. In a parallel study, functionally-defined neurons would be studied using the same protocol as in the first aim, comparing neuronal properties in control and neuropathic rats before and after pregabalin injection,

Reference List

Aanonsen LM, Kajander KC, Bennett GJ, Seybold VS, 1992. Autoradiographic analysis of 125I-substance P binding in rat spinal cord following chronic constriction injury of the sciatic nerve1. Brain Res. 596: 259-268.

Amaya F, Wang H, Costigan M, Allchorne AJ, Hatcher JP, Egerton J, Stean T, Morisset V, Grose D, Gunthorpe MJ, Chessell IP, Tate S, Green PJ, Woolf CJ, 2006. The voltage-gated sodium channel Na(v)1.9 is an effector of peripheral inflammatory pain hypersensitivity. J. Neurosci. 26: 12852-12860.

Batbayar B, Nagy G, Kovesi G, Zelles T, Feher E, 2004. Morphological basis of sensory neuropathy and neuroimmunomodulation in minor salivary glands of patients with Sjogren's syndrome . Arch. Oral Biol. 49: 529-538.

Bian D, Ossipov MH, Zhong C, Malan TP, Jr., Porreca F, 1998. Tactile allodynia, but not thermal hyperalgesia, of the hindlimbs is blocked by spinal transection in rats with nerve injury. Neurosci. Lett. 241: 79-82.

Cahill CM, Coderre TJ, 2002. Attenuation of hyperalgesia in a rat model of neuropathic pain after intrathecal pre- or post-treatment with a neurokinin-1 antagonist. Pain 95: 277-285.

Campbell JN, Meyer RA, 2006. Mechanisms of neuropathic pain. Neuron 52: 77-92.

Carlson JD, Maire JJ, Martenson ME, Heinricher MM, 2007. Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. J. Neurosci. 27: 13222-13231.

Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De KP, De KY, 2003. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424: 938-942.

Devor M, 2006. Sodium channels and mechanisms of neuropathic pain. J. Pain 7: S3-S12.

Devor M, 1983. Nerve pathophysiology and mechanisms of pain in causalgia. J. Auton. Nerv. Syst. 7: 371-384.

Djouhri L, Lawson SN, 2001. Increased conduction velocity of nociceptive primary afferent neurons during unilateral hindlimb inflammation in the anaesthetised guinea-pig. Neuroscience 102: 669-679.

Dooley DJ, Taylor CP, Donevan S, Feltner D, 2007. Ca2+ channel alpha2delta ligands: novel modulators of neurotransmission . Trends Pharmacol. Sci. 28: 75-82.

Fang X, McMullan S, Lawson SN, Djouhri L, 2005. Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. J. Physiol 565: 927-943.

Field MJ, McCleary S, Hughes J, Singh L, 1999. Gabapentin and pregabalin, but not morphine and amitriptyline, block both static and dynamic components of mechanical allodynia induced by streptozocin in the rat. Pain 80: 391-398.

Field MJ, Oles RJ, Lewis AS, McCleary S, Hughes J, Singh L, 1997. Gabapentin (neurontin) and S-(+)-3-isobutylgaba represent a novel class of selective antihyperalgesic agents. Br. J. Pharmacol. 121: 1513-1522.

Fink K, Meder W, Dooley DJ, Gothert M, 2000. Inhibition of neuronal Ca(2+) influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. Br. J. Pharmacol. 130: 900-906.

Finnerup NB, Sindrup SH, Jensen TS, 2007. Chronic neuropathic pain: mechanisms, drug targets and measurement. Fundam. Clin. Pharmacol. 21: 129-136.

Gardell LR, Vanderah TW, Gardell SE, Wang R, Ossipov MH, Lai J, Porreca F, 2003. Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation. J. Neurosci. 23: 8370-8379.

Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, Ansel JC, Olerud JE, 2002. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. J. Surg. Res. 108: 122-128.

Han DW, Kweon TD, Lee JS, Lee YW, 2007. Antiallodynic effect of pregabalin in rat models of sympathetically maintained and sympathetic independent neuropathic pain . Yonsei Med. J. 48: 41-47.

Hawksley H, 2006. Managing pain after shingles: a nursing perspective. Br. J. Nurs. 15: 814-818.

Henry JL, 1976. Effects of substance P on functionally identified units in cat spinal cord. Brain Res. 114: 439-451.

Hill R, 2000. NK1 (substance P) receptor antagonists--why are they not analgesic in humans? Trends Pharmacol. Sci. 21: 244-246.

Hofmann HA, De VJ, Siegling A, Spreyer P, Denzer D, 2003. Pharmacological sensitivity and gene expression analysis of the tibial nerve injury model of neuropathic pain. Eur. J. Pharmacol. 470: 17-25.

Jang JH, Nam TS, Paik KS, Leem JW, 2004. Involvement of peripherally released substance P and calcitonin gene-related peptide in mediating mechanical hyperalgesia in a traumatic neuropathy model of the rat. Neurosci. Lett. 360: 129-132.

Katz EJ, Gold MS, 2006. Inflammatory hyperalgesia: a role for the C-fiber sensory neuron cell body? J. Pain 7: 170-178.

Kauppila T, Kontinen VK, Wei H, Jyvasjarvi E, Pertovaara A, 2002. Cutaneous vascular responses evoked by noxious stimulation in rats with the spinal nerve ligation-induced model of neuropathy. Brain Res. Bull. 58: 21-26.

Kovelowski CJ, Ossipov MH, Sun H, Lai J, Malan TP, Porreca F, 2000. Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain neuropathic pain in the rat. Pain 87: 265-273.

Lang PM, Schober GM, Rolke R, Wagner S, Hilge R, Offenbacher M, Treede RD, Hoffmann U, Irnich D, 2006. Sensory neuropathy and signs of central sensitization in patients with peripheral arterial disease. Pain 124: 190-200.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Lynch JJ, III, Wade CL, Zhong CM, Mikusa JP, Honore P, 2004. Attenuation of mechanical allodynia by clinically utilized drugs in a rat chemotherapy-induced neuropathic pain model. Pain 110: 56-63.

Malcangio M, Ramer MS, Jones MG, McMahon SB, 2000. Abnormal substance P release from the spinal cord following injury to primary sensory neurons 1. Eur. J. Neurosci. 12: 397-399.

Merskey H, Lindblom U, Mumford JM, Nathan PW, Sunderland S. Pain Terms. A current list with definitions and notes on usage. In: Merskey H, Bolduk N. Eds. Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain**.** Second Edition, Part III, IASP Press, Seattle, WA, USA. 1994.

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ, 2002. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J. Neurosci. 22: 6724-6731.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K, 1995. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J. Neurosci. 15: 7633-7643.

Ossipov MH, Hong ST, Malan P, Jr., Lai J, Porreca F, 2000. Mediation of spinal nerve injury induced tactile allodynia by descending facilitatory pathways in the dorsolateral funiculus in rats. Neurosci. Lett. 290: 129-132.

Pertovaara A, Kontinen VK, Kalso EA, 1997. Chronic spinal nerve ligation induces changes in response characteristics of nociceptive spinal dorsal horn neurons and in their descending regulation originating in the periaqueductal gray in the rat. Exp. Neurol. 147: 428-436.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan TP, Jr., Ossipov MH, Lappi DA, Lai J, 2001. Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. J. Neurosci. 21: 5281-5288.

Richards RL, 1967. Causalgia. A centennial review. Arch. Neurol. 16: 339-350.

Saade NE, Al AH, Abdel BS, Safieh-Garabedian B, Atweh SF, Jabbur SJ, 2006a. Transient attenuation of neuropathic manifestations in rats following lesion or reversible block of the lateral thalamic somatosensory nuclei. Exp. Neurol. 197: 157-166.

Saade NE, Al AH, Chalouhi S, Baki SA, Jabbur SJ, Atweh SF, 2006b. Spinal pathways involved in supraspinal modulation of neuropathic manifestations in rats. Pain 126: 280-293.

Sandkuhler J, Liu X, 1998. Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. Eur. J. Neurosci. 10: 2476-2480.

Saxby Pridmore, 2002**.**Managing Chronic Pain: A Biopsychosocial Approach. Informa Healthcare Published: 2002

Schaible HG, 2007. Peripheral and central mechanisms of pain generation. Handb. Exp. Pharmacol.: 3-28.

Sindrup SH, Graf A, Sfikas N, 2006. The NK1-receptor antagonist TKA731 in painful diabetic neuropathy: a randomised, controlled trial. Eur. J. Pain 10: 567-571.

Sotgiu ML, Biella G, 2000. Contribution of central sensitization to the pain-related abnormal activity in neuropathic rats. Somatosens. Mot. Res. 17: 32-38.

Suzuki R, Rahman W, Hunt SP, Dickenson AH, 2004. Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. Brain Res. 1019: 68-76.

Swamydas M, Skoff AM, Adler JE, 2004. Partial sciatic nerve transection causes redistribution of pain-related peptides and lowers withdrawal threshold. Exp. Neurol. 188: 444-451.

Taylor CP, Angelotti T, Fauman E, 2007. Pharmacology and mechanism of action of pregabalin: the calcium channel alpha2-delta (alpha2-delta) subunit as a target for antiepileptic drug discovery. Epilepsy Res. 73: 137-150.

Uehara K, Yamagishi S, Otsuki S, Chin S, Yagihashi S, 2004. Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice. Diabetes 53: 3239-3247.

Urban LA, Fox AJ, 2000. NK1 receptor antagonists--are they really without effect in the pain clinic? Trends Pharmacol. Sci. 21: 462-464.

Wallin J, Schott E, 2002. Substance P release in the spinal dorsal horn following peripheral nerve injury. Neuropeptides 36: 252-256.

Yang RH, Xing JL, Duan JH, Hu SJ, 2005. Effects of gabapentin on spontaneous discharges and subthreshold membrane potential oscillation of type A neurons in injured DRG. Pain 116: 187-193.

CHAPTER 2

Changes in functional properties of A-type but not C-type sensory neurons in vivo in a rat model of peripheral neuropathy

Authors: Yong Fang Zhu, Qi Wu, James L Henry

Corresponding author: Qi Wu, PhD
James L Henry, PhD
Department of Psychiatry and Behavioural Neurosciences,
McMaster University,
1200 Main St. West, HSC 4N35
Hamilton, Ontario, Canada, L8N 3Z5,
Tel: +1-905-525-9140, extension 24750,
FAX: 905-4522-8844
Email address: qwu1975@gmail.com
jhenry@mcmaster.ca

Preface

Significance to thesis

This study demonstrated the successful reproduction of the peripheral neuropathic model. This study also demonstrated all functionally defined mechanosensitive neurons changes in this model in action potential (AP) configuration, conduction velocity (CV) and receptive field properties to mechanical stimuli. This study showed significant changes in Aβ-fiber sensory neurons including Aβ-fiber low-threshold mechanoreceptor (LTM) neurons but not classical C-fiber high-threshold mechanoreceptor (HTM) neurons, which implicates a possible role of Aβ-fiber non-nociceptive primary sensory neurons in the pathogenesis of peripheral neuropathic pain.

Authors' contribution

Yong Fang Zhu did the electrophysiological experiments, the behavioral tests, and model induction. She also analyzed the data, performed statistical analyses, wrote the initial draft of the manuscript, and worked on refining this draft and the revision based on editorial review.

Qi Wu helped technological setting up. James L. Henry conceived of, designed, and coordinated the study. He also worked on refining this draft and the revision based on editorial review.

Abstract

The aim of the present study was to compare primary sensory neurons in control and in a neuropathic pain animal model in order to understand which mechanosensitive neuron types underlie change associated with peripheral neuropathy. On the basis of intracellular recordings *in vivo* from somata, L4 sensory DRG neurons were categorized according to AP configuration, CV and receptive field properties to mechanical stimuli. In a peripheral neuropathy model compared to control rats, a specific population of Aβ-fiber LTM neurons, which respond normally to innocuous mechanical stimuli, exhibited differences in AP configuration and CV. No abnormal CV, AP configuration and tactile sensitivity of C-fiber sensory neurons were encountered. This study provides evidence for defining a potential role of Aβ-fiber LTM neurons that might contribute to peripheral neuropathic pain.

2.1. Introduction

Neuropathic pain is initiated or caused by a primary lesion or dysfunction in the nervous system (Finnerup et al., 2007; Hawksley, 2006) and includes postherpetic neuralgia, trigeminal neuralgia, diabetic neuropathy, spinal cord injury, cancer and chemotherapy, stroke as well as some degenerative neurological diseases (Ro and Chang, 2005). In contrast to nociceptive pain, neuropathic pain is described as spontaneous burning pain with accompanying hyperalgesia and allodynia (Richards, 1967). Tactile allodynia is a common stimulus-evoked response and is defined as pain resulting from a light touch that ordinarily does not elicit a painful response.

It has become evident that peripheral neuropathic pain is characterized by membrane ectopic activity that is thought to be generated in both damaged as well as neighboring intact/surviving fibers of primary sensory neurons (Devor, 2009).

Previous studies have shown that ectopic activity may arise from the DRG soma, the axon and the peripheral nerve terminals (Amaya et al., 2006; Campbell and Meyer, 2006; Katz and Gold, 2006; Liu et al., 2000; Schaible, 2007; Yang et al., 2005) and that prolonged responses to sensory inputs of dorsal horn neurons in neuropathic rats are reduced by local anesthetic application to the peripheral sensory nerve (Pitcher and Henry, 2002). These abnormal activities of peripheral neurons are suggested to play a role as a pain signal and as an inducer of central sensitization observed in animal models of peripheral neuropathy (Campbell and Meyer, 2006; Devor, 1983; Devor, 2006; Lang et al., 2006).

However, it is still not clear which functional subgroup of sensory neurons is involved in altering nociceptive scores in various animal models of peripheral neuropathic pain, and particularly whether or not nociceptors are involved. The major concepts or hypotheses in this regard are clearly described in a recent review by Devor (Devor, 2009). One hypothesis is the "excitable nociceptor hypothesis", which is based on a reduced response threshold in nociceptive sensory neurons. Another hypothesis is that ectopic activity in non-nociceptive sensory neurons is abnormally "amplified" in the spinal cord (Campbell et al., 1988). Both hypotheses embrace the observation that C-fiber or Aβ-fiber sensory neurons carry the ectopic activity in models of peripheral neuropathy. However, so far most of the previous studies were based on axotomized neurons *in vitro* that were anatomically and/or only partially functionally identified. Identifying axotomized DRG neurons as nociceptive or non-nociceptive is questionable as they are disconnected from their sensory receptors.

In our earlier studies on the Mosconi & Kruger rat model of neuropathic pain (Mosconi and Kruger, 1996), in which a polyethylene cuff is placed around a sciatic

nerve unilaterally, we observed hypersensitivity to tactile stimuli, as assessed in the von Frey test (Pitcher et al., 1999); this is generally considered to be the animal equivalent of 'allodynia' in humans (Merskey and Bolduk, 1994). Extracellular electrophysiological recordings made in this model showed an elevated discharge of wide dynamic range (WDR) dorsal horn neurons in response to both noxious and innocuous mechanical stimulation of peripheral cutaneous receptive fields (Pitcher and Henry, 2004; Pitcher and Henry, 2008). Further, in acutely spinalized rats, the hyperactivity of dorsal horn neurons was blocked by application of lidocaine to the sciatic nerve (Pitcher and Henry, 2008). Together, this evidence suggests that increased WDR neuron discharge may be maintained by increased or exaggerated input from primary sensory neurons that can be directly stimulated from peripheral cutaneous receptive fields or are spontaneously active. Therefore, to understand the possible contribution of primary sensory neurons to tactile hypersensitivity in this model, we recorded intracellularly from DRG somata in *in vivo* electrophysiological experiments, and fully characterized each neuron on the basis of several parameters, including the configuration of the APs, the CV of the axon and activation of the respective sensory receptive field. Comparisons were then made with the same properties of DRG neurons recorded from naive control rats.

We report here that LTM neurons associated with Aβ-fibers in particular undergo changes in functional properties and thus might play a role as an essential trigger of tactile hypersensitivity in the Mosconi and Kruger model and possibly to tactile allodynia after peripheral nerve injury; small neurons and high-threshold large neurons exhibited either no change or only minor changes in these properties.

2.2. Methods

All experimental procedures conformed to the Guide to the Care and Use of Laboratory Rats, Vols. 1 and 2, of the Canadian Council on Animal Care, and all protocols were approved by the McMaster University Animal Review Ethics Board. At the end of the acute electrophysiological experiment, the animal was euthanized without recovery by an anesthetic overdose.

Experimental rats and neuropathic surgery

Young male Sprague-Dawley rats (obtained from Charles River Inc. St. Constant, QC, Canada) weighing 170-200 g were used. Rats were divided into two groups, naive control and neuropathic model groups. A peripheral neuropathy was induced according to the method previously described in detail (Mosconi and Kruger, 1996; Pitcher et al., 1999; Pitcher and Henry, 2004). Under anesthesia with a mixture of ketamine (Ketamine; 5 mg/100g; Bimeda-MTC Animal Health Inc.; Cambridge, ON, Canada), xylazine (Rompun; 0.5 mg/100g; Bayer HealthCare, Toronto, ON, Canada), and acepromazine (Atravet; 0.1 mg/100g; Ayerst Veterinary Laboratories, Guelph, ON, Canada) given i.p., the right sciatic nerve was exposed at the mid-thigh level. Two cuffs of 0.5mm polyethylene (PE 90) tubing (Intramedic PE-90, Fisher Scientific Ltd., Whitby, Ontario, Canada) were inserted around the exposed nerve about 1 mm apart. The wound was then sutured in two layers, muscle and skin. Antibiotic ointment (Furacin; nitrofurazone 0.2%; Vetoquinol N.-A. Inc.; Lavaltrie, QC, Canada) was applied over the wound, and 0.01 ml/100g of antibacterial injectable solution (Bayer HealthCare) was injected subcutaneously (s.c.). Rats were given 1 ml saline s.c., ocular lubricant, and placed under a heating lamp until they recovered from the anesthetic, and then returned to their home cages.

von Frey test of paw withdrawal threshold

In all cases, the von Frey test was run on the same day as the recording day before the rats were anesthetized for the acute electrophysiological experiment; this was done to confirm that each animal had developed the tactile hypersensitivity that characterizes this model. To quantify mechanical sensitivity of the foot, brisk foot withdrawal in response to normally innocuous mechanical stimuli were measured as described previously (Kim and Chung, 1992). The rats were placed in a transparent Plexiglas box with a clear Plexiglas floor, containing 0.5 cm diameter holes spaced 1.5 cm apart to allowed full access to the paws (Pitcher et al., 1999; Pitcher and Henry, 2002; Pitcher and Henry, 2004). Rats were allowed to habituate to the box for approximately 15 min, when cage exploration and major grooming activities had ceased.

von Frey filaments (Stoelting Co., Wood Dale, IL, USA) were applied to the plantar surface of the hind paw to determine the withdrawal threshold. A von Frey filament was applied 5 times (for 3-4sec each, at 3 sec intervals) to a different spot on each hind paw. For each rat, these filaments were applied in ascending order until a clear withdrawal response was observed. When this occurred, the next lightest filament was applied following the same procedure. A 50% withdrawal response threshold was derived according to responses to this testing regimen (Chaplan et al., 1994), using the up-down method of Dixon (Dixon, 1980). Brisk foot withdrawal in response to these mechanical stimuli was interpreted as indicating mechanical hypersensitivity.

Intracellular recording in vivo

Details of the surgical preparation and intracellular recording approaches have been reported previously (Wu and Henry, 2009; Wu and Henry, 2010). In brief, the

rat was initially anesthetized with the anesthetic mixture described above. The right jugular vein was cannulated for i.v. infusion of drugs. The rat was then fixed in a stereotaxic frame and the vertebral column rigidly clamped at the L2 and L6 vertebral levels. The right femur was fixed by a customized clamp onto the stereotaxic frame to minimize movement of the DRG during mechanical searching for receptive fields on the leg. The L4 DRG was selected for study as it contains large numbers of hind leg afferent somata. A laminectomy was performed to expose the ipsilateral L4 DRG. The L4 dorsal root was sectioned close to the spinal cord and placed on a bipolar electrode (FHC, Bowdoinham, ME, USA) used for stimulation purposes. The exposed spinal cord and DRG were covered with warm paraffin oil at 37^{o} C to prevent drying. Rectal temperature was maintained at ~37^{o}C using a temperature controlled infrared heating lamp.

For recording, each rat was anesthetized at a surgical level with sodium pentobarbital (20 mg/kg; CEVA SANTE ANIMAL, Libourne, France) given. The rat was mechanically ventilated via a tracheal cannula using a Harvard Ventilator (Model 683, Harvard apparatus, Quebec, Canada). The ventilation parameters were adjusted so that end-tidal CO_2 concentration was maintained around 40-50 mmHg, as measured using a CapStar-100 End-Tidal CO2 analyzer (CWE, Ardmore, PA, USA). Immediately before the start of recording, an initial 1 mg/kg dose of pancuronium (Sandoz, Boucherville, QC, Canada) was given to eliminate muscle tone. The effects of pancuronium were allowed to wear off periodically to confirm a surgical level of anesthesia; this was monitored by observing pupil diameter and any response to noxious pinch of a forepaw. Supplements of pentobarbital and pancuronium were given at a dose of 1/3 approximately each hour via the jugular cannula.

Intracellular recordings from somata in the exposed DRGs were made with borosilicate glass micropipettes (1.2 mm outside diameter, 0.68 mm inside diameter; Harvard Apparatus, Holliston MA, USA). The electrodes were pulled using a Brown-Flaming puller (model p-87; Sutter Instrument Co., Novota, CA, USA). These electrodes were filled with 3 M KCl (DC resistance 50-70 MΩ). Signals were recorded with a Multiclamp 700B amplifier (Molecular Devices, Union City CA, USA) and digitized on-line via Digidata 1322A interface (Molecular Devices, USA) with pClamp 9.2 software (Molecular Devices). The microelectrode was advanced using an EXFO IW-800 micromanipulator (EXFO, Montreal, QC, Canada) in 2 μm steps until a hyperpolarization of at least 40 mV suddenly appeared. Once a stable membrane potential had been confirmed, a single stimulus was applied to the dorsal roots to provoke an AP; with the aid of the protocol editor function in pClamp 9.2 software, a somatic AP was evoked by stimulation with a single rectangular voltage pulse.

DRG neuron classification

Neurons were divided into three groups on the basis of dorsal root CV. The CV range for C-fiber sensory neurons was ≤0.8 m/s, for Aδ-fiber neurons it was 1.5-6.5 m/s and for Aβ-fiber sensory neurons it was >6.5 m/s, as defined elsewhere (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010).

The sensory receptive properties of each DRG neuron were examined using hand-held mechanical stimulators and classified as previously described (Lawson et al., 1997; Leem et al., 1993; Wu and Henry, 2009; Wu and Henry, 2010). The differentiation of HTM neurons vs. LTM neurons was based on their sensory

properties identified during receptive field searching. HTM neurons responded to noxious stimuli including noxious pressure, pinch, and probing with fine forceps, a sharp needle, coarse-toothed forceps, or coarse flat forceps, whereas LTM neurons responded to innocuous stimuli such as a moving brush, light pressure with a blunt object, light manual tap or vibration. Neurons that did not respond to any of the non-noxious or noxious mechanical stimuli were classified as unresponsive as previously described (Lawson et al., 1997).

Besides the threshold of activation, the rate of adaption and the tissue location of the receptive field were other major factors used to further classify Aβ-fiber LTM neurons as guard/field hair neurons (GF), glabrous skin neurons, Pacinian neurons, slowly adapting neurons (SA) and muscle spindle neurons (MS). GF neurons were rapidly adapting cutaneous neurons. Glabrous and Pacinian neurons were both rapidly adapting non-hair neurons, and were named rapidly adapting neurons (RA). SA neurons were slowly adapting cutaneous neurons. MS neurons were slowly adapting neurons with deep subcutaneous receptive fields activated by deep tissue manipulation of the muscle belly but not by cutaneous stimulation.

AP configuration

The first evoked AP in each neuron was used to determine any differences in configuration between control and neuropathic rats. Criteria for acceptance of neurons in the analysis included a stable Vm more negative than ~40mV with a somatic spike evoked by dorsal root stimulation that was > 40mV.

Variables in AP configuration included resting membrane potential (Vm), AP amplitude, AP duration at base (APdB), AP rise time (APRT), AP fall time (APFT), maximum AP rising rate (MRR) , maximum AP falling rate (MFR),

afterhyperpolarization amplitude (AHPA), and afterhyperpolarization duration to 50% recovery (AHP50).

Conduction velocity

The distance from the stimulation site (cathode) to the recording site (center of the DRG) was measured at the end of the experiment to determine the conduction distance. This was then used to calculate the CV of the dorsal root axon associated with each neuron.

Mechanical sensitivity test during intracellular recording

The mechanical sensitivity of HTM neurons was determined individually using calibrated von Frey filaments as described previously (Boada and Woodbury, 2007; Leem et al., 1993; Shim et al., 2005). Briefly, after functional classification of a neuron using the hand-held mechanical stimulators, von Frey filaments were applied to the identified receptive field and the mechanical activation threshold of each neuron was expressed as the minimum force (g) necessary to evoke impulses on the most sensitive spot on the skin. The mechanical forces exerted with the calibrated von Frey filaments used in this study were a set of von Frey filaments exerting pressures of 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10, 15, 26, 60, 100, 180 and 300g; tip diameters ranged from 1.65 to 6.65 mm.

Statistical analysis

Mann Whitney t-test was used for comparison between control and neuropathic rats in various subtypes and for various parameters. All statistical tests and graphing were done using Prism4 software (Graphpad, La Jolla, CA, USA). $P<0.05$ was considered to indicate a significant difference as shown in the graphs.

2.3. Results

Withdrawal threshold in the von Frey test

Behavioral tests of tactile hypersensitivity were made from a total of 124 rats (60 control and 64 neuropathic rats). Stimulation of the plantar surface of the hind paw with von Frey filaments evoked a withdrawal response in control rats, with hairs exerting pressures of 10-100 g. Three weeks after cuff ligation of the sciatic nerve on the right side, model rats fully developed behavioral signs of neuropathic pain on the affected hind limb; filaments to which the control rats showed no withdrawal response, i.e. 0.001 -6.0 g, in neuropathic rats evoked a clear withdrawal of the nerve-injured hind limb. Furthermore, the withdrawal was greatly exaggerated in amplitude and duration, and it was frequently accompanied by licking of the paw. Withdrawal thresholds were 14.82 ± 0.180 g in control rats (N = 60) and 5.65 ± 1.337 g in neuropathic rats (N = 64); comparison of the data indicated $p < 0.0001$.

Electrophysiological recording

Intrasomal recordings in these rats were made from a total of 399 L4 DRG neurons (175 neurons in control rats and 224 neurons in neuropathic rats). In control rats these included 33 C-fiber (21 HTM, 8 LTM and 4 unresponsive neurons), 22 Aδ-fiber (11 HTM, 7 LTM, 4 unresponsive neurons), 120 Aβ-fiber (26 HTM, 86 LTM and 8 unresponsive neurons). In neuropathic rats these included 40 C-fiber (28 HTM, 8 LTM, and 4 unresponsive neurons), 20 Aδ-fiber (7 HTM, 9 LTM, and 4 unresponsive neurons), and 164 Aβ-fiber (39 HTM, 116 LTM and 9 unresponsive neurons). All neurons included in these results met the inclusion criteria described above. Examples of APs recorded from individual neuron types are illustrated in Figure 2.1. With respect to Aβ-fiber LTM neurons, 20 GF neurons were recorded in control and 25 in neuropathic rats. Similarly, 20 RA neurons were recorded in control

and 24 in neuropathic rats; 10 SA neurons were recorded in control rats and 14 in neuropathic rats; 36 MS neurons were recorded in control rats and 53 in neuropathic rats.

Action potential configuration

The various AP configuration parameters of corresponding subclasses in each CV group were compared between control and neuropathic rats. All data are shown in Table 2.1 and the scatter plots of Figures 2.2-2.4.

Resting membrane potential

Values of Vm for all neurons included in this study are given in Table 2.1. Vm of all subtypes of C-fiber sensory neurons was similar in control and neuropathic rats; this was the case for HTM neurons (N = 21 in control rats, vs. N = 28 in neuropathic rats; P = 0.506), for LTM neurons (N = 8 in control rats vs. N = 8 in neuropathic rats; P = 0.658); and for unresponsive neurons (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.993).

In all subtypes of Aδ-fiber sensory neurons, Vm was also similar between control and neuropathic rats. This was the case for HTM neurons (N = 11 in control rats vs. N = 7 neurons in neuropathic rats; P = 0.986), for LTM neurons (N = 7 in control rats vs. N = 9 in neuropathic rats; P = 0.072), and for unresponsive (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.807) neurons.

In Aβ-fiber HTM neurons and unresponsive neurons, Vm also showed similar values in control and neuropathic rats: HTM neurons (N = 26 in control rats vs. N = 39 in neuropathic rats; P = 0.886), unresponsive neurons (control, N = 8 in control rats vs. N = 9 in neuropathic rats; P = 0.596).

However, Vm in Aβ-fiber LTM neurons in neuropathic rats was more

depolarized than that in control rats, including both cutaneous and deep neurons: CUT neurons (N = 50 in control rats vs. neuropathic, N = 63; P = 0.042); MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.044). These data are illustrated in Figure 2.2A.

Action potential amplitude

There were no significant differences in AP amplitude between control and neuropathic rats in any subtype of neurons in any CV group, as illustrated in Table 2.1.

For example, in C-fiber sensory neurons there were no differences between HTM neurons in control rats (N = 21 neurons) vs. neuropathic rats (N = 28 neurons; P = 0.093). Similarly, for LTM neurons there was no difference between control (N = 8 neurons) and neuropathic (N = 8 neurons; P = 0.957) rats, and for unresponsive neurons there was no significant difference between control (N = 4 neurons) and neuropathic (N = *4* neurons) rats (P = 0.381).

With respect to Aδ-fiber sensory neurons, AP amplitude of HTM neurons was the same in control and neuropathic rats (N = 11 and N = 7 neurons, respectively; P = 0.442). Similarly, in LTM neurons AP amplitude was the same in control and neuropathic rats (N = 7, N = 9 neurons, respectively; P = 0.351) and in unresponsive neurons AP amplitude was the same in control and neuropathic (N = 4 neurons each; P = 0.644) rats.

In Aβ-fiber sensory neurons, AP amplitude was the same between groups for all neuron types, including HTM neurons (N = 26 in control rats vs. N = 39 in neuropathic rats; P = 0.912), CUT neurons (N = 50 in control rats vs. N = 63 in neuropathic rats; P = 0.970), MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.442) and unresponsive neurons (N = 8 in control rats vs. N = 9

in neuropathic rats; $P = 0.508$). The data are shown in Figure 2.2B.

Action potential duration at base

APdB differed between control and neuropathic rats in some neuron types, Aβ- and Aδ-fiber neurons in particular, as shown in Table 2.1.

Thus, none of the subgroups of C-fiber sensory neurons exhibited a difference between control and neuropathic rats, including HTM neurons ($N = 21$ in control rats vs. $N = 28$ in neuropathic rats; $P = 0.574$), LTM neurons ($N = 8$ in control rats vs. $N = 8$ in neuropathic rats; $P = 0.636$) and unresponsive neurons ($N = 4$ in control rats vs. $N = 4$ in neuropathic rats; $P = 0.871$).

Of Aδ-fiber sensory neurons, only HTM neurons exhibited a difference between control and neuropathic rats ($N = 11$ in control rats vs. $N = 7$ in neuropathic rats; $P = 0.035$). No difference was seen between control and neuropathic rats for LTM neurons ($N = 7$ in control rats vs. $N = 9$ in neuropathic rats; $P = 0.147$) or unresponsive neurons (control, $N = 4$ vs. neuropathic, $N = 4$; $P = 0.161$).

In marked contrast to C-fiber sensory neurons, all Aβ-fiber sensory neuron groups were different. Aβ-fiber sensory neurons exhibited a longer AP duration at base in neuropathic rats compared to controls, including Aβ-fiber HTM neurons ($N = 26$ in control rats vs. $N = 39$ in neuropathic rats; $P < 0.001$), CUT neurons ($N = 50$ in control rats vs. $N = 63$ in neuropathic rats ; $P = 0.004$), MS neurons ($N = 36$ in control rats vs. $N = 53$ in neuropathic rats; $P = 0.022$) and unresponsive neurons ($N = 8$ in control rats vs. $N = 9$ in neuropathic rats ; $P = 0.029$). The data are shown in Figure 2.3A.

Action potential rise time

APRT did not differ between control and neuropathic rats in either C-fiber

sensory neurons or Aδ-fiber sensory neurons. However, a significantly longer APRT was observed in the subgroups of Aβ-fiber sensory neurons, with the exception of the unresponsive neurons. The data are presented in Table 2.1.

Thus, in C-fiber sensory neurons, APRT was the same in control and neuropathic rats, including HTM neurons (N = 21 in control rats vs. N = 28 in neuropathic rats; P = 0.680), LTM neurons (N = 8 in control rats vs. N = 8 in neuropathic rats; P = 0.883) and unresponsive neurons (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.976).

Similarly, in Aδ-fiber sensory neurons, APRT was the same in control and neuropathic rats, including HTM neurons (N = 11 in control rats vs. N = 7 in neuropathic rats; P = 0.869), LTM neurons (N = 7 in control rats vs. N = 9 in neuropathic rats; P = 0.229) and unresponsive neurons (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.244).

Types of Aβ-fiber sensory neuron that exhibited a statistically significant difference in APRT between control and neuropathic rats included HTM neurons (N = 26 in control rats vs. N = 39 in neuropathic rats; P = 0.049), CUT neurons (N = 50 in control rats vs. N = 63 in neuropathic rats; P = 0.002) and MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.015). Unresponsive Aβ-fiber sensory neurons did not show a difference between control and neuropathic rats (N = 8 in control rats vs. N = 9 in neuropathic rats; P = 0.189). The data are shown in Figure 2.3B.

Action potential fall time

APFT also showed a differential effect on different neuron types between control and neuropathic rats. The data are presented in Table 2.1.

Thus, C-fiber sensory neurons did not show a difference, whether HTM neurons (N = 21 in control rats vs. N = 28 in neuropathic rats; P = 0.651), LTM neurons (N = 8 in control vs. N = 8 in neuropathic rats; P = 0.733) or unresponsive neurons (N = 4 in control vs. N = 4 in neuropathic rats; P = 0.937).

In Aδ-fiber sensory neurons both HTM and LTM neurons exhibited longer APFT in neuropathic than control rats; HTM neurons (N = 11 in control rats vs. N = 7 in neuropathic rats; P = 0.018), LTM neurons (N = 7 in control rats vs. N = 9 in neuropathic rats; P = 0.035). In contrast, unresponsive neurons were the same in both control and neuropathic rats (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.647).

In Aβ-fiber sensory neurons, all but the unresponsive neuron type displayed longer APFT in neuropathic compared to control rats. Thus, longer APFT were observed in neuropathic rats for HTM neurons (N = 26 in control rats vs. N = 39 in neuropathic rats; P = 0.001), CUT neurons (N = 50 in control rats vs. N = 63 in neuropathic rats; P = 0.022) and MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.046). APFT in unresponsive Aβ-fiber sensory neurons were the same in control and neuropathic rats (N = 8 in control rats vs. N = 9 in neuropathic rats; P = 0.094). These data are shown in Figure 2.3C.

Maximum rising rate

MRR was significantly slower in some but not all subtypes of neuron, as shown in Table 2.1.

C-fiber sensory neurons did not show a difference, whether HTM neurons (N = 21 in control rats vs. N = 28 in neuropathic rats; P = 0.735), LTM neurons (N = 8 in control rats vs. N = 8 in neuropathic rats; P = 0.592) or unresponsive neurons (N = 4

in control rats vs. $N = 4$ in neuropathic rats; $P = 0.901$).

Aδ-fiber LTM neurons displayed a slower MRR in neuropathic rats compared to control rats ($N = 7$ in control rats, vs. $N = 9$ in neuropathic rats; $P = 0.033$). Neither HTM neurons ($N = 11$ in control rats vs., $N = 7$ in neuropathic rats; $P = 0.331$) nor unresponsive neurons ($N = 4$ in control rats vs. $N = 4$ in neuropathic rats; $P = 0.320$) differed between neuropathic or control rats with respect to MRR.

Of Aβ-fiber sensory neurons, all showed slower MRR in neuropathic rats compared to control rats, with the exception of the unresponsive neurons. Thus, slower MRR was observed in HTM neurons (control, $N = 61$ vs. neuropathic, $N = 39$; $P = 0.028$), CUT neurons (control, $N = 50$ vs. neuropathic, $N = 63$; $P = 0.002$) and MS neurons (control, $N = 36$ vs. neuropathic, $N = 53$; $P = 0.023$); the unresponsive neurons displayed similar MRR in both groups of rat (control, $N = 8$ vs. neuropathic, $N = 9$; $P = 0.202$). The data are shown in Figure 2.3D.

Maximum falling rate

MFR was also significantly slower in some neuron types in neuropathic rats compared to controls. Table 2.1 shows the data.

As above, such changes were not observed in C-fiber sensory neurons, including HTM neurons ($N = 21$ in control rats vs. $N = 28$ in neuropathic rats; $P = 0.535$), LTM neurons ($N = 8$ in control rats vs. $N = 8$ in neuropathic rats; $P = 0.287$) and unresponsive neurons ($N = 4$ in control rats vs. $N = 4$ in neuropathic rats; $P = 0.280$).

Aδ-fiber sensory neurons showed a different distribution of slowing of MFR in the neuron types compared to that seen above with MRR. For example, LTM neurons displayed a slower MFR in neuropathic rats compared to control rats ($N = 7$

in control rats vs. $N = 9$ in neuropathic rats; $P = 0.013$). HTM neurons also showed a slowing of MRF ($N = 11$ in control rats vs. $N = 7$ in neuropathic rats; $P = 0.044$). However, unresponsive neurons did not show a difference in MFR between rats ($N = 4$ in control rats, vs. $N = 4$ in neuropathic rats; $P = 0.631$).

Aβ-fiber sensory neurons also displayed a neuron-type based grouping of differences between control and neuropathic rats in MFR. MFR was different in HTM neurons ($N = 26$ in control rats vs. $N = 39$ in neuropathic rats; $P = 0.011$) and MS neurons ($N = 36$ in control rats vs. $N = 53$ in neuropathic rats; $P = 0.001$), while there was no difference in MFR in CUT neurons ($N = 50$ in control rats vs. $N = 63$ in neuropathic rats; $P = 0.164$) or in unresponsive neurons ($N = 8$ in control rats vs. $N = 9$ in neuropathic rats; $P = 0.127$). The data are shown in Figure 2.3E.

Afterhyperpolarization amplitude

A significant difference in AHPA between neuropathic and control rats was seen only in two populations of Aβ-fiber neurons. These neuron types were Aβ-fiber HTM neurons and unresponsive neurons.

Thus, of the C-fiber sensory neurons, none showed a difference between control and neuropathic rats, including the HTM neurons ($N = 21$ in control rats vs. $N = 28$ in neuropathic rats; $P = 0.421$), LTM neurons ($N = 8$ in control rats, vs. $N = 8$ in neuropathic rats; $P = 0.506$) and unresponsive neurons ($N = 4$ in control rats vs. $N = 4$ in neuropathic rats; $P = 0.880$).

Similarly, of the sensory neurons in the Aδ-fiber range, none showed a difference between control and neuropathic rats, including HTM neurons ($N = 11$ in control rats vs, $N = 7$ in neuropathic rats; $P = 0.951$), LTM neurons ($N = 7$ in control rats vs., $N = 9$ in neuropathic rats; $P = 0.454$) and unresponsive neurons ($N = 4$ in

control rats vs. $N = 4$ in neuropathic rats; $P = 0.761$).

In the Aβ-fiber sensory neuron range, AHPA differed in HTM neurons (N = 26 in control rats vs. N = 39 in neuropathic rats; P = 0.0101) and unresponsive neurons (N = 8 in control rats vs. N = 9 in neuropathic rats; P = 0.048), but did not change in CUT (N = 50 in control rats vs. N = 63 in neuropathic rats; P = 0.304) or MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.708). The data are shown in Figure 2.4A.

Afterhyperpolarization duration to 50% recovery

AHP50 was significantly different between control and neuropathic rats but only in LTM MS neurons.

None of the C-fiber sensory neurons showed a difference between control and neuropathic rats, including the HTM neurons (N = 21 in control rats vs. N = 28 in neuropathic rats; P = 0.941), LTM neurons (N = 8 in control rats vs. N = 8 in neuropathic rats; P = 0.786) and unresponsive neurons (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.937).

Of the sensory neurons in the Aδ-fiber range, again none showed a difference between control and neuropathic rats, including HTM neurons (N = 11 in control rats vs. N = 7 in neuropathic rats; P = 0.677), LTM neurons (N = 7 in control rats vs. N = 9 in neuropathic rats; P = 0.560) and unresponsive neurons (N = 4 in control rats vs. N = 4 in neuropathic rats; P = 0.926).

Of the sensory neurons in the Aβ-fiber range, AHP50 was longer only in the MS neurons (N = 36 in control rats vs. N = 53 in neuropathic rats; P = 0.003). There was no difference between control and neuropathic rats in the other types of neuron in the Aβ-fiber range, including HTM neurons (N = 26 in control rats vs. N = 39 in

neuropathic rats; P = 0.318), CUT neurons (N = 50 in control vs. N = 63 in neuropathic rats; P = 0.839) and unresponsive neurons (N = 8 in control rats vs. N = 9 in neuropathic rats; P = 0.893). The data are shown in Figure 2.4B

Conduction velocity

CV was studied as it reflects properties of the axon rather than of the soma, which was the aim of the data presented above. Figure 2.5 illustrates the distributions of CVs for individual neurons in each neuron type in control and neuropathic rats. Comparison of the CV between these groups of rats in each sensory neuron type of C-fiber and Aδ-fiber did not show a significant difference. However, in the Aβ-fiber range, LTM neurons showed significantly slower CV in neuropathic rats.

Thus, in C-fiber sensory neurons, CV was 0.59 ± 0.040 m/s (N = 21) and 0.60 ± 0.036 m/s (N = 28) in control and neuropathic HTM neurons, respectively (P = 0.835). In LTM neurons, CV was 0.47 ± 0.058 m/s (N = 8) and 0.50 ± 0.033 m/s (N = 8) in control and neuropathic rats, respectively (P = 0.685). In unresponsive neurons, CV was 0.60 ± 0.054 m/s (N = 4) and 0.61 ± 0.094 m/s (N = 4) in control and neuropathic rats, respectively (P = 0.946).

In Aδ-fiber sensory neurons, CV was 4.32 ± 0.514 m/s (N = 11) and 4.86 ± 0.505 m/s (N = 7) in control and neuropathic HTM neurons, respectively (P = 0.487), while it was 5.17 ± 0.705 m/s (N = 7) and 3.91 ± 0.500 m/s (N = 9) in control and neuropathic LTM neurons, respectively (P = 0.154) and was 3.21 ± 0.766 m/s (N = 4) and 4.14 ± 1.041 m/s (N = 4) in control and neuropathic unresponsive neurons, respectively (P = 0.496).

In Aβ-fiber sensory neurons, CV was 12.42 ± 0.548 m/s (N = 26) in control HTM neurons and 11.38 ± 0.504 m/s (N = 39) in neuropathic HTM neurons (P =

0.179), it was 17.02 ± 0.469 m/s (N = 50) and 14.70 ± 0.548 m/s (N = 63) in control and neuropathic CUT neurons, respectively (P = 0.002), it was 18.76 ± 0.638 m/s (N = 36) and 16.69 ± 0.599 m/s (N = 53) in control and neuropathic MS neurons, respectively (P = 0.023) and it was 12.67 ± 0.831 m/s (N = 8) in control vs. 11.15 ± 1.088 m/s (N = 9) in neuropathic unresponsive neurons (P = 0.292).

Activation of the sensory receptors

Activation threshold of sensory receptors associated with different neuron types was studied throughout the entire hind-leg. This was done to complement the data reported above regarding properties of the soma and the axon. Almost all of the neurons studied with identifiable receptive field could be classified into one of the categories according to the type of stimulus; Table 2.2 shows the locations of the receptive fields of the neurons recorded.

C-fiber sensory neurons in neuropathic rats did not show differences in the threshold of activation of the receptive field compared to those in control rats. C-fiber LTM neurons (N = 8 in control; N = 8 in neuropathic rats) were activated by slow brushing on the receptive field or lightly stretching of the skin surrounding the receptive field. Neither stimulus produced any discharge in C-fiber HTM neurons (N = 21 in control rats, N = 28 in neuropathic rats), which were activated only by stimuli in the noxious range. The distributions of C-fiber HTM and LTM neuron receptive fields were relatively evenly distributed over the hind leg, each with relatively small receptive field sizes.

Similarly, none of the Aδ-fiber sensory neurons in neuropathic rats showed any difference in the activation of the receptive field when data from control and neuropathic rats were compared. For Aδ-fiber HTM neurons (N = 11 in control; N =

7 in neuropathic rats), the receptive fields usually consisted of several small spots and were found on the foot, calf and thigh. All Aδ-fiber HTM neurons responded to noxious stimuli. Aδ-fiber LTM neurons tested in this way were D-hair neurons (N = 7 in control; N = 9 in neuropathic rats). They were activated by von Frey filaments (≤ 0.16 g) and were found on the foot, calf and thigh.

For Aβ-fiber HTM neurons (N = 26 in control rats, N = 39 in neuropathic rats), the distribution of receptive fields was also over the entire hind leg. All responded the noxious stimuli, including noxious stimulation deeply toward the bone. Aβ-fiber LTM neurons, GF neurons (N = 20 in control rats, N = 25 in neuropathic rats) were distributed entirely over hairy skin and were activated by lightly stimulating the tip of the hair/moving a group of hairs. RA glabrous neurons (N = 6 in control rats, N = 6 in neuropathic rats) were activated by lightly stimulating glabrous skin of the foot with blunt objects; these neurons responded to these stimuli with a brief, quickly adapting discharge. Pacinian neurons (N = 14 in control rats, N = 18 in neuropathic rats) were activated by gently tapping on the experiment table and, with searching mechanical stimuli, were found over the entire hind leg. SA neurons (N = 10 in control rats, N = 14 in neuropathic rats) were activated by lightly stimulating skin surrounding the nails in the foot, except one control neuron with a receptive field on the ankle joint. MS neurons were activated by touching along the muscle belly or changing joint position (N = 36 in control rats, N = 53 in neuropathic rats). One particular abnormality observed was found in two Aβ-fiber sensory neurons in neuropathic rats; in response to current injection into the cell body, the response of these neurons (N = 2) resembled that of MS neurons or SA neurons in terms of the relatively slow adaptation, but the receptive-field characteristics of these neurons were similar to other hair neurons with

low thresholds to stimulation of hairs (≤ 0.008g von Frey filament stimulation; these neurons are not included in this study).

Previous studies have shown that the thresholds of activation of most nociceptors in the feet of control rats are higher than 14 mN (1.43 g) (Leem et al., 1993). To identify whether HTM neurons showed any differences in mechanical sensitivity in this model, we also measured the mechanical threshold of a selected number of HTM neurons in control and neuropathic rats. The mechanical threshold for C-fiber HTM neurons was within a range of 2-100 g in control rats (N = 14) vs. 6-100 g in neuropathic rats (N = 15). Mechanical thresholds for Aδ-fiber HTM neurons were in a range 4-60 g in control rats (N = 8) and 4-60 g in neuropathic rats (N = 6). Mechanical thresholds for Aβ-fiber HTM neurons were in a range of 4-60 g in control rats (N = 8) vs. 2-100 g in neuropathic rats (N = 6). None of the HTM neurons in neuropathic rats showed a mechanical threshold of activation below 1.4 g.

2.4. Discussion

Classification of DRG neurons

The aim of the present study was to compare primary sensory neurons in control and in neuropathic rats in order to identify changes in peripheral neurons associated with tactile hypersensitivity. Interest was particularly in the type of neuron exhibiting change.

To achieve this aim, DRG sensory neurons were classified during intracellular *in vivo* electrophysiological experiments according to parameters reported previously by other laboratories to distinguish DRG neuron types, including: 1) the configuration of the AP, 2) the CV, and 3) the response properties to application of natural stimuli to peripheral receptive fields, such as threshold of activation and adaptation (Djouhri

et al., 1998; Fang et al., 2005; Lawson et al., 1997; Leem et al., 1993; Wu and Henry, 2009; Wu and Henry, 2010). With this classification, each DRG mechanosensitive neuron could be functionally classified and each could be distinguished as nociceptive (HTM), non-nociceptive (LTM) and unresponsive mechanoreceptors. CV criteria for classification in the present study were also based on those described previously by Lawson et al. (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010); these criteria were followed because the experimental approach in the present study most closely approximated those in Lawson's previous reports, including similar surgical procedures, recording techniques and recording setups.

The differentiation based on the CV ranges of Aβ-, Aδ- and C-fiber sensory neurons, and the differentiation between the HTM neurons and LTM neurons in control rats were clearly maintained in this neuropathic animal model. For example, Aβ-, Aδ- and C-fiber sensory neurons in neuropathic rats still appeared to conduct in three widely separate ranges. In neuropathic rats, HTM neurons in the C-fiber, Aδ-fiber and Aβ-fiber sensory neurons ranges exhibited a relatively depolarized resting membrane potential, a relatively higher AP amplitude, relatively slower AP kinetics (i.e. longer AP duration, AP rise and fall times, slower AP maximum rising and falling rates), a relatively higher AHP amplitude and a longer 50% AHP recovery time than exhibited by LTM neurons. Thus, other than the few neurons that are described in the results and excluded in this classification, all neurons were clearly classified in both control and neuropathic rats.

Differences in properties of neuron types

In the present study, when comparing control to neuropathic rats, none of the

small-diameter C-fiber DRG neurons with identifiable receptive fields exhibited any differences in any of the properties investigated here, including both LTM and HTM neurons. In contrast, DRG A-type neurons, in particular Aβ-fiber LTM neurons in neuropathic rats, showed significant differences in electrophysiological properties compared to those in control rats, manifesting as decreased CV, a more depolarized Vm and slower AP kinetics.

Our results demonstrating changes mainly in the large-diameter primary sensory neurons are compatible with those of several groups investigating other types of rat neuropathic pain models, which showed that most ectopic discharge is generated in large-diameter fast conducting myelinated Aβ-fiber sensory neurons after nerve injury (Han et al., 2000; Khan et al., 2002; Liu et al., 2000; Ma et al., 2003; Tal et al., 1999). In fact, some features such the longer AP duration, rise time and fall time and lack of AHP changes in A-fiber sensory neurons in our neuropathic rats are similar to the well-described differences in A-fiber sensory neurons after peripheral axotomy (Abdulla and Smith, 2001; Kim et al., 1998; Liu and Eisenach, 2005; Ma et al., 2003; Stebbing et al., 1999) and neighboring intact DRG neurons recorded in L5 neurons in the spinal nerve ligation model of peripheral neuropathy (Ma et al., 2003). The decreased CV of A-fiber sensory neurons is similar to that reported in A-fiber sensory neurons after chronic constriction injury of the sciatic nerve (Gabay and Tal, 2004). Somewhat different magnitudes of changes in these parameters in the different reports might be attributable to the many factors, such as different sensory neurons targeted (“injured” vs. “intact” neurons) and to different animal models employed (nerve-section axotomy model, spinal nerve ligation model, peripheral nerve section model, chronic constriction injury model and compression of the DRG model, etc.).

Different techniques, such as sharp microelectrodes *in vivo* or *in vitro* in some experiments vs. patch electrodes to study dissociated cells in others, and different chosen animal species, strain, age, sex and time between the initial model induction and electrophysiological experiment may have also contributed to differences in the magnitude of the changes in different reports (Abdulla and Smith, 2001).

CVs and AP properties of unresponsive neurons were not significantly different from those of HTM in the C-fiber, Aδ-fiber and Aβ-fiber ranges, comparing control to neuropathic rats. These types of neuron were thus probably HTM neurons with inaccessible receptive fields or were very high-threshold HTM units, as described earlier by Lawson et al. (Lawson et al., 1997). However, although unresponsive neurons in neuropathic rats undergo electrophysiological changes compared to control rats, measurement of mechanical sensitivity was not possible as theses neurons were not responsive to any mechanical stimulation of the cutaneous receptive field, and thus they might not be related to tactile allodynia.

Correlation of changes in Aβ-type LTMs with tactile hypersensitivity

While this evidence argues against C-fiber sensory neurons contributing to tactile allodynia after model induction, this has sometimes been assumed to involve HTM neurons as a possible contribution. Similarly, while there is evidence for Aβ-fiber sensory neurons contributing to tactile allodynia after nerve or tissue injury, this has sometimes been assumed to involve LTM neurons as a possible contribution (Devor, 2009).

AP configuration, CV and peripheral mechanical activation threshold of all subtypes of DRG neuron were systemically evaluated in this study, as each of these parameters might reflect changed electrophysiological properties in various parts of

the primary sensory neuron, such as the soma, the axon and the receptive field, respectively. These features of peripheral neurons have been previously described and have been thought to play a role in generating the pain signal in several animal models of peripheral neuropathy (Campbell and Meyer, 2006; Devor, 2006; Devor, 2009; Lang et al., 2006).

Although it is widely believed that afferent C-fiber input is a necessary condition for the induction and maintenance of central sensitization, none of the C-fiber sensory neurons in this study showed any difference in electrophysiological properties. Perhaps most important, no C-fiber HTM neurons showed any decrease in mechanical sensitivity when tested with von Frey filaments.

On the other hand, A type neurons, especially Aβ-fiber LTM neurons in neuropathic rats showed significant differences in electrophysiological properties. Notably, our pervious studies in this same model showed nociceptive response in WDR dorsal horn neurons to innocuous mechanical stimulation (Pitcher and Henry, 2004) and it was further proposed that ectopic activity recorded from WDR dorsal horn neurons was mediated via myelinated sensory neurons (Pitcher and Henry, 2008). Thus the present results provide further support for a possible role of Aβ-fiber LTM neurons in the behavioral tactile hypersensitivity exhibited in this model, as these neurons remained connected to their normal impulse generating site and respond to normal innocuous mechanical stimuli, which are essential for defining tactile sensitivity.

Our finding adds to the body of evidence that C-fiber DRG neurons are likely not related to the tactile allodynia and raise the possibility that LTM neurons of Aβ-fiber DRG neurons fulfill this role. The mechanisms underlying the

electrophysiological changes in Aβ-fiber LTM neurons that could induce neuropathic pain or allodynia remain to be determined. One possible explanation is that some Aβ-fiber LTM neurons undergo phenotypic changes and take up a new role in nociception, and began to convey signals along novel pathways leading to activation of spinal nociceptive mechanisms (Devor, 2009). There is evidence that released mediators such as substance P (SP), calcitonin gene-related peptide (CGRP), brain-derived neurotrophic factor (BDNF) and neuropeptide Y released by A-type neurons might trigger changes in the responsiveness of postsynaptic neurons and rewiring of sensory pathways at the first sensory synapse in the spinal cord (Lever et al., 2001; Ma and Bisby, 1998; Malcangio et al., 2000; Michael et al., 1999; Miki et al., 1998; Noguchi et al., 1995; Weissner et al., 2006).

We speculate here that there may be an altered supply of such factors in the neurons that have undergone the changes reported here. These changes may therefore constitute a mechanism leading to the pain, dysesthesia and allodynia that commonly accompany peripheral neuropathy.

Cellular mechanisms of underlying differences in A-type neurons

These differences in neurons in neuropathic rats might be due to membrane remodeling, thus altering the intrinsic electrogenic properties of the neuronal membrane in those neuron types exhibiting changes. There are three major ion channels, voltage-gated Na^+, Ca^{2+} and K^+ channels, which play major roles in determining electrogenic properties of neurons. For example, Na^+ channels can modulate resting membrane potential in DRG neurons and Na^+ channels are also likely to have a major influence on the AP rising phase and therefore on AP duration(Waxman et al., 1999). Ca^+ inward currents likely contribute to the falling

phase inflections in HTM neurons (Djouhri et al., 1998; Yoshida et al., 1978). K^+ channels have been reported to contribute to the repolarization phase in LTM neurons (Djouhri et al., 1998). Differences in expression and/or activation of both voltage-gated and Ca^{2+}-activated K^+ channels have been reported as likely to contribute to the AHP (Djouhri et al., 1998). Thus, alterations in the level of expression, cellular localization, distribution or activation/kinetics of each of these ion channel types might lead to the changes in AP configuration in neuropathic rats.

Changes in the activity of sodium channels and the consequence of these changes have been reported in various neuropathic pain models. Immunohistochemical studies have demonstrated a reorganization of the levels of expression and distribution of various sodium channels in neuropathic animal models (Cummins et al., 2007; Devor, 2006; Rogers et al., 2006). The expression of some sodium channel subtypes in DRG cell bodies is diminished following nerve injury, while others appear *de novo* and yet others are distributed to different parts of the neuron (Hains et al., 2003). Changes in the activity of calcium channels and potassium channels have also been reported (Aurilio et al., 2008). For example, voltage-clamp studies of isolated currents in dissociated axotomized DRG cells have revealed an upregulation of a tetrodotoxin-sensitive (TTX-S) Na^+ current and a downregulation of a tetrodotoxin-resistant (TTX-R) Na^+ current (Black et al., 1999; Rizzo et al., 1995; Sleeper et al., 2000; Waxman et al., 1999), together with a reduction of K^+ and Ca^{2+} currents(Abdulla et al., 2001; Baccei and Kocsis, 2000; Everill and Kocsis, 1999).

Explanations for the mechanisms underlying these changes in membrane channel expression might be based on many factors, such as nerve growth factor

(NGF) or glial cell line-derived neurotrophic factor (GDNF), cytokines or other inflammatory mediators released by immune cells and Schwann cells (Cui et al., 2000; Gherardini et al., 1999; Li et al., 2000; Ramer et al., 1997; Shamash et al., 2002; Sommer and Schafers, 1998; Wagner and Myers, 1996). NGF and GDNF are important neurotrophic factors for maintaining normal function of sensory neurons and may influence AP electrogenesis and neuronal excitability in DRG neurons via regulating ionic currents.

Demyelination, which has been proposed to result in membrane remodeling, also should be considered as a possible driving force of change, as demyelination has been reported to lead to ion channel redistribution (Kretschmer et al., 2002). In normal conditions, newly synthesized Na^+ channels are transported in endocytoplasmatic vesicles along the axons to be expressed only on specific target sites such as nodes of Ranvier and nerve terminal endings. As a consequence of the neuronal damage in this model, this target-specific transfer may be altered and the channels in transit may be redistributed in any remaining part of the membrane, particularly in DRGs and demyelinated patches (Aurilio et al., 2008). In fact, our data show that the decreased conduction velocity of Aβ-fiber LTM neurons might be due to demyelination so as to be compatible with the change in AP shape in theses neurons following peripheral neuropathy.

Some of these findings or other factors might be contributing in various ways to the changes we have observed in the shape of the AP of different A-type neurons. In fact, the more heavily myelinated Aβ-fiber LTM neurons in this neuropathic model might be more affected by such comprehensive factors and thus showed significant changes in functional properties.

2.5. Conclusions

The purpose of this study was to examine the electrophysiological properties and mechanical sensitivity of the different types of DRG neurons in control vs. neuropathic rats. Comparing previous reports, this is the first study providing evidence showing changes in functional properties of sensory DRG neurons with identifiable receptive fields. In this model, there were no differences in C-fiber sensory neurons between control and model rats. However, there were significant and possibly important differences in DRG neurons associated with Aβ-fibers and Aδ-fibers, especially Aβ-fiber LTM neurons. These findings are unique and unexpected because Aβ-fiber LTM neurons are normally considered to be non-nociceptive neurons. We interpret these data to suggest that A-type but not C-type primary sensory neurons in this model of peripheral neuropathy may be involved in generating the tactile hypersensitivity seen in these rats in the von Frey behavioral test.

2.6. Tables and figures

Table 2.1. Comparison of properties of high-threshold mechanoreceptive and low-threshold mechanoreceptive DRG neurons between control and neuropathic rats

CV range	Receptor class	Model	n	CV (m/s)	Vm (-mV)	AP A (mV)	APdB (ms)	APRT (ms)	APFT (ms)	MRR (mV/ms)	MFR (mV/ms)	AHPA (mV)	AHP50 (ms)
C	HTM	Control	21	0.59±0.040	62.12±2.130	83.80±2.092	3.76±0.247	1.47±0.092	2.27±0.181	146.5±8.587	60.89±3.390	8.73±0.877	11.14±1.888
		NeP	28	0.60±0.036	60.35±1.626	77.58±2.725	3.99±0.296	1.41±0.111	2.40±0.210	151.5±10.81	58.04±3.039	9.68±0.771	10.95±1.614
				p=0.835	p=0.506	p=0.093	p=0.574	p=0.680	p=0.651	p=0.735	p=0.535	p=0.421	p=0.941
	LTM	Control	8	0.47±0.058	72.79±1.866	70.89±3.526	3.37±0.273	1.28±0.096	2.16±0.177	161.5±18.85	73.94±1.437	1.40±0.437	10.05±1.054
		NeP	8	0.50±0.033	71.62±1.817	71.10±2.915	3.19±0.267	1.30±0.052	2.26±0.236	173.4±10.91	71.27±1.84	1.78±0.347	9.68±0.861
				p=0.685	p=0.658	p=0.957	p=0.636	p=0.883	p=0.733	p=0.592	p=0.287	p=0.506	p=0.786
	Unresponsive	Control	4	0.60±0.054	59.67±5.840	82.71±4.650	3.74±0.429	1.45±0.246	2.18±0.826	147.7±22.08	68.29±5.938	8.67±3.346	11.56±2.278
		Nep	4	0.61±0.094	59.59±6.306	74.37±7.491	3.95±1.139	1.44±0.334	2.10±0.686	154.0±43.60	59.51±4.406	8.09±1.659	11.86±2.856
				p=0.946	p=0.993	p=0.381	p=0.871	p=0.976	p=0.937	p=0.901	p=0.280	p=0.880	p=0.937
Aσ	HTM	Control	11	4.32±0.514	53.96±3.875	74.70±3.300	2.49±0.289	1.03±0.123	1.40±0.204	193.5±13.69	94.53±10.12	9.51±1.494	9.68±2.241
		NeP	7	4.86±0.505	53.86±3.341	78.75±3.829	3.87±0.604	1.06±0.075	2.66±0.518	168.3±23.21	62.09±9.594	9.68±2.564	8.32±1.910
				p=0.487	p=0.986	p=0.442	p=0.035*	p=0.869	p=0.018*	p=0.331	p=0.044*	p=0.951	p=0.677
	LTM	Control	7	5.17±0.705	70.65±4.122	66.92±3.867	1.76±0.155	0.77±0.081	0.98±0.098	202.8±15.22	123.1±7.946	5.72±1.695	3.22±0.628
		NeP	9	3.91±0.500	62.23±2.126	62.59±2.602	2.51±0.412	0.93±0.092	1.91±0.341	159.1±11.34	89.84±8.396	7.44±1.463	4.14±1.255
				p=0.154	p=0.072	p=0.351	p=0.147	p=0.229	p=0.035*	p=0.033*	p=0.013*	p=0.454	p=0.560
	Unresponsive	Control	4	3.21±0.766	54.12±3.227	75.26±5.250	1.98±0.262	0.88±0.15	1.09±0.167	195.5±17.24	86.23±21.145	8.54±3.340	12.87±3.639
		NeP	4	4.14±1.041	53.21±1.545	79.11±5.968	2.71±0.619	1.17±0.06	1.28±0.386	163.1±26.37	74.371±10.165	10.34±3.344	13.3±2.652
				p=0.496	p=0.807	p=0.644	p=0.161	p=0.244	p=0.647	p=0.320	p=0.631	p=0.761	p=0.926
Aβ	HTM	Control	26	12.42±0.548	62.80±2.280	75.18±2.116	1.73±0.061	0.71±0.041	1.02±0.032	262.9±14.87	141.9±9.230	9.39±0.614	10.69±1.551
		NeP	39	11.38±0.504	63.21±1.783	75.47±1.674	2.45±0.146	0.86±0.054	1.58±0.132	211.5±11.07	94.47±9.224	6.57±0.765	8.98±0.931
				p=0.179	p=0.886	p=0.912	P<0.001***	p=0.049*	p=0.001**	p=0.028*	p=0.011*	p=0.010*	p=0.318
	LTM (CUT)	Control	50	17.02±0.469	68.63±1.100	63.09±1.508	1.25±0.038	0.49±0.019	0.76±0.028	262.4±8.735	142.7±4.855	5.51±0.493	5.42±0.834
		NeP	63	14.70±0.548	65.63±0.962	63.16±1.292	1.48±0.065	0.57±0.019	0.90±0.050	226.2±7.947	131.7±5.882	4.89±0.365	5.65±0.743
				p=0.002**	p=0.042*	p=0.970	p=0.004*	p=0.002**	p=0.022*	p=0.002**	p=0.164	p=0.304	p=0.839
	LTM (MS)	Control	36	18.76±0.638	66.47±1.704	57.83±0.929	0.96±0.032	0.43±0.017	0.53±0.022	258.4±12.44	186.0±8.476	6.73±0.678	1.58±0.114
		NeP	53	16.69±0.599	62.60±1.055	56.63±1.107	1.10±0.045	0.49±0.016	0.62±0.033	225.9±8.019	155.0±5.126	7.07±0.581	2.20±0.146
				p=0.023*	p=0.044*	p=0.442	p=0.022*	p=0.015*	p=0.046*	p=0.023*	p=0.001**	p=0.708	p=0.003**
	Unresponsive	Control	8	12.67±0.831	63.48±2.774	81.95±2.107	1.63±0.062	0.63±0.021	1.03±0.048	258.4±13.18	133.5±7.025	8.34±0.719	10.26±3.545
		NeP	9	11.15±1.088	61.03±3.482	79.13±3.438	2.29±0.251	0.77±0.093	1.53±0.260	221.4±23.38	104.8±15.48	5.23±1.207	9.74±1.805
				p=0.292	p=0.596	p=0.508	p=0.029*	p=0.189	p=0.094	p=0.202	p=0.127	p=0.048*	p=0.893

Statistical tests for each variable were made in all subgroups comparing neurons in control and neuropathic rats. The means ± S.D. of variables measured are given in the

table. The value of P (mean-Whitney) is shown below each section. Abbreviations: n, the number of neurons in each group; CV, conduction velocity; Vm, resting membrane potential; APA, action potential amplitude; APdB, action potential duration at base; APRT, action potential rise time; APFT, action potential fall time; MRR, maximum rising rate; MFR, maximum falling rate; AHPA, afterhyperpolarization amplitude; AHP50, after hyperpolarization duration at 50% recovery; HTM, high-threshold mechanoreceptor neurons; LTM, low-threshold mechanoreceptor neurons; UN, unresponsive neurons; MS, muscle spindle neurons. The asterisks show the level of significance of the difference between neurons in control and neuropathic rats: *$p<0.05$, **$P<0.01$; ***$p<0.001$.

Table 2.2. Locations of receptive fields of high-threshold mechanoreceptors and low-threshold mechanoreceptors in both the control and neuropathic rats

CV range	Receptor class	Model	n	Foot	Calf	Thigh	Ankle joint	Knee joint
C	HTM	Control	21	8	5	4	2	2
		NeP	28	12	4	6	4	2
	LTM	Control	8	5	2	1	/	/
		NeP	8	6	/	2	/	/
Aδ	HTM	Control	11	5	3	3	/	1
		NeP	7	4	2	1	/	/
	LTM	Control	7	2	3	2	/	/
		NeP	9	2	4	3	/	/
Aβ	HTM	Control	26	4	6	8	4	4
		NeP	39	3	9	12	3	2
	LTM (GF)	Control	20	4	6	8	1	1
		NeP	25	3	5	13	2	1
	LTM (RA)	Control	20	10	1	2	1	2
		NeP	24	9	1	3	2	1
	LTM (SA)	Control	10	9	/	/	1	/
		NeP	14	12	/	/	2	/
	LTM (MS)	Control	36	2	25	9	/	/
		NeP	53	3	40	10	/	/

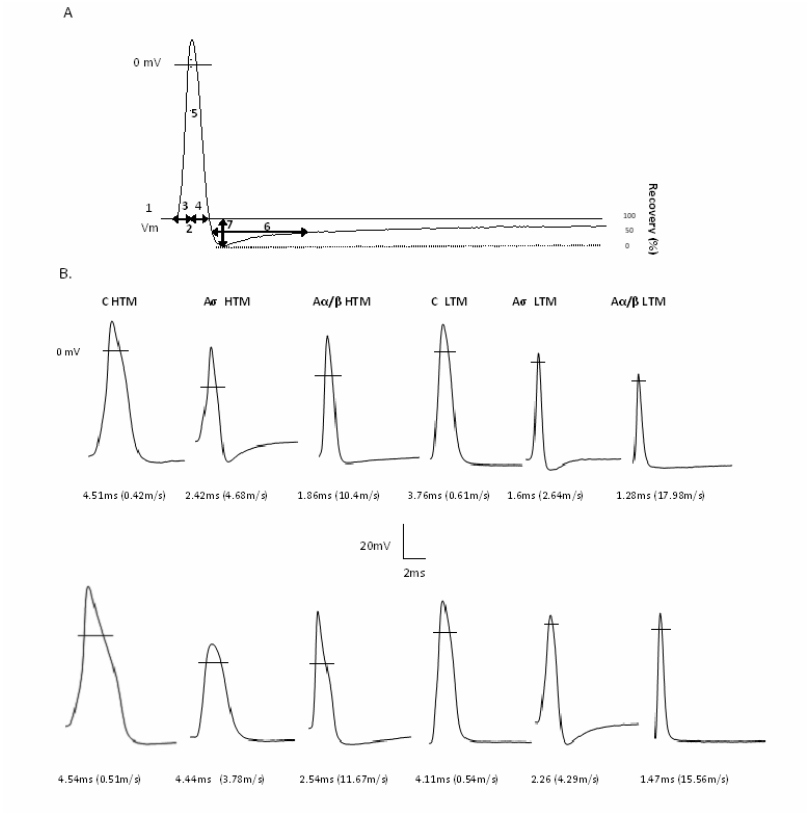

Figure 2.1. Examples of APs recorded from mechanoreceptive neurons. A. Representative intracellular somatic AP of an A-fiber neuron evoked by electrical stimulation of the dorsal root showing the electrophysiological parameters measured, including 1, resting membrane potential (Vm), 2, AP duration at base (APdB), 3, AP rise time (RT), 4, AP fall time (FT), 5, AP amplitude (APA), 6, AHP duration to 50% recovery (AHP50), 7, and AHP amplitude below Vm (AHPA). In addition, maximum rising and falling rates, (dV/d*t*) max, were measured from the differential trace of the AP. **B**. Somatic APs evoked by dorsal root stimulation and recorded intracellularly from 12 mechanosensitive neurons selected to represent the mean AP duration values for each of the different groups of neuron in control (upper) and neuropathic (lower) rats. The AP duration and (CV) for each neuron are given to the below each record. The horizontal lines across the APs indicate zero membrane potential.

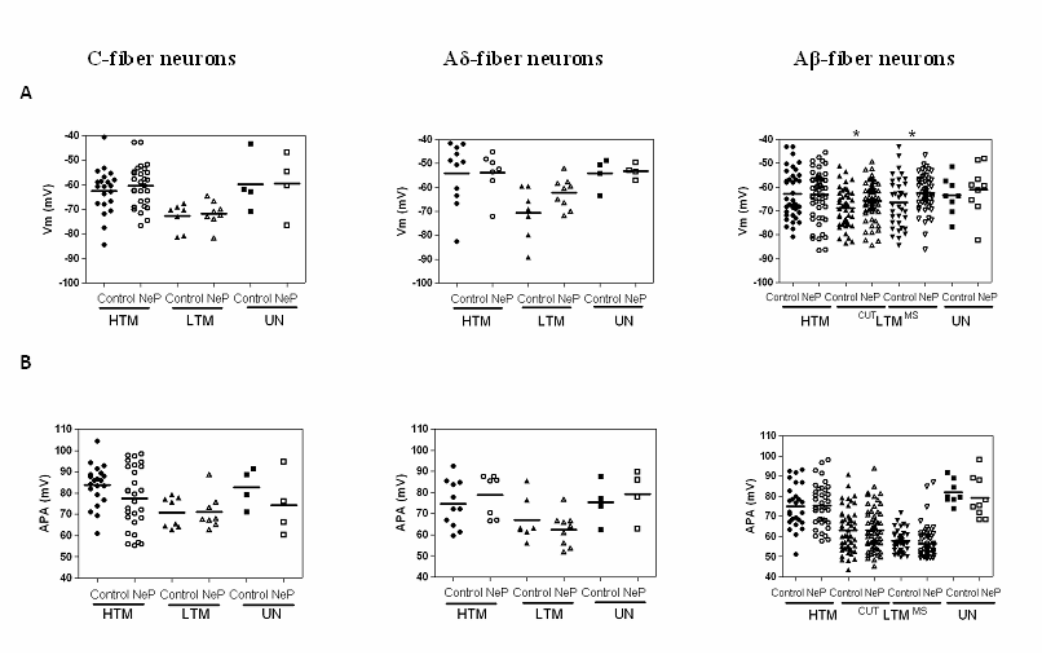

Figure 2.2. Comparison of AP resting membrane potential and amplitude of DRG neurons between control and neuropathic rats. Scatter plots show the distribution of the variables with the median (horizontal line) superimposed in each case. Vm, resting membrane potential AP; APA, action potential amplitude; HTM, high-threshold mechanoreceptor neurons; LTM, low-threshold mechanoreceptor neurons; UN, unresponsive neurons; CUT, Aβ-fiber LTM neurons including guard/field hair neurons (GF), rapidly adapting neurons (RA) and slowly adapting neurons (SA); MS, muscle spindle neurons. Asterisks above the graph indicate the significant differences between control and neuropathic rats: *p<0.05, **P<0.01; ***p<0.001. The absence of an asterisk indicates the lack of a statistically significant difference.

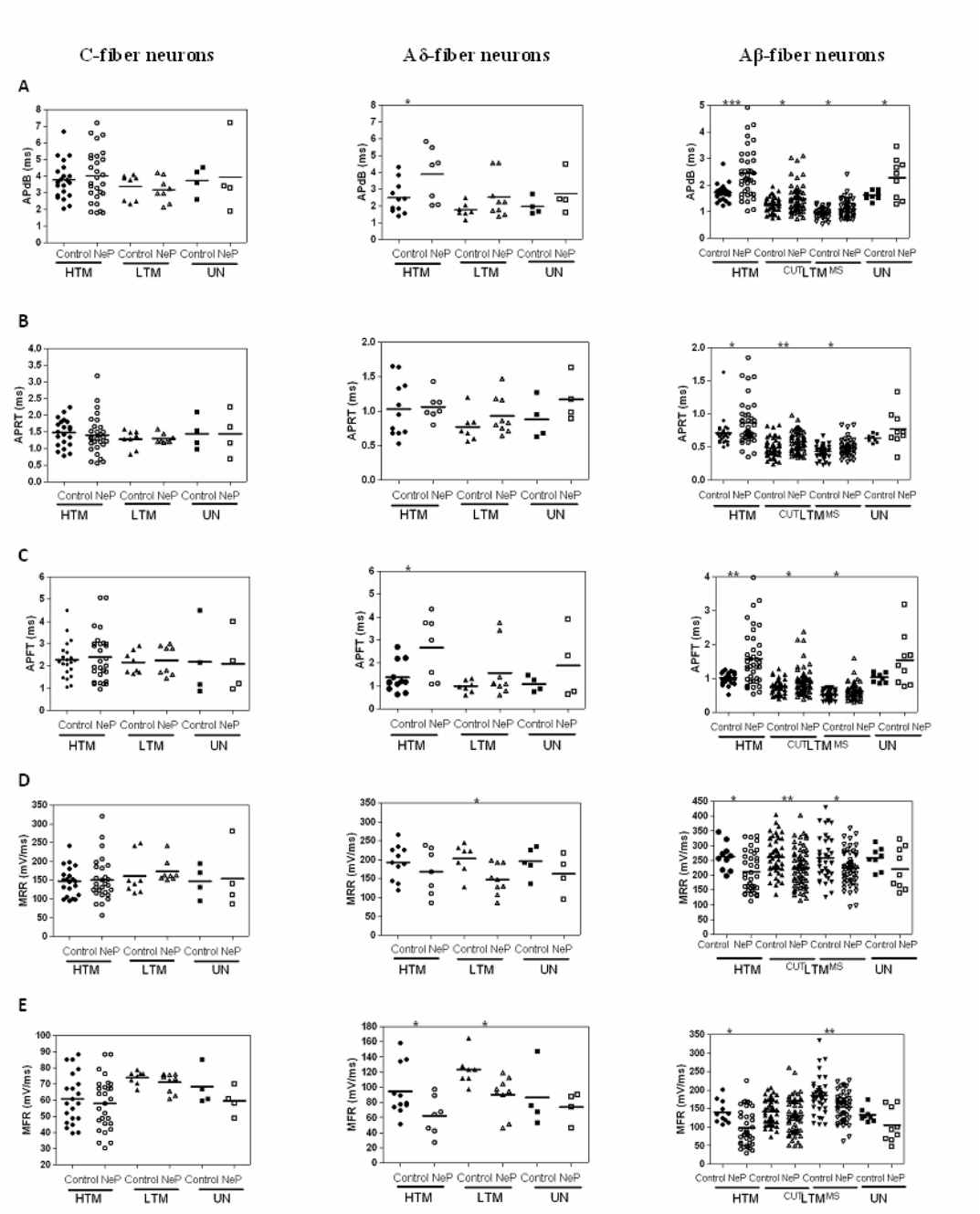

Figure 2.3. Comparison of AP dynamic parameters of DRG neurons between control and neuropathic rats. Scatter plots show the distribution of the variables with the median (horizontal line) superimposed in each case. APdB, action potential

duration at base; APRT, action potential rise time; APFT, action potential fall time; MRR, maximum AP rising rate; MFR, maximum AP falling rate. Other details are the same as in Figure 2.2. Asterisks above the graph indicate the significant differences between control and neuropathic rats: *$p<0.05$, **$P<0.01$; ***$p<0.001$. The absence of an asterisk indicates the lack of a statistically significant difference.

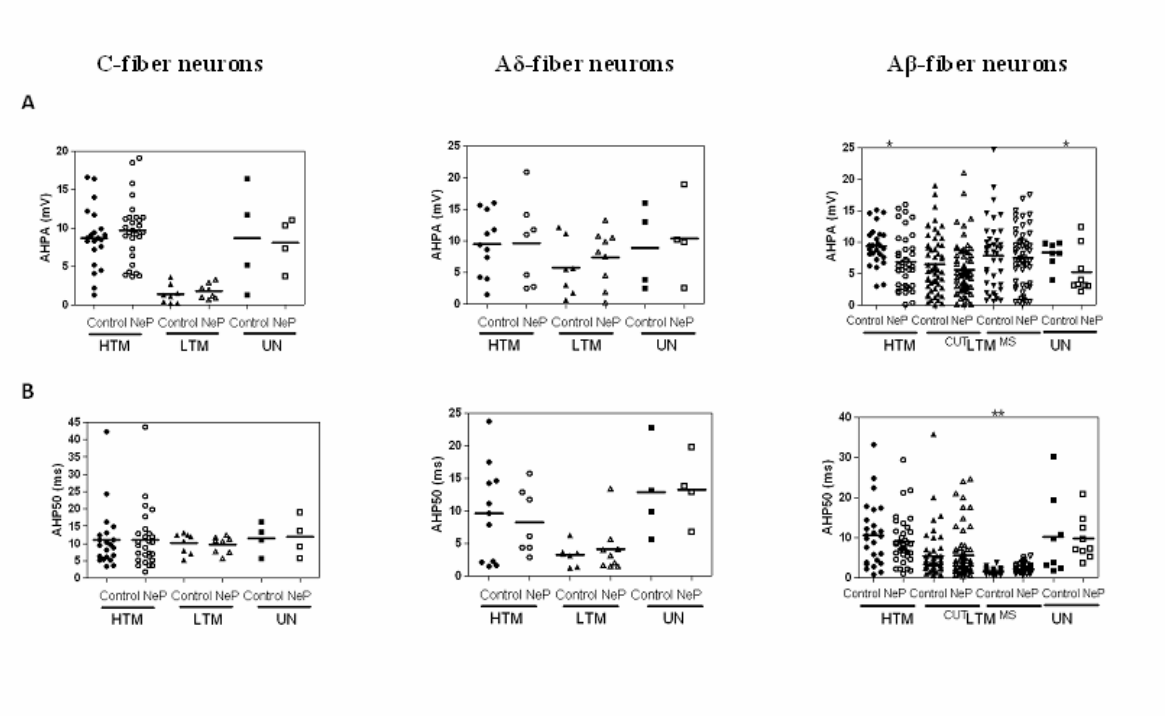

Figure 2.4. Comparison of AHP variables of DRG neurons between control and neuropathic rats. Scatter plots show the distribution of the variables with the median (horizontal line) superimposed in each case. AHPA, afterhyperpolarization amplitude; AHP50, afterhyperpolarization duration to 50% recovery. Other details are the same as in Figure 2.2. Asterisks above the graph indicate the significant differences between control and neuropathic rats: *p<0.05, **P<0.01; ***p<0.001. The absence of an asterisk indicates the lack of a statistically significant difference.

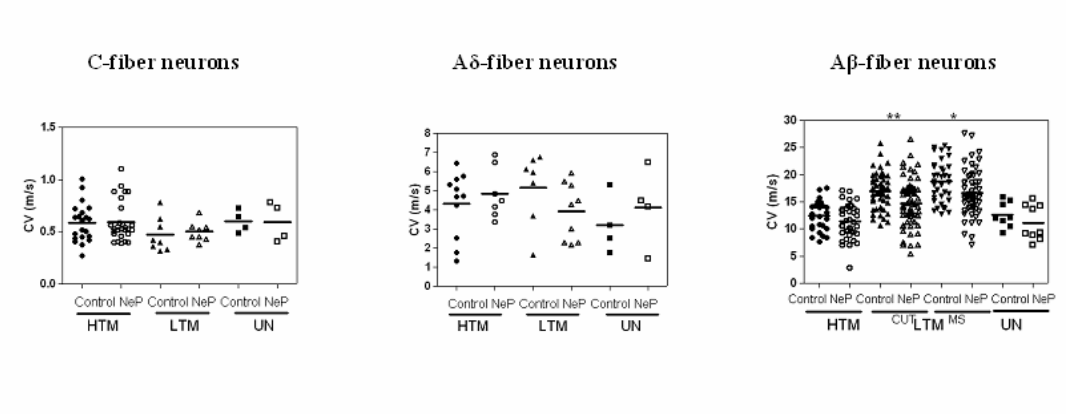

Figure 2.5. Comparison of dorsal root conduction velocity of DRG neurons between control and neuropathic rats. Scatter plots show the distribution of the variables with the median (horizontal line) superimposed in each case. CV, conduction velocity. Details are the same as in Figure 2.2. Asterisks above the graph indicate the significant difference between control and neuropathic rats: *p<0.05, **P<0.01; ***p<0.001. The absence of an asterisk indicates the lack of a statistically significant difference.

Reference List

Abdulla FA, Smith PA, 2001. Axotomy- and autotomy-induced changes in the excitability of rat dorsal root ganglion neurons. J. Neurophysiol. 85: 630-643.

Amaya F, Wang H, Costigan M, Allchorne AJ, Hatcher JP, Egerton J, Stean T, Morisset V, Grose D, Gunthorpe MJ, Chessell IP, Tate S, Green PJ, Woolf CJ, 2006. The voltage-gated sodium channel Na(v)1.9 is an effector of peripheral inflammatory pain hypersensitivity. J. Neurosci. 26: 12852-12860.

Aurilio C, Pota V, Pace MC, Passavanti MB, Barbarisi M, 2008. Ionic channels and neuropathic pain: physiopathology and applications. J. Cell Physiol 215: 8-14.

Baccei ML, Kocsis JD, 2000. Voltage-gated calcium currents in axotomized adult rat cutaneous afferent neurons. J. Neurophysiol. 83: 2227-2238.

Black JA, Cummins TR, Plumpton C, Chen YH, Hormuzdiar W, Clare JJ, Waxman SG, 1999. Upregulation of a silent sodium channel after peripheral, but not central, nerve injury in DRG neurons. J. Neurophysiol. 82: 2776-2785.

Boada MD, Woodbury CJ, 2007. Physiological properties of mouse skin sensory neurons recorded intracellularly in vivo: temperature effects on somal membrane properties. J. Neurophysiol. 98: 668-680.

Campbell JN, Meyer RA, 2006. Mechanisms of neuropathic pain. Neuron 52: 77-92.

Campbell JN, Raja SN, Meyer RA, Mackinnon SE, 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32: 89-94.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL, 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53: 55-63.

Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderoth B, 2000. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. Pain 88: 239-248.

Cummins TR, Sheets PL, Waxman SG, 2007. The roles of sodium channels in nociception: Implications for mechanisms of pain. Pain 131: 243-257.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Devor M, 1983. Nerve pathophysiology and mechanisms of pain in causalgia. J. Auton. Nerv. Syst. 7: 371-384.

Devor M, 2006. Sodium channels and mechanisms of neuropathic pain. J. Pain 7: S3-S12.

Dixon WJ, 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20: 441-462.

Djouhri L, Bleazard L, Lawson SN, 1998. Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J. Physiol 513 (Pt 3): 857-872.

Everill B, Kocsis JD, 1999. Reduction in potassium currents in identified cutaneous afferent dorsal root ganglion neurons after axotomy. J. Neurophysiol. 82: 700-708.

Fang X, McMullan S, Lawson SN, Djouhri L, 2005. Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. J. Physiol 565: 927-943.

Gabay E, Tal M, 2004. Pain behavior and nerve electrophysiology in the CCI model of neuropathic pain. Pain 110: 354-360.

Gherardini G, Lundeberg T, Cui JG, Eriksson SV, Trubek S, Linderoth B, 1999. Spinal cord stimulation improves survival in ischemic skin flaps: an experimental study of the possible mediation by calcitonin gene-related peptide. Plast. Reconstr. Surg. 103: 1221-1228.

Hains BC, Klein JP, Saab CY, Craner MJ, Black JA, Waxman SG, 2003. Upregulation of sodium channel Nav1.3 and functional involvement in neuronal hyperexcitability associated with central neuropathic pain after spinal cord injury. J. Neurosci. 23: 8881-8892.

Han HC, Lee DH, Chung JM, 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain 84: 253-261.

Katz EJ, Gold MS, 2006. Inflammatory hyperalgesia: a role for the C-fiber sensory neuron cell body? J. Pain 7: 170-178.

Khan GM, Chen SR, Pan HL, 2002. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. Neuroscience 114: 291-299.

Kim SH, Chung JM, 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50: 355-363.

Kim YI, Na HS, Kim SH, Han HC, Yoon YW, Sung B, Nam HJ, Shin SL, Hong SK, 1998. Cell type-specific changes of the membrane properties of peripherally-axotomized dorsal root ganglion neurons in a rat model of neuropathic pain. Neuroscience 86: 301-309.

Kretschmer T, Happel LT, England JD, Nguyen DH, Tiel RL, Beuerman RW, Kline DG, 2002. Accumulation of PN1 and PN3 sodium channels in painful human

neuroma-evidence from immunocytochemistry. Acta Neurochir. (Wien.) 144: 803-810.

Lang PM, Schober GM, Rolke R, Wagner S, Hilge R, Offenbacher M, Treede RD, Hoffmann U, Irnich D, 2006. Sensory neuropathy and signs of central sensitization in patients with peripheral arterial disease. Pain 124: 190-200.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Leem JW, Willis WD, Chung JM, 1993. Cutaneous sensory receptors in the rat foot. J. Neurophysiol. 69: 1684-1699.

Lever IJ, Bradbury EJ, Cunningham JR, Adelson DW, Jones MG, McMahon SB, Marvizon JC, Malcangio M, 2001. Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. J. Neurosci. 21: 4469-4477.

Li Y, Dorsi MJ, Meyer RA, Belzberg AJ, 2000. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 85: 493-502.

Liu B, Eisenach JC, 2005. Hyperexcitability of axotomized and neighboring unaxotomized sensory neurons is reduced days after perineural clonidine at the site of injury. J. Neurophysiol. 94: 3159-3167.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Ma C, Shu Y, Zheng Z, Chen Y, Yao H, Greenquist KW, White FA, LaMotte RH, 2003. Similar electrophysiological changes in axotomized and neighboring intact dorsal root ganglion neurons. J. Neurophysiol. 89: 1588-1602.

Ma W, Bisby MA, 1998. Increase of calcitonin gene-related peptide immunoreactivity in the axonal fibers of the gracile nuclei of adult and aged rats after complete and partial sciatic nerve injuries. Exp. Neurol. 152: 137-149.

Malcangio M, Ramer MS, Jones MG, McMahon SB, 2000. Abnormal substance P release from the spinal cord following injury to primary sensory neurons. Eur. J. Neurosci. 12: 397-399.

Merskey H, Lindblom U, Mumford JM, Nathan PW, Sunderland S. 1994.Pain Terms**.** A current list with definitions and notes on usage. In: Merskey H, Bolduk N. Eds. Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain. Second Edition, Part III, IASP Press, Seattle, WA, USA.

Michael GJ, Averill S, Shortland PJ, Yan Q, Priestley JV, 1999. Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. Eur. J. Neurosci. 11: 3539-3551.

Miki K, Fukuoka T, Tokunaga A, Noguchi K, 1998. 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 82: 1243-1252.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K, 1995. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J. Neurosci. 15: 7633-7643.

Pitcher GM, Henry JL, 2002. Second phase of formalin-induced excitation of spinal dorsal horn neurons in spinalized rats is reversed by sciatic nerve block. Eur. J. Neurosci. 15: 1509-1515.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Pitcher GM, Henry JL, 2008. Governing role of primary afferent drive in increased excitation of spinal nociceptive neurons in a model of sciatic neuropathy. Exp. Neurol. 214: 219-228.

Pitcher GM, Ritchie J, Henry JL, 1999. Nerve constriction in the rat: model of neuropathic, surgical and central pain. Pain 83: 37-46.

Ramer MS, French GD, Bisby MA, 1997. Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. Pain 72: 71-78.

Richards RL, 1967. Causalgia. A centennial review. Arch. Neurol. 16: 339-350.

Rizzo MA, Kocsis JD, Waxman SG, 1995. Selective loss of slow and enhancement of fast Na+ currents in cutaneous afferent dorsal root ganglion neurones following axotomy. Neurobiol. Dis. 2: 87-96.

Rogers M, Tang L, Madge DJ, Stevens EB, 2006. The role of sodium channels in neuropathic pain. Semin. Cell Dev. Biol. 17: 571-581.

Schaible HG, 2007. Peripheral and central mechanisms of pain generation. Handb. Exp. Pharmacol.: 3-28.

Shamash S, Reichert F, Rotshenker S, 2002. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J. Neurosci. 22: 3052-3060.

Shim B, Kim DW, Kim BH, Nam TS, Leem JW, Chung JM, 2005. Mechanical and heat sensitization of cutaneous nociceptors in rats with experimental peripheral neuropathy. Neuroscience 132: 193-201.

Sleeper AA, Cummins TR, Dib-Hajj SD, Hormuzdiar W, Tyrrell L, Waxman SG, Black JA, 2000. Changes in expression of two tetrodotoxin-resistant sodium channels and their currents in dorsal root ganglion neurons after sciatic nerve injury but not rhizotomy. J. Neurosci. 20: 7279-7289.

Sommer C, Schafers M, 1998. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res. 784: 154-162.

Stebbing MJ, Eschenfelder S, Habler HJ, Acosta MC, Janig W, McLachlan EM, 1999. Changes in the action potential in sensory neurones after peripheral axotomy in vivo. Neuroreport 10: 201-206.

Tal M, Wall PD, Devor M, 1999. Myelinated afferent fiber types that become spontaneously active and mechanosensitive following nerve transection in the rat. Brain Res. 824: 218-223.

Wagner R, Myers RR, 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73: 625-629.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA, 1999. Sodium channels and pain. Proc. Natl. Acad. Sci. U. S. A 96: 7635-7639.

Weissner W, Winterson BJ, Stuart-Tilley A, Devor M, Bove GM, 2006. Time course of substance P expression in dorsal root ganglia following complete spinal nerve transection. J. Comp Neurol. 497: 78-87.

Wu Q, Henry JL, 2009. Delayed onset of changes in soma action potential genesis in nociceptive A-beta DRG neurons in vivo in a rat model of osteoarthritis. Mol. Pain 5: 57.

Wu Q, Henry JL, 2010. Changes in Abeta non-nociceptive primary sensory neurons in a rat model of osteoarthritis pain. Mol. Pain 6: 37.

Yang RH, Xing JL, Duan JH, Hu SJ, 2005. Effects of gabapentin on spontaneous discharges and subthreshold membrane potential oscillation of type A neurons in injured DRG. Pain 116: 187-193.

Yoshida S, Matsuda Y, Samejima A, 1978. Tetrodotoxin-resistant sodium and calcium components of action potentials in dorsal root ganglion cells of the adult mouse. J. Neurophysiol. 41: 1096-1106.

CHAPTER 3

Excitability of Aβ sensory neurons is altered in an animal model of peripheral neuropathy

Authors: Yong Fang Zhu, James L Henry

Corresponding author: James L Henry, PhD
Department of Psychiatry and Behavioral Neurosciences,
McMaster University,
1200 Main St. West, HSC 4N35
Hamilton, Ontario, Canada, L8N 3Z5,
Tel: +1-905-525-9140, extension 24750,
FAX: 905-4522-8844
Email address: jhenry@mcmaster.ca

Preface

Significance to thesis

This study demonstrated the altered excitability of Aβ-fiber high-threshold mechanoreceptor (HTM) neurons and low-threshold mechanoreceptor (LTM) neurons, especially muscle spindle (MS) neurons, which associated with peripheral neuropathy. This study suggested that these neurons may contribute to symptoms of tactile allodynia and spontaneous pain following peripheral nerve injury in humans.

Authors' contribution

Yong Fang Zhu did the electrophysiological experiments, the behavior test, and model induction. She also analyzed the data, performed statistical analyses, wrote the initial draft of the manuscript, and worked on refining this draft and the revision based on editorial review.

James L. Henry conceived of, designed the study. He also worked on refining this draft and the revision based on editorial review.

Abstract

Altered excitability of primary sensory neurons may be may be one of the mechanisms underlying pain following a peripheral neuropathy. Studies aimed at investigating the role of primary sensory neurons in neuropathic pain models have so far mostly focused on small diameter neurons despite evidence of ectopic activity of Aβ-fiber sensory neurons in models of peripheral neuropathy. We therefore used intracellular recordings from the somata of DRG neurons *in vivo* using the Mosconi and Kruger model of neuropathic pain to identify changes in neurons associated with fast-conducting large diameter Aβ-fiber sensory neurons. These sensory neurons in model rats exhibited altered excitability, consisting of decreased mechanical activation threshold of the periphery and abnormal discharge patterns of action potentials (APs) from stimulation of the receptive field, decreased current activation threshold of the soma and a greater number of APs evoked by intracellular current injection, increased current activation threshold of dorsal roots by dorsal root stimulation as well as longer refractory interval and a delayed AP response to the second of paired-pulse dorsal root stimulation. The data suggest increased excitability of receptive field and somata but decreased excitability of dorsal root axons. These observations provide the first demonstration of altered excitability of Aβ-fiber HTM and LTM neurons, especially MS neurons in an animal model of peripheral neuropathy, and suggest that changes in these neurons may contribute to symptoms of spontaneous pain and tactile allodynia following peripheral nerve injury in humans.

3.1. Introduction

Neuropathic pain is associated with exaggerated responses to painful stimuli (hyperalgesia), pain provoked by innocuous stimulation (allodynia), abnormal

spontaneous sensations (dysesthesia) and/or a spontaneous burning pain (Bonica, 1968; Bonica, 1973; Devor, 1983; Richards, 1967). Currently, the mechanisms of neuropathic pain following peripheral nerve injury remain unresolved and thus mechanism-based treatments are lacking. Evidence from axotomy models of peripheral neuropathic pain suggests that injured sensory neurons and perhaps also those of neighboring uninjured neurons in these models can generate ectopic spiking neuronal activity (Devor, 2009). These ectopic discharges are suggested to enter the spinal cord to alter central sensory processing by sensitizing dorsal horn neurons (Devor, 2009; Lang et al., 2006; Campbell and Meyer, 2006). Therefore, understanding the characteristics of ectopic discharge in DRG neurons in neuropathic rats may provide insights into mechanisms underlying neuropathic pain in humans.

The physiological consequences of peripheral nerve damage associated with neuropathic pain readouts in animal models have provided detailed information suggesting an involvement of C-fiber sensory neurons in mediating the functional changes in these models. This is consistent with the classic concept that pain and central sensitization are largely due to activity in C-fiber sensory neurons. However, in several animal models of peripheral neuropathy, the bulk of the ectopic activity has been reported to occur in thickly myelinated, large diameter axons (Han et al., 2000; Liu et al., 2000; Tal et al., 1999; Ma et al., 2003; Khan et al., 2002). In some of our recent studies on the Mosconi and Kruger animal model of peripheral neuropathy, which focused on the configuration of the somata AP, the CV of the axon and activation of the respective sensory receptor fields, it was observed that the major changes occurred in large Aβ-fiber DRG neurons, with only minor changes in Aδ-fiber sensory neurons, and no changes in C-fiber sensory neurons (Chapter 2).

Thus, to explore the possibility that Aβ-fiber sensory neurons may contribute to sensory afferent ectopic activity and potentially to neuropathic pain, the present study was designed to use intracellular recording *in vivo* to examine further the excitability of defined subpopulations of functionally identified DRG neurons after a peripheral nerve injury. We aimed to determine the excitability and measure any ectopic activity in Aβ-fiber sensory neurons, and to determine whether changes in excitability occur in the soma, the axon or the peripheral receptors.

3.2. Methods

All experimental procedures were approved by the McMaster University Animal Research Ethics Board and rats were treated in accordance with the Guide to the Care and Use of Experimental Rats, Vols. 1 and 2, of the Canadian Council on Animal Care.

Experimental rats and model induction

Young male Sprague-Dawley rats (obtained from Charles River Inc. St. Constant, QC, Canada) weighing 170-200 g were used. Rats were divided into two groups, control and neuropathic model groups. A peripheral neuropathy was induced according to the method previously described in detail (Pitcher et al., 1999; Mosconi and Kruger, 1996; Chapter 2). Briefly, under anesthesia with a mixture of ketamine (Ketamine; 5 mg/100g; Bimeda-MTC Animal Health Inc.; Cambridge, ON, Canada), xylazine (Rompun; 0.5 mg/100g; Bayer HealthCare, Toronto, ON, Canada), and acepromazine (Atravet; 0.1 mg/100g; Ayerst Veterinary Laboratories, Guelph, ON, Canada) given i.p., the right sciatic nerve was exposed at the mid-thigh level. Two cuffs of 0.5mm polyethylene tubing (Intramedic PE-90, Fisher Scientific Ltd., Whitby, Ontario, Canada) were inserted around the exposed nerve and placed approximately 1

mm apart. The incision was then sutured in two layers, muscle and skin, and the rats allowed to recover from anesthesia. Antibiotic ointment (Furacin; nitrofurazone 0.2%; Vetoquinol N.-A. Inc.; Lavaltrie, QC, Canada) was applied over the sutured incision, and 0.01 ml/100g of antibacterial injectable solution (Bayer HealthCare, Toronto, ON, Canada) was injected subcutaneously (s.c.). Rats were given 1 ml saline s.c., and ocular lubricant, and placed under a heating lamp until they recovered from the anesthetic. They were then returned to their home cages.

von Frey test of paw withdrawal threshold

In all cases, the von Frey test was run on the same day as the recording day before the rats were anesthetized for the acute electrophysiological experiment; this was to confirm the establishment of a behavioural tactile hypersensitivity, a hallmark of this model of peripheral neuropathic pain. To quantify mechanical sensitivity of the foot, brisk foot withdrawal in response to application of von Frey filaments was measured as described previously (Kim and Chung, 1992; Chapter 2). The rats were placed in a transparent Plexiglas box with a clear Plexiglas floor, containing 0.5 cm diameter holes spaced 1.5 cm apart to allowed full access to the paws (Pitcher and Henry, 2004). Each rat was allowed to habituate to the box for approximately 15 min, until cage exploration and major grooming activities had ceased.

Calibrated von Frey filaments (Stoelting Co., Wood Dale, IL, USA) were applied to the plantar surface of the hind paw to determine the withdrawal threshold. Each von Frey filament was applied 5 times (for 3-4sec each, at 3 sec intervals) to a different spot on each hind paw. These filaments were applied in ascending order, beginning with the finest filament, until a clear withdrawal response was observed. When this occurred, the next lightest filament was applied following the same

procedure. A 50% withdrawal response threshold was derived according to responses to this testing regimen (Chaplan et al., 1994) using the up-down method of Dixon (Dixon, 1980). A brisk foot withdrawal in response to these innocuous mechanical stimuli was considered the mechanical threshold.

Intracellular recording in vivo

Approaches to the animal preparation and intracellular recording techniques have been reported previously (Wu and Henry, 2009; Wu and Henry, 2010; Chapter 2). In brief, each animal was initially anesthetized with ketamine mixture described above. The right jugular vein was cannulated for i.v. infusion of drugs. A cannula was inserted into the trachea. The rat was then fixed in a stereotaxic frame and the vertebral column rigidly clamped at the L2 and L6 vertebrae. The right femur was fixed by a customized clamp onto the stereotaxic frame to minimize movement of the DRG during mechanical searching for receptive fields on the leg. The L4 DRG was selected for study as it contains one of the largest numbers of the hind leg afferent somata (Wu and Henry, 2009; Wu and Henry, 2010). A laminectomy was performed to expose the ipsilateral L4 DRG. The L4 dorsal root was sectioned close to the spinal cord, allowing a 12-15 mm length, and the proximal end was placed on a bipolar electrode (FHC, Bowdoinham, ME, USA) used for stimulation purposes. The exposed spinal cord and DRG were covered with warm paraffin oil at 37^{o} C to prevent drying.

The rat was mechanically ventilated via the tracheal cannula using a Harvard Ventilator (Model 683, Harvard apparatus, Quebec, Canada). The ventilation parameters were adjusted so that end-tidal CO_2 concentration was maintained around 40-50 mmHg, as measured using a CapStar-100 End-Tidal CO2 analyzer (CWE,

Ardmore, PA, USA). Rectal temperature was maintained at ~37^{o}C using a temperature controlled infrared heating lamp. Immediately before the start of recording, the animal was given 20 mg/kg of Na pentobarbital (CEVA SANTE ANIMAL, Libourne, France), and supplemental doses of 10mg/kg of pentobarbital were given each hour through the carotid cannula to maintain a surgical level of anesthesia. In addition, just before recording, each animal was also given an initial 1 mg/kg dose of pancuronium bromide (Pavulon, Sandoz, Boucherville, QC, Canada) to eliminate muscle tone. Supplemental doses of pentobarbital and pancuronium were added with at initial dose of 1/3 about each hour via the carotid cannula.

Intracellular recordings from somata in the exposed DRG were made with borosilicate glass micropipettes (1.2 mm outside diameter, 0.68 mm inside diameter; Harvard Apparatus, Holliston MA, USA). The electrodes were pulled using a Brown-flaming puller (model p-87; Sutter Instrument CO., Novota, CA, USA) and were filled with 3 M KCl (DC resistance 50-70 MΩ). Signals were recorded with a Multiclamp 700B amplifier (Molecular Devices, Union City CA, USA) and digitized on-line via Digidata 1322A interface (Molecular Devices, USA) with pClamp 9.2 software (Molecular Devices, USA). The microelectrode was advanced using an EXFO IW-800 micromanipulator (EXFO, Montreal, QC, Canada) in 2 μm steps until a hyperpolarization of at least 40 mV suddenly appeared. Once a stable membrane potential had been confirmed, a stimulus was applied to the dorsal roots to provoke an AP; with the aid of the protocol editor function in pClamp 9.2 software, a somatic AP was evoked by stimulation with a single rectangular voltage pulse.

DRG neuron classification

The criteria for neuron classification were based on those reported in previous

in studies (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010; Chapter 2). Neurons were divided into three groups according to dorsal root CVs: C-fiber sensory neurons (<=0.8mm/ms), Aδ-fiber neurons (1.5-6.5mm/ms) and Aβ-fiber neurons (>6.5mm/ms). Compared to other criteria from other groups (Handwerker et al., 1991; Leem et al., 1993), these criteria most closely matched the present study, including similar surgical procedure, recording technique and setting, etc.

The sensory receptive properties of each DRG neuron were examined using hand-held mechanical stimulators and classified as previously described (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Leem et al., 1993; Wu and Henry, 2009; Wu and Henry, 2010). The threshold of activation, the depth of the receptive field and the pattern of adaption were the major factors to further classify neurons into LTM, HTM and unresponsive neurons.

LTM neurons were further classified using soft brush, light pressure with a blunt object, light tap and vibration. Many Aβ-fiber neurons LTM neurons are cutaneous (CUT), and include guard/field hair neurons (GF), glabrous skin neurons, Pacinian neurons, slowly adapting neurons (SA). GF neurons were rapidly adapting cutaneous neurons. Glabrous and Pacinian neurons were both rapidly adapting non-hair neurons, and were named rapidly adapting neurons (RA). SA neurons were slowly adapting cutaneous neurons. One further group in Aß-fiber LTM neurons with deeper receptive fields, that were very sensitive to light pressure and/or leg movement and often showed ongoing activity, were classified as muscle spindle (MS) neurons.

HTM neurons responded to noxious stimuli including noxious pinch and application of sharp objects such as the end of a syringe needle. Neurons that did not

respond to any of the innocucous or noxious mechanical stimuli listed above were classed as unresponsive (Lawson et al., 1997). Heat nociceptors and specific cooling receptors were not included in this study due to the very low numbers of such neurons (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997).

Stimulation from different sites of sensory neurons

The various parts of the primary afferents were stimulated to determine excitability measured as evoked APs in the soma, including stimulation of the soma by direct depolarizing current injection, electrical stimulation of the dorsal roots using bipolar stimulating electrodes and activation of the peripheral receptive field.

Soma - To quantify soma excitability, with the aid of the protocol editor function in pClamp 9.2 software (Molecular Devices), the threshold of depolarizing current pulses injected into the soma was determined by lying current pulses of 100 ms in increments of 0.05 nA through the recording electrode until an AP was elicited or until a maximum current of 4nA was reached. The excitability of the soma was also evaluated by comparing the number of APs evoked by injecting defined current pulses to the DRG soma; three intracellular current injections of 20 ms each were delivered with 1, 1.5 and 2 nA.

Dorsal roots - Dorsal root excitability was measured by determining the chronaxie curve (threshold-duration), which was defined by delivering the minimum current that would elicit an AP in the soma to the dorsal root using current pulse durations of 0.1, 1, 2, 4 and 6 ms. The stimulation pulse was delivered from an S940/910 stimulus adaptor/isolator (Dagan, Minneapolis, MN. USA). Dorsal root activity was also tested by measuring the refractory interval of two APs to paired-pulse stimulation. Stimulation started with search stimuli with coarse interval steps

down of 1ms, followed by final interval steps at 0.1ms. Stimulus pairs had intervals of 1-80 ms and this interval was decreased gradually at final interval steps with a pause of 4s between pairs of stimuli. For each neuron, the recording with smallest RI (refractory interval) was chosen for analysis.

Peripheral receptive field - The mechanical sensitivity of DRG neurons was determined individually with calibrated von Frey filaments as described by other groups (Lawson et al., 1997; Leem et al., 1993). After identification of the receptor type for classification of DRG neurons, the size and localization of the receptive field using von Frey filaments applied to the receptive field were determined. Mechanical threshold of these neurons was determined as the minimum force, in g, necessary to evoke APs. In most cases, von Frey filaments were applied to the most sensitive spot on the skin. For Aβ-fiber G-hair and Aδ D-hair neurons, the von Frey filaments were applied to the tips of the hairs, while for Aβ field-hair neurons the von Frey filament were applied the skin next to the base of the hairs. For MS neurons, von Frey filaments were applied the skin along the region the muscle belly and the joint. Neurons that were not responsive to von Frey filaments were excluded in this part of the study. The mechanical stimuli forces exerted with the calibrated von Frey filaments used in this study were a set of von Frey filaments (0.008, 0.02, 0.04,0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0 ,8.0, 10, 15, 26, 60, 100, 180 and 300g; 1.65-6.65 mm in tip-diameters).

Statistical analysis

The data are represented as means ± SEMs. A Mann Whitney t-test was used for comparison of the response properties of the neurons between control and neuropathic rats unless otherwise stated. All statistical tests and graphing were done

using Prism4 software (Graphpad, La Jolla, CA, USA). P-value is indicated in the graphs and $P < 0.05$ was considered to indicate a significant difference.

3.3. Results

Withdrawal response in the behavioral von Frey test

Stimulation of the plantar surface of the hind paw with von Frey filaments evoked a withdrawal response in control rats, with hairs exerting pressures of 10-100 g. Three weeks after cuff implantation on the sciatic nerve on the right side, the rats fully developed behavioral signs of mechanical hypersensitivity on the affected hind limb, a parallel to the tactile allodynia reported by people with neuropathic pain. Neuropathic rats responded to filaments that the control rats ignored, i.e. 0.001 - 6.0 g, which evoked a clear withdrawal on the nerve-injured side. Furthermore, in neuropathic rats the withdrawal was exaggerated in amplitude and duration, and it was frequently accompanied by licking of the paw. Withdrawal thresholds were 14.82 ± 0.180 g in control rats ($N = 60$) and 5.65 ± 1.337 g in neuropathic rats ($N = 64$). Comparison of the data indicated a difference between these groups $P < 0.0001$. The data are shown in Figure 3.1 (same data used in Chapter 2).

Excitability of the receptive field measured by responses to application of von Frey filaments

To determine whether changes in properties of peripheral receptors might contribute to the mechanical hypersensitivity that characterizes this model, von Frey filaments were applied to the peripheral receptive fields of neurons studied in the electrophysiological recordings. Calibrated von Frey filaments were applied to the identified receptive field areas as a tactile stimulation and the minimum filament that elicited an AP in the soma was recorded.

The mechanical thresholds with von Frey filaments of all Aβ-fiber sensory neurons during electrophysiological recording are shown in Figure 3.2. The mechanical thresholds of GF (N = 14), RA (N = 15), SA (N = 9), MS (N = 25) and Aβ-fiber HTM (N = 16) neurons in control rats were within the range 0.008-2 g, 0.07-2 g, 0.07-2 g, 0.16-60 g and 4-60 g, respectively. In neuropathic rats, the thresholds of GF (N = 16), RA (N = 16) neurons were the same as in control rats. However, in neuropathic rats the thresholds of SA (N = 9), MS (N = 28) and HTM (N = 22) neurons were shifted lower, to 0.008-2 g, 0.02-60 g and 2-60 g, respectively. The mean mechanical threshold of the MS neurons was significantly lower in neuropathic rats than in control rats: 19.48 ± 4.459 g in control vs. 5.64 ± 2.311g in neuropathic rats (P = 0.006). The other subtypes of Aβ-fiber sensory neurons showed no significant difference. Mean thresholds of GF neurons were 0.40 ± 0.170 g in control and 0.18 ± 0.124 g in neuropathic rats (P = 0.291). Those in RA neurons were 0.60 ± 0.170 g vs. 0.37 ± 0.118 g in control and neuropathic rats, respectively (P = 0.267). Those in SA neurons were 0.98 ± 0.237 g in control rats and 0.52 ± 0.200 g in neuropathic rats (P = 0.155). Those in HTM neurons in control rats were 22.31 ± 5.674 g vs.19.08 ± 3.948 g in neuropathic rats (P = 0.632).

Some neurons in neuropathic rats exhibited an ongoing discharge consisting of fast excitatory APs. For example, C-fiber cool neurons usually discharge at room temperature and MS neurons can discharge when the leg is fixed in an extended position in both control and neuropathic rats. In the present study, ongoing discharge was recorded from C-fiber cool neurons and Aβ-fiber MS neurons in both control (N = 62) and neuropathic rats (N = 70). As this study focused on differences in responses evoked by applied stimuli between control and model rats, these neurons were

excluded from the present analysis.

In neuropathic rats, 6 of 39 Aβ-fiber HTM neurons, and 1 of 10 Aβ-fiber GF neurons demonstrated certain electrical signs that are reasonably interpreted as ongoing discharge (Figure 3.3). These action potentials could be evoked by stimulation of the receptive field, and discharge persisted for several minutes after removal of the stimulus from the receptive field. It is important to note that in control rats, none of the 26 Aβ-fiber HTM neurons or of the 50 Aβ-fiber CUT neurons demonstrated any ongoing discharge following application of peripheral stimuli.

Excitability of the soma measured by responses to injection of depolarizing current

The AP responses to intracellular depolarizing current pulse injection were tested to determine whether there is a difference in soma excitability induced by the peripheral neuropathy. Figure 3.4 illustrates the threshold currents that elicited APs in control and neuropathic rats. Compared to the control group, Aβ-fiber HTM neurons and Aβ-fiber MS neurons in neuropathic rats showed a significant difference from controls; activation threshold of HTM neurons was 2.430 ± 0.526 nA (N = 15) vs. 0.87 ± 0.284 nA (N = 15) in control and neuropathic rats, respectively (P = 0.023). Activation threshold of MS neurons was 0.60 ± 0.138 nA in control (N = 17) and 0.18 ± 0.124 g in neuropathic (N = 17) rats (P = 0.021). There was no significant difference in Aβ-fiber CUT neurons in control vs. model rats (1.14 ± 0.062 nA, N = 16, in control rats vs. 0.87 ± 0.154 nA, N = 16, in neuropathic rats; P = 0.129).

The effects of peripheral neuropathy on repetitive discharge were analyzed quantitatively by frequency-current analysis. Figure 3.5A shows the number of APs elicited with different current strengths, comparing control vs. neuropathic rats. Compared to the control group, Aβ-fiber HTM neurons and Aβ-fiber MS neurons in

the neuropathic rats showed a significant difference, while Aβ-fiber CUT neurons showed no difference.

For Aβ HTM neurons, the results were based on 25 neurons in control rats and 38 neurons in neuropathic rats. With a 1 nA, 20 ms current injection, the number of APs in control rats was 0.08 ± 0.055 and in neuropathic rats it was 0.34 ± 0.087 (P = 0.027). With a 1.5nA, 20ms current injection, the number of APs in control rats was 0.24 ± 0.087 while neuropathic rats this was 0.68 ± 0.137 (P = 0.018). With a 2nA, 20ms current injection, the number of APs in control rats was 0.28 ± 0.092 while neuropathic rats it was 0.79 ± 0.132 (P = 0.005).

For MS neurons, the results were based on 25 neurons in control rats and 28 neurons in neuropathic rats. With a 1 nA, 20 ms current injection, the number of APs in control rats was 1.28 ± 0.345 while in neuropathic rats it was 3.72 ± 0.397 (P<0.0001). With a 1.5nA, 20 ms current injection, the number of APs in control rats was 2.40 ± 0.378 and 4.66 ± 0.411 in neuropathic rats (P = 0.002). With a 2nA, 20ms current injection, the number of APs in control rats was 3.31 ± 0.448 while it was 5.44 ± 0.400 in neuropathic rats (P = 0.001).

For Aβ-fiber CUT neurons, the results were based on 66 neurons in control rats and 66 in neuropathic rats. With the 1nA, 20 ms current injection, the number of spikes in control rats was 0.12 ± 0.040 and neuropathic rats was 0.136 ± 0.052 (P = 0.8193). With the 1.5nA, 20ms current injection, the number of spikes in control rats was 0.28 ± 0.070 and 0.30 ± 0.081 in neuropathic rats (P = 0.888). With the 2nA, 20ms current injection, the number of APs in control rats was 0.59 ± 0.112 and neuropathic rats it was 0.62 ± 0.120 (P = 0.854).

Figure 3.5B shows typical discharge patterns elicited in MS neurons by 2nA

current pulses with a duration 20ms. In this figure, control rats showed 7 APs while neuropathic rat showed 9 APs with the same current pulse injection, which was the maximal number of APs observed in both groups using 2nA current pluses.

Excitability of the axon measured by responses to dorsal root stimulation

The responses to axonal stimulation by delivering single current pulses to the dorsal root were examined. Dorsal root excitability was determined as the chronaxie curve (threshold-duration), which was derived by determining the minimum current applied to the dorsal root that evoked a soma AP with pulse durations of 0.1ms, 1ms, 2ms, 4ms and 6ms. The data are shown in Figure 3.6. The amount of current required to evoke APs was less in all neuronal types in the neuropathic rats. Rheobase was higher by 64.52% in HTM neurons, 15.68% in MS neurons and 24.04% in CUT neurons,

To investigate the fast process of recovery from an AP in each subtype of afferent neuron, paired-pulse stimuli were delivered to the dorsal root. The RI of Aβ neurons showed a significant difference between neuropathic and control rats. Figure 3.7A displays the RI distributions for individual neurons in each Aβ-fiber sensory neuron subtype in control and neuropathic rats. In control rats, Aβ-fiber HTM neurons had the longest RI (3.05 ± 0.530 ms, $N = 8$). MS neurons had the shortest RI (0.55 ± 0.052 ms, $N = 22$). CUT neurons had an intermediate RI (1.25 ± 0.132 ms, $N = 18$). In neuropathic rats, under the same stimulation conditions, it was found that RI in all Aβ-fiber sensory neurons was significantly greater compared with neurons in control rats. RI in MS neurons in neuropathic rats was 1.14 ± 0.17 ms, $N = 28$ ($P = 0.0038$). RI in CUT neurons was 2.15 ± 0.152 ms, $N = 29$ ($P = 0.0002$). Aβ-fiber HTM neurons also showed significantly greater RI (6.93 ± 1.045 ms, N = 15; $P =$

0.017).

Figure 3.7B (a-b) shows representative examples of MS neuron responses in control and neuropathic rats in response to paired-pulse stimuli. Figure 7B (c-d) shows examples of responses observed in MS neurons in neuropathic rats. In these cases, MS neurons not only showed a greater RI between two APs, but also showed a delayed second AP response to the second stimulus. This delayed response disappeared when the inter-stimulus interval was increased (Figure 3.7B (e)).

Figure 3.8 shows APs recorded in one MS neuron in a neuropathic rat evoked by soma current injection and by dorsal root paired-pulse stimulation, to compare the minimum inter-AP intervals evoked by each of these two types of activation. Figure 3.8A shows an inter-AP interval of 2.6~ 2.9 ms in response to paired-pulse dorsal root stimulation. The actual delayed response interval was at least 5.57 ms and failed response interval was at 2.6, 2.6 ms. Figure 3.8B shows 7 responses with individual inter-AP intervals of 2.71~3.24 ms by direct soma stimulation by injection of depolarizing current (2nA, 20ms). For this MS neuron in a neuropathic rat the minimum inter-AP interval in response to intracellular injection stimulation was 2.71 ms while that in response to paired-pulse stimulation of the dorsal root was 5.57 ms. This implies that the axon exhibited a decreased excitability compared to the soma. Further, although this neuron exhibited a delayed response to paired-pulse stimulation it showed 7 APs in response to the intracellular injection of current (2nA, 20ms), which is higher than the average of response in neuropathic rats (5.44 ± 0.440 ms). This implies an attenuated excitability of the axon that did not affect the enhanced excitability in the soma. Thus, we can conclude that the ectopic discharge characteristics of this neuropathic model are an increased excitability of DRG but an

attenuated decreased excitability of the dorsal root axon.

3.4. Discussion

General characteristics of neuropathic afferent neurons

Sensitization is often considered to play a major role in the neural basis of hyperalgesia and allodynia associated with peripheral neuropathy. The present study determined the excitability and patterns of ectopic activity in functionally defined DRG neurons, including electrophysiological properties under different conditions of neuronal activation. In addition, some neurons were provoked to display ectopic activity, including otherwise quiescent neurons. A systematic correlation of neuronal type with expression of ectopic activity indicated that Aβ-fiber sensory neurons, including LTM and HTM neurons, exhibited ectopic activity in neuropathic rats, whereas these neurons did not display ectopic activity in control rats. This principle conclusion is consistent with our previous study on this model.

These data are also consistent with previous reports on neuropathic rat models. For example, it has been shown that after nerve injury, sensitization of primary sensory neurons is characterized not only by abnormal discharges but also by having a lowered activation threshold and by exhibiting exaggerated responses to various stimuli (Blumberg and Janig, 1984; Devor et al., 1994; Liu et al., 2000; Abdulla and Smith, 2001; Kajander et al., 1992; Kajander and Bennett, 1992; Wall and Devor, 1983).

Here we report several signs of altered excitability, specifically of Aβ DRG neurons. For example, ongoing discharge was generated ectopically specifically in both Aβ-fiber HTM and Aβ-fiber LTM neurons. Further, a reduction was also observed in the mechanical response threshold of some subtypes of Aβ-fiber sensory

neuron in the neuropathic rats when the distribution of mechanical thresholds receptive fields of the individual neurons was compared. This suggests a change in excitability of peripheral terminal receptors. Intracellular current injection into the soma of Aβ DRG neurons generated significantly more APs in neuropathic rats than in controls, which suggests that there is a change in excitability of Aβ DRG neuron somata as well.

While the difference in the soma AP response in Aβ DRG neurons between control and neuropathic rats suggests that a constitutively enhanced hyperexcitability occurs in these neurons, it is important to point out that in contrast there was an increased current threshold for activation of dorsal roots as well as a delay in the latency of the second AP in response to pair-pulse dorsal root stimulation at shorter inter-pulse intervals. This suggests an attenuated excitability of Aβ axons in the dorsal root. This appears to be a novel finding. To our knowledge, previous studies have not compared changes in excitability in soma, axon and peripheral receptor in this or in other animal models of chronic pain.

Possible mechanisms underlying changes in excitability in different parts of* Aβ *DRG neurons

The cellular mechanisms underlying generation of ectopic activity and the changes in activation threshold of the soma, axon and peripheral sensory receptor in Aβ DRG neurons are not clear. It is generally presumed that ectopic discharge results from the classical Hodgkin-Huxley repetitive firing process. The ability of theses neurons to sustain repetitive discharge depends on all three major neuronal ion channels, voltage-gated Na^+, Ca^{2+} and K^+ channels. Alterations in the level of expression, distribution, cellular localization, and activation kinetics of each of these

channel types might be involved in determination of these changes in excitability. Thus, they may have contributed to the difference in excitability between soma, axon and peripheral receptor.

It is also worth considering how injury to the axon may lead to changes in excitability in the somata and the peripheral endings. While no clear answers immediately come to mind, some studies on models of peripheral neuropathy might offer some insight. For example, Walters and Ambron (Walters and Ambron, 1995) hypothesized that axonal injury might unmask nuclear localization signals in certain axoplasm proteins at the injured site. It was suggested that this caused the transport of cytokines, nerve growth (NGF) or glial cell line-derived neurotrophic factor (GDNF), tumor necrosis factor (TNF) or other inflammatory mediators released by immune cells and Schwann cells (Cui et al., 2000; Gherardini et al., 1999; Li et al., 2000; Ramer et al., 1997; Shamash et al., 2002b; Sommer and Schafers, 1998; Wagner and Myers, 1996), to be transported retrogradely to the somata and activate transcription factors, which then induce the alternation in expression of neuropeptides, receptors and ion channels in the somata. This in turn induces increased excitability in the DRG somata. In fact, a previous study provided evidence that DRG somata exhibit *de novo* expression of TTX-sensitive type III Na^+ channels, a reduction of TTX-resistant Na^+ current, and a change in K^+ and Ca^+ currents in different neuropathic animal models (Black et al., 1999; Baccei and Kocsis, 2000; Rizzo et al., 1995; Abdulla et al., 2001; Everill and Kocsis, 1999; Nakagawa et al., 2010; Sleeper et al., 2000; Waxman et al., 1999).

The mechanisms underlying the changes in the activation threshold of the peripheral terminals might be related also to alterations in the release of cytokines and

growth factors. Previous studies have shown that TNF-α is upregulated during Wallerian degeneration (Shamash et al., 2002a) and that exogenous administration of TNF-α produces a direct activation and sensitization of injured afferent fibers (Schafers et al., 2003). When exposed to NGF, sensory neurons undergo an increase in either the firing of APs evoked by a ramp of the depolarizing current (Zhang et al., 2002; Zhang and Nicol, 2004) or in currents evoked by the application of capsaicin (Shu and Mendell, 1999).

There is no clear explanation for the changes in excitability of the MS neurons. Muscle spindles are the sensory endings primarily responsible for initiation of proprioception. These receptors can be exquisitely sensitive, responding to light tapping, vibration or pressure applied to the skin overlying the muscle spindle within the muscle belly (Macefield, 2005). However, unless there are adequate changes in muscle length, the muscle spindle will not be excited. In the present study, we observed a significantly decreased mechanical threshold of MS neurons. However, the mechanisms underlying this change in excitability remain to be determined.

Although there was no direct evidence to explain the attenuated excitability of dorsal root axons in response to direct electrical stimulation, previous studies have shown that the behavioral and cellular effects of nerve injury to the central branches, such as partial dorsal rhizotomy, were different from those induced by injury to the peripheral branches, such as chronic constriction injury of the sciatic nerve. For example, Sheen et al. failed to observe a neuropathic pain behavior after a dorsal rhizotomy (Sheen and Chung, 1993), while Song et al. found that partial dorsal rhizotomy produced significantly less severe hyperalgesia (Song et al., 2003). These studies suggest that the dorsal root might generate different injury signals and might

transport such signals to the spinal cord rather than to DRG somata (Song et al., 2003). Based on our results, we further suggest that the attenuated excitability of dorsal root axons might act as a" negative injury signal", while the increased excitability of somata and peripheral receptors might act as a "positive injury signal" to the central nervous system in this model of peripheral neuropathic pain. Future studies are necessary to test this suggestion.

Consequences of ectopic activity of Aβ neuron

In this model, a significant correlation was found between several variables of ectopic discharge and pain behaviors. The "positive" correlation along with the "negative" correlation, as shown in this study, suggest that ectopic discharge may be important for maintaining neuropathic pain behavior.

Our previous study in this model showed that innocuous touch stimulation of the cutaneous receptive field induces an excessively prolonged afterdischarge in spinal wide dynamic range neurons and found that this unusually prolonged afterdischarge of dorsal horn neurons is reduced by peripheral nerve block proximal to the site of the formalin injection (Pitcher and Henry, 2004; Pitcher and Henry, 2008). This present study has shown that Aβ DRG neurons exhibit ectopic discharge, which includes a prolonged afterdischarge firing pattern in response to direct soma and receptive field stimulation. Thus, it seems reasonable to suggest that the prolonged nature of the afterdischarge in Aβ-fiber sensory neurons in this model of peripheral neuropathy may be due to excessive peripheral drive and that mechanical allodynia, and perhaps also the burning pain, associated with peripheral neuropathy may be due to ectopic activity in Aβ-fiber sensory neurons delivering exaggerated excitation to nociceptive neurons in the spinal cord.

Thus, based on our findings we propose that the hyperalgesia and dysesthesia experienced by people with peripheral neuropathy might be at least partially due to sustained repetitive discharge of Aβ-fiber sensory neurons and prolongation of excitation from these sensory neurons in response to light tactile or noxious stimulation of the skin. We further postulate that the ectopic activity of Aβ-fiber sensory neurons that develops in neuropathic rats may be a critical factor in signaling mechanical allodynia and may contribute to symptoms of allodynia and spontaneous pain following peripheral nerve injury in humans.

Overall, our data provide specific evidence for a major contribution of afferent ectopic activity in Aβ-fiber sensory neurons deriving from multiple regions of these neurons, including the receptive field and the somata, possibly modified by altered excitability of dorsal root axons, resulting in altered spinal nociceptive mechanisms in this animal model of painful peripheral neuropathy.

3.5. Conclusions

This study has demonstrated changes in excitability of Aβ-fiber HTM and LTM primary sensory neurons in the Mosconi and Kruger model of peripheral neuropathic pain and those different sites of these sensory neurons exhibit different changes in excitability after sciatic nerve injury. These differences consisted of a reduced mechanical activation threshold and abnormal prolonged discharges following receptive field stimulation, a reduced current activation threshold and a greater number of APs evoked by intracellular current injection into the some, yet an increased current activation threshold and delayed recovery kinetics in dorsal root axons. This study provides new evidence to support a role for injury-induced plasticity of primary sensory neurons in this model of neuropathic pain and also

indicate that changes to different regions of the sensory neurons might have different effects on hyperalgesia or allodynia.

3.6. Tables and Figures

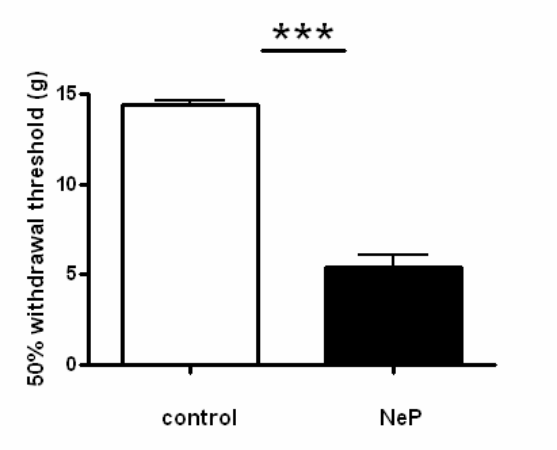

Figure 3.1. Comparison of 50% withdrawal threshold between control and neuropathic rats. Withdrawal threshold to mechanical indentation of the plantar surface of the ipsilateral hind paw with von Frey filaments, recorded on the same day immediately before the acute electrophysiological experiment, during the third week after model induction, with control (N = 60) and neuropathic (N = 64) rats (NeP).

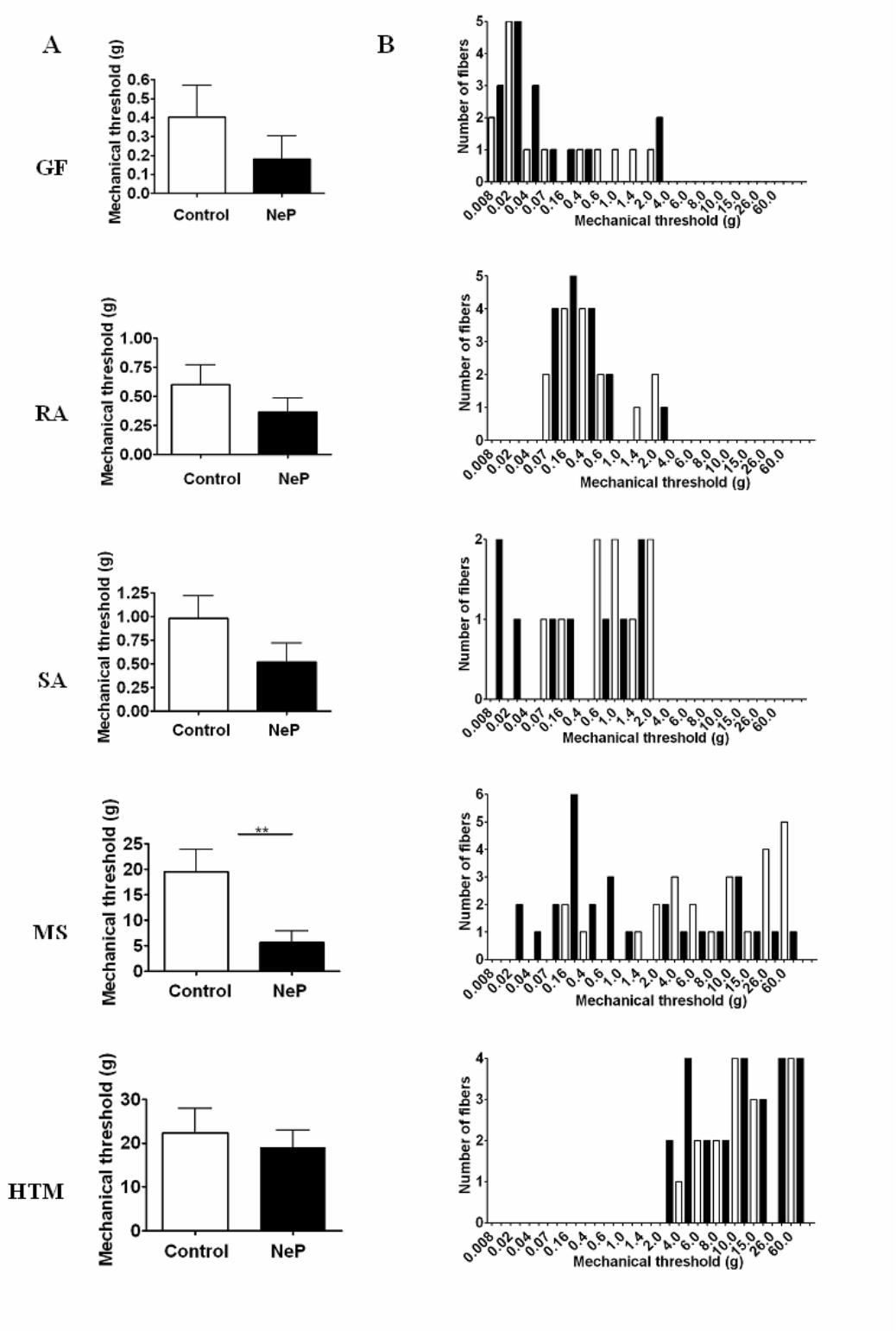
A
B
GF
RA
SA
MS
HTM
Mechanical threshold (g)
Control
NeP
**
Number of fibers
0.008
0.02
0.04
0.07
0.16
0.4
0.6
1.0
1.4
2.0
4.0
6.0
8.0
10.0
15.0
26.0
60.0

Figure 3.2. Comparison of the mechanical response threshold of the Aβ-fiber sensory neurons to application of von Frey filaments to the peripheral receptive field of control and neuropathic rats. The column on the left shows the mean mechanical response thresholds of GF, RA, SA, MS and HTM neurons in control (open bar) and neuropathic (NeP: filled bars) rats. The mechanical threshold of MS neurons was significantly lower in neuropathic than in control rats. The column on the right shows the distribution of the mechanical response thresholds of these neurons. There is a leftward shift in the distribution of the SA and MS neurons in the neuropathic rats compared with the control rats.

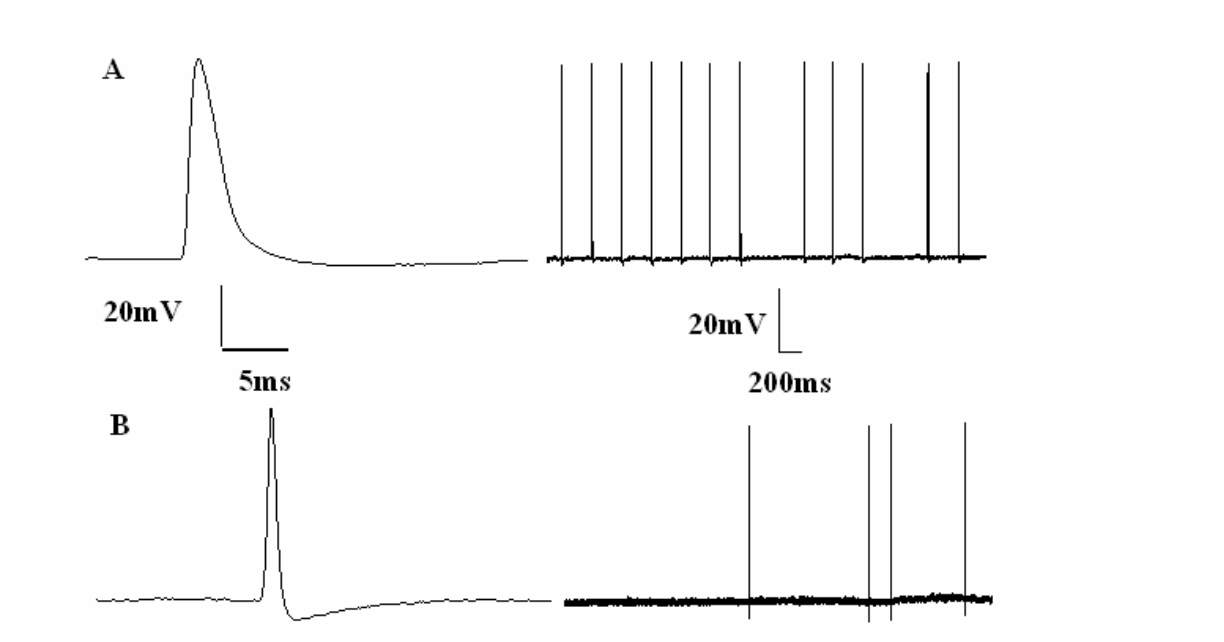

Figure 3.3. Ongoing discharge in neuropathic rats. This figure shows on-going discharge in DRG neurons in neuropathic rats: an Aβ-fiber HTM neuron (A), Aβ-fiber LTM hair neuron (B). The APs on the left are the first spike in the ongoing discharge shown on the right at a slower sweep speed.

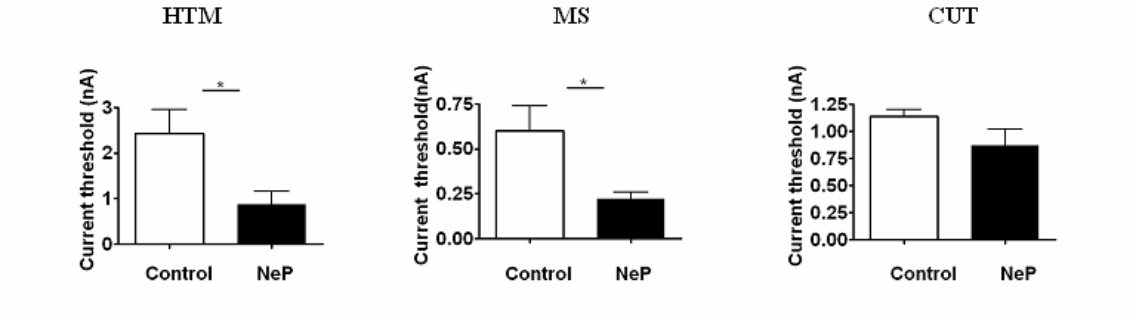

Figure 3.4. Comparison of the activation threshold of Aβ-fiber sensory neurons in response to intracellular current injection, between control and neuropathic rats. The current threshold was defined as the minimum current required evoking an AP by intracellular current injection. Excitability of the DRG somata was significantly increased, as indicated by the decreased activation threshold in MS and HTM neurons. CUT, cutaneous neurons, including Aβ-fiber LTM GF, RA, and SA neurons. Asterisks above the graph indicate the significant difference between control and neuropathic rats: *$p<0.05$. Mann Whitney t-test.

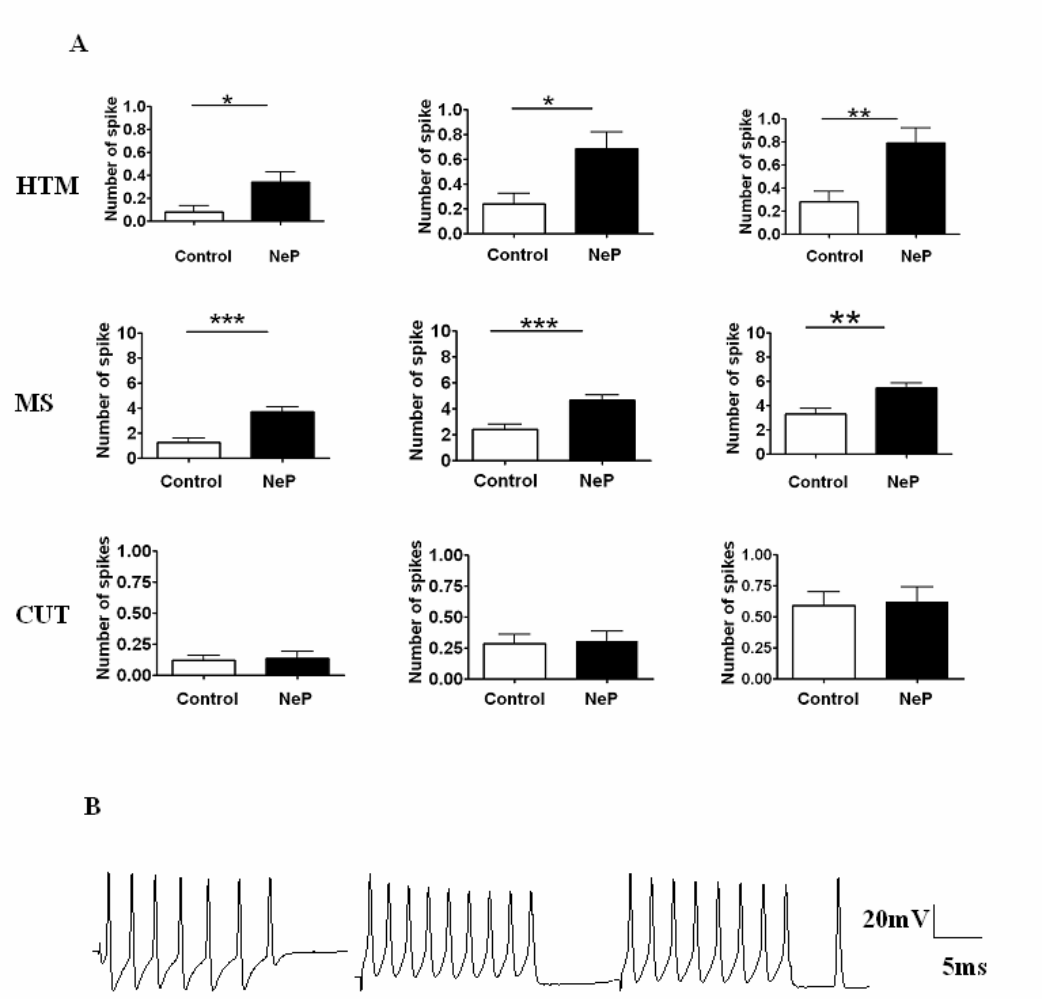

Figure 3.5. A comparison the repetitive discharge characteristics of DRG cells produced by intracellular current injection. The discharge was evoked by injecting a series of depolarizing current pulses into DRG soma through the recording electrode. Differences in the discharges of the 3 categories of Aβ-fiber neuron are summarized. **A**. Colum bars showing the number of APs evoked by different magnitudes of intracellular depolarizing current injection. Left, 1nA, 20ms. Mid, 1.5 nA, 20ms. Right, 2 nA, 20ms. **B**. Representative examples of raw recordings to show the greater number of APs evoked by intracellular current injection in MS neurons.

APs were evoked by current pulses of 2nA, 20ms. Left, control rat. Mid, neuropathic rat. Right, neuropathic rat. Details of the abbreviation are as indicated in Figure 3.4. Asterisks above the graph indicate a significant difference between control and neuropathic rats. *p<0.05, **P<0.01; ***p<0.001. Mann Whitney t-test

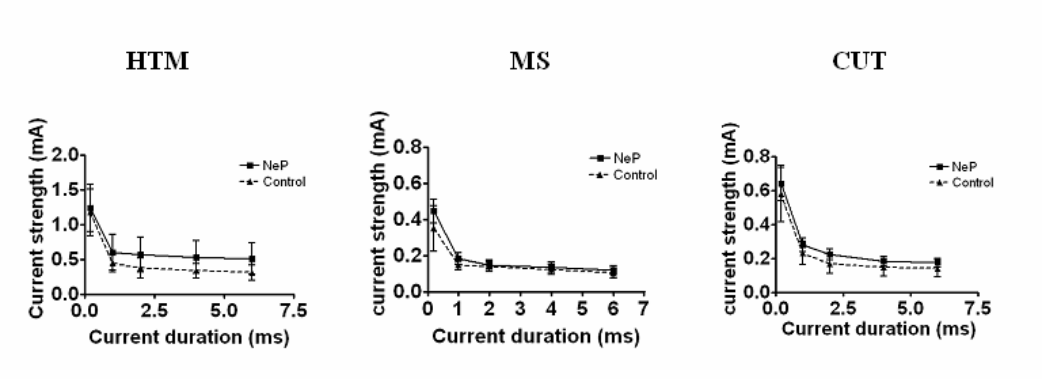

Figure 3.6. Comparison of current activation threshold of the Aβ-fiber sensory neurons in response to stimulation of the dorsal roots, between control and neuropathic rats. Dorsal root current threshold was defined by the chronaxie curve (threshold-duration), which was determined as the minimum stimulus current to the dorsal root sufficient to evoke a soma AP with pulses of 0.1ms, 1ms, 2ms, 4ms and 6ms duration. Figures show a reduction in the rheobase in DRG neurons in neuropathic rats. Details of abbreviations are as indicated in Figures 3.4.

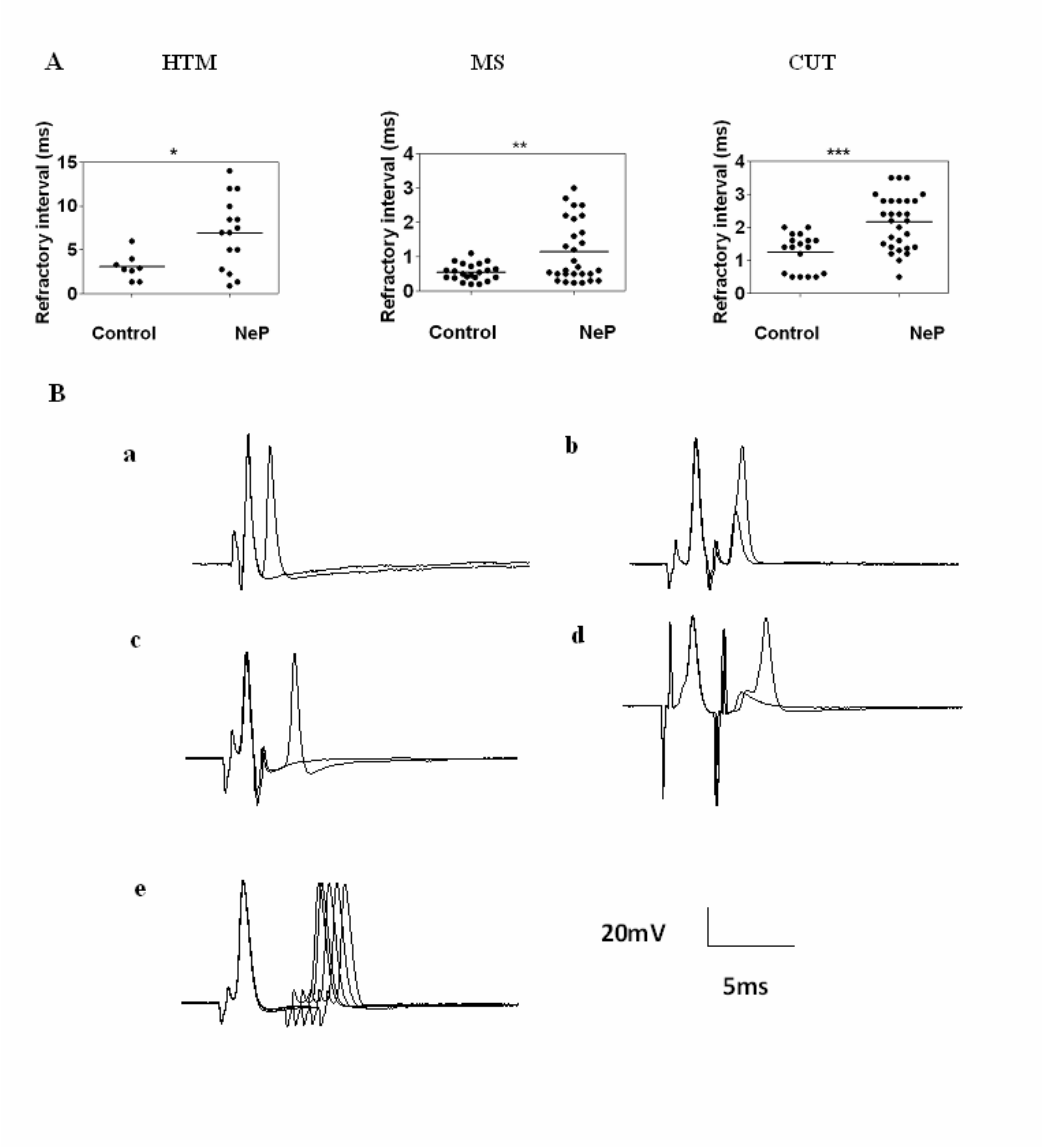

Figure 3.7. Comparison of the repetitive discharge characteristics of DRG cells evoked by dorsal root stimulation between control and neuropathic (NeP) rats. Dorsal root repetitive discharge characteristics were tested by measuring the refractory interval of two APs elicited by paired pulse stimulation of the dorsal roots. **A**. Scatter plots showing the distribution of the RI variables with the median (horizontal line) superimposed in each case. Asterisks above the graph indicate a significant difference between control and neuropathic rats. *p<0.05, **P<0.01;

***p<0.001. Abbreviations are as indicated in Figures 3.4. **B**. Representative examples of somatic APs evoked by paired pulse dorsal root stimulation with variable inter-stimulus intervals. (a) a MS neuron in a control rat (interval 0.3, 0.2ms), (b) a MS neuron in a neuropathic rat (interval at 1.8, 2ms). The data show a longer RI in neuropathic rats. (c, d) MS neurons in neuropathic rats (left: interval: 1.6, 1.5 ms; right: interval: 2.5, 2.6ms). The data show not only a longer refractory interval but also a delayed second AP response to the second stimulus. (e) a MS neuron in a neuropathic rat (interval. 5.5, 5, 4.5, 4, 3.5, 3 ms). The data show that the second AP failed at an interval of 3ms, the second AP was delayed at 3.5 ms and the second AP was elicited when at an interval of 4 ms.

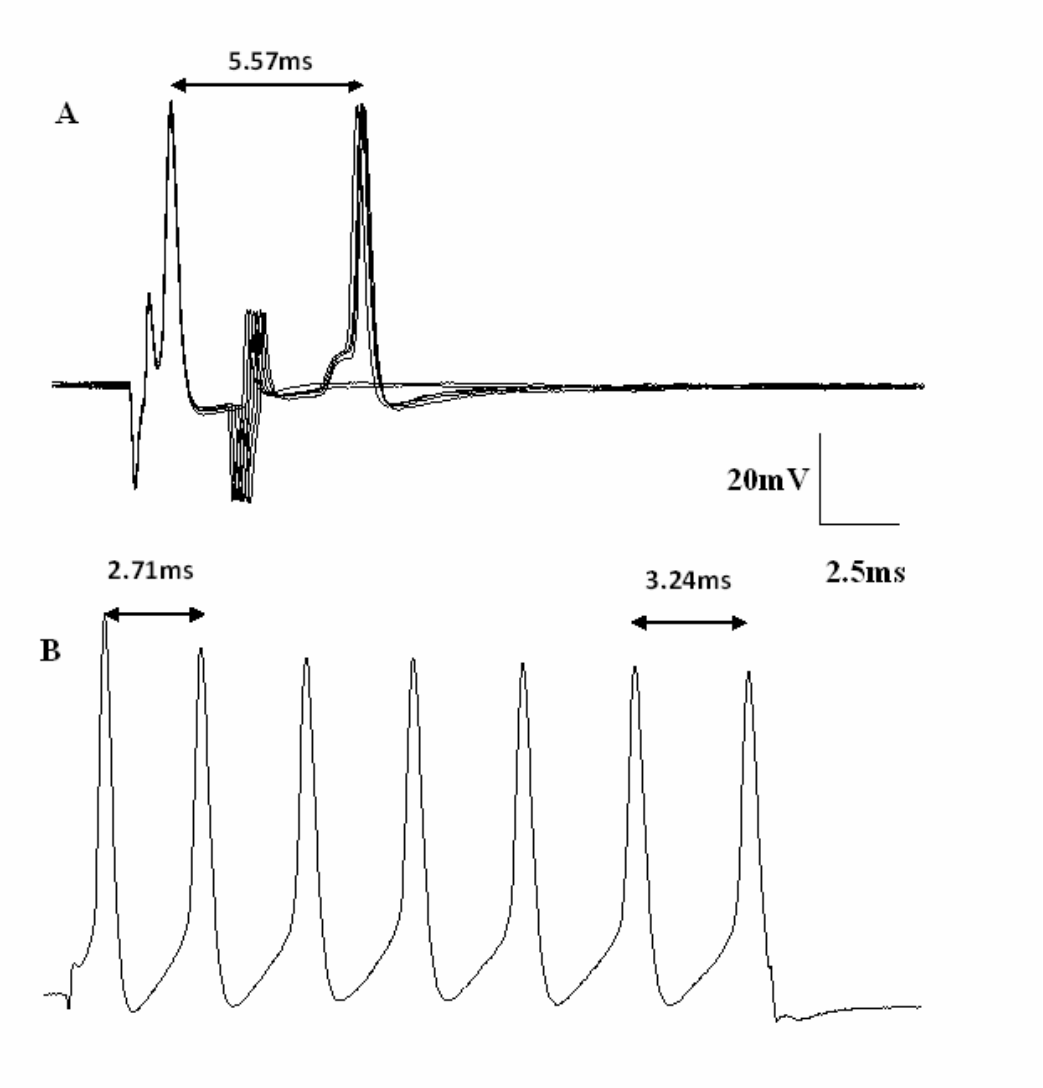

Figure 3.8. Comparison of APs evoked by dorsal root paired-pulse stimulation and by soma current injection. The figure shows APs in one specific MS in a neuropathic animal evoked by dorsal root paired pulse stimulation and by soma current injection. **A**. shows a delayed second response by 2.6~ 2.9 ms at intervals of paired dorsal root stimulation and a failed response at 2.5, 2.6 ms. The actual delayed response interval was at least 5.57ms. **B.** shows 7 APs in response to depolarizing current injection, with individual AP intervals of 2.71~3.24 ms.

Reference List

Abdulla FA, Stebbing MJ, Smith PA, 2001. Effects of substance P on excitability and ionic currents of normal and axotomized rat dorsal root ganglion neurons. Eur. J. Neurosci. 13: 545-552.

Baccei ML, Kocsis JD, 2000. Voltage-gated calcium currents in axotomized adult rat cutaneous afferent neurons. J. Neurophysiol. 83: 2227-2238.

Black JA, Cummins TR, Plumpton C, Chen YH, Hormuzdiar W, Clare JJ, Waxman SG, 1999. Upregulation of a silent sodium channel after peripheral, but not central, nerve injury in DRG neurons. J. Neurophysiol. 82: 2776-2785.

Bonica JJ, 1968. [Causalgia and other sympathetic reflex dystrophies]. Rev. Esp. Anestesiol. Reanim. 15: 394-396.

Bonica JJ, 1973. Causalgia and other reflex sympathetic dystrophies. Postgrad. Med. 53: 143-148.

Campbell JN, Meyer RA, 2006. Mechanisms of neuropathic pain. Neuron 52: 77-92.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL, 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53: 55-63.

Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderoth B, 2000. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. Pain 88: 239-248.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Devor M, 1983. Nerve pathophysiology and mechanisms of pain in causalgia. J. Auton. Nerv. Syst. 7: 371-384.

Dixon WJ, 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20: 441-462.

Djouhri L, Bleazard L, Lawson SN, 1998. Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J. Physiol 513 (Pt 3): 857-872.

Everill B, Kocsis JD, 1999. Reduction in potassium currents in identified cutaneous afferent dorsal root ganglion neurons after axotomy. J. Neurophysiol. 82: 700-708.

Fang X, McMullan S, Lawson SN, Djouhri L, 2005. Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. J. Physiol 565: 927-943.

Gherardini G, Lundeberg T, Cui JG, Eriksson SV, Trubek S, Linderoth B, 1999. Spinal cord stimulation improves survival in ischemic skin flaps: an experimental study of the possible mediation by calcitonin gene-related peptide. Plast. Reconstr. Surg. 103: 1221-1228.

Han HC, Lee DH, Chung JM, 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain 84: 253-261.

Handwerker HO, Kilo S, Reeh PW, 1991. Unresponsive afferent nerve fibres in the sural nerve of the rat. J. Physiol 435: 229-242.

Khan GM, Chen SR, Pan HL, 2002. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. Neuroscience 114: 291-299.

Kim SH, Chung JM, 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50: 355-363.

Lang PM, Schober GM, Rolke R, Wagner S, Hilge R, Offenbacher M, Treede RD, Hoffmann U, Irnich D, 2006. Sensory neuropathy and signs of central sensitization in patients with peripheral arterial disease. Pain 124: 190-200.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Leem JW, Willis WD, Chung JM, 1993. Cutaneous sensory receptors in the rat foot. J. Neurophysiol. 69: 1684-1699.

Li Y, Dorsi MJ, Meyer RA, Belzberg AJ, 2000. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 85: 493-502.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Ma C, Shu Y, Zheng Z, Chen Y, Yao H, Greenquist KW, White FA, LaMotte RH, 2003. Similar electrophysiological changes in axotomized and neighboring intact dorsal root ganglion neurons. J. Neurophysiol. 89: 1588-1602.

Macefield VG, 2005. Physiological characteristics of low-threshold mechanoreceptors in joints, muscle and skin in human subjects. Clin. Exp. Pharmacol. Physiol 32: 135-144.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Nakagawa K, Takeda M, Tsuboi Y, Kondo M, Kitagawa J, Matsumoto S, Kobayashi A, Sessle BJ, Shinoda M, Iwata K. 2010. Alteration of primary afferent activity following inferior alveolar nerve transection in rats. Mol. Pain 3: 6:9.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Pitcher GM, Henry JL, 2008. Governing role of primary afferent drive in increased excitation of spinal nociceptive neurons in a model of sciatic neuropathy. Exp. Neurol. 214: 219-228.

Pitcher GM, Ritchie J, Henry JL, 1999. Nerve constriction in the rat: model of neuropathic, surgical and central pain. Pain 83: 37-46.

Ramer MS, French GD, Bisby MA, 1997. Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. Pain 72: 71-78.

Richards RL, 1967. Causalgia. A centennial review. Arch. Neurol. 16: 339-350.

Rizzo MA, Kocsis JD, Waxman SG, 1995. Selective loss of slow and enhancement of fast Na+ currents in cutaneous afferent dorsal root ganglion neurones following axotomy. Neurobiol. Dis. 2: 87-96.

Schafers M, Lee DH, Brors D, Yaksh TL, Sorkin LS, 2003. Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation. J. Neurosci. 23: 3028-3038.

Shamash S, Reichert F, Rotshenker S, 2002a. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J. Neurosci. 22: 3052-3060.

Sheen K, Chung JM, 1993. Signs of neuropathic pain depend on signals from injured nerve fibers in a rat model. Brain Res. 610: 62-68.

Shu X, Mendell LM, 1999. Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci. Lett. 274: 159-162.

Sleeper AA, Cummins TR, Dib-Hajj SD, Hormuzdiar W, Tyrrell L, Waxman SG, Black JA, 2000. Changes in expression of two tetrodotoxin-resistant sodium channels and their currents in dorsal root ganglion neurons after sciatic nerve injury but not rhizotomy. J. Neurosci. 20: 7279-7289.

Sommer C, Schafers M, 1998. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res. 784: 154-162.

Song XJ, Vizcarra C, Xu DS, Rupert RL, Wong ZN, 2003. Hyperalgesia and neural excitability following injuries to central and peripheral branches of axons and somata of dorsal root ganglion neurons. J. Neurophysiol. 89: 2185-2193.

Tal M, Wall PD, Devor M, 1999. Myelinated afferent fiber types that become spontaneously active and mechanosensitive following nerve transection in the rat. Brain Res. 824: 218-223.

Wagner R, Myers RR, 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73: 625-629.

Walters ET, Ambron RT, 1995. Long-term alterations induced by injury and by 5-HT in Aplysia sensory neurons: convergent pathways and common signals? Trends Neurosci. 18: 137-142.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA, 1999. Sodium channels and pain. Proc. Natl. Acad. Sci. U. S. A 96: 7635-7639.

Wu Q, Henry JL, 2009. Delayed onset of changes in soma action potential genesis in nociceptive A-beta DRG neurons in vivo in a rat model of osteoarthritis. Mol. Pain 5: 57.

Wu Q, Henry JL, 2010. Changes in Abeta non-nociceptive primary sensory neurons in a rat model of osteoarthritis pain. Mol. Pain 6: 37.

Zhang YH, Nicol GD, 2004. NGF-mediated sensitization of the excitability of rat sensory neurons is prevented by a blocking antibody to the p75 neurotrophin receptor. Neurosci. Lett. 366: 187-192.

Zhang YH, Vasko MR, Nicol GD, 2002. Ceramide, a putative second messenger for nerve growth factor, modulates the TTX-resistant Na(+) current and delayed rectifier K(+) current in rat sensory neurons. J. Physiol 544: 385-402.

CHAPTER 4

Substance P is expressed in Aβ low-threshold mechanoreceptor neurons in an animal model of peripheral neuropathy

Authors: Yong Fang Zhu, Yu Fang Wang
Alexander K, Ball, James L Henry

Corresponding author: Yu Fang Wang
Alexander k, Ball, PhD
James L Henry, PhD
Department of Psychiatry and Behavioural Neurosciences,
McMaster University,
1200 Main St. West, HSC 4N35
Hamilton, Ontario, Canada, L8N 3Z5,
Tel: +1-905-525-9140, extension 24750,
FAX: 905-4522-8844
Email address: wangyu@mcmaster.ca
akball@mcmaster.ca
jhenry@mcmaster.ca

Preface

Significance to thesis

This study demonstrated *de novo* expression of substance P (SP) in Aβ-fiber low-threshold mechanoreceptor (LTM) muscle spindle (MS) neurons of DRG somata in the neuropathic pain model. This finding added further weight to the accumulated evidence that plasticity of Aβ-fiber LTM neurons can contribute to nerve injury-induced pain by expressing SP.

Authors' contribution

Yong Fang Zhu did the electrophysiological experiments, the behavior test, model induction, and DRG preparation for immunohistochemical examination. She also analyzed the data, performed statistical analyses, wrote the initial draft of the manuscript, and worked on refining this draft and the revision based on editorial review.

Yu Fang Wang coordinated immunohistochemical procedure. Alexander K. Ball supervised, coordinated the immunohistochemical part of study. James L. Henry conceived of, designed the study. Both of them worked on refining this draft and the revision based on editorial review.

Abstract

It has been reported that the *de novo* expression of SP in large myelinated primary sensory neurons in animal models of neuropathic pain might be involved in development and maintenance of neuropathic pain. To date there has been no study of SP distribution in functionally-classified DGR neurons *in vivo* in an animal model of neuropathic pain. We therefore used intracellular recordings from the somata of L4 dorsal root ganglion (DRG) neurons *in vivo* using the Mosconi and Kruger model of peripheral neuropathy. Following the acute electrophysiological experiment that functionally classified DRG neurons, these neurons were injected intracellularly with neurobiotin to identify the physiologically characterized cell. The DRG were then extracted, fixed and stained for SP immunoreactivity. The aim of this study was to gain information on the functional properties of different neuron types, with a focus on large diameter neurons, in order to determine the type and percentage of this neuron population that expresses SP, and to compare their numbers in control vs. neuropathic rats. Our results showed greater expression of SP in Aβ-fiber LTM neurons in neuropathic rats, which add further weight to the evidence that plasticity of Aβ-fiber sensory neurons may contribute to nerve injury-induced pain by expressing SP.

4.1. Introduction

Peripheral nerve injury in humans may lead to abnormal sensations and neuropathic pain. Although it is thought that alternations in central neuronal processing may play an important role in the development of such pathological pain, recent evidence has suggested that changes in peripheral primary sensory neurons may also contribute. One possible mechanism whereby primary afferents might

influence spinal or supraspinal nociceptive neurons following peripheral nerve injury is to change the expression profile of neuropeptides released from primary afferent terminals in the spinal cord which would influence the processing of nociceptive information (Hokfelt et al., 1994). In recent years, much emphasis has been placed on the peptide SP, which is usually localized to large dense core vesicles in a subset of small-diameter C-fiber sensory neurons and a limited number of Aδ-fiber neurons (Lawson et al., 1997; McCarthy and Lawson, 1989). SP is released from the central terminals of these afferent neurons in the spinal cord in response of noxious stimulation (Brodin et al., 1990; Duggan et al., 1988; McCarson and Goldstein, 1991) and selectively excites nociceptive neurons in the sensory dorsal horn (Henry, 1976).

SP and NK-1 receptors have been implicated in animal models of neuropathic pain. While it has been reported that nerve injury results in downregulation of SP in unmyelinated C fibers (Hokfelt et al., 1994), it has also been proposed that nerve injury can lead to *de novo* expression of SP in the large-diameter Aß-fiber sensory neurons (Marchand et al., 1994; Noguchi et al., 1994; Noguchi et al., 1995; Weissner et al., 2006). Noguchi et al. (1995) demonstrated that after sciatic nerve transection some large- and medium-sized DRG neurons are labeled for preprotachykinin (PPT) mRNA, which codes for SP, whereas PPT mRNA in small DRG cells is depressed. Coincident with these data, immunohistochemistry showed an increase in SP immunoreactivity in large myelinated fibers in the dorsal root. More recently, Malcangio showed that a low-intensity electrical stimulus, sufficient only to activate the Aβ axons, applied to the dorsal root *in vitro* induced the release of SP in the spinal cord weeks after spinal nerve lesion (Malcangio et al., 2000). Although some studies reported that NK-1 receptor antagonists failed to reduce nociceptive scores in animal

models of neuropathic pain (Malcangio and Tomlinson, 1998; Urban and Fox, 2000; Yamamoto and Yaksh, 1992), other studies have reported positive effects of NK-1 receptor antagonists, which has been shown to attenuate tactile hypersensitivity in such models in the guinea pig (Campbell et al., 1998; Gonzalez et al., 2000) and rat (Cahill and Coderre, 2002; Coudore-Civiale et al., 1998; Cumberbatch et al., 1998; Gonzalez et al., 2000). Furthermore, mechanical hypersensitivity induced by spinal nerve ligation is reduced in NK-1 knockout mice (Mansikka et al., 2000). Pitcher and Henry (Pitcher and Henry, 2004) have observed that touch-evoked afterdischarges in dorsal horn neurons were apparently driven by myelinated sensory neurons and were blocked by NK-1 receptor antagonists in the Mosconi and Kruger model (Mosconi and Kruger, 1996).

In view of electrophysiological recordings of spinal neurons, which have demonstrated that iontophoretic application of SP elicits firing of nociceptive specific neurons (Henry, 1976; De Koninck and Henry, 1991), and that SP selectively excites nociceptive dorsal horn neurons (Salter and Henry, 1991), these results lead to the notion that upregulation of the peptide in Aß-fiber sensory neurons may be responsible for a peripheral drive of excitation of nociceptive mechanisms in the spinal cord and may thereby contribute to hyperalgesia and allodynia that appear after certain types of peripheral nerve injury (Devor, 2009). To date, though, it has not been determined whether there is any greater expression of SP in functionally classified DGR neurons *in vivo*. For these reasons, we undertook an examination of SP expression in identified DRG neurons classified on the basis of both electrophysiological and functional criteria. The experiments were done on control and neuropathic rats, by making stable intracellular recordings from DRG neurons in

the anesthetized rat. Once functionally classified, neurons were injected intracellularly with neurobiotin and were counterstained for SP immunoreactivity. The aim of this study was to gain information on characterized DRG neuron types with a focus on large-diameter Aß-fiber sensory neurons and to determine the type and percentage of the population that expresses SP, comparing control to neuropathic rats.

4.2. Methods

Experimental rats and surgery for model induction

Young male rats (Sprague Dawley; Harlan Laboratories) weighing 170-200 g were used. Rats were divided into two groups: control and neuropathic rats. The rats were kept one week before the surgery. A peripheral neuropathy was then induced according to the method previously described in detail (Mosconi and Kruger, 1996; Chapter 2). Briefly, under anesthesia (ketamine, 5 mg/100 g, xylazine, 0.5 mg/100 g and aceptomazine, 0.1 mg/100g, i.p.), the right sciatic nerve was exposed at the mid-thigh level by a lateral approach. Two 0.5mm polyethylene cuffs (Intramedic PE-90, Fisher Scientific Ltd., Whitby, ON, Canada) were inserted around the nerve with a spacing of about 1 mm. The muscle and skin were then sutured in two layers. Antibiotic ointment (Furacin; nitrofurazone 0.2%, Vétoquinol Canada Inc., Lavaltrie, QC, Canada) was applied over the wound, and 0.01 ml/100g of antibacterial injectable solution (Bayer HealthCare, Toronto, ON, Canada) was injected subcutaneously (s.c.). Rats were given 1 ml saline s.c. and ocular lubricant, and were placed under a heating lamp until they recovered from the anaesthetic. Rats were then returned to their home cage.

For the neuropathic group, rats were examined on the same day as the

recording day for behavioral signs indicating a mechanical hypersensitivity, prior to receiving anesthesia for the acute electrophysiological experiment. To quantify mechanical sensitivity of the foot, brisk foot withdrawal was determined in response to normally innocuous mechanical stimuli applied with von Frey filaments, as described previously (Kim and Chung, 1992). The rats were placed in a transparent Plexiglas box with a clear Plexiglas floor, containing 0.5 cm diameter holes that were spaced 1.5 cm apart, allowing full access to the paws. Accommodation to the testing chamber was allowed for approximately 15 min, until cage exploration and major grooming activities ceased. The von Frey filaments (Stoelting; Wood Dale, IL, USA) were applied to the soft tissue of the plantar surface of the hind paw to determine the withdrawal threshold. The filament was applied 10 times for 3-4 sec at 3 sec intervals, to each hind paw. A 50% withdrawal response threshold was derived according to the test pattern (Chaplan et al., 1994). Brisk foot withdrawals in response to innocuous mechanical stimuli (under 5 g of 50% withdrawal response threshold) were interpreted as mechanical activation threshold. Those rats showing clear mechanical hypersensitivity were considered as neuropathic model rats and were used in the electrophysiological study.

Intracellular recording

The animal preparation and intracellular recordings followed those reported previously (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010; Chapter 2). Briefly, rats were initially anesthetized with the ketamine mixture described above. The right jugular vein was cannulated for i.v. infusion of drugs and cannula was inserted into the trachea to facilitate ventilation. The rat was then fixed in a stereotaxic frame and the vertebral

column rigidly clamped at the L2 and L6 vertebral levels. The right femur was fixed using a customized clamp onto a stereotaxic frame to minimize movement of the DRG during the mechanical search for receptive fields on the leg. Because the L4 DRG contains one of the largest numbers of the hind leg afferent somata it was selected for study. A laminectomy was performed to expose the ipsilateral L4 DRG and the L4 dorsal root was sectioned close to the spinal cord before being placed on a bipolar electrode (FHC, Bowdoinham, ME, USA)for stimulation purposes. The exposed spinal cord and DRG were covered with warm paraffin oil at 37^{o} C to prevent drying.

For recording, each rat was anesthetized at a surgical level with pentobarbital (20 mg/kg; Ceva Sante Animal, Liourne, France). Rectal temperature was maintained at ~37^{o}C using a temperature controlled infrared heating lamp. The rat was mechanically ventilated via the tracheal cannula using a Harvard Ventilator (Model 683, Harvard apparatus, Quebec, Canada). The ventilation parameters were set so that the end-tidal CO_2 concentration was maintained around 40-50 mmHg, measured using a CapStar-100 End-Tidal CO_2 analyzer (CWE, Ardmore, PA, USA). Immediately before the start of recording, an initial 1 mg/kg dose of pancuronium bromide (Sandoz, Boucherville, QC, Canada) was given i.v. to eliminate muscle tone. Throughout the experiment the effects of pancuronium were allowed to wear off periodically to confirm a surgical level of anesthesia. This was determined by observing pupil diameter and any response to the noxious pinch of a forepaw. Supplements of pentobarbital and pancuronium were added with at initial dose of 1/3 approximately each hour of the experimental procedure via the jugular cannula.

Intracellular recordings from somata in the exposed DRG were made using

borosilicate glass micropipettes (1.2 mm outside diameter, 0.68 mm inside diameter; Harvard Apparatus, Holliston MA, USA). The electrodes were pulled using a Brown-Flaming puller (model p-87; Sutter Instrument CO., Novota, CA, USA). These electrodes were filled with 3 M KCl (DC resistance 50-70 MΩ). Signals were recorded with a Multiclamp 700B amplifier (Molecular Devices, Union City CA, USA) and digitized on-line using a Digidata 1322A interface (Molecular Devices) with pClamp 9.2 software (Molecular Devices). The microelectrode was advanced using an EXFO IW-800 micromanipulator (EXFO, Montreal, QC, Canada) in 2 μm steps until a hyperpolarization of at least 40 mV suddenly appeared. Once a stable membrane potential had been confirmed, a single stimulus was applied to the dorsal roots to provoke an action potential (AP), which was used as one of the criteria for classification of neurons.

DRG neuron classification

In addition to the AP configuration, the sensory receptive properties of neurons were also used for functional classification. This was done using hand-held mechanical stimulators and neurons were classified as previously described (Djouhri et al., 1998; Lawson et al., 1997; Chapter 2). Dorsal root conduction velocities (CVs) were also measured to classify neurons as C-fiber (<=0.8mm/ms), Aδ-fiber (1.5-6.5mm/ms) or Aβ-fiber (>6.5mm/ms) neurons according to criteria published earlier (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010). Neurons were further subdivided into three main groups.

LTM neurons were identified by their excitatory responses to the application of a soft brush, light pressure with a blunt object, light tap, or vibration. Many Aß-fibers LTM neurons were cutaneous (CUT), and included guard/field hair neurons

(GF), glabrous skin neurons, Pacinian neurons, and slowly adapting neurons (SA). One further group of Aß-fiber LTM neurons, with deeper receptive fields that were very sensitive to light pressure and/or leg movement and often showed ongoing activity, were classified as muscle spindle (MS) neurons.

HTM neurons were those that responded to noxious stimuli, including noxious pinch and application of sharp objects such as the tip of a syringe needle applied to the receptive field.

Neurons that did not respond to any of the innocuous or noxious mechanical stimuli listed above were classed as unresponsive as defined elsewhere (Lawson et al., 1997). Heat nociceptors and cool receptors were not included in this study.

Intracellular labeling

After physiological characterization of electrophysiological and functional properties, selected neurons were stained by injecting neurobiotin by iontophoresis. Recording microelectrodes were filled with 4% neurobiotin (Vector Laboratories, Burlingame, CA, USA) in 1M KCL. Dye was ejected from the electrode by pulses of positive current (1nA at 2Hz) for periods up to 2 min, while confirming a stable membrane potential every 30s.

To prevent labeled cells from being improperly identified, a maximum of two neurons were filled in each experiment. They were always at different depths within the DRG and they were located at opposite ends of the ganglia. This was particularly important in the present study because for each neuron it was necessary to reliably correlate electrophysiological and functional properties with SP immunoreactivity in histological sections. Neurons were rejected from the data set if their location and depth did not match, or if more cells than expected were labeled.

Histochemical procedures

The DRG containing neurobiotin-injected neurons was surgically extracted and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) at pH 7.4 and left overnight at 4^{o}C. The DRG was cryoprotected with 30% sucrose for 24 hrs at 4^{o}C.

Each DRG was then superficially stained for 30 sec with 1% toluidine blue for easy identification, mounted in Tissue-Tek® (OCT compound; PELCO International, P.O. Box 492477, Redding, CA, USA), and cryostat sectioned. Frozen transverse DRG sections, 25μm thick, were serially cut and collected, and were studied with indirect immunofluorescence staining. The sections were washed 3 x in 0.4% Triton X-100 in PBS for 15 min to remove OCT compound and incubated with blocking solution containing 10% normal goat serum at room temperature for 2 hours. The sections were then incubated with rabbit anti-substance P antibody (SP; 1:500; Chemicon International Inc, Temecula, CA, USA) at 4^{o}C overnight. The excess antibody was washed from sections in PBS and incubated with Alexa Fluor 488 labeled goat anti-rabbit IgG (1:500; Invitrogen Canada Inc. Burlington, ON, Canada) for SP diluted in 1:500 and NeutrAvidin Texas Red 1:100 for neurobiotin at room temperature for 2 hrs. After washing with PBS, the sections were mounted with mounting medium containing DAPI (Vector Laboratories). Optimal dilution of the SP antibody was determined after staining using different dilutions of the antibody at 1:100, 1:500, 1:1000, and 1:10000. Control experiments consisted of staining sections of DRG where the primary antibody was eliminated from the incubating solution. No SP-immunoreactive cells were observed in sections where the primary antibody was omitted. Neurobiotin labeled cells (Texas Red) were not visible using the 488nm epifluorescence filter set or laser excitation used to visualize SP-IR, when

the primary antibody was omitted.

Substance P quantification

Tissue sections were viewed by laser scanning confocal microscopy using a Carl Zeiss LSM 510 microscope equipped with both Argon laser 488nm and HeNe1 laser 543nm, and filtered for the detection of Alexa Fluor 488 and Texas Red using single tracking mode to avoid bleed through with dual excitation. Images in optimal depth of focus were recorded and stored. The same settings for the microscope configureation, laser intensity, and photomultiplier were used between imaging sessions using the Reuse function of the software. The quantification of SP in neurobiotin-injected cells was acquired from profiles in the LSM 510 imaging software and scored as follows.

A ratiometric technique was used to account for potential variations in antibody staining even though the exact protocol was used for each experiment. For each section, the 0% intensity (n) was taken as the mean intensity of three neurons deemed to be unquestionably negative in the immediate vicinity of the labeled cell. The 100% intensity (p) was the mean of the three values from the most intensely labeled cell in the section. The mean of the three intensity values from the dye-labeled cell provided the value t. The relatively intensity of the cell was calculated as (t-n)/(p-n) and expressed as a percentage.

Neurons with relative intensity >15% were judged subjectively as clearly positive and <10% were judged subjectively as clearly negative by three viewers. Those neurons classified with 10-15% relative intensity were border line and were considered as negative for simplified description. Cell size was estimated from the cross-section diameter of the largest section, which contained the nucleus, through the

dye-labeled neuron and classified as small (< 25μm diameter), medium (25-35μm diameter), or large (> 35μm diameter) sized.

Statistical analysis

The data were represented as mean ± SEMs. Mann Whitney t-test was used for comparison of the SP distributions of the neurons between control and neuropathic rats. All statistical tests and graphing were done using Prism4 software (Graphpad, La Jolla, CA, USA). Each P-value is indicated in the graphs and $P<0.05$ was considered to indicate a significant difference.

4.3. Results

This report is based on an analysis of 80 neurons that were characterized electrophysiologically to determine their function, and subsequently labeled for analysis by immunohistochemistry. These included 38 neurons in control rats and 42 neurons in neuropathic rats. Examples of identified DRG neurons showing typical positive and negative immunohistochemical staining for SP are illustrated in Figure 4.1.

In 17 C-fiber neurons, positive SP immunoreactive material appeared in 4 of 6 control and 4 of 7 neuropathic HTM neurons. Negative SP was observed in 2 of 2 control and 2 of 2 neuropathic LTM neurons.

In 8 Aδ-fiber neurons, 1 of 2 control and 2 of 3 neuropathic HTM neurons showed SP immunoreactive material, and 1 of 1 control and 2 of 2 neuropathic LTM neurons showed negative SP.

A total of 55 Aβ-fiber sensory neurons were successfully classified and labeled. One HTM neuron of 2 in control and 1 of 3 in neuropathic rats showed SP-positive immunoreactive material. In control rats, none of the 9 CUT neurons and

none of 16 MS neurons showed any SP-positive immunoreactive material. However, in neuropathic rats, 1 of 11 CUT neurons showed slight SP positive intensity (14.10%). Importantly, in neuropathic rats, 4 of 14 MS neurons showed SP- positive immunoreactive material, which ranged from 18.41% to 85.61%. Figure 4.2 shows that the expression of SP was significantly greater in MS neurons of neuropathic rats (control rats: $N = 16$, mean 4.18 ± 0.84; neuropathic rats: $N = 14$, mean 18.34 ± 6.96; $P = 0.039$). The other groups did not shown significant differences between control and neuropathic rats. The comparison of SP-expression intensity in different types of neuron between control and neuropathic groups is shown in Figure 4.3 and Table 4.1.

Size distributions of all neurons studied showing those with unambiguous SP labeling appears in Figure 4.4. The mean cell-diameter range was Aβ-fiber > Aδ-fiber > C-fiber, a pattern similar to that previously established in rat DRGs. The diameters of C-fiber cells were in the range 20-30 μm; the majority of positive C-fiber labeled cells had diameters <25 μm. The diameters of Aδ-fiber cells were in the range 20-30μm, with 1 positive HTM cell <25 μm and 1 positive HTM cell in the 25-30μm range. The diameters of Aβ cells were in the range 25-65 μm (placing them in medium (25-35 μm) and large (>35 μm) size range, with 2 positive HTM fibers in the range 25-30 μm. In this study, 4 MS neurons in neuropathic rats showing SP positive labeling were medium or large size, with 2 neurons located in the 25-30 μm range, 1 neuron in the 30-35 μm range and 1 neuron in the 35-40 μm range. As shown in Figure 4.4, smaller cells including all neuron types were more likely to show SP, in both control and neuropathic rats.

The total number of DRG cells with clearly positive SP immunoreactive material as judged by 3 viewers was counted in every section of ten ipsilateral L4

DRGs (Figure 4.5). The distribution of SP expression in different sized neurons was calculated based on total 30-33 sections of each DRG, from 5 neuropathic and 5 control rats. As shown in Table 4.2, the fraction of neurons with positive SP was about 22%-28%. There was no difference between control and neuropathic rats (control: 25.15 ± 0.856, N = 5; neuropathic 26.66 ± 0.383, N = 5; P = 0.309). However, in comparison with the control rats, the percentage of SP immunoreactive material in the small-sized neurons in neuropathic rats was significantly less (control: 15.70 ± 0.049, N = 5; neuropathic 15.42 ± 0.100, N = 5; P = 0.007), while that in the medium-sized and large-sized neurons had a tendency for an increase (control: 6.72 ± 0.590, N = 5; neuropathic: 8.00 ± 0.354 , N = 5; P = 0.099 in medium-sized neurons and control: 2.73 ± 0.276, N = 5; neuropathic: 3.24 ± 0.261, N = 5; P = 0.095 in large-sized neurons; respectively). This calculation was robust as there was considerable overlap of the cell size. However, the calculation biased by overlapping might be diminished by statistical analysis based on the same distribution samples. Lack of clarity regarding the cell size would lead two conclusions: on the one hand, the actual percentage of small-sized neurons in neuropathic rats would be lower as increased SP expression in medium- and large-sized neurons would be counted in the small-size group, and the number of medium-sized neurons in neuropathic rats might be contributed to from large cells.

4.4. Discussion

Previous studies in several neuropathic pain models have suggested there is a phenotypic switch in large-diameter Aβ primary sensory neurons such that they *de novo* express SP (Marchand et al., 1994; Noguchi et al., 1994; Noguchi et al., 1995; Weissner et al., 2006). Concomitantly, small-diameter C-fiber sensory neurons appear

to downregulate SP (Hokfelt et al., 1994). Our results show changes in SP-like immunoreactive material in the same direction as these changes. However, so far, most of the previous studies on SP expression in animal models of peripheral neuropathy have been based on axotomized neurons *in vitro* which were anatomically and/or only partially functionally identified. To our knowledge, there is no direct evidence regarding the changes in SP expression observed in functionally defined DRG neurons following experimental peripheral neuropathy. The present study provides a direct experimental approach to gather such evidence. Once a specific neuron was fully classified functionally, it was injected intracellularly with an identifiable dye, the ganglion extracted and a comparative analysis was done of SP expression of that particular neuron with neurons of other sizes, in both control and neuropathic rats. This project thus provided a direct way to determine SP expression alterations in single, functionally defined sensory neurons, which is not accessible by any other approach.

It has been reported that there is a broad spectrum of staining intensities for SP in guinea pig DRGs (Lawson et al., 1997). Although we cannot totally exclude the possibility that some neurons with relatively low SP intensities may have been excluded from analysis, the patterns reported here show a clear difference in the intensity of labeling across different groups of neurons. The fraction of neurons in control rats with positive SP (>15% intensity) was 6/38 of the total sample. The value of this fraction is lower than the estimated 25% which was reported for afferent DRG neurons in the rat (Lawson, 1997) and our report based on total DRG section (22%-28%). This might due to the bias of selection of the neurons and the low sample number. However, the distribution of SP expression in fully classified neurons with

specific type and size is similar to the distribution that has been reported in other studies (Lawson et al., 1997). These comparable results lend circumstantial support to our labeling and adequacy of sampling.

An important conclusion of this study is that in neuropathic rats. some Aβ-fiber LTM neurons express SP. Our data demonstrate that the SP is synthesized in Aβ-fiber MS neurons. It is unclear whether there is SP expression in other Aβ-fiber LTM neurons. However, in the present study, the SP intensity of one cutaneous Aβ LTM neuron was in the 10%-15% range, which is in the borderline between our classification of negative and positive intensity. This raises the possibility that SP expression in these Aβ-fiber LTM neurons might be much lower than that in MS neurons and thus cannot be detected.

SP is an important peptide responsible for nociceptive transmission from the periphery to the central nervous system (Henry, 1976; Henry, 1978; Wiesenfeld-Hallin, 1986). Although the absence of SP does not preclude its nociceptive function, as not all types of HTM nociceptive DRG somata had demonstrable SP, unquestionably SP immunoreactivity was a feature of neurons that were activated solely by noxious stimuli. Thus it is possible to argue that the presence of SP in a primary afferent neuron projecting to somatic tissue may classify that cell as nociceptive (Lawson et al., 1997). Notably, in our previous study in the same model we observed that touch-evoked afterdischarge in dorsal horn neurons was apparently driven by myelinated afferents and was blocked by an NK-1 receptor antagonist (Pitcher and Henry, 2004). Thus, this *de novo* SP expression in Aß-fiber LTM neurons may contribute to tactile allodynia, and is consistent with its innate low activation threshold characteristics.

In our model, it is unlikely that the Aδ- and C-fiber sensory neurons would contribute to SP release evoked by low-intensity stimulation as SP content of small-sized neurons is reduced after such injury and there was no reduction in the threshold of HTM neurons (data are shown in chapter 2).

The basis for the differences in the degree of SP expression between control and neuropathic rats remains unknown. It may be that phenotypic changes in sensory neurons with large-cell bodies from being nonpeptidergic neurons to peptidergic neurons might be induced by nerve inflammation, such as Wallerian degeneration after nerve inflammation (Djouhri et al., 2006; Neumann et al., 1996). It also may be that axonal injury might alter nuclear localization signals of axonplasmic proteins at the injured site (Walters and Ambron, 1995). Both of these changes might cause abnormal release of cytokines, nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), tumor necrosis factor (TNF) or other inflammatory mediators from immune cells and Schwann cells (Cui et al., 2000; Gherardini et al., 1999; Li et al., 2000; Shamash et al., 2002; Sommer and Schafers, 1998; Wagner and Myers, 1996). It has been reported that a spinal nerve lesion induces expression of NGF in DRG satellite cells, primarily those surrounding large-diameter neuronal profiles (Zhou et al., 1999). However, it has also been reported that the loss of target-derived NGF might be responsible for the decrease in SP content in C-fiber sensory neurons after axotomy (Verge et al., 1995), suggesting that this type of negative signaling does not underlie *de novo* synthesis of SP in Aβ neurons. Thus, whether or not such NGF signaling mechanism is related to different functional type neurons remains to be determined. As the MS neuron is the main type of neuron that might be undergoing this type of phenotypic change in this model, it is consistent with our

previous electrophysiological studies in which MS neurons exhibited the most ectopic discharges. One necessary factor for these neurons is neurotrophin-3 (NT3) and therefore NT3 may be sufficient to promote the differentiation of MS neurons (Oakley and Karpinski, 2002). Thus, whether NT3 might be involved in changing the properties of MS neurons needs further investigation.

4.5. Conclusions

In this study, we have described a functional index of SP expression in all identified DRG neurons. Our direct evidence to show greater expression of SP in Aβ-fiber LTM neurons of the DRG in the neuropathic pain model adds further weight to the accumulating evidence that plasticity of Aβ-fiber sensory neurons may contribute to nerve injury-induced pain by expressing SP that is then released into the sensory dorsal horn by low- intensity stimuli to the peripheral receptive fields.

4.6. Tables and figures

Table 4.1. SP and identified DRG neurons.

DRG neurons classification	SP(+/-)		SP intensity(%)	
	Control	NeP	Control	NeP
C HTM	4/6	4/7	45.20 (7-100)	51.86(7-100
C LTM	0/2	0/2	5.09(3-7)	48.29(3-9)
Aσ HTM	1/2	2/3	31.69(6-58)	29.90(2-66)
Aσ LTM	0/1	0/2	3	4.53 (1-7)
Aα/β HTM	1/2	1/3	37.69(3-72)	21.55(7-57)
Aα/β LTM				
Cutaneous	0/9	0/11	3.24(0-9)	3.59(0-14)
Muscle spindle	0/16	4/14	4.18(0-10)	18.34(0-85)
Total	6/38	11/42		

Different types of sensory neuron wer categorized according to conduction velocity and receptive properties (see text). For each group the ratio of positive to negative neurons (+/-) is provided with the mean SP relative intensity, with the minimum and maximum relative intensity for that group of neurons in parentheses.

Table 4.2. Distribution of substance P expression in different sized neurons.

		Total SP negative cell (%)	Total SP positive cell (%)	Total SP positive small cell (%)	Total SP positive medium cell (%)	Total SP positive large cell (%)
Control	1	72.43	27.57	15.82	8.19	3.56
	2	75.4	24.6	15.79	6.6	2.21
	3	77.25	22.75	15.57	4.75	2.43
	4	74.45	26.55	15.72	7.62	3.21
	5	75.72	24.27	15.61	6.42	2.24
	Mean ± SEM	75.05 ± 0.795	25.15 ± 0.856	15.70 ± 0.049	6.72 ± 0.590	2.73 ± 0.276
NeP	1	72.87	27.13	15.02	8.49	3.62
	2	73.5	26.5	15.45	8.57	2.48
	3	74.54	25.46	15.56	7.14	2.76
	4	72.24	27.76	15.51	8.67	3.58
	5	73.55	26.45	15.54	7.13	3.78
	Mean ± SEM	73.34 ± 0.383	26.66 ± 0.383	15.42 ± 0.100	8.00 ± 0.354	3.24 ± 0.261
	p value	0.1508	0.3095	0.0079	0.099	0.0952

Distribution of SP expression in different sized neurons was calculated based on a total of 33-35 sections of L4 DRGs, from 5 neuropathic and 5 control rats. For each group, the percentage of SP positive or negative neurons is provided. Cell size was classified as small (<25μm diameter), medium (25-35μm) or large (>35μm). P value indicates difference between control and neuropathic rats. Mann Whitney test

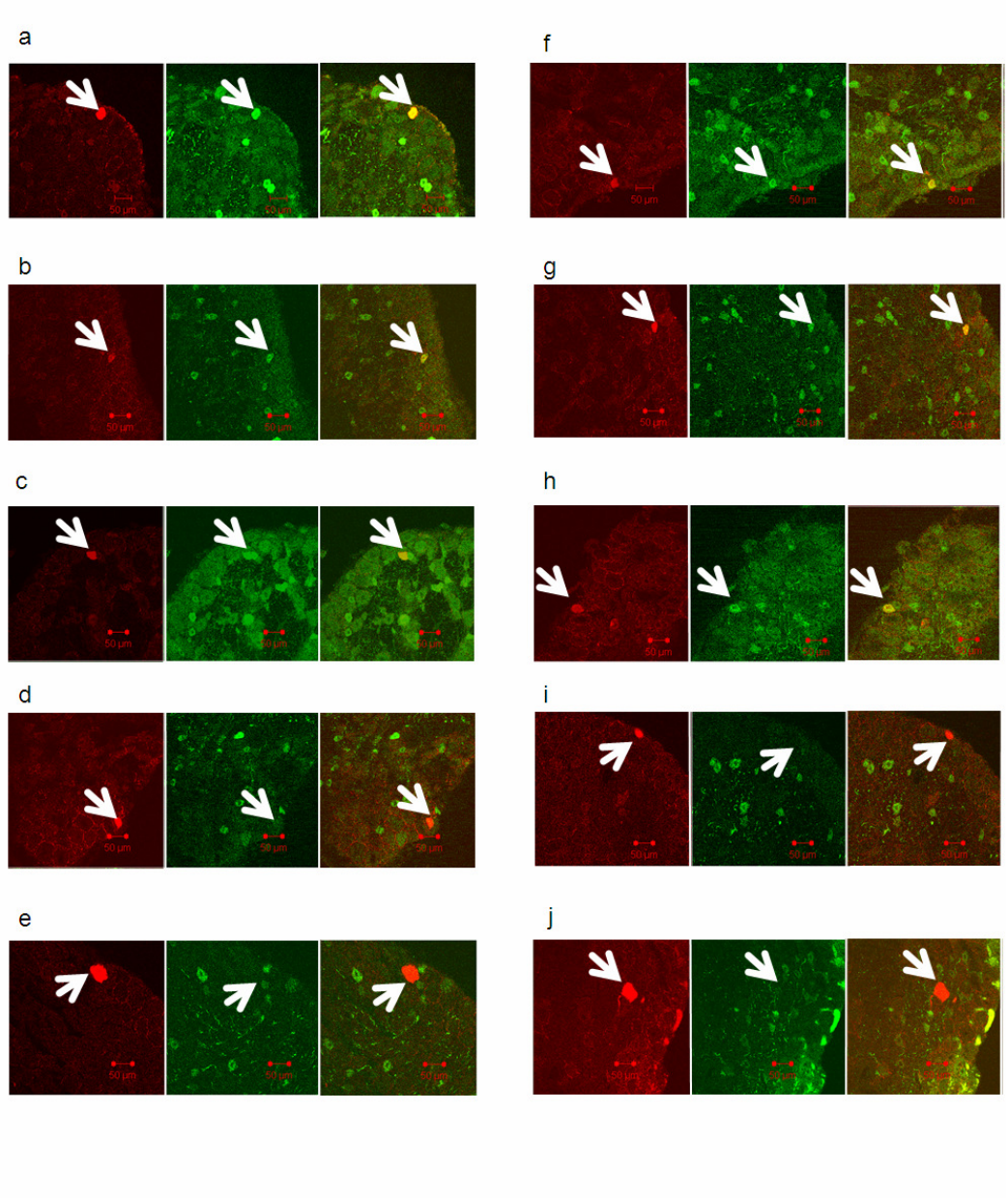

Figure 4.1. Localization of substance P in identified DRG neurons. Micrographs showing representative neurobiotin labeled DRG neurons in sections labeled for SP (a-j). Each section (a-j) consists of three images:cells filled with neurobiotin (left) are shown in red (Texas Red), cells immunoreactive for SP (middle) are shown in green

(Alexa 488), and the merged image (right) shows colocalization (yellow). On the left are fluorescence images of dye-injected neurons in control rats (a-e). The images from neuropathic rats are on the right (f-j). All sections illustrated are cut through the nucleus of the cell. Neurons were categorized according to conduction velocity and receptive properties (see text). a, C-fiber HTM neuron, which was positive for SP. b, Aδ-fiber HTM neuron, which was positive for SP. c, Aβ-fiber HTM neuron, which was positive for SP. d, C-fiber LTM neuron, which was negative for SP. e, Aβ-fiber LTM neuron, which was negative for SP. f, C-fiber HTM neuron, which was positive for SP. g. Aδ-fiber HTM neuron, which was positive for SP. h. Aβ-fiber HTM neuron, which was positive for SP. i. C-fiber LTM neuron, which was negative for SP. j. Aβ-fiber LTM neuron, which was negative for SP. Abbreviation: HTM, high threshold mechanical neuron; LTM, low threshold mechanical neuron; CUT, Aβ LTM including guard/field hair neuron (GF), rapidly adapting neuron (RA) and slowly adapting neuron (SA); MS, Aβ LTM MS neuron. Scale bar, 50 μm.

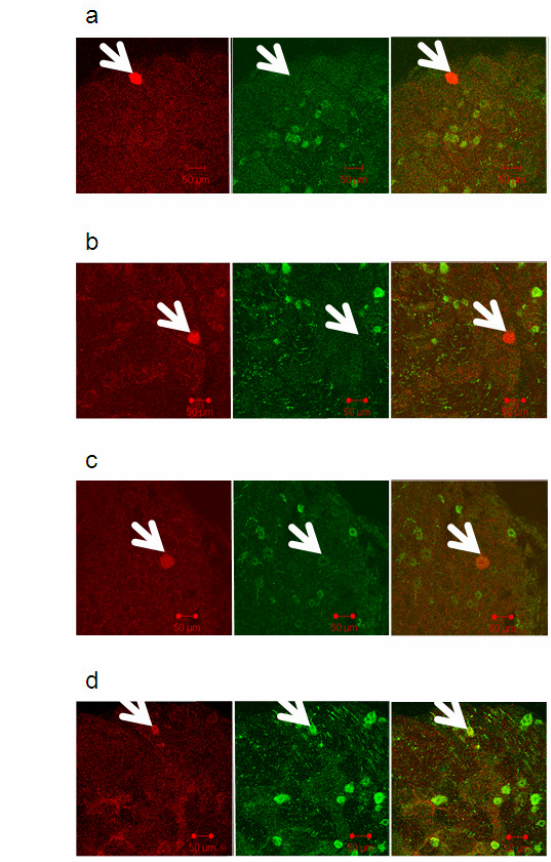

Figure 4.2. Control and neuropathic rats differ in the expression of SP by muscle spindle neurons. Sections are organized as described in Figure 1. a, a neuron in a control animal (cell diameter in range 35-40μm), which was negative for SP. b, a neuron in a neuropathic animal (cell diameter in range 35-40μm), which was negative for SP. c, a neuron in a neuropathic animal (cell diameter in range 35-40μm), which was positive for SP. d, a neuron in a neuropathic animal (cell diameter in range 25-35μm), which was positive for substance P. Scale bar, 50 μm.

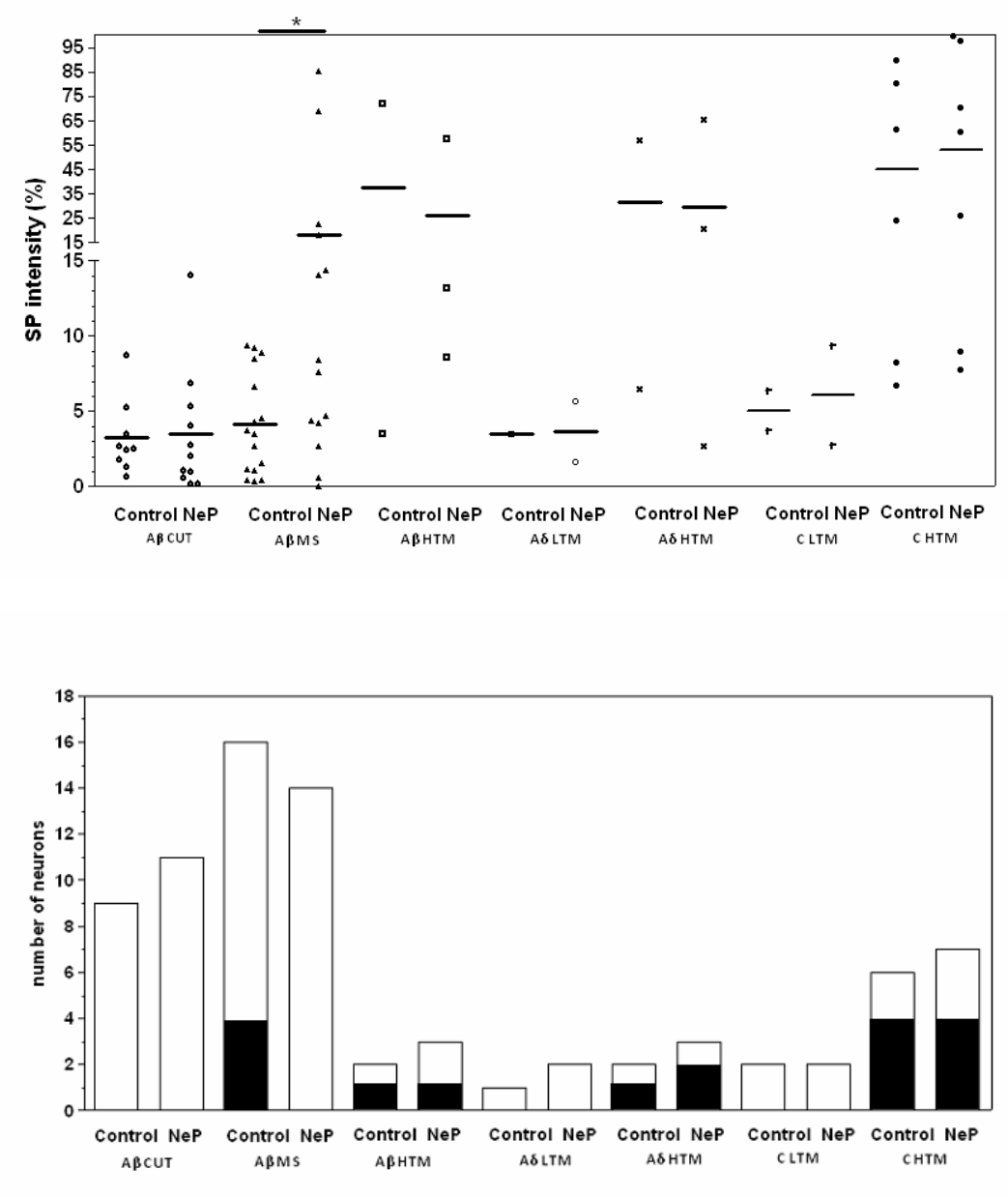

Figure 4.3. Quantification of SP in identified control and neuropathic DRG neurons. Scatter graphs in A show the distribution of SP intensity (the relative intensity of each cell as a percentage of the intensity of the most intensely labeled cell in the respective section), with the median (horizontal line) superimposed in each

case. Stacked bar charts in B show the proportions of neurons in A that classified as positive for SP (> 15%), represented as filled bars, and those classified as negative for SP (< 15%), represented as open bars. Details of abbreviations are as in Figure 4.1. The asterisk above the graph indicates a significant difference between control and neuropathic rats: *$p<0.05$. Mann Whitney t-test

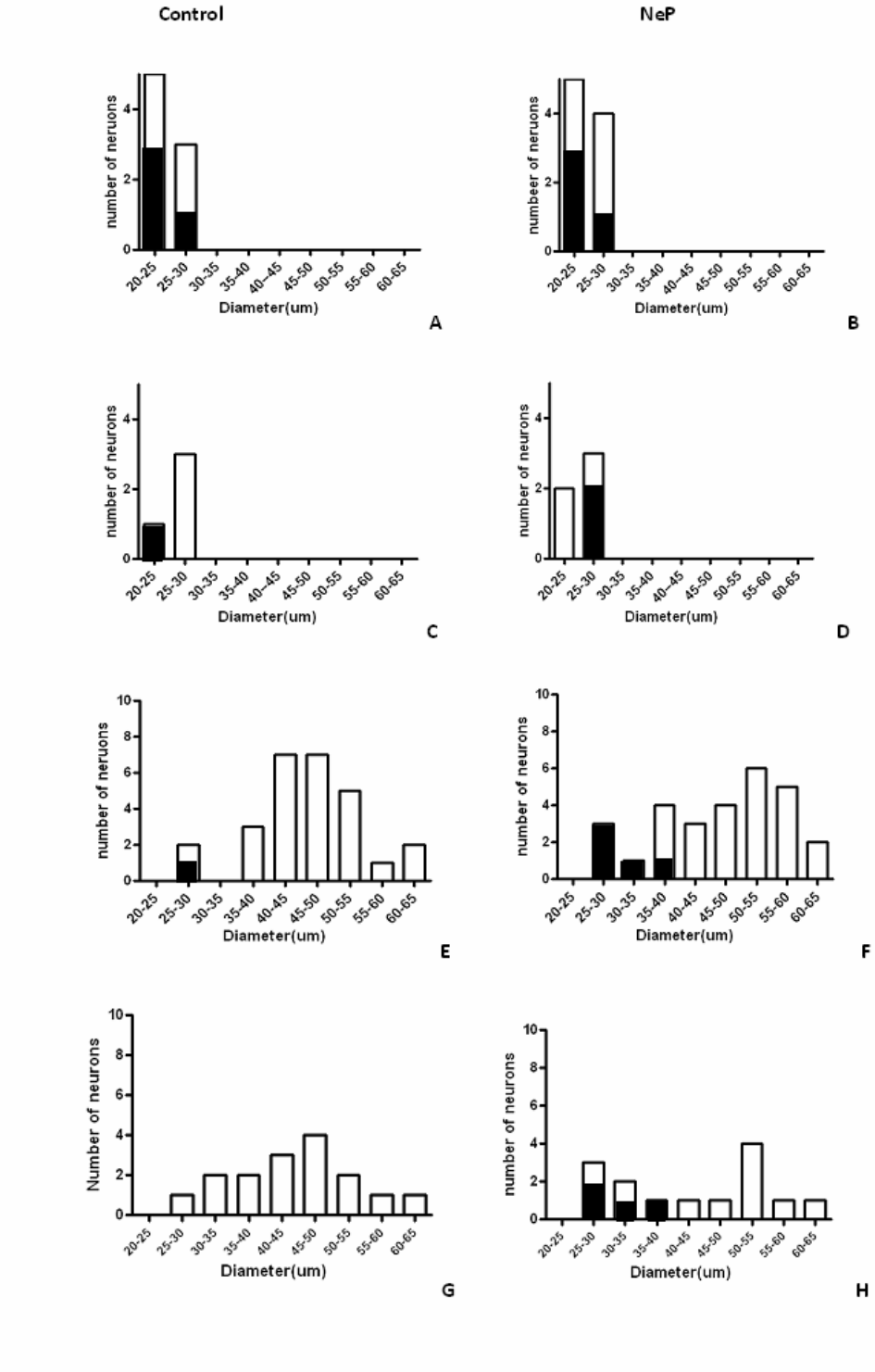
Control
NeP
number of neruons
numbeer of neruons
number of neurons
Number of neurons
Diameter(um)
20-25
25-30
30-35
35-40
40-45
45-50
50-55
55-60
60-65
A
B
C
D
E
F
G
H

Figure 4.4. Cell sizes and SP in control and neuropathic DRG neurons. Histograms showing the largest cell diameter range of cells expressing SP. Stacked bar charts show the proportions of neurons in Figure 4.3 that classified as positive for SP (>15%), represented as filled bars, and those classified as negative for SP (<15%), represented as open bars. Details of abbreviations are as in Figure 4.1.

A, C-fiber sensory neurons in control rats. B, C-fiber sensory neurons in neuropathic rats. C, Aσ-fiber neurons in control rats. D, Aσ-fiber neurons in neuropathic rats. E. Aβ-fiber neurons in control rats. F, Aβ-fiber neurons in neuropathic rats. G, Aβ-fiber LTM MS neurons in control rats. H, Aβ-fiber LTM MS neurons in neuropathic rats.

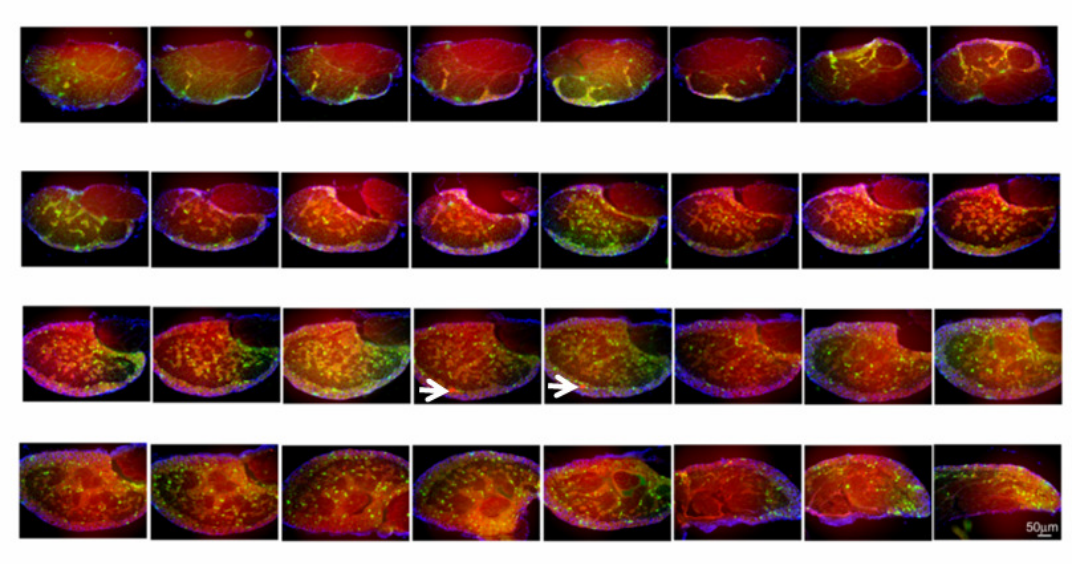

Figure 4.5. Localization of SP expression in serial DRG sections. Representative frozen DRG sections, 25µm thick, were serially collected and studied with indirect immunofluorescence staining for SP. The dye-labeled cell is indicated by arrowheads.

Reference list

Brodin E, Linderoth B, Goiny M, Yamamoto Y, Gazelius B, Millhorn DE, Hokfelt T, Ungerstedt U, 1990. In vivo release of serotonin in cat dorsal vagal complex and cervical ventral horn induced by electrical stimulation of the medullary raphe nuclei. Brain Res. 535: 227-236.

Cahill CM, Coderre TJ, 2002. Attenuation of hyperalgesia in a rat model of neuropathic pain after intrathecal pre- or post-treatment with a neurokinin-1 antagonist. Pain 95: 277-285.

Campbell EA, Gentry CT, Patel S, Panesar MS, Walpole CS, Urban L, 1998. Selective neurokinin-1 receptor antagonists are anti-hyperalgesic in a model of neuropathic pain in the guinea-pig. Neuroscience 87: 527-532.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL, 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53: 55-63.

Coudore-Civiale MA, Courteix C, Eschalier A, Fialip J, 1998. Effect of tachykinin receptor antagonists in experimental neuropathic pain. Eur. J. Pharmacol. 361: 175-184.

Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderoth B, 2000. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. Pain 88: 239-248.

Cumberbatch MJ, Carlson E, Wyatt A, Boyce S, Hill RG, Rupniak NM, 1998. Reversal of behavioural and electrophysiological correlates of experimental peripheral neuropathy by the NK1 receptor antagonist GR205171 in rats. Neuropharmacology 37: 1535-1543.

De Koninck Y, Henry JL, 1991. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. Proc. Natl. Acad. Sci. U. S. A 88: 11344-11348.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Djouhri L, Bleazard L, Lawson SN, 1998. Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J. Physiol 513 (Pt 3): 857-872.

Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN, 2006. Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. J. Neurosci. 26: 1281-1292.

Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ, 1988. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Res. 451: 261-273.

Fang X, McMullan S, Lawson SN, Djouhri L, 2005. Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. J. Physiol 565: 927-943.

Gherardini G, Lundeberg T, Cui JG, Eriksson SV, Trubek S, Linderoth B, 1999. Spinal cord stimulation improves survival in ischemic skin flaps: an experimental study of the possible mediation by calcitonin gene-related peptide. Plast. Reconstr. Surg. 103: 1221-1228.

Gonzalez MI, Field MJ, Hughes J, Singh L, 2000. Evaluation of selective NK(1) receptor antagonist CI-1021 in animal models of inflammatory and neuropathic pain. J. Pharmacol. Exp. Ther. 294: 444-450.

Henry JL, 1978. [Role of peptides in central neural transmission: substance P, bradykinin and beta-endorphine]. Ann. Anesthesiol. Fr. 19: 391-398.

Henry JL, 1976. Effects of substance P on functionally identified units in cat spinal cord. Brain Res. 114: 439-451.

Hokfelt T, Zhang X, Wiesenfeld-Hallin Z, 1994. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. Trends Neurosci. 17: 22-30.

Kim SH, Chung JM, 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50: 355-363.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Li Y, Dorsi MJ, Meyer RA, Belzberg AJ, 2000. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 85: 493-502.

Malcangio M, Ramer MS, Jones MG, McMahon SB, 2000. Abnormal substance P release from the spinal cord following injury to primary sensory neurons. Eur. J. Neurosci. 12: 397-399.

Malcangio M, Tomlinson DR, 1998. A pharmacologic analysis of mechanical hyperalgesia in streptozotocin/diabetic rats. Pain 76: 151-157.

Mansikka H, Sheth RN, DeVries C, Lee H, Winchurch R, Raja SN, 2000. Nerve injury-induced mechanical but not thermal hyperalgesia is attenuated in neurokinin-1 receptor knockout mice. Exp. Neurol. 162: 343-349.

Marchand JE, Wurm WH, Kato T, Kream RM, 1994. Altered tachykinin expression by dorsal root ganglion neurons in a rat model of neuropathic pain. Pain 58: 219-231.

McCarson KE, Goldstein BD, 1991. Release of substance P into the superficial dorsal horn following nociceptive activation of the hindpaw of the rat. Brain Res. 568: 109-115.

McCarthy PW, Lawson SN, 1989. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. Neuroscience 28: 745-753.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Neumann S, Doubell TP, Leslie T, Woolf CJ, 1996. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 384: 360-364.

Noguchi K, Dubner R, De LM, Senba E, Ruda MA, 1994. Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons. J. Neurosci. Res. 37: 596-603.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K, 1995. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J. Neurosci. 15: 7633-7643.

Oakley RA, Karpinski BA, 2002. Target-independent specification of proprioceptive sensory neurons. Dev. Biol. 249: 255-269.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Salter MW, Henry JL, 1991. Responses of functionally identified neurones in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin. Neuroscience 43: 601-610.

Shamash S, Reichert F, Rotshenker S, 2002. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J. Neurosci. 22: 3052-3060.

Sommer C, Schafers M, 1998. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res. 784: 154-162.

Urban LA, Fox AJ, 2000. NK1 receptor antagonists--are they really without effect in the pain clinic? Trends Pharmacol. Sci. 21: 462-464.

Verge VM, Richardson PM, Wiesenfeld-Hallin Z, Hokfelt T, 1995. Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. J. Neurosci. 15: 2081-2096.

Wagner R, Myers RR, 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73: 625-629.

Walters ET, Ambron RT, 1995. Long-term alterations induced by injury and by 5-HT in Aplysia sensory neurons: convergent pathways and common signals? Trends Neurosci. 18: 137-142.

Weissner W, Winterson BJ, Stuart-Tilley A, Devor M, Bove GM, 2006. Time course of substance P expression in dorsal root ganglia following complete spinal nerve transection. J. Comp Neurol. 497: 78-87.

Wiesenfeld-Hallin Z, 1986. Substance P and somatostatin modulate spinal cord excitability via physiologically different sensory pathways. Brain Res. 372: 172-175.

Wu Q, Henry JL, 2009. Delayed onset of changes in soma action potential genesis in nociceptive A-beta DRG neurons in vivo in a rat model of osteoarthritis. Mol. Pain 5: 57.

Wu Q, Henry JL, 2010. Changes in Abeta non-nociceptive primary sensory neurons in a rat model of osteoarthritis pain. Mol. Pain 6: 37.

Yamamoto T, Yaksh TL, 1992. Effects of intrathecal capsaicin and an NK-1 antagonist, CP,96-345, on the thermal hyperalgesia observed following unilateral constriction of the sciatic nerve in the rat. Pain 51: 329-334.

Zhou XF, Deng YS, Chie E, Xue Q, Zhong JH, McLachlan EM, Rush RA, Xian CJ, 1999. Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. Eur. J. Neurosci. 11: 1711-1722.

CHAPTER 5

Pregabalin reduces action potential amplitude in sensory neurons in control and neuropathic rats

Authors: Yong Fang Zhu, Kiran Yashpal, James L Henry

Corresponding author: Kiran Yashpal, PhD
James L Henry, PhD
Department of Psychiatry and Behavioural Neurosciences,
McMaster University,
1200 Main St. West, HSC 4N35
Hamilton, Ontario, Canada, L8N 3Z5,
Tel: +1-905-525-9140, extension 24750,
FAX: 905-4522-8844
Email address: yashpal@mcmaster.ca
jhenry@mcmaster.ca

Preface

Significance to thesis

This study demonstrated the acute antiallodynic effects of pregabalin on tactile allodynia in a neuropathic model. This study demonstrated that intravenously (i.v.) administered pregabalin attenuated both control and neuropathic model, with selective reduction of sodium currents in dorsal root ganglion (DRG) sensory neurons.

Authors' contribution

Yong Fang Zhu did the electrophysiological experiments, the behavior test, and model induction. She also analyzed the data, performed statistical analyses, wrote the initial draft of the manuscript, and worked on refining this draft and the revision based on editorial review.

Kiran Yashpal helped intravenously tail injection. James L. Henry conceived of, designed, and coordinated the study. He also worked on refining this draft and the revision based on editorial review.

Abstract

Pregabalin is thought to bind to the voltage-dependent α2δ calcium channel subunit and modulates the release of neurotransmitters, resulting in analgesic effects in neuropathic pain. To understand the possible changes in primary sensory neurons in an animal model of peripheral neuropathy, and effects of pregabalin on these neurons, we studied the antinociceptive effects of pregabalin on tactile allodynia in a neuropathic model. A peripheral neuropathy was induced by inserting a polyethylene cuff around the sciatic nerves in the rat. Pregabalin was administered i.v. at incremental doses until an antinociceptive effect was observed. Tactile sensitivity was assessed using von Frey filaments. The data show that pregabalin dose-dependently raises tactile withdrawal threshold in both control and neuropathic rats. The electrophysiological data suggest that pregabalin acts by depressing action potential (AP) amplitude of primary sensory neurons including both low-threshold mechanoreceptor (LTM) neurons and high-threshold mechanoreceptor (HTM) neurons but no effect on low-threshold cutaneous neurons (CUT).

5.1. Introduction

Neuropathic pain is a consequence of disease, trauma or dysfunction of central or peripheral neurons. Clinically, neuropathic pain is resistant to conventional treatment. However, it is reported that the novel compound pregabalin (Lyrica), which is structurally related to the antiepileptic drug gabapentin (Neurontin) (Taylor et al., 2007; Field et al., 2006), has a broad analgesic effect on mechanical and thermal hypersensitivity associated with nerve injury (Field et al., 2006; Field et al., 2001; Field et al., 1999). Pregabalin has been proven to be effective in patients with painful diabetic peripheral neuropathy and postherpetic neuralgia (Dworkin et al.,

2003; Farrar et al., 2001), and has also been reported to have antinociceptive activity in rodent models of neuropathic pain, including peripheral neuropathic pain and central neuropathic pain (Fehrenbacher et al., 2003). The exact analgesic mechanism of action of pregabalin has yet to be elucidated. However, pregabalin, like gabapentin, has been reported to be a selective, high-affinity ligand for the auxiliary α2δ subunit of voltage-gated calcium channels (VGCCs), and acts on this subunit to regulate the conductance of this channel (Dooley et al., 2007; Field et al., 2006; Taylor et al., 2007). It has been reported that pregabalin reduces the depolarization-induced calcium influx at nerve terminals, resulting in a reduction of the presynaptic release of excitatory neurotransmitters, including glutamate, substance P (SP) and calcitonin gene-related peptide (CGRP) (Fink et al., 2002; Han et al., 2007).

While it is inherent in theories regarding the mechanisms of the analgesic action of pregabalin that the site of the action is on the central terminals of sensory neurons in the spinal cord and brain stem (Dooley et al., 2007; Taylor et al., 2007), this may not be exclusively the case. The α2δ subunit of the VGCC is reported to be upregulated in DRG neurons in the partial sciatic ligation model of neuropathic pain (Newton et al., 2001) and calcium mobilization has been implicated in activation of visceral nociceptors by mast cell mediators from patients with inflammatory bowel disease (Barbara et al., 2007). Inference with calcium influx in peripheral nerve terminals has been demonstrated to reduce the severity of inflammation-induced pain readouts (Asfaha et al., 2007) and a calcium-dependent activation of some of the transient receptor potential channels has been reported (Doerner et al., 2007). These results have led to the belief that pregabalin might have a potential effect on primary sensory neurons via different analgesic sites and mechanisms.

As the primary sensory neuron is a site of pain generation in neuropathic pain states (Devor, 2009; Liu and Eisenach, 2005; Yang et al., 2005; Campbell et al., 1988; Katz and Gold, 2006; Schaible, 2007), the present study focused on the possibility that there is a peripheral site of action of pregabalin. In this study, DRG neurons were functionally defined using electrophysiological recordings in vivo. Acute antinociceptive effects of pregabalin were examined in neurons in control and neuropathic model rats to compare neuronal properties before and after pregabalin administration (i.v.).

5.2. Methods

Experimental rats and model induction

Young male Sprague-Dawley rats (obtained from Charles River Inc. St. Constant, QC, Canada) weighing 170-200 g were used. Rats were divided into two groups, control and neuropathic model groups. A peripheral neuropathy was induced according to the method previously described in detail (Pitcher and Henry, 2002; Pitcher et al., 1999; Mosconi and Kruger, 1996; Chapter 2). Under anesthesia with a mixture of ketamine (Ketamine; 5 mg/100g; Bimeda-MTC Animal Health Inc.; Cambridge, ON, Canada), xylazine (Rompun; 0.5 mg/100g; Bayer HealthCare, Toronto, ON, Canada), and acepromazine (Atravet; 0.1 mg/100g; Ayerst Veterinary Laboratories, Guelph, ON, Canada) given i.p., the right sciatic nerve was exposed at the mid-thigh level. Two cuffs of 0.5mm polyethylene (PE 90) tubing (Intramedic PE-90, Fisher Scientific Ltd., Whitby, Ontario, Canada) were inserted around the exposed nerve about 1 mm apart. The wound was then sutured in two layers, muscle and skin. Antibiotic ointment (Furacin; nitrofurazone 0.2%; Vetoquinol N.-A. Inc.; Lavaltrie, QC, Canada) was applied over the wound, and 0.01 ml/100g of

antibabacterial injectable solution (Bayer HealthCare, Toronto, ON, Canada) was injected subcutaneously (s.c.). Rats were given 1 ml saline s.c. and ocular lubricant, and were placed under a heating lamp until they recovered from the anaesthetic. They were then returned to their home cages.

von Frey test of paw withdrawal threshold

In all cases, the von Frey test was run on the same day as the acute electrophysiological experiment, before the rats were anesthetized. To quantify mechanical sensitivity of the foot, brisk foot withdrawal in response to normally innocuous mechanical stimuli was measured as described previously (Pitcher and Henry, 2002; Pitcher et al., 1999; Kim et al., 1998; Chapter 2). The rats were placed in a transparent Plexiglas box with a clear Plexiglas floor, containing 0.5 cm diameter holes spaced 1.5 cm apart to allowed full access to the paws (Pitcher and Henry, 2002; Pitcher and Henry, 2004; Pitcher et al., 1999). Rats were allowed to habituate to the box for approximately 15 min, until cage exploration and major grooming activities had ceased. von Frey filaments (Stoelting Co., Wood Dale, IL, USA) were then applied to the plantar surface of the hind paw to determine the withdrawal threshold. A von Frey filament was applied 5 times for 3-4sec each, at intervals of 3 sec intervals, each to a different spot on the hind paw. For each rat, these filaments were applied in ascending order until a clear withdrawal response was observed. The mechanical forces exerted with the calibrated von Frey filaments used in this study were 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10, 15, 26, 60, 100, 180 and 300 g. Tip diameters were within the range 1.65-6.65 mm.

Intracellular recording in vivo

The animal preparation and intracellular recording approaches have been

reported previously (Wu and Henry, 2009; Wu and Henry, 2010; Chapter 2). In brief, each rat was initially anesthetized with the mixture described above. The right jugular vein was catheterized for i.v. infusion of drugs and a cannula was inserted into the trachea. The rat was fixed in a stereotaxic frame and the vertebral column rigidly clamped at the L2 and L6 vertebral levels. To minimize movement of the DRG during mechanical searching for receptive fields on the leg the right femur was fixed by a customized clamp to the stereotaxic frame. The L4 DRG was selected for study because it contains a large number of hind leg afferent somata. A laminectomy was performed to expose the ipsilateral L4 DRG. The L4 dorsal root was cut close to the spinal cord and placed on a bipolar electrode (FHC, Bowdoinham, ME, USA) for stimulation to evoke an antidromic AP in the DRG somata. The exposed spinal cord and DRG were covered with warm paraffin oil at 37^{o} C to prevent drying.

The rat was mechanically ventilated via the tracheal cannula using a Harvard Ventilator (Model 683, Harvard apparatus, Quebec, Canada). The ventilation parameters maintained end-tidal CO_2 concentration around 40-50 mmHg, as measured using a CapStar-100 End-Tidal CO2 analyzer (CWE, Ardmore, PA, USA). Rectal temperature was maintained at ~37^{o}C using a temperature controlled infrared heating lamp. Immediately before the start of recording, an initial 1 mg/kg dose of pancuronium (Sandoz, Boucherville, QC, Canada) was given to eliminate muscle tone. The effects of pancuronium were allowed to wear off periodically to confirm a surgical level of anesthesia; this was monitored by observing pupil diameter and any response to noxious pinch of a forepaw. Anesthetic was maintained at the surgical level with pentobarbital (20 mg/kg; CEVA SANTE ANIMAL, Liourne, France). Supplements of pentobarbital and pancuronium were added with at initial dose of 1/3

about each hour via the jugular vein.

Intracellular recordings from somata in the exposed L4 DRG were made with borosilicate glass micropipettes (1.2 mm outside diameter, 0.68 mm inside diameter; Harvard Apparatus, Holliston MA, USA). The electrodes were pulled using a Brown-Flaming puller (model p-87; Sutter Instrument CO., Novota, CA, USA). These electrodes were filled with 3 M KCl (DC resistance 50-70 MΩ). Signals were recorded with a Multiclamp 700B amplifier (Molecular Devices, Union City CA, USA) and digitized on-line using a Digidata 1322A interface (Molecular Devices, USA) with pClamp 9.2 software (Molecular Devices, USA). The microelectrode was advanced using an EXFO IW-800 micromanipulator (EXFO, Montreal, QC, Canada) in 2 μm steps until a hyperpolarization of at least 40 mV suddenly appeared. Once a stable membrane potential had been confirmed, with the aid of the protocol editor function in pClamp 9.2 software a somatic AP was evoked by stimulation of the dorsal root with a single rectangular voltage pulse.

DRG neuron classification

Neurons were divided into three groups according to dorsal root CVs. As described earlier (Djouhri et al., 1998; Fang et al., 2005; Lawson et al., 1997; Wu and Henry, 2009; Wu and Henry, 2010; Chapter 2) the CV range for C-fiber sensory neurons was <=0.8mm/ms, for Aδ-fiber neurons it was (1.5-6.5mm/ms) and for Aβ-fiber sensory neurons it was >6.5mm/ms.

The sensory receptive properties of each DRG neuron were examined using hand-held mechanical stimulators applied to the periphery. Neurons were classified as previously described (Lawson et al., 1997; Leem et al., 1993; Wu and Henry, 2010; chapter 2). The differentiation of HTM neurons and LTM neurons was based on their

sensory properties identified during receptive field searching. HTM neurons responded to noxious stimuli including noxious pressure, pinch, and probing with fine forceps, a sharp needle, coarse-toothed forceps, or coarse flat forceps. LTM neurons responded to innocuous stimuli by moving brush, light pressure with a blunt object, light manual tap, vibration. Neurons that did not respond to any of the innocuous or noxious mechanical stimuli were classed as unresponsive as defined in another group (Lawson et al., 1997).

The threshold of activation, the rate of adaption and the tissue location of the receptive field were factors to further classify Aβ-fiber LTM neurons as cutaneous neurons, which included guard/field hair neurons (GF), glabrous neurons, Pacinian neurons, slowly adapting (SA) neurons and muscle spindle neurons (MS) neurons. GF neurons were rapidly adapting cutaneous neurons. Glabrous and Pacinian neurons were rapidly adapting non-hair neurons and were named rapidly adapting neurons (RA). SA neurons were slowly adapting cutaneous neurons. MS neurons were slowly adapting neurons with deep receptive fields

Action potential configuration

The first evoked AP in each neuron was used to determine any differences in configuration between control and neuropathic rats. Criteria for acceptance of neurons in the analysis included a stable Vm more negative than ~40mV and a somatic spike evoked by dorsal root stimulation that was >40mV.

Variables in AP configuration included resting membrane potential (Vm), AP amplitude (APA), AP duration at base (APdB), AP rise time (APRT), AP fall time (APFT), maximum AP rising rate (MRR) , maximum AP falling rate (MFR), afterhyperpolarization amplitude (AHPA) and afterhyperpolarization duration at 50%

recovery (AHP50).

Conduction velocity

The distance from the stimulation site (cathode) to the recording site (centre of the DRG) was measured at the end of the experiment to determine the conductance distance and thereby calculate the conduction velocity of the axon associated with each neuron recorded.

Pregabalin administration

Pregabalin was dissolved in 0.9% normal saline, and administered by i.v. injection. During behavioral testing, rats were anesthetized by inhalation using isoflurane in oxygen. Injections were performed via the tail vein.

During the electrophysiological experiments, injections were via the jugular catheter. Pregabalin was given in a dose range of 0-5mg/kg; 0mg/kg dose served the control test. Rats were tested before and at 5, 15, 30, 45 and 60 min after administration.

Statistical analysis

Dates were presented as the Mean ± SEM. The antinociceptive effects of pregaballin were analyzed by Mann Whitney t-test for comparison before and after pregabalin administration, both in control and neuropathic rats for various subtypes and for various parameters. All statistical tests and graphing were done using Prism4 software (Graphpad, La Jolla, CA, USA). The P-value is indicated in the graphs and $P<0.05$ was considered to indicate a significant difference.

5.3. Results

Behavioral testing

From one week to 30 days after unilateral cuff ligation of the sciatic nerve, the

rats fully developed behavioral signs of neuropathic pain on the affected hind limb. In control rats, stimulation of the plantar surface of the ipsilateral hind paw with von Frey filaments evoked a withdrawal response from filaments exerting pressures of 6-76g of force. In model rats, filaments exerting pressures of 0.4 to 0.6 g of force, which control rats ignore, evoked a clear withdrawal of the nerve-injured hind paw. Furthermore, the withdrawal in neuropathic rats was greatly exaggerated in amplitude and duration, and it was frequently accompanied by licking of the paw.

A total of 30 rats was tested, 15 control and 15 neuropathic rats, for the antinociceptive effects of i.v. administered pregabalin at doses of 0, 1, 5 mg/kg (each 5 rats). Figure 5.1 showed the time course of the effect of pregabalin comparing data from control and neuropathic model rats. The data showed that withdrawal threshold was altered by pregabalin administration not only in neuropathic rats but also in control rats. The maximum effect was reached 5-30 min after i.v. administration. However, these results might be affected by the inhalation anesthetic procedure from observing 0 mg/kg control test. Thus, it appeared that the 5 mg/kg dose of pregabalin but not the 1mg/kg dose increased mechanical threshold in both models.

Electrophysiological experiments

In this study, APs were evoked by delivering single current pulses to the dorsal root (for measuring the soma AP configuration and axon conduction velocity), by intracellular injection of depolarizing current (for determining the soma current activation threshold) and by natural simulation of the peripheral receptive field (for determining the mechanical threshold of the receptive field).

A total of 19 neurons were recorded in neuropathic rats, including 12 Aβ-fiber LTM (6 CUT neurons, 6 MS neurons), 3 C-fiber HTM, 1 Aδ-fiber HTM and 3 Aβ

HTM neurons. In control rats a total of 16 neurons was recorded, including 12 Aβ-fiber LTM neurons (5 CUT neurons and 7 for MS neurons), 2 C-fiber HTM, 2 Aβ-fiber HTM neurons. In agreement previous studies presented in chapter 2, recordings of membrane electrophysiological properties of DRG somata indicated increased neuronal excitability, depolarized membrane potential and increased AP duration in neuropathic model neurons.

The cellular responses to peripheral stimulation before and after 5mg/ml i.v. administration were tested. Pregabalin had an effect on neurons in both control and neuropathic rats, with a different effect on different types of neuron. Various parameters of corresponding subclasses in different CV groups were compared before and after pregabalin administration, both in control and neuropathic rats, as shown in Table 5.1.

Effects of pregabalin on Aβ-fiber LTM cutaneous neurons

There were no changes in any of the parameters studied in control Aβ-fiber CUT neurons after pregabalin administration. Compared with neurons in control rats, in neuropathic rats, while there were no changes in soma excitability, CV, mechanical threshold of the receptive field and most AP electrophysiological parameters following administration of pregabalin, AHPA was significantly less after administration of pregabalin, as shown in Table 5.1 and Figure 5.2.

Effects of pregabalin on Aβ-fiber LTM MS neurons

In both control and neuropathic rats, several electrophysiological parameters of Aβ-fiber MS neurons showed a significant difference following administration of pregabalin. For example, APA and APRT were less and greater, respectively, AHP showed a tendency for shortening, and the current activation threshold of the soma

was greater. CV and mechanical activation threshold of the receptive field remained unchanged. The data are presented in Table 5.1 and illustrated in Figure 5.3.

Figure 5.4 shows one example of an MS neuron that underwent changes induced by pregabalin administration. Over a succession of 1 min tests, monitoring the ongoing discharge, 5 min after pregabalin administration this MS neuron showed a decrease in amplitude of the AP and the AHP.

Figure 5.5 showed one example of the responses of another MS neuron to dorsal root stimulation, to intracellular current injection and to peripheral stimulation, to illustrate the consistency of the configuration of the AP as well as the effect of administration of pregabalin.

Effects of pregabalin on HTM neurons

Similar to the effects of administration of pregabalin on Aβ-fiber MS neurons, in Aβ-fiber HTM neurons AP electrophysiological parameters showed significant changes, both in control and neuropathic rats. The data are shown in Table 5.1. The typical response of a cell was a single AP in response to a single stimulus. However, 5 min after i.v. administration of pregabalin, in some HTM neurons it was no longer possible to elicit an AP and in others a truncated AP only could be elicited. Figure 5.6 shows examples of these changes in response to dorsal root stimulation.

In these HTM neurons, although there was no response later after pregabalin administration, during the period when an AP could be elicited in response to dorsal root stimulation, CV and mechanical threshold of receptive field were unchanged (data are not shown).

5.4. Discussion

The data from this study indicate that 5 mg/kg of pregabalin given i.v.

increases tactile withdrawal threshold in awake naive (control) and neuropathic rats and depresses APA of primary sensory neurons recorded intracellularly in the DRG. These effects appear to include both LTM and HTM neurons but no effect was observed on Aβ-fiber CUT neurons. Further, the inhibition appears to be on the soma rather than the axon or the nerve terminal in the periphery, as CV and mechanical activation threshold of the receptive field did not appear to change after administration of pregabalin. The decrease in APA is most easily explained as a loss of a sodium spike in the rising phase of the AP, as will be discussed below. The present study thus presents data that may require a reassessment of the current thought on the mechanism of action of pregabalin as primarily an inhibition of a calcium current at the cellular level.

Decreased sodium current and calcium current after administration of pregabalin

In Aβ-fiber CUT neurons, the data demonstrated that no AP or AHP changes occurred with administration of pregabalin in control rats. However, in neuropathic rats there was a tendency for the AHP to shorten following administration of pregabalin. In Aβ-fiber MS neurons, and all C-fiber Aδ- and Aβ-HTM neurons, the data showed significantly decreased APA, diminished AHP, and attenuated excitability in both control and neuropathic rats. As decreased sodium current might contribute the longer depolarization phase and reduction of the APA (Waxman et al., 1999), and reducing calcium current might have resulted in diminishing the AHP phase by reducing a calcium-dependent potassium current (Djouhri et al., 1998). Thus, our results suggest decreased sodium current and decreased calcium current after administration of pregabalin on Aβ-fiber CUT neurons in neuropathic rats, and on all Aβ-fiber MS and HTM neurons in both control and neuropathic rats.

Calcium channel α2δ-1 subunit unregulated in LTM neurons in neuropathic rats

Previous studies have reported that pregabalin has a high affinity for the α2δ-1 subunit of VDCCs and thus reduced intra-calcium current (Field et al., 2006; Taylor et al., 2007). Changes in the AHP observed in this study suggest that the calcium channel α2δ-1 subunit might be expressed mainly in HTM and MS neurons, but less in CUT neurons in control rats. However, after peripheral neuropathy, CUT neurons might upregulate α2δ-1 subunit expression (might not related for reduction of inward calcium current in neuropathy). These results correspond to the findings of a previous study. Using in situ hybridization analysis, Newton et al., showed 70%, 40% and 19% of small, medium and large neuronal profiles, respectively, expressed α2δ-1 mRNA from L5 DRGs in sham-operated rats and an increase in the α2δ-1 subunit appearing in medium and large neuronal profiles in a peripheral neuron injury model (Newton et al., 2001).

Sodium channel or calcium channel is functionally relevant to the inhibitory action of pregabalin

Although the previous study provided evidence that the α2δ subunit of the VGCC is the major binding protein for pregabalin in the central nerve system (CNS) (Fehrenbacher et al., 2003; Field et al., 2006; Taylor et al., 2007), the effects on the rising phase of the AP in the present study suggest that the analgesic effect of pregabalin on neuropathic pain may also be mediated, at least in part, by its peripheral inhibitory action on some sodium channels. It is not yet known whether the sodium channel is functionally relevant to the inhibitory action of pregabalin. However, a sodium channel, especially a TTX-resistant sodium channel, might mediate effects via a calcium current in other neurons and in muscle (De Laet et al., 2002; Linsdell and

Moody, 1995; Zamponi and French, 1995).

In the present study, pregabalin might suppress a TTX-resistant sodium channel indirectly via inhibiting a calcium channel. Small neurons, including C- and Aδ-fiber neurons, mostly express TTX-resistant sodium channels, while medium and large neurons, including Aβ-fiber sensory neurons mostly express TTX-sensitive channels or co-express TTX-sensitive and TTX-resistant channels (Liu et al., 2001). This would be consistent with results reported here that C- and Aδ-fiber neurons were most significantly affection by pregabalin.

Suppressive effects by pregabalin on both control and neuropathic rats

Although reduction of an inward calcium current may diminish AHP and thus increase excitability (Kanai et al., 2004), diminishing a sodium current may result in deceased excitability (Liu et al., 2001). Our electrophysiological results demonstrated that pregabalin attenuated peripheral excitability in both control and neuropathic rats, which consisted with our behavior data showing antinociceptive effects of pregabalin administration in both control and neuropathic rats. In fact, the antinociceptive effect of pregabalin, which is mainly considered to be due to a spinal action on VGCCs, may be still related to this model.

However, a pregabalin effect at a peripheral site could be expected to reduce sensory traffic in the neuropathic model, but pregabalin also produced effects on neurons in control rats. Possibly, these responses to pregabalin may balance other pathologic nerve function. A side-effect in terms of clinical practice might be related to the effects of pregabalin in both control rats and neuropathic model rats.

5.5. Conclusions

In summary, as current thought on the mechanisms of the analgesic effects of

pregabalin on neuropathic pain focuses on inhibition of the calcium channel α2δ-1 subunit, which is distributed in different types of DRG neurons and unregulated in LTM neurons in neuropathic rats, the current study, showing pregabalin depressing primary sensory neurons in both control and neuropathic rats, a suppressive effect on HTM and LTM neurons but not on CUT neurons as well as an apparent inhibition of a sodium spike without an apparent concomitant inhibition of a calcium spike, suggests that current thought needs further investigation at the primary sensory neuron level.

5.6. Tables and figures

Table 5.1. Effects of pregabalin on electrophysiological parameters of DRG sensory neurons in control and neuropathic rats.

NeP Neuron									
	Aβ-LTM CUT (n=6)			Aβ-LTM MS (n=6)			HTM (n=7)		
	BL	pregabalin		BL	pregabalin		BL	pregabalin	
CV (m/s)	15.48 ± 1.795	14.58 ± 1.842	p=0.734	18.03 ± 1.112	17.36 ± 1.316	p=0.691	N	N	
Vm (mV)	-65.49±2.438	-63.82 ± 4.128	p=0.836	-61.09 ± 3.029	-63.02 ± 2.685	p=0.643	-59.39 ± 3.329	-64.03 ± 3.571	p=0.371
APA (mV)	64.46 ± 4.861	58.00 ± 5.699	p=0.365	61.34 ± 8.702	33.78 ± 6.071	p=0.026*	N	N	
APDb (ms)	1.98 ± 0.376	2.41 ± 0.576	p=0.541	1.35 ± 0.21	2.45± 0.505	p=0.041*	N	N	
APRT (ms)	0.77 ± 0.162	1.09 ± 0.299	p=0.425	0.57 ± 0.121	1.38 ± 0.314	p=0.015*	N	N	
APFT ms)	1.21 ± 0.234	1.31 ± 0.278	p=0.654	0.77 ± 0.095	1.06 ± 0.191	p=0.213	N	N	
AHPA (mV)	6.66 ± 1.159	2.94 ± 1.002	p=0.042*	6.08 ± 1.620	1.41 ± 1.284	p=0.049*	N	N	
AHP50% (ms)	3.81 ± 0.853	2.19 ± 0.817	p=0.220	2.59 ± 0.949	0.50 ± 0.288	p=0.069	N	N	
soma current threshold (nA)	1.20 ± 0.339	1.21 ± 0.334	p=0.981	0.44 ± 0.229	1.48 ± 0.453	p=0.074	N	N	
receptive field threshold (g)	0.69± 0.370	0.69± 0.370	p=1	4.42 ± 2.723	4.42 ± 2.723	p=1	N	N	

Control Neuron									
	Aβ-LTM CUT (n=5)			Aβ-LTM MS (n=7)			C-HTM (n=4)		
	BL	pregabalin		BL	pregabalin		BL	pregabalin	
CV (m/s)	19.11 ± 4.836	18.50 ± 3.931	p=0.925	19.10 ± 0.874	17.53 ± 0.825	p=0.229	N	N	
Vm (mV)	-72.11 ± 4.842	-72.02 ± 3.103	p=0.981	-74.76 ± 4.844	-75.72 ± 7.723	p=0.919	-63.82 ± 0.1500	-64.44 ± 3.585	p=0.880
APA (mV)	61.91 ± 9.763	60.47 ± 9.360	p=0.922	57.59 ± 2.806	36.43 ± 8.180	p=0.012*	N	N	
APDb (ms)	1.51 ± 0.277	1.46 ± 0.309	p=0.909	1.11 ± 0.089	1.52 ± 0.131	p=0.033*	N	N	
APRT (ms)	0.60 ± 0.143	0.69 ± 0.173	p=0.715	0.45± 0.049	0.69 ± 0.079	p=0.028*	N	N	
APFT ms)	0.91± 0.15	0.77 ± 0.16	p=0.673	0.67 ± 0.051	0.84 ± 0.163	p=0.335	N	N	
AHPA (mV)	8.12 ± 1.959	7.37 ± 1.812	p=0.792	7.22 ± 2.598	1.32 ± 0.855	p=0.047*	N	N	
AHP50% (ms)	4.86 ± 2.575	4.91 ± 2.765	p=0.973	2.96 ± 0.830	0.69± 0.431	p=0.051	N	N	
soma current threshold (nA)	1.50 ± 0.289	1.53± 0.260	p=0.942	1.21 ± 0.247	2.72 ± 0.183	p<0.001***	N	N	
receptive field threshold (g)	1.13 ± 0.466	1.13 ± 0.466	p=1	8.25 ± 2.780	8.25 ± 2.780	p=1	N	N	

Asterisks indicate the significant difference for comparison before and 15 min after pregabalin administration, both in control and neuropathic rats for various subtypes and for various parameters. *p<0.05, **P<0.01; ***p<0.001 as determined by Mann Whitney t test. In these HTM neurons, since there was no response later after pregabalin administration in some neurons, the parameters of AP and AHP were not

compared in this table. CV, conduction velocity; Vm, resting membrane potential; APA, action potential amplitude; APdB, action potential duration at base; APRT, action potential rise time; APFT, action potential fall time; AHPA, afterhyperpolarization amplitude; AHP50, afterhyperpolarization duration to 50%. Current threshold is the minimum current by intracellular injection of depolarizing current that elicited an AP. Mechanical threshold is the minimum force in grams that elicited an AP by a von Frey filament applied to the peripheral receptive field.

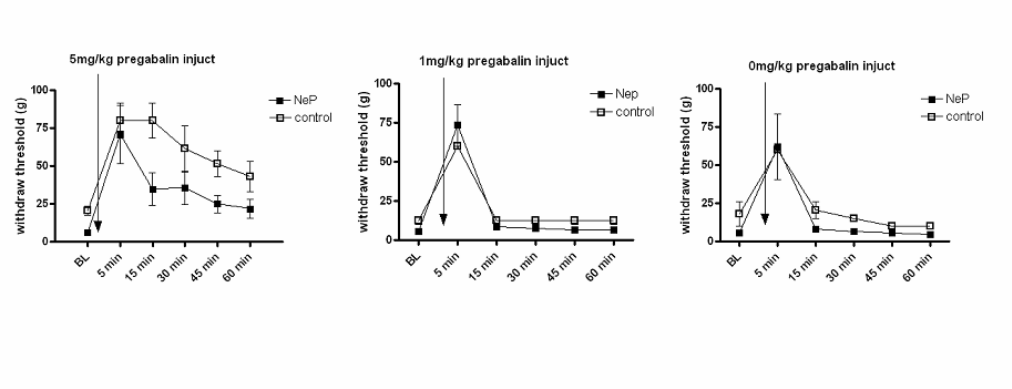

Figure 5.1. Mechanical withdrawal threshold in the von Frey test after i.v. administration of 5, 1 and 0 mg/kg of pregabalin, at various times following administration. Data are derived from five control and five neuropathic model rats. Each point represents the Mean ± SEM. BL: baseline withdrawal threshold before pregabalin.

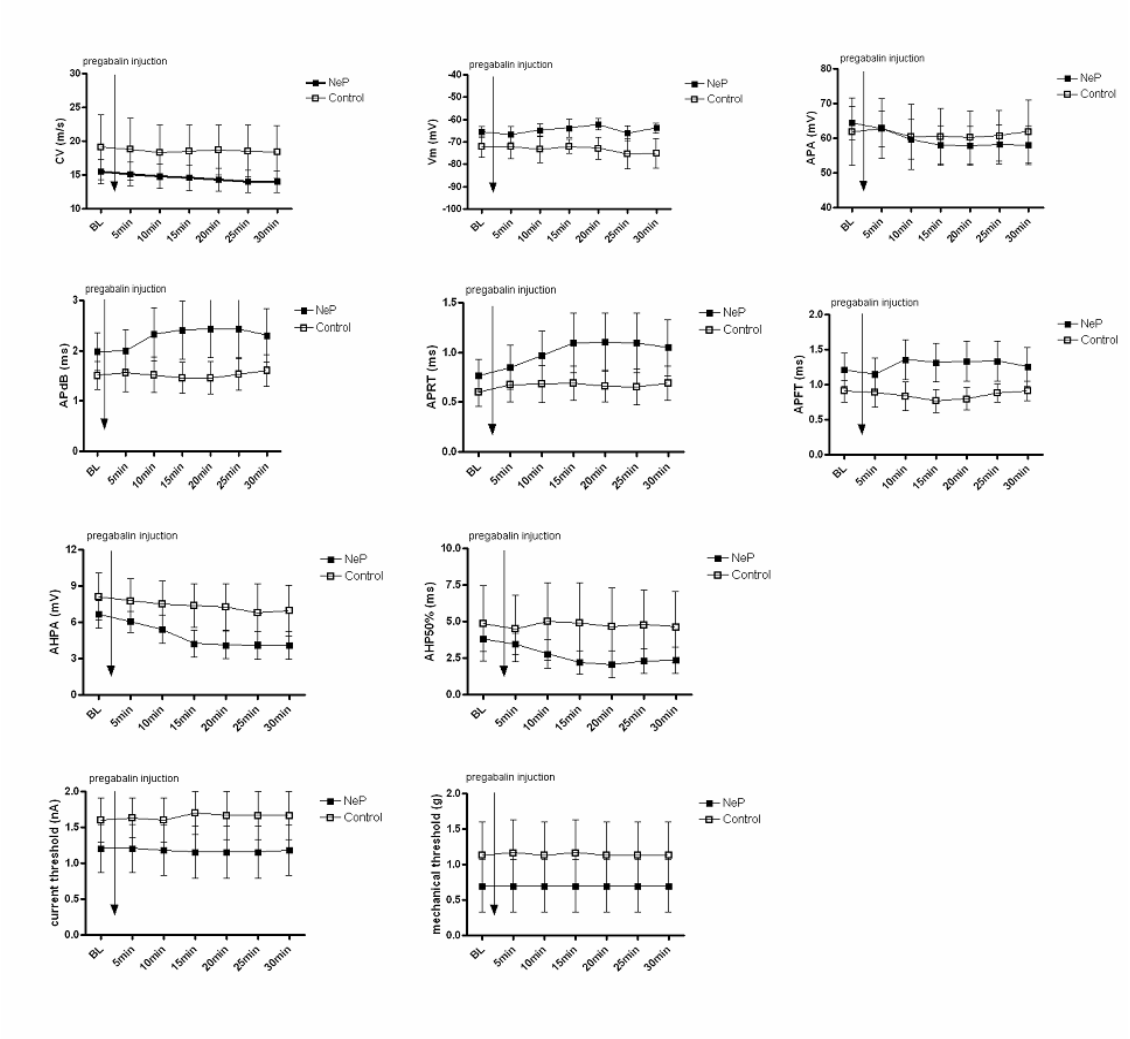

Figure 5.2. Effects of i.v. administration of pregabalin on electrophysiological properties of Aβ-fiber LTM cutaneous neurons. Aβ-fiber LTM CUT neurons were including guard/field hair neurons (GF), rapidly adapting (RA) neurons and slowly adapting (SA) neurons. Recordings were made intracellularly from L4 DRG neurons. The figure showed the changes in the various parameters monitored at different times after pregabalin administration; up to 30 min. Details and abbreviations are as in the legend to table 5.1.

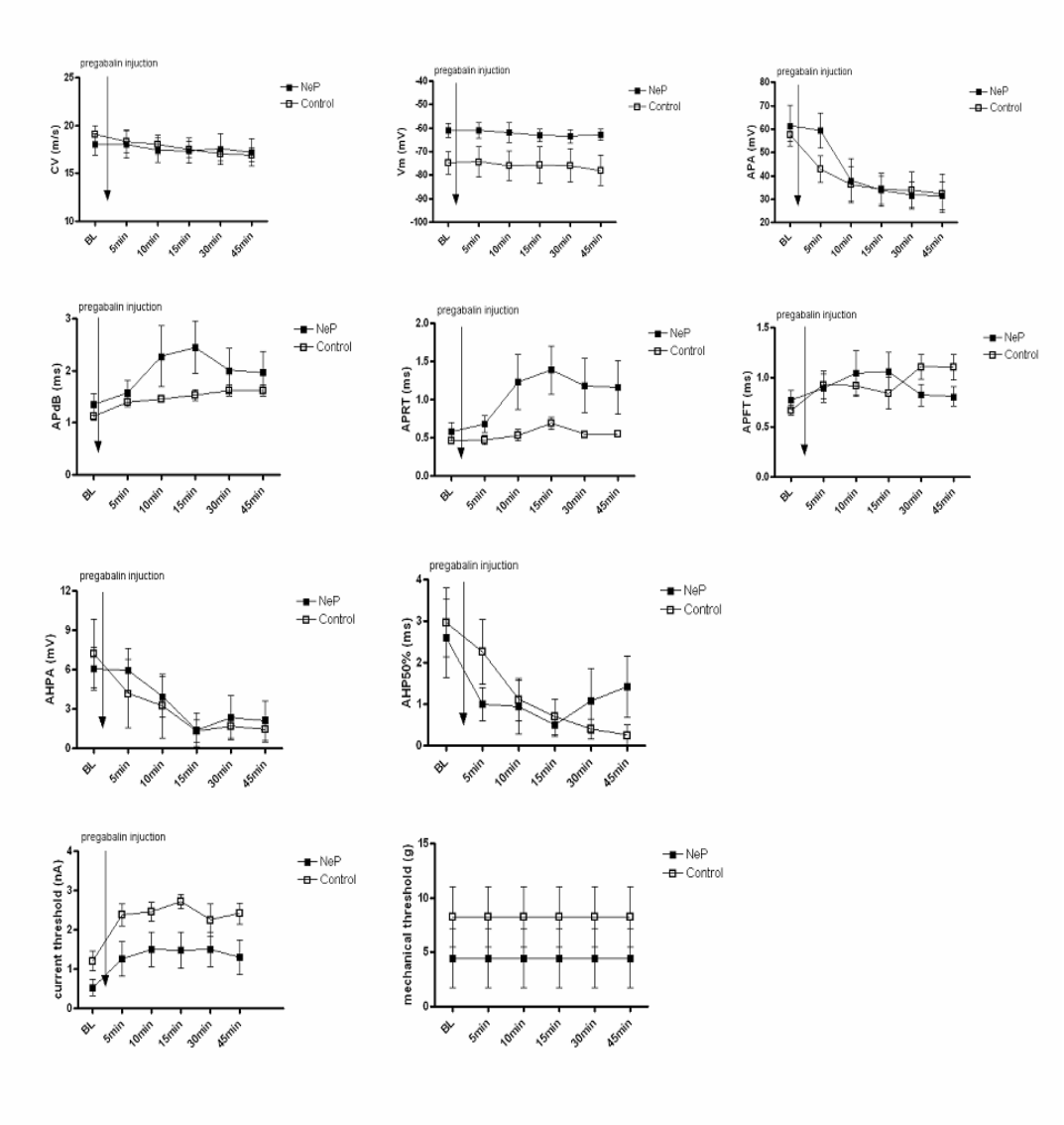

Figure 5.3. Effects of i.v. administration of pregabalin on electrophysiological properties of Aβ-fiber muscle spindle neurons. Details and abbreviations are as in the legend to table 5.2.

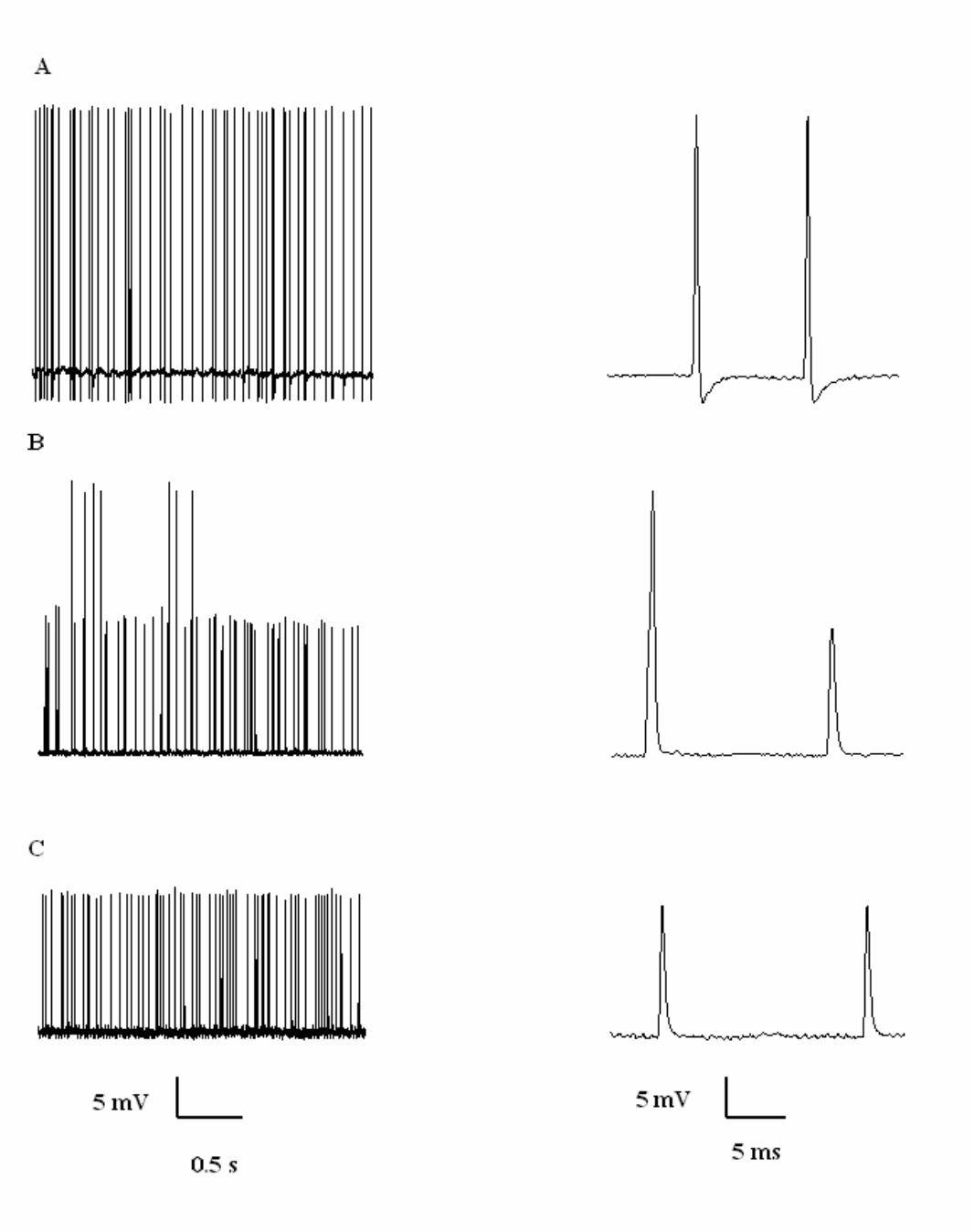

Figure 5.4. Example to show pregabalin effect ongoing discharge of Aβ-fiber muscle spindle neurons. Amplitude of AP, AHP discharge changed over time in this neuron. A, before pregabalin administration; B, 5 min after pregabalin administration; C, 15 min after pregabalin administration**.**

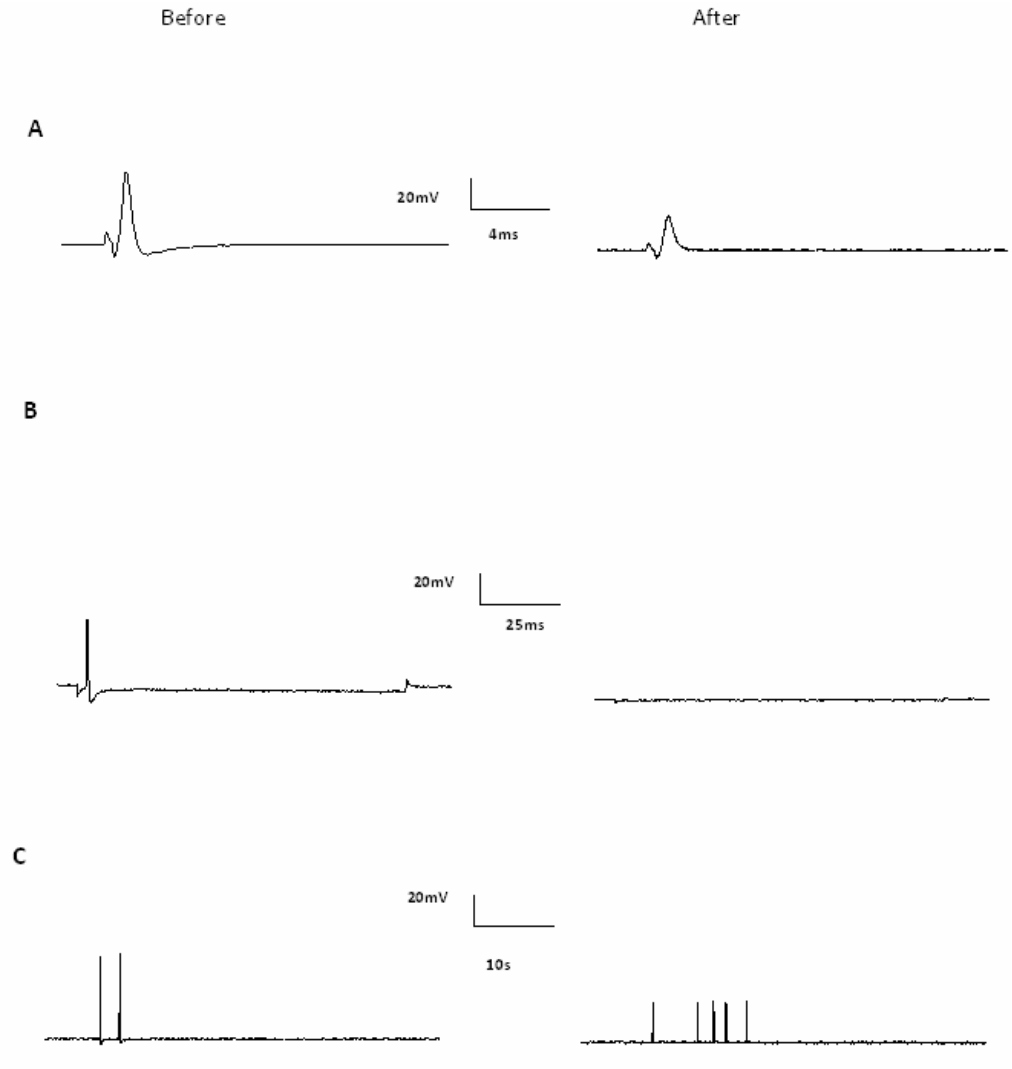

Figure 5.5. Examples of intracellular recordings illustrating the somatic APs by different peripheral site stimulations from Aβ-fiber muscle spindle neurons before and 15 min after pregabalin administration in a neuropathic rat. A, APs evoked by dorsal root stimulation (0.8mA, 0.3ms); B, APs evoked by intracellular depolarizing current injection (1nA, 100ms); C, APs evoked by von Frey stimulation of the receptive field.

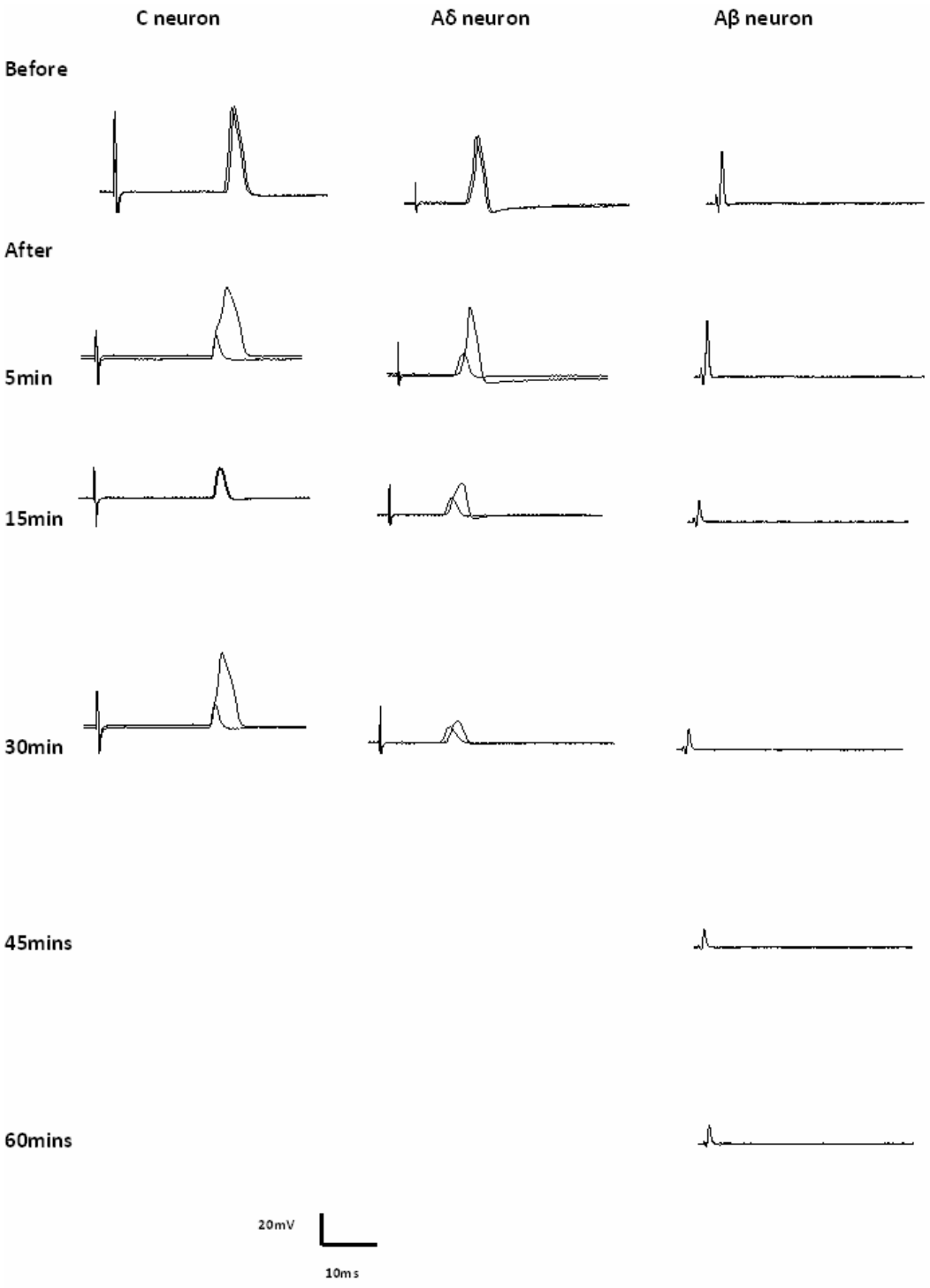
C neuron
Aδ neuron
Aβ neuron
Before
After
5min
15min
30min
45mins
60mins
20mV
10ms

Figure 5.6. Examples of intracellular recordings illustrating effects of pregabalin administration on different types of HTM neurons. left: C-fiber HTM neuron in NeP model (CV=0.59 m/s); middle: Aδ-fiber HTM neuron in NeP model (CV= 1.51m/s); right: Aβ-fiber HTM neuron in control model (CV=20.71m/s). Figure showed before pregabalin administration and after 5mins, 15mins, 30mins, 45mins and 60mins pregabalin administration, individually.

Reference List

Asfaha S, Cenac N, Houle S, Altier C, Papez MD, Nguyen C, Steinhoff M, Chapman K, Zamponi GW, Vergnolle N, 2007. Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. Br. J. Pharmacol. 150: 176-185.

Barbara G, Wang B, Stanghellini V, de GR, Cremon C, Di NG, Trevisani M, Campi B, Geppetti P, Tonini M, Bunnett NW, Grundy D, Corinaldesi R, 2007. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 132: 26-37.

Campbell JN, Raja SN, Meyer RA, Mackinnon SE, 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32: 89-94.

De Laet A, Cornelissen W, Adriaensen D, Van Bogaert PP, Scheuermann DW, Timmermans JP, 2002. Ca2+ involvement in the action potential generation of myenteric neurones in the rat oesophagus. Neurogastroenterol. Motil. 14: 161-172.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Djouhri L, Bleazard L, Lawson SN, 1998. Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J. Physiol 513 (Pt 3): 857-872.

Doerner JF, Gisselmann G, Hatt H, Wetzel CH, 2007. Transient receptor potential channel A1 is directly gated by calcium ions. J. Biol. Chem. 282: 13180-13189.

Dooley DJ, Taylor CP, Donevan S, Feltner D, 2007. Ca2+ channel alpha2delta ligands: novel modulators of neurotransmission. Trends Pharmacol. Sci. 28: 75-82.

Dworkin RH, Corbin AE, Young JP, Jr., Sharma U, LaMoreaux L, Bockbrader H, Garofalo EA, Poole RM, 2003. Pregabalin for the treatment of postherpetic neuralgia: a randomized, placebo-controlled trial. Neurology 60: 1274-1283.

Fang X, McMullan S, Lawson SN, Djouhri L, 2005. Electrophysiological differences between nociceptive and non-nociceptive dorsal root ganglion neurones in the rat in vivo. J. Physiol 565: 927-943.

Farrar JT, Young JP, Jr., LaMoreaux L, Werth JL, Poole RM, 2001. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. Pain 94: 149-158.

Fehrenbacher JC, Taylor CP, Vasko MR, 2003. Pregabalin and gabapentin reduce release of substance P and CGRP from rat spinal tissues only after inflammation or activation of protein kinase C. Pain 105: 133-141.

Field MJ, Bramwell S, Hughes J, Singh L, 1999. Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones? Pain 83: 303-311.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D, 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc. Natl. Acad. Sci. U. S. A 103: 17537-17542.

Field MJ, Oles RJ, Singh L, 2001. Pregabalin may represent a novel class of anxiolytic agents with a broad spectrum of activity. Br. J. Pharmacol. 132: 1-4.

Fink K, Dooley DJ, Meder WP, Suman-Chauhan N, Duffy S, Clusmann H, Gothert M, 2002. Inhibition of neuronal Ca(2+) influx by gabapentin and pregabalin in the human neocortex. Neuropharmacology 42: 229-236.

Han DW, Kweon TD, Lee JS, Lee YW, 2007. Antiallodynic effect of pregabalin in rat models of sympathetically maintained and sympathetic independent neuropathic pain. Yonsei Med. J. 48: 41-47.

Kanai A, Sarantopoulos C, McCallum JB, Hogan Q, 2004. Painful neuropathy alters the effect of gabapentin on sensory neuron excitability in rats. Acta Anaesthesiol. Scand. 48: 507-512.

Katz EJ, Gold MS, 2006. Inflammatory hyperalgesia: a role for the C-fiber sensory neuron cell body? J. Pain 7: 170-178.

Kim YI, Na HS, Kim SH, Han HC, Yoon YW, Sung B, Nam HJ, Shin SL, Hong SK, 1998. Cell type-specific changes of the membrane properties of peripherally-axotomized dorsal root ganglion neurons in a rat model of neuropathic pain. Neuroscience 86: 301-309.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Leem JW, Willis WD, Chung JM, 1993. Cutaneous sensory receptors in the rat foot. J. Neurophysiol. 69: 1684-1699.

Linsdell P, Moody WJ, 1995. Electrical activity and calcium influx regulate ion channel development in embryonic Xenopus skeletal muscle. J. Neurosci. 15: 4507-4514.

Liu B, Eisenach JC, 2005. Hyperexcitability of axotomized and neighboring unaxotomized sensory neurons is reduced days after perineural clonidine at the site of injury. J. Neurophysiol. 94: 3159-3167.

Liu X, Zhou JL, Chung K, Chung JM, 2001. Ion channels associated with the ectopic discharges generated after segmental spinal nerve injury in the rat. Brain Res. 900: 119-127.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Newton RA, Bingham S, Case PC, Sanger GJ, Lawson SN, 2001. Dorsal root ganglion neurons show increased expression of the calcium channel alpha2delta-1 subunit following partial sciatic nerve injury. Brain Res. Mol. Brain Res. 95: 1-8.

Pitcher GM, Henry JL, 2002. Second phase of formalin-induced excitation of spinal dorsal horn neurons in spinalized rats is reversed by sciatic nerve block. Eur. J. Neurosci. 15: 1509-1515.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Pitcher GM, Ritchie J, Henry JL, 1999. Nerve constriction in the rat: model of neuropathic, surgical and central pain. Pain 83: 37-46.

Schaible HG, 2007. Peripheral and central mechanisms of pain generation. Handb. Exp. Pharmacol.: 3-28.

Taylor CP, Angelotti T, Fauman E, 2007. Pharmacology and mechanism of action of pregabalin: the calcium channel alpha2-delta (alpha2-delta) subunit as a target for antiepileptic drug discovery. Epilepsy Res. 73: 137-150.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA, 1999. Sodium channels and pain. Proc. Natl. Acad. Sci. U. S. A 96: 7635-7639.

Wu Q, Henry JL, 2010. Changes in Abeta non-nociceptive primary sensory neurons in a rat model of osteoarthritis pain. Mol. Pain 6: 37.

Wu Q, Henry JL, 2009. Delayed onset of changes in soma action potential genesis in nociceptive A-beta DRG neurons in vivo in a rat model of osteoarthritis. Mol. Pain 5: 57.

Yang RH, Xing JL, Duan JH, Hu SJ, 2005. Effects of gabapentin on spontaneous discharges and subthreshold membrane potential oscillation of type A neurons in injured DRG. Pain 116: 187-193.

Zamponi GW, French RJ, 1995. Sodium current inhibition by internal calcium: a combination of open-channel block and surface charge screening? J. Membr. Biol. 147: 1-6.

CHAPTER 6

Pregabalin: early daily treatment induces persisting antinociception in a rat model of peripheral neuropathic pain

Authors: Yong Fang Zhu, James L Henry

Corresponding author: James L Henry, PhD
Department of Psychiatry and Behavioural Neurosciences,
McMaster University,
1200 Main St. West, HSC 4N35
Hamilton, Ontario, Canada, L8N 3Z5,
Tel: +1-905-525-9140, extension 24750,
FAX: 905-4522-8844
Email address: jhenry@mcmaster.ca

Preface

Significance to thesis

This study demonstrated pain relief efficacy of pregabalin in the management of early peripheral neuropathy. This study demonstrated that intraperitoneally *(*i.p.*)* administered pregabalin resulted in a rapid-onset and relatively persisted effects improvement in tactile allodynia. This study also demonstrated that the rats with early treatment pregabalin was more favorable compared with rats with late treatment.

Authors' contribution

Yong Fang Zhu did i.p. injections, the behavioral test, and model induction. She also analyzed the data, performed statistical analyses, wrote the initial draft of the manuscript, and worked on refining this draft and the revision based on editorial review.

James L. Henry conceived of, designed, and coordinated the study. He also worked on refining this draft and the revision based on editorial review.

Abstract

Peripheral neuropathic pain is known to develop within days following surgical induction in animal models, but remains poorly understood and treated in humans, where diagnosis and treatment tend to be delayed after nerve injury. The aim of this study was to evaluate the antinociceptive effectiveness of administration of pregabalin during the onset of an animal model of peripheral neuropathic pain. The Mosconi and Kruger (1996) model of peripheral neuropathy was induced in three groups of rats. One group was otherwise untreated. A second group was given alternating weeks of daily administration of saline or pregabalin over a four week period. The third group was given daily administration of pregabalin during the first week, then saline during the second and third weeks, followed by pregabalin again during the fourth week. At the end of each week mechanical withdrawal threshold was determined using the von Frey test. Daily administration of pregabalin resulted in a markedly reduced mechanical withdrawal threshold in groups two and three, and this threshold remained reduced for the duration of the study. The third group exhibited a much-reduced decrease in mechanical threshold at the end of the first week and this remained reduced through the remainder of the study. The results demonstrate that early, daily treatment with pregabalin prevents full development of mechanical hypersensitivity in this animal model of peripheral neuropathic pain and it is suggested that pregabalin may be an important early treatment to prevent the development of neuropathic pain following neurotrauma.

6.1. Introduction

Peripheral neuropathy is a frequent and common result of a wide range of conditions including diabetes, nerve root compression, herpes zoster infection, cancer

chemotherapy and stroke, thus affecting millions of people around the world. In contrast to nociceptive pain, neuropathic pain is described as a spontaneous burning pain and an exaggerated response to noxious stimuli (hyperalgesia) that may be provoked by innocuous stimulation (allodynia) (Devor, 1983; Richards, 1967). It is recognized that the time of onset of mechanical hypersensitivity in animal models of peripheral neuropathy, homologous to the allodynia reported in humans with peripheral neuropathy, can be evoked early within days following surgical model induction (Pitcher et al., 1999). Therefore, early recognition of peripheral neuropathy and early treatment may be important components of care after nerve injury.

Many patients with neuropathic pain respond poorly to conventional analgesics. However, it has been reported that the novel compound pregabalin (Lyrica), which is structurally related to the antiepileptic drug gabapentin (Neurontin) (Taylor et al., 2007; Field et al., 2006), has a broad analgesic effect on mechanical and thermal hypersensitivity associated with nerve injury (Field et al., 2006; Field et al., 2001; Field et al., 1999). These effects are thought to be mediated by its binding to the α2δ subunit of voltage-gated calcium channels (VGCCs), which are thought to decrease pain by modulating the influx of calcium ions into hyperexcited neurons with a subsequent reduction in the central release of excitatory neurotransmitters such as glutamate and substance P (SP).

Based on the demonstrated effects of pregabalin in animal models of peripheral neuropathy (Park er al., 2010; Kumar et al., 2010), we sought to determine whether pregabalin is effective in the treatment of early peripheral neuropathic pain and whether effects may persist long term after the end of pregabalin administration. The aim of this study was to evaluate the possible long-lasting antinociceptive

effectiveness of pregabalin in the early management of sensory symptoms of peripheral neuropathy.

6.2. Methods

Animals

Young male Sprague-Dawley rats (obtained from Charles River Inc., St. Constant, QC, Canada) weighing 170-200 g were used. All procedures were in compliance with The Care and Use of Experimental Animals, Vols. 1 and 2, of the Canadian Council on Animal Care. All experimental protocols were approved in advance by the McMaster University Animal Review Ethics Board.

Model induction

A peripheral neuropathy was induced according to the method previously described in detail (Pitcher and Henry, 2002; Pitcher et al., 1999; Mosconi and Kruger, 1996; Chapter 2). Under anesthesia with a mixture of ketamine (Ketamine; 5 mg/100g; Bimeda-MTC Animal Health Inc.; Cambridge, ON, Canada), xylazine (Rompun; 0.5 mg/100g; Bayer HealthCare, Toronto, ON, Canada) and acepromazine (Atravet; 0.1 mg/100g; Ayerst Veterinary Laboratories, Guelph, ON, Canada) given i.p., the right sciatic nerve was exposed via a lateral approach at the mid-thigh level. Two cuffs of 0.5mm polyethylene (PE 90) tubing (Fisher Scientific Ltd., Whitby, Ontario, Canada) were inserted around the exposed nerve about 1 mm apart. The exposure was then sutured in two layers and antibiotic ointment (Furacin; nitrofurazone 0.2%; Vetoquinol N.-A. Inc.; Lavaltrie, QC, Canada) was applied over the suture; then 0.01 ml/100g of antibabacterial injectable solution (Bayer HealthCare, Toronto, ON, Canada) was given subcutaneously (s.c.). Animals were given ocular lubricant and 1 ml saline s.c. and were placed under a heating lamp for recovery from

anesthetic. They were then returned to their home cages.

Measurement of mechanical withdrawal threshold

The onset of tactile hypersensitivity in this model usually occurs early 1-7 days after induction of the model (Pitcher and Henry, 1999). To determine mechanical sensitivity of the foot, brisk withdrawal in response to normally innocuous mechanical stimuli was measured as described previously (Kim et al., 1998). At the end of each seven-day period, the rats were placed in a transparent Plexiglas box with a clear Plexiglas floor, containing 0.5 cm diameter holes spaced 1.5 cm apart to allow full access to the paws (Pitcher and Henry, 2002; Pitcher and Henry, 2004; Pitcher et al., 1999). . Rats were allowed to habituate until cage exploration and grooming activities had ceased. von Frey filaments (Stoelting Co., Wood Dale, IL, USA) were applied to the plantar surface of the hind paw ipsilateral to the cuffs to determine the withdrawal threshold. A von Frey filament was applied 5 times for 3-4 sec, each at 3 sec intervals, to a different spot in ascending order until a clear withdrawal response was observed; when this occurred, the next lightest filament was applied following the same procedure. A 50% withdrawal response threshold was derived according to responses to this testing regimen (Chaplan et al., 1994) using the up-down method of Dixon (Dixon, 1980). Brisk foot withdrawal in response to these innocuous mechanical stimuli was interpreted as indicating tactile withdrawal threshold.

Pregabalin treatment

Pregabalin was dissolved in 0.9% normal saline and administered by intraperitoneal (i.p.) injection. The dose was 30 mg/kg per day. Rats were separated into three Groups, each of which was comprised of 6 rats. After model induction, Group 1 remained otherwise untreated and acted as an intervention control group.

Group 2 was treated with saline daily throughout the first week, followed by daily administration of pregabalin throughout second week. Saline was then given daily throughout the third week and finally pregabalin again daily throughout the fourth week. Group 3 was treated with pregabalin daily throughout the first week, followed by daily saline injection throughout the second and third weeks; this was followed by pregabalin administration daily throughout the fourth week. Rats were assessed for tactile sensitivity in the von Frey test each seven days, before and 1 hour after pregabalin or saline administration.

Statistical analysis

Statistical significance between three groups in every week measurement was determined by one-way ANOVA, and analysis for specific comparisons was performed by a t-test when an effect was identified. All statistical tests and graphing were done using Prism4 software (Graphpad, La Jolla, CA, USA). $P<0.05$ was considered to indicate a significant difference.

6.3. Results

Figure 6.1 illustrates the changes of mechanical withdrawal thresholds of the three groups of rats in the von Frey test on day 7 of each respective week. Figure 6.1A showed measurement data before administration while Figure 6.1B showed measurement data after 1 hour administration on each respective week.

Weekly comparison of effect with saline and pregabalin administration among three groups

Table 6.1 showed the comparison of mean tactile threshold and P value of each group in every week.

Before surgery, rats in Group 1, Group 2 and Group 3 exhibited characteristic

normal behaviors. Mechanical withdrawal thresholds were 14.50 ± 0.500 g (Group 1), 14.57 ± 0.429 g (Group 2) and 14.14 ± 0.553 g (Group 3), which showed no significant differences between three groups: $P = 0.9150$ between Group 1 and Group 2; $P = 0.6462$ between Group 1 and Group 3; $P = 0.5517$ between Group 2 and Group 3).

In the end of the first week measurement, 7 days after model induction, rats in Group 1 exhibited characteristic neuropathic pain behaviors, measured as decreased mechanical withdrawal threshold. The calculated mean tactile threshold value was 4.19 ± 0.538 (comparing before surgery 14.50 ± 0.500 g, $P < 0.0001$). Rats in Group 2 also exhibited characteristic neuropathic pain behaviors 7 days after receiving daily saline injection, the calculated mean tactile threshold values was 3.71 ± 0.747 g before saline injection and 3.63 ± 0.654 g after 1 hour saline injection at seventh day (comparing before surgery: 14.57 ± 0.429 g; both $P < 0.0001$). Rats in Group 3, the group that received pregabalin daily through the first week**,** exhibited significantly attenuated tactile allodynia, the mean tactile threshold value at the end of this first week was 9.81 ± 0.444 g and 12.76 ± 0.843 g before and after 1 hour injection. For comparison three groups in this first weekend measurement, there is no significant difference between Group 1 and Group 2 ($P = 0.6224$ before saline injection; $p = 0.5341$ after saline injection, respectively); However, Group 3 were significantly different from the control Group 2 and Group 1 ($P < 0.0001$ in all each other comparison).

In the end of second week measurement, with no treatment, Group 1 rats exhibited no significant difference compared to value after the first week. The calculated mean tactile threshold values was 4.49 ± 0.549 g (comparing before

surgery 14.50 ± 0.500 g, P = 0.7015). Group 2 rats, which were administered pregabalin for this week, were 5.67 ± 0.979 g in tactile mean threshold before seventh day pregabalin injection, and were 7.46 ± 0.823 g after pregablin injection. Group 3 rats received daily injection of saline in this week. The mean threshold values decreased to 7.91 ± 1.164 g and 8.10 ± 1.120 g before and after seventh day saline injection measurement. For comparison three groups in the end of second week measurement, the values of Group 2 showed no significant difference from Group 1 before pregabalin injection (P = 0.3407) and significant difference after pregabalin injection (P = 0.0148), which thus showed ineffective when daily administration is initiated after the end of the first week . The values of Group 3 were significantly different from the respective values in the untreated Group 1, which thus showed a persisting effect of the first week of pregabalin administration (P = 0.0293; P = 0.0194; respectively). There is no difference between Group 2 and Group 3 in this week measurement.

In the end of third week measurement, with no treatment, Group 1 still showed no significant difference compared to value after the first week (before and after were both 4.15 ± 0.544 g; P = 0.9559). Group 2, after daily saline injection through the third week, the mean threshold before and after seventh day injection was 4.80 ± 0.999 g and 5.02 ± 1.011 g, Group 3, which received daily injection of saline through the third week, the threshold values decreased to 6.92 ± 1.422 g and 6.80 ± 1.466 g before and after seventh day injection measurement. For comparison three group in third weekend measurement, there were no significant differences between each other.

In the end of forth week measurement, Group 1 still showed no significant difference compared to value after the first week (4.12 ± 0.368 g; P = 0.9185) with no

treatment. Mean tactile threshold of Group 2, after daily pregabalin administration through the fourth week, was 5.36 ± 0.722 g before seventh day pregabalin injection and was 6.78 ± 0.837 g after 1 hour pregablin injection measurement. Mean threshold value of Group 3, after pregabalin injection in the fourth week, was increased to 7.98 ± 1.571 g before seventh day injection measurement and 10.01 ± 1.601 g after seventh day injection measurement. Comparison three groups in the fourth weekend measurement, there is no significant difference between Group 1 and Group 2 before injection measurement (P = 0.1747), but there is significant difference after injection (P = 0.0193). There are significant difference between Group 1 and Group 3 (before injection measurement, P = 0.0487, after injection measurement, P = 0.0068). Between Group 2 and Group 3, There is no significance before injection measurement, (P = 0.1559), and after injection measurement (P = 0.0987).

Some specific comparison of pregabalin administration between Group 2 and Group 3

In table 6.2, it is showed the comparison of antinociceptive effects of i.p. administered pregabalin with early treatment vs. late treatment; with single phase vs. multiple phase treatment; and also with acute vs. long-term treatment.

The values of Group 2 in end of second week showed significant difference from the values of Group 3 in end of first week, before and after pregabalin injection measurement (P = 0.0023, P = 0.0007, respectively) .The values of Group 2 in the fourth week showed no significant difference from the values of Group 3 in the fourth week, before and after pregabalin injection measurement (P = 0.1891; P = 0.0987., respectively).

The effect of first doses of pregabline administration showed no significant difference with second doses administration, both in group 2 (before injection: P = 0.8059, after injection: P = 0.5723) and in Group 3 (before injection: P = 0.2636, after injection: P =

0.1554).

The acute effects were determined by comparing before and after pregabalin administration on same day. Group 2 showed no difference in both second week and forth week administration ($P = 0.1864$ and $P = 0.2241$, respectively). Group 3 showed significant difference on the first week administration ($P = 0.0093$), but not on the forth week administration ($P = 0.3586$).

Discussion

The present study aimed at determining the effect of pregabalin administration, in a nerve injury animal model of peripheral neuropathic pain, when it was administered daily during the onset period of model development after nerve injury compared to when it was administered daily beginning the second week after nerve injury. The data show that when administered daily during the first week pregabalin is effective in reducing the tactile hypersensitivity that characterizes this model but is ineffective when daily administration is initiated after the end of the first week. This suggests that pregabalin is acting on mechanisms involved in the developing model but are not involved once the model has become established. The implications are that pregabalin may be a particularly effective treatment in preventing development of pain when administered immediately following nerve trauma.

Importantly, the data also show that if pregabalin is administered daily throughout the first week after nerve injury, even when treatment ends there is a persisting beneficial effect in reducing the tactile hypersensitivity. This supports the notion that pregabalin is acting on mechanisms involved in establishing tactile hypersensitivity but also suggests that if these initial mechanisms can be suppressed there is a persisting beneficial treatment effect. The implication for human

neuropathic pain is that pregabalin may be a particularly effective treatment in preventing the full expression of neuropathic pain following nerve trauma.

The mechanism of mechanical hypersensitivity is complex and is not completely understood. Evidence exists that pain following peripheral nerve injury is maintained by histological and functional changes in Aβ primary sensory neurons. Further, ectopic activity in sensory neurons is thought to arise from the DRG, along the axons or in the peripheral nerve terminals (Devor, 2006; Liu et al., 2000; Amaya et al., 2006; Yang et al., 2005; Katz and Gold, 2006; Schaible, 2007; Chapter 2-4). A constant barrage of synaptic activity from Aβ primary sensory neurons is thought to induce or maintain central sensitization observed in animal models of neuropathic pain (Lang et al., 2006; Campbell and Meyer, 2006; Campbell et al., 1988). Thus, inhibition of mechanical hypersensitivity could result from either the prevention of central sensitization or the blockade of low-threshold inputs.

Peripheral nerve injury induces up-regulation of expression of the α2δ-1 subunit located presynaptically and postsynaptically in the spinal dorsal horn and in medium and large dorsal root ganglion neurons (Newton et al., 2001). Previous studies have shown that pregabalin has a high affinity to the α2δ-1 subunit of voltage-dependent calcium channels and reduced calcium current (Field et al., 2006; Taylor et al., 2007). Reducing the calcium current at nerve terminals is thought to result in a reduction of the presynaptic release of excitatory neurotransmitters, including glutamate, noradrenaline, SP and CGRP (Fink et al., 2002; Han et al., 2007). Our previous study on the acute effect of pregabalin on peripheral DRG neurons also suggested that the analgesic effect of pregabalin on neuropathic pain may be mediated, at least in part, by is peripheral inhibitory action on sodium current (chapter 5). SP

and perhaps CGRP release from Aβ terminals is an attractive and viable mechanism that could underlie Aβ fiber-induced pain (Devor, 2009). Our recent data suggest that the peripheral mechanism of hypersensitivity might be mediated by a sodium channel as it can be blocked with low concentrations of local sodium channel anesthetic (Pitcher and Henry, 2002). Thus, inhibition of the release of SP and other excitatory amino acids from primary afferents and inhibition of sodium channel activity by pregabalin binding, possibly in association with the calcium channel α2δ-1 subunit, could result in reduced tactile hypersensitivity.

Neurotransmitters released from afferent terminals during ectopic activity may also have postsynaptic effects beyond the moment-to moment modulation of the membrane potential (Devor, 2009). For example, there is evidence that released mediators such as SP, CGRP and neuropeptide Y might trigger longer-term changes in the responsiveness of postsynaptic neurons (Woolf and Salter, 2000; Obata et al., 2004). In this way, Aβ-fiber sensory neurons which begin to synthesize and release some of the very transmitters that might be not only implicated in mediating nociception by moment-to moment peripheral drive of spinal nociceptive neurons, but also of the ability in having trigger and maintain central sensitization over a long-term course (Devor, 2009). Thus, pregabalin binding at calcium channel α2δ-1 subunit could result in reduced tactile hypersensitivity long term.

On the basis of currently available information it is not clear how early treatment with pregabalin has more potent antinociceptive effects compared to late treatment beginning one week after nerve injury. However, the time of onset of ectopic activity in Aβ neurons matches well with the time of onset of tactile hypersensitivity (Devor, 2009; Han et al., 2000; Liu et al., 2000; Schaible, 2007).

Further, our earlier study in the same model showed that early cuff removal leads to recovery (Dableh and Henry, manuscript in press, J Pain Research). Together, these data suggest that early ectopic activity might be important in the development of tactile hypersensitivity, and perhaps allodynia in humans and that early treatment might inhibit mechanisms leading to generation of ectopic activity in peripheral sensory neurons and thus reduce the development of central sensitization.

6.5. Conclusions

Neuropathic pain is notoriously severe and refractory to medical treatment. Our studies show that the administration of pregabalin results in a rapid-onset improvement in reducing the development of tactile hypersensitivity but early treatment is of paramount importance. The outcome of rats with early daily treatment with pregabalin was more favorable compared with rats with the same daily treatment initiated one week after peripheral nerve injury. It is suggested that controlled studies be launched to investigate pregabalin as an approved drug for the immediate treatment of peripheral nerve injury and perhaps of neurotrauma more broadly.

6.6. Tables and figures

Table 6.1. Weekly comparison of mean tactile threshold and P value of each group. A. showed data before administration at seventh day measurement. B. showed data 1 hour after administration at seventh day measurement. The detail asterisks explanations see Figure 6.1.

A

	baseline	1st week	2nd week	3rd week	4th week
Grroup 1 Mean ± SEM (g)	14.50 ± 0.500	4.19 ± 0.538	4.49 ± 0.549	4.15 ± 0.544	4.12 ± 0.368
Grroup 2 Mean ± SEM (g)	14.57 ± 0.429	3.71 ± 0.747	5.67 ± 0.979	4.80 ± 0.999	5.36 ± 0.722
Grroup 3 Mean ± SEM (g)	14.14 ± 0.553	9.81 ± 0.444	7.91 ± 1.164	6.92 ± 1.422	7.98 ± 1.571
Group 1 vs. Group 2 P value	0.9150	0.6224	0.3407	0.5943	0.1747
Group 1 vs. Group 3 P value	0.6462	p<0.0001¤¤¤	0.0293¤	0.1158	0.0487¤
Group 2 vs. Group 3 P value	0.5517	p<0.0001***	0.1669	0.2464	0.1559

B

	baseline	1st week	2nd week	3rd week	4th week
Grroup 1 Mean ± SEM (g)	14.50 ± 0.500	4.19 ± 0.538	4.49 ± 0.549	4.15 ± 0.544	4.12 ± 0.368
Grroup 2 Mean ± SEM (g)	14.57 ± 0.429	3.63 ± 0.654	7.46 ± 0.823	5.02 ± 1.011	6.78 ± 0.837
Grroup 3 Mean ± SEM (g)	14.14 ± 0.553	12.76 ± 0.843	8.10 ± 1.120	6.80 ± 1.466	10.01 ± 1.601
Group 1vs. Group 2 P value	0.915	0.5341	0.0148Δ	0.4841	0.0193Δ
Group 1 vs. Group 3 P value	0.6462	p<0.0001¤¤¤	0.0194¤	0.1403	0.0068¤¤
Group 2 vs. Group 3 P value	0.5517	p<0.0001***	0.6534	0.3374	0.0987

Table 6.2. Specific comparison of antinociceptive effects of i.p. administered pregabalin with early treatment vs. late treatment; with single phase vs. multiple phase treatment; and also with acute vs. long-term treatment.

before injection	First phase doses	Second phase doses	first doses vs. second doses P values
Group 2 Mean ± SEM	5.67 ± 0.979 g	5.36 ± 0.722 g	0.8059
Group 3 Mean ± SEM	9.81 ± 0.444 g	7.98 ± 1.571 g	0.2636
Group 2 vs. Group 3 before injection P value	0.0023**	0.1891	
after injection			
Group 2 Mean ± SEM	7.46 ± 0.823 g	6.78 ± 0.837 g	0.5723
Group 3 Mean ± SEM	12.76 ± 0.843 g	10.01 ± 1.601 g	0.1554
Group 2 vs. Group 3 after injection P value	0.0007***	0.0987	
before vs. after injection			
Group 2 before vs. after P value	0.1864	0.2241	
Group 3 before vs. after P value	0.0093**	0.3586	

First doses (first one week adminstration), Second doses (second one week adminstration). *p<0.05, **P<0.01, ***p<0.001 showed any significant differences

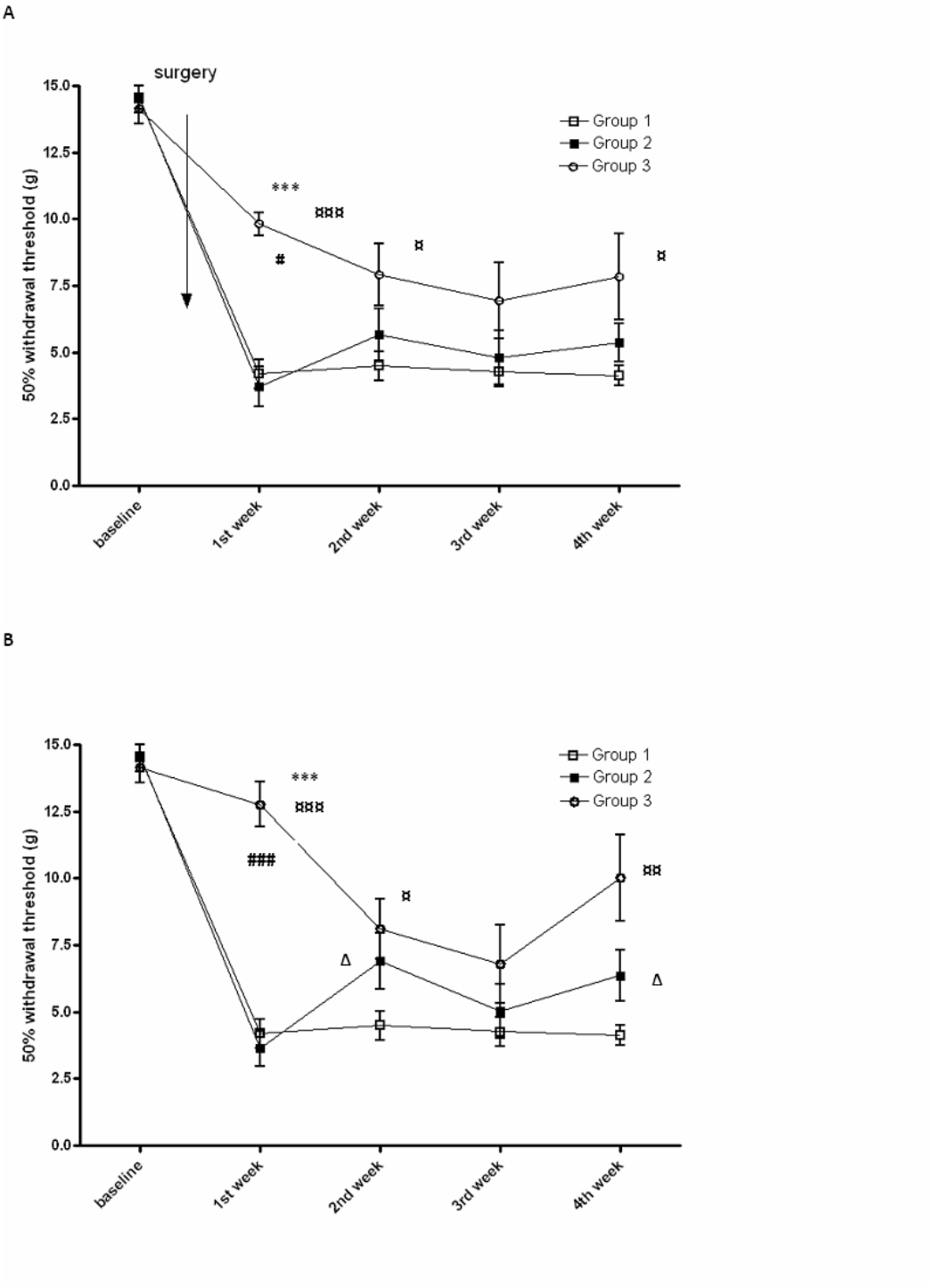

Figure 6.1. Comparison of the antinociceptive effects of IP-administered pregabalin in the Mosconi and Kruger animal model of peripheral neuropathic pain, comparing early treatment vs. late treatment. A showed measurement data

before administration on day 7 of each respective week. B showed measurement data after 1 hour administration on day 7 of each respective week. Each point represents the mean ± SEM mechanical withdrawal threshold for 6 rats in each group. After surgically inducing the peripheral neuropathy, the rats in Group 1 were untreated. Rats in group 2 were treated daily with saline during the first week, then pregabalin during week 2, saline during week 3 and pregabalin during week 4. Rats in Group 3 were treated daily with pregabalin during the first week, saline during weeks 2 and 3, and pregabalin during week 4. *p<0.05, **P<0.01, ***p<0.001 showed any significant differences between Group 3 and Group 2, $^{¤}$p< 0.05, $^{¤¤}$p< 0.01, $^{¤¤¤}$p< 0.001 showed any significant difference between Group 3 and Group 1; $^{\Delta}$p< 0.05 showed any significant difference between Group 2 and Group 1. $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 showed any significant differences between Group 3 and Group 2 after one week pregabalin injection. The absence of asterisks indicates the absence of any significant difference.

Reference List

Amaya F, Wang H, Costigan M, Allchorne AJ, Hatcher JP, Egerton J, Stean T, Morisset V, Grose D, Gunthorpe MJ, Chessell IP, Tate S, Green PJ, Woolf CJ, 2006. The voltage-gated sodium channel Na(v)1.9 is an effector of peripheral inflammatory pain hypersensitivity. J. Neurosci. 26: 12852-12860.

Campbell JN, Meyer RA, 2006. Mechanisms of neuropathic pain. Neuron 52: 77-92.

Campbell JN, Raja SN, Meyer RA, Mackinnon SE, 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32: 89-94.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL, 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53: 55-63.

Devor M, 2006. Sodium channels and mechanisms of neuropathic pain. J. Pain 7: S3-S12.

Devor M, 1983. Nerve pathophysiology and mechanisms of pain in causalgia. J. Auton. Nerv. Syst. 7: 371-384.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Dixon WJ, 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20: 441-462.

Dooley DJ, Donovan CM, Meder WP, Whetzel SZ, 2002. Preferential action of gabapentin and pregabalin at P/Q-type voltage-sensitive calcium channels: inhibition of K+-evoked [3H]-norepinephrine release from rat neocortical slices. Synapse 45: 171-190.

Field MJ, Bramwell S, Hughes J, Singh L, 1999. Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones?. Pain 83: 303-311.

Field MJ, Cox PJ, Stott E, Melrose H, Offord J, Su TZ, Bramwell S, Corradini L, England S, Winks J, Kinloch RA, Hendrich J, Dolphin AC, Webb T, Williams D, 2006. Identification of the alpha2-delta-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. Proc. Natl. Acad. Sci. U. S. A 103: 17537-17542.

Field MJ, Oles RJ, Singh L, 2001. Pregabalin may represent a novel class of anxiolytic agents with a broad spectrum of activity. Br. J. Pharmacol. 132: 1-4.

Fink K, Dooley DJ, Meder WP, Suman-Chauhan N, Duffy S, Clusmann H, Gothert M, 2002. Inhibition of neuronal Ca(2+) influx by gabapentin and pregabalin in the human neocortex. Neuropharmacology 42: 229-236.

Han DW, Kweon TD, Lee JS, Lee YW, 2007. Antiallodynic effect of pregabalin in rat models of sympathetically maintained and sympathetic independent neuropathic pain. Yonsei Med. J. 48: 41-47.

Han HC, Lee DH, Chung JM, 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain 84: 253-261.

Kajander KC, Bennett GJ, 1992. Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neurons. J. Neurophysiol. 68: 734-744.

Katz EJ, Gold MS, 2006. Inflammatory hyperalgesia: a role for the C-fiber sensory neuron cell body? J. Pain 7: 170-178.

Kim YI, Na HS, Kim SH, Han HC, Yoon YW, Sung B, Nam HJ, Shin SL, Hong SK, 1998. Cell type-specific changes of the membrane properties of peripherally-axotomized dorsal root ganglion neurons in a rat model of neuropathic pain. Neuroscience 86: 301-309.

Kumar N, Laferriere A, Yu JS, Leavitt A, Coderre TJ. 2010. Evidence that pregabalin reduces neuropathic pain by inhibiting the spinal release of glutamate. J Neurochem 113(2):552-61.

Lang PM, Schober GM, Rolke R, Wagner S, Hilge R, Offenbacher M, Treede RD, Hoffmann U, Irnich D, 2006. Sensory neuropathy and signs of central sensitization in patients with peripheral arterial disease. Pain 124: 190-200.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000a. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000b. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Mosconi T, Kruger L, 1996. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. Pain 64: 37-57.

Newton RA, Bingham S, Case PC, Sanger GJ, Lawson SN, 2001. Dorsal root ganglion neurons show increased expression of the calcium channel alpha2delta-1 subunit following partial sciatic nerve injury. Brain Res. Mol. Brain Res. 95: 1-8.

Obata K, Yamanaka H, Dai Y, Mizushima T, Fukuoka T, Tokunaga A, Noguchi K, 2004. Differential activation of MAPK in injured and uninjured DRG neurons following chronic constriction injury of the sciatic nerve in rats. Eur. J. Neurosci. 20: 2881-2895.

Park HJ, Joo HS, Chang HW et al. 2010. Attenuation of neuropathy-induced allodynia following intraplantar injection of pregabalin. Can J Anaesth: 57(7):664-71.

Pitcher GM, Henry JL, 2004. 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. 186: 173-197.

Pitcher GM, Henry JL, 2002. Second phase of formalin-induced excitation of spinal dorsal horn neurons in spinalized rats is reversed by sciatic nerve block. Eur. J. Neurosci. 15: 1509-1515.

Pitcher GM, Ritchie J, Henry JL, 1999. Nerve constriction in the rat: model of neuropathic, surgical and central pain. Pain 83: 37-46.

Richards RL, 1967. Causalgia. A centennial review. Arch. Neurol. 16: 339-350.

Schaible HG, 2007. Peripheral and central mechanisms of pain generation. Handb. Exp. Pharmacol.: 3-28.

Taylor CP, Angelotti T, Fauman E, 2007. Pharmacology and mechanism of action of pregabalin: the calcium channel alpha2-delta (alpha2-delta) subunit as a target for antiepileptic drug discovery. Epilepsy Res. 73: 137-150.

Woolf CJ, Salter MW, 2000. Neuronal plasticity: increasing the gain in pain. Science 288: 1765-1769.

Yang RH, Xing JL, Duan JH, Hu SJ, 2005. Effects of gabapentin on spontaneous discharges and subthreshold membrane potential oscillation of type A neurons in injured DRG. Pain 116: 187-193.

CHAPTER 7

General discussion and conclusions

Significance of the study

A common symptom of peripheral neuropathy is pain evoked in response to light touch of the skin, termed "tactile allodynia". While the underlying mechanisms remain to be clarified, at present there are two hypotheses. One is the classic “excitable nociceptor hypothesis”, which involves a reduced response threshold in nociceptive C-fiber sensory neurons. Another hypothesis suggests ectopic impulse activity is generated in low-threshold mechanoreceptor Aβ-fiber sensory neurons and that this activity is abnormally “amplified” in the spinal cord, a process termed "central sensitization" (Devor, 2009; Campbell et al., 1988). The hypothesis implicating C-fiber sensory neurons in causing neuropathic pain is mainly based on the abnormal spontaneous activity observed in these neurons. However, several neuropathic model studies have shown that Aβ-fiber sensory neurons undergo significant changes in their electrophysiological properties as well as other phenotypic changes, such as the expression of substance P (SP) (Han et al., 2000; Liu et al., 2000; Tal et al., 1999; Khan et al., 2002). Further studies have shown that Aβ-fiber neurons, not C-fiber sensory neurons might be the major drivers of stimulus-evoked neuropathic pain and tactile allodynia {Devor, 2009; Devor et al., 2006; Lang et al., 2006; Campbell et al., 1988}.

However, a critically important question is which functional group of dorsal root ganglion (DRG) sensory neurons displays these changes, and particularly whether or not nociceptors undergo the greatest changes. Most previous electrophysiological observations were conducted in axotomized neurons. Identifying the function of axotomized DRG neurons is a difficult task as they are disconnected

from their sensory receptors.

In our present study, we choose a radical departure from the common avenues exploring the mechanism of neuropathic pain by the development of electrophysiological methods of intracellular recording *in vivo*, together with functional classification of each neuron as well as immunohistochemical analysis *in vitro*. The compelling reason is that each neuron studied can be classified functionally based on its response to natural stimulation of the peripheral receptive field as well as other criteria such as axonal conduction velocity, configuration of the action potential, etc. This offers us a unique opportunity to test individual type-specific neuron excitability to correlate with the activation threshold in the receptor terminal, which is used to directly define neuropathic pain in animal models. We have recorded from approximately one hundred DRG neurons; most were fully functionally defined with their receptive field, and then dye-injected and recovered in histological sections of the DRG, and successfully examined for SP presence, as well as any pregabalin effect.

Our perspectives of the study

Although some of the quiescent neurons from our model may have still intact axons with no changes in the characteristics, the systematic check of the functional properties of the different neuronal groups lead to two principal conclusions. First, none of the C-fiber DRG neurons show changes in the properties investigated. The most important evidence is that none of the high threshold C-fiber mechanoreceptors showed any increase in sensitization during von-Frey testing in electrophysiological recording. Second, the most significant changes were in DRG neurons associated with large diameter, myelinated fibers especially Aβ-fiber LTM neurons.

Our results provide specific evidence that Aβ-fiber sensory neurons may provide a major contribution to ectopic activity, suggesting that the changes were deriving from multiple regions of the sensory neuron, including (1) the receptive field, which showed increasing sensitization, (2) the axon of the dorsal root, which showed decreased conduction velocity and decreased excitability, and (3) the DRG somata, which directly reflected changed action potential configuration, enhanced excitability and abnormal SP expression. Thus the present results strongly point to a possible role of Aβ-fiber LTMs for the behavioral tactile hypersensitivity in this model as these neurons are still connected to their normal impulse generating site and respond to normal innocuous mechanical stimuli, which is essential for defining tactile sensitivity.

Ectopic discharge in Aβ-fiber sensory neurons in itself is not expected to be painful. However, the time of onset of hyperactivity in these neurons was simultaneous with the time of onset of tactile allodynia (Sun et al., 2005). The degree of ectopic firing was correlated to the degree of pain behavior (Liu et al., 2000). The exacerbation of the ectopic discharge was parallel to the stimulation of tactile pain (Blumberg and Janig, 1984; Devor et al., 1994; Liu et al., 2000; Kajander et al., 1992; Kajander and Bennett, 1992; Wall and Devor, 1983). In fact, drugs which suppress ectopic firing also suppress neuropathic pain (Devor, 2006; Pitcher et al., 1999). In addition, ectopic discharge such as ongoing discharge enhances the barrage, depolarizes postsynaptic neurons, and brings their resting potential closer to firing threshold (Devor, 2009). Thus, a large proportion of the excess discharge in the dorsal horn in neuropathic models is due to direct drive from the periphery (Devor, 2009). All these observations constitute a priori evidence that the ectopic firing is a

cause of the pain arising from peripheral neuropathy.

SP is typically found only in neurons that are activated by noxious stimuli, that is, in nociceptive neurons (Lawson et al., 1997). It is an important peptide responsible for nociceptive transmission from the peripheral to the central nervous system (Brodin et al., 1990; Duggan et al., 1988; Henry, 1976; Henry, 1978; Malcangio and Tomlinson, 1998). Thus, it is an important contribution of the present study that SP was found in Aβ-fiber LTM neurons, as this provides functional evidence to support the possibility of directly driving postsynaptic pain signaling pathways in the spinal cord. SP released from afferent terminals during ectopic activity may also have postsynaptic effects beyond the moment-to moment modulation of the membrane potential (Devor, 2009). There is evidence that release of mediators such as CGRP and NPY might trigger longer-term changes in the responsiveness of postsynaptic neurons (Woolf and Salter, 2000; Obata et al., 2004).

In summary, Aβ-fiber sensory neurons undergo a change in their electrical discharge characteristics and also in the neurotransmitter complement that they express in this model of peripheral neuropathy. This dual phenotypic switching renders these neurons capable of directly driving central pain circuitry and trigger and maintain central sensitization in neuropathy (Devor, 2009).

Future work of the study

The cellular changes responsible for these ectopic activities in neuropathic neurons are not known in detail. Our current understanding of the process based on previous studies and hypotheses is as follows. Axon injury might unmask nuclear localization signals in certain axonplasmic proteins at the injured site. This blocks the normal retrograde flow of some transport cytokins, nerve growth factor (NGF), glial

cell line-derived neurotrophic factor (GDNF), tumor necrosis factor (TNF), or other inflammatory mediators released by immune cells and Schwann cells between the periphery and the sensory cell body, which then induce the alteration in expression of neuropeptides, receptors and ion channels in somata, and export of proteins by axoplasmic transport to both peripheral and central axon endings (Li et al., 2000; Cui et al., 2000; Shamash et al., 2002; Sommer and Schafers, 1998; Gherardini et al., 1999; Wagner and Myers, 1996).

Thus, future work will be required to determine the details governing expression and properties of how these target factors undergo alternations in expression, product disposition, and activity kinetics that together to affect resonance properties of primary DRG sensory neurons. This may be achieved by *in vivo* intracellular recording and *in vitro* patch clamp recording, and single-cell RT-PCR analysis to investigate the correlation between the changes in functionally-defined DRG neurons from in vivo experiments and changes in the expression of these target factors and properties identified in in vitro experiments on the same neuron.

Conclusions of the study

The purpose of this study was to examine the electrophysiological properties, the immunohistochemical distribution, and pharmacological sensitivity to pregabalin of the different types of DRG neuron in control vs. neuropathic rats. Comparing previous reports, this is the first study providing definitive evidence showing changes in functional properties of sensory DRG neurons with identifiable receptive fields. In this model, there were no differences in C-fiber sensory neurons between control and model rats. However, there were significant differences in DRG neurons associated with Aβ-fibers, especially Aβ-fiber LTM neurons. This finding is unique and

unexpected because Aβ-fiber LTM neurons are normally considered to be non-nociceptive sensory neurons. We interpret these data to suggest that A-type, but not C-type neurons in this model of peripheral neuropathy may be involved in generating the tactile hypersensitivity seen in these rats in the von Frey behavioral test.

Reference List

Blumberg H, Janig W, 1984. Discharge pattern of afferent fibers from a neuroma. Pain 20: 335-353.

Brodin E, Linderoth B, Goiny M, Yamamoto Y, Gazelius B, Millhorn DE, Hokfelt T, Ungerstedt U, 1990. In vivo release of serotonin in cat dorsal vagal complex and cervical ventral horn induced by electrical stimulation of the medullary raphe nuclei. Brain Res. 535: 227-236.

Campbell JN, Raja SN, Meyer RA, Mackinnon SE, 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32: 89-94.

Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderoth B, 2000. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. Pain 88: 239-248.

Devor M, 2006. Centralization, central sensitization and neuropathic pain. Focus on "sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons". J. Neurophysiol. 96: 522-523.

Devor M, 2009. Ectopic discharge in Abeta afferents as a source of neuropathic pain. Exp. Brain Res. 196: 115-128.

Devor M, Janig W, Michaelis M, 1994. Modulation of activity in dorsal root ganglion neurons by sympathetic activation in nerve-injured rats. J. Neurophysiol. 71: 38-47.

Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ, 1988. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Res. 451: 261-273.

Gherardini G, Lundeberg T, Cui JG, Eriksson SV, Trubek S, Linderoth B, 1999. Spinal cord stimulation improves survival in ischemic skin flaps: an experimental study of the possible mediation by calcitonin gene-related peptide. Plast. Reconstr. Surg. 103: 1221-1228.

Han HC, Lee DH, Chung JM, 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain 84: 253-261.

Henry JL, 1976. Effects of substance P on functionally identified units in cat spinal cord. Brain Res. 114: 439-451.

Henry JL, 1978. [Role of peptides in central neural transmission: substance P, bradykinin and beta-endorphine]. Ann. Anesthesiol. Fr. 19: 391-398.

Kajander KC, Bennett GJ, 1992. Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neuron. J. Neurophysiol. 68: 734-744.

Kajander KC, Wakisaka S, Bennett GJ, 1992. Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat. Neurosci. Lett. 138: 225-228.

Khan GM, Chen SR, Pan HL, 2002. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. Neuroscience 114: 291-299.

Lawson SN, Crepps BA, Perl ER, 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J. Physiol 505 (Pt 1): 177-191.

Li Y, Dorsi MJ, Meyer RA, Belzberg AJ, 2000. Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 85: 493-502.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M, 2000. Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85: 503-521.

Malcangio M, Tomlinson DR, 1998. A pharmacologic analysis of mechanical hyperalgesia in streptozotocin/diabetic rats. Pain 76: 151-157.

Obata K, Yamanaka H, Dai Y, Mizushima T, Fukuoka T, Tokunaga A, Noguchi K, 2004. Differential activation of MAPK in injured and uninjured DRG neurons following chronic constriction injury of the sciatic nerve in rats. Eur. J. Neurosci. 20: 2881-2895.

Pitcher GM, Ritchie J, Henry JL, 1999. Nerve constriction in the rat: model of neuropathic, surgical and central pain. Pain 83: 37-46.

Shamash S, Reichert F, Rotshenker S, 2002. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J. Neurosci. 22: 3052-3060.

Sommer C, Schafers M, 1998. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res. 784: 154-162.

Sun Q, Tu H, Xing GG, Han JS, Wan Y, 2005. Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation. Exp. Neurol. 191: 128-136.

Tal M, Wall PD, Devor M, 1999. Myelinated afferent fiber types that become spontaneously active and mechanosensitive following nerve transection in the rat. Brain Res. 824: 218-223.

Wagner R, Myers RR, 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73: 625-629.

Wall PD, Devor M, 1983. Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. Pain 17: 321-339.

Woolf CJ, Salter MW, 2000. Neuronal plasticity: increasing the gain in pain. Science 288: 1765-1769.