CHANGES OF DREAM AND BDNF PROTEINS EXPRESSIONS, PRO-INFLAMMATORY AND OXIDATIVE STRESS LEVELS IN SPINAL CORD OF STREPTOZOTOCIN-INDUCED PAINFUL DIABETIC NEUROPATHY RATS UPON MINOCYCLINE AND IFENPRODIL TREATMENTS

CHE AISHAH NAZARIAH BINTI ISMAIL

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by

CHE AISHAH NAZARIAH BINTI ISMAIL

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αCREM	α-cyclic AMP-responsive promoter elements
ADP	adenosine diphosphate
AGE	advanced glycosylation end product
ANOVA	analysis of variance
AP-1	activator protein-1
APS	ammonium persulphate
ASC	protein-containing apoptosis-associated speck-like protein containing caspase recruitment domain
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CAKβ	cell-adhesion kinase-β
CaMKII	calcium-calmodulin-dependent protein kinase II
CARD	caspase recruitment domain
CCI	chronic constriction injury
CCR2	cysteine-cysteine chemokine receptor-2
CFA	complete Freund's adjuvant
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin-gene related peptide
Cl^-	chloride ion
CM	centromedial nucleus
CNS	central nervous system

CR	cytokine receptor
CRE	cyclic adenosine monophosphate response element
CREB	cyclic adenosine monophosphate response element binding protein
CREM	cyclic adenosine monophosphate response element-responsive promoter elements
Cu/Zn	copper/zinc
CX_3 CR1	CX_3 chemokine receptor 1
DAB	diaminobenzidine
DAMPs	damage-associated molecular pattern molecules
DM	diabetes mellitus
DN	diabetic neuropathy
DNA	deoxyribonucleic acid
DPN	diabetic peripheral neuropathy
DREAM	Downstream Regulatory Element Antagonist Modulator
DRE	Downstream Regulatory Element
DRG	dorsal root ganglion
EAA	excitatory amino acid
EDTA	ethylenediamine-tetraacetic acid
e.g.	for example
ELISA	enzyme-linked immunoabsorbent assay
EphB	Ephrin B
ERK	extracellular-signal-regulated kinase
Fe/Mg	ferrous/magnesium
FIND	find domain

GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A
$GABA_B$	γ-aminobutyric acid type B
GFAP	glial fibrillary acidic protein
GPCR	G-protein coupled receptors
GSH	glutathione
HIV	human-immunodeficiency virus
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
IDDM	insulin-dependent diabetes mellitus
IDV	integrated density value
i.e.	that is
IL-1β	interleukin-1β
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
JNK	Jun-nuclear kinase
KCC2	potassium-chloride contransporter-2
KCHiP3	Kv channel-interacting protein 3
KCl	potassium chloride
LC	lateral nucleus
LDL	low-density lipoprotein
LIF	leukaemia inhibitory factor
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1

MDA	malondialdehyde
MDvc	ventral region of dorsal medial nucleus
Mg^{2+}	magnesium ion
mRNA	messenger ribonucleotide acid
Na^{+}	sodium ion
NO	nitric oxide
Na_2HPO_4	di-sodium hydrogen phosphate
$Na_2HPO_4.7H_2O$	di-sodium hydrogen phosphate heptahydrate
NaCl	sodium chloride
NACHT	nucleotide binding domain
NAD	nucleotide binding domain-associated domain
NAD^{+}	oxidized form of nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate
$NaH_2PO_4.H_2O$	sodium dihydrogen phosphate anhydrous
NaOH	sodium hydroxide
NF-κB	nuclear factor-κB
NGF	nerve growth factor
Ni	nickel
NIDDM	non-insulin dependent diabetes mellitus
NLRP3	Pyrin domain at N-terminus, followed by NACHT domain, NAD and LRR rich repeats domain at C-terminus
NMDA	N-methyl-D-aspartate
NRM	nucelus raphe magnus
O_2-	superoxide anion

OD	optical density
$OH^{\cdot}$	hydroxyl radical
$^{\cdot}O\text{-}O^{\cdot}$	highly active singlet oxygen
O_2	oxygen
OX-42	microglial cell marker
PAG	periaqueductal gray
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular pattern molecules
PB	phosphate buffer
PBS	phosphate buffered saline
PDN	painful diabetic neuropathy
PDZ	Post-synaptic density protein, Drosophila disc large tumour suppressor (Dlg1) and Zonula occludents-1 (zo-1) proteins
PFA	paraformaldehyde
Phospho-ERK	phosphorylation of extracellular-regulated kinase
PKA	protein kinase A
PKC	protein kinase C
PO	posterior nucleus
PSD-95	postsynaptic density protein-95
PTP	dephosphorylation of tyrosine phosphatase
PYD	pyrin domain
P-ser896-NR1	NR1 phosphorylated at Ser896
RACK1	receptor for activated C kinase 1
RAGE	receptor of advanced glycosylation end product
RNA	ribonucleic acid

ROS	reactive oxygen species
RVM	rostro ventromedial medulla
SI	primary somatosensory cortex of postcentral gyrus
SII	secondary somatosensory cortex
SAP90	synapse-associated protein 90
SCN	sciatic nerve cryoneurolisis
SDS	sodium duodecyl sulfate
S.E.M.	standard error of mean
SFKs	Src family of kinases
SG	substantia gelatinosa
SHI	Src homology I
SH2	Src homology 2
SH3	Src homology 3
SOD	superoxide dismutase
SOD 1	copper-zinc superoxide dismutase
SOD 2	manganese-superoxide dismutase
SPSS	Statistical Package for Social Sciences
STZ	streptozotocin
T cells	transmission cells
TBARS	thiobarbituric acid reactions
TBS	tris buffer saline
TBST	tris buffer saline-tween 20
TBS/Tx	tris buffer saline-triton X-100
TCA	trichloroacetic acid
TEMED	N, N, N'N'-tetramethylenediamine

TGF-β	transforming growth factor-β
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor-α
TrkB	tropomysin-related kinase B
Tyr	tyrosine kinase
VEGF	vascular endothelial growth factor
VmPO	posterior division of the ventromedial nucleus
VPI	ventroposterior
VPL	ventroposterolateral
VPM	ventroposteromedial
WB	western blot
WDR	wide-dynamic-range

PERUBAHAN EKSPRESI PROTEIN DREAM DAN BDNF, SITOKIN PRO-KERADANGAN DAN TEKANAN OKSIDATIF DALAM KORDA SPINA TIKUS NEUROPATI DIABETES YANG MENYAKITKAN YANG DIARUHKAN OLEH STREPTOZOTOCIN DENGAN RAWATAN MINOCYCLINE DAN IFENPRODIL

ABSTRAK

Neuropati diabetes (DN) merupakan komplikasi jangka panjang penyakit diabetes melitus (DM) yang menyebabkan kesakitan (PDN) atau sebaliknya (non-PDN). Kajian ini bertujuan untuk meneroka peranan (i) penanda bio protein (keseluruhan subunit NR2B (NR2B) dan subunit NR2B yang telah difosforilasi (phospho-NR2B) daripada reseptor NMDA, pengaktifan mikroglia, protein BDNF dan DREAM), (ii) sitokin pro-keradangan (IL-1β dan TNF-α) dan (iii) status stres oksidatif (MDA, SOD dan 'catalase') dalam patogenesis DN pada korda spina tikus diabetes yang diaruhkan oleh 'streptozotocin'. Seratus enam puluh lapan ekor tikus jantan Sprague-Dawley dibahagikan kepada tujuh kumpulan (n=24), terdiri daripada kumpulan bebas diabetes (S+CB), kumpulan kawalan PDN (S+STZ), kumpulan kawalan tidak mengalami PDN (non-PDN), kumpulan PDN dirawat dengan minocycline (M80 dan M160) atau ifenprodil (I0.5 dan I1.0). DM diaruhkan dengan satu suntikan 'streptozotocin' (60mg/kg). Ujian tingkah laku kesakitan seperti Von Frey, plat-panas dan formalin dilakukan untuk menilai 'allodynia' sentuhan, hiperalgesia terhadap haba dan bahan kimia. Rawatan 'saline', minocycline (80 atau 160µg sehari) atau ifenprodil (0.5 atau 1.0µg sehari) diberikan secara suntikan intratekal selama tujuh hari. Selepas itu, kesakitan keradangan kronik telah diaruhkan dengan suntikan formalin dan tikus-tikus tersebut telah dikorbankan tiga hari

kemudian. Bahagian pembesaran lumbar pada korda spina tikus dibedah keluar untuk tujuan penganalisaan. Keputusan kajian menunjukkan tikus PDN mengalami 'allodynia' sentuhan dan hiperalgesia terhadap bahan kimia tetapi tidak terhadap haba, yang mana simptom tersebut telah direncat oleh minocycline dan ifenprodil. Sementara itu, tikus non-PDN tidak mengalami 'allodynia' sentuhan dan hiperalgesia terhadap haba mahupun bahan kimia. Ekspresi protein NR2B, phospho-NR2B, pengaktifan mikroglia, BDNF dan DREAM menunjukkan peningkatan drastik pada bahagian ipsilateral dan kontralateral korda spina dalam kumpulan (S+STZ) dan keputusan ini bertentangan dengan keputusan daripada kumpulan non-PDN. Minocycline dan ifenprodil berjaya mengurangkan ekspresi NR2B, phospho-NR2B, BDNF, DREAM dan pengaktifan mikroglia pada bahagian ipsilateral dan kontralateral korda spina tikus PDN bergantung pada dos yang diberikan. Tambahan pula, kumpulan-kumpulan (S+STZ) dan non-PDN menunjukkan peningkatan yang signifikan terhadap kadar TNF-α namun menunjukkan tiada perubahan pada kadar IL-1β. Minocycline telah berjaya merencatkan peningkatan rembesan kedua-dua sitokin tersebut manakala ifenprodil telah merencatkan peningkatan kadar TNF-α namun meningkatkan pula kadar IL-1β. Selain itu, kadar MDA meningkat secara signifikan dalam kumpulan-kumpulan (S+STZ) dan non-PDN. Pengurangan aktiviti enzim 'catalase' dengan tiada perubahan pada aktiviti enzim SOD dikesan dalam kumpulan (S+STZ) manakala peningkatan aktiviti enzim 'catalase' dengan pengurangan aktiviti enzim SOD dilihat dalam kumpulan non-PDN. Minocycline dan ifenprodil mengurangkan kadar MDA dan meningkatkan aktiviti enzim-enzim 'catalase' dan SOD pada korda spina. Secara tuntasnya, minocycline dan ifenprodil berkesan merawat PDN melalui aktiviti-aktiviti anti-kesakitan, anti-oksida dan anti-keradangan yang telah ditunjukkan dalam penyelidikan ini.

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ABSTRACT

Diabetic neuropathy (DN) is a long-term complication of diabetes mellitus (DM) which could be painful (PDN) or non-painful (non-PDN). This study aimed to explore the effect of minocycline and ifenprodil on the (i) proteins expressions of NR2B subunit (NR2B) and phosphorylated NR2B subunit (phospho-NR2B) of N-methyl-D-aspartate (NMDA) receptors, microglial activation, brain-derived neurotrophin factor (BDNF) and Downstream Regulatory Element Antagonist Modulator (DREAM) proteins), (ii) pro-inflammatory cytokines (interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) and (iii) oxidative stress markers (malondialdehyde (MDA), superoxide dismutase (SOD) and catalase) in the pathogenesis of DN in the spinal cord of streptozotocin-induced diabetic rats. One hundred and sixty-eight Sprague-Dawley male rats were assigned into seven groups (n=24) consisting of non-diabetic control (S+CB), diabetic PDN control (S+STZ), diabetic non-PDN control (non-PDN), minocycline-treated PDN groups (M 80 and M 160) and ifenprodil-treated PDN groups (I 0.5 and I 1.0). DM was induced with a single streptozotocin injection at 60 mg/kg. Nociceptive behavioural tests such as Von Frey, hot-plate and formalin tests were conducted to assess tactile allodynia, thermal hyperalgesia and chemical hyperalgesia respectively. Treatment of either saline, minocycline (80 µg/day or 160 µg/day) or ifenprodil (0.5 µg/day or 1.0 µg/day) was administered intrathecally for seven days. Chronic inflammatory pain was induced

with formalin injection before being sacrificed three days later. The spinal cord lumbar enlargement region was collected for immunohistochemistry, Western Blot (WB) and enzyme-linked immunoabsorbent assay (ELISA) analyses. The results showed that PDN rats developed tactile allodynia and chemical hyperalgesia but not thermal hyperalgesia, in which were prevented by minocycline and ifenprodil at both lower and higher doses used. Meanwhile, non-PDN group showed lower tactile allodynia, thermal and chemical hyperalgesia. There was significant higher NR2B, activated microglia, BDNF and DREAM proteins ipsilaterally and contralaterally by immunohistochemistry and WB analyses in (S+STZ) group, in which the results were reduced in non-PDN group. Minocycline and ifenprodil at both lower and higher doses significantly attenuated the expressions and mean relative NR2B, phospho-NR2B, BDNF, DREAM proteins levels and activated microglial positive neurons in a dose-dependent manner. Furthermore, (S+STZ) and non-PDN groups showed a significant higher TNF-α level. Minocycline inhibited both cytokines. Moreover, MDA level was significantly higher in (S+STZ) and non-PDN groups. Significant lower catalase enzyme activity with insignificant SOD enzyme activity was detected in (S+STZ) group whilst marked higher catalase activity with lower SOD enzyme activity were detected in non-PDN group. Minocycline and ifenprodil attenuated MDA level and lead to higher catalase and SOD activities in the spinal cord. In conclusion, minocycline and ifenprodil is effective to combat PDN through their strong anti-nociceptive, anti-oxidant and anti-inflammatory activities as has been shown in this study.

CHAPTER ONE

INTRODUCTION

1.1 Diabetic neuropathy

Neuropathic pain is one of the critical problems in clinical medicine as it is not easy to cure. It is pathological and defined as a chronic pain state resulting from injury or disease of neurons in peripheral or central nervous system (CNS). Neuropathic pain may result either from acute events (e.g amputation and spinal cord injury) or systemic disease (e.g diabetes, viral infection and cancer) (Zhuo et al., 2011, Ji et al., 2017). Patients with neuropathic pain experience devastated suffering as this pathological pain is hugely resistant to currently available analgesics. Neuropathic pain is characterized by unusual response to somatic sensory stimulation. The patients experiencing peripheral neuropathies may feel pain from stimuli which are non-noxious in normal condition such as a soft touch on the skin or by changes in temperature. They also suffer from enhanced responses to pain stimuli. In fact, neuropathic pain treatment costs billions dolars annually and most drugs focus on reducing the neuronal hyperexcitability either peripherally or centrally. However, currently-used medication such as tricyclic antidepressants and the "gold standard" gabapentin that are the mainstay of neuropathic pain treatment, has been shown to be limited in efficacy and produces several side-effects in a number of patients (Childers and Baudy, 2007; Kukkar et al., 2013).

One of the devastating diseases classified under neuropathic pain is diabetic peripheral neuropathy (DPN). Diabetic neuropathy is a late complication of diabetes mellitus (DM) of either Type I or II. A study from the Mayo Clinic revealed that diabetic neuropathies are common in diabetic patients affecting approximately 66%

with insulin-dependent DM (Type I DM) and 59% in patients with non-insulin-dependent DM (Type II DM) (Sadosky et al., 2008). Moreover, PDN is reported to affect approximately 18% of adult diabetic patients compared with a minimum of 30% of patients with overall diabetic peripheral neuropathy (Spallone and Greco, 2013). In fact, numerous types of diabetic neuropathy have been reported including cranial, truncal, focal limb and amyotrophic neuropathy (Jensen et al., 2006). However, in specific, the sensory neuropathy, which is also referred to as distal symmetric sensory polyneuropathies, may be further classified as being acute or chronic. But, chronic form is actually the most common occurrence of diabetic neuropathy including painful diabetic neuropathy (PDN), which occupies most of the available epidemiologic data (Sadosky et al., 2008).

Neuropathic pain has raised the question of whether a completely different strategy is needed and could offer an alternative approach to obtain a better treatment outcome. Pain expert panel has identified a number of deficiencies in the available clinical data that need serious attention, including the lack of understanding of the underlying mechanisms of PDN and the need for studies to investigate the treatment effects on different symptoms of PDN such as allodynia, hyperalgesia and spontaneous pain (Jensen et al., 2006). Thus, it is crucial to deeply explore and understand the pathogenesis underlying PDN together with the discovery of new molecular targets that potentially give some hope to the patients and also strengthen the demand for alternative way for curing neuropathic pain.

In year 1991, the glial cell has become the centre of researchers' attention since an animal model of neuropathic pain was reported to stimulate spinal astrocytes activation (Garrison et al., 1991). During that period, the drugs experimented also demonstrated attenuation towards numbers of astrocytic activation in the rat model of

sciatic nerve constriction injury (Garrison et al., 1994). Beginning from that, glial cell activation was believed to be strongly connected with the development of neuropathic pain. To be specific, microglial activation in neuropathic pain has been widely investigated and research discovered evidence that glial cell are the key players in the creation and maintenance of several types of neurodegenerative diseases. The discovery of spinal cord glial cells which strongly implicates pain processing enhances the understanding of pain, including understanding the glia-neuron interactions. In fact, as microglia could possibly modulate pain, it is vital to understand the mechanisms of microglial activation on the pathogenesis of neuropathic pain, especially on PDN. Inhibiting the microglial activation could potentially one of the possible ways to combat the development of PDN. Therefore, understanding this mechanism leading to the pathogenesis of PDN should be well-studied.

Apart from that, N-methyl-D-aspartate (NMDA) receptors activation has been implicated in the initiation and maintenance of central sensitization during the pain states. Understanding the molecular and cellular mechanisms of NMDA receptors may give some hope to the pain analgesic development targeting these receptors to combat PDN. In brief, NMDA receptors are composed of three subunits: NR1, NR2 (A, B, C and D) and NR3 (A and B) subunits which play different roles whether in pain transmission or in learning and memory. NR2B subunit of NMDA receptor is reported to be abundantly expressed in the spinal dorsal horn and have more implications for neuropathic pain (Ma and Hangreaves, 2000). Since NR2B subunit receptor are located at the extra synaptic sites, it is possible that its effect differs from the other types of NMDA receptor subunits (Parsons and Raymond, 2014). The development of drugs specifically targeting this site may aid in alleviating pain in patients with PDN. Last but not least, the previous studies on the mechanisms of neuropathic pain revealed

the involvement of signalling neuromodulators such as brain-derived neurotrophic factor (BDNF) and downstream regulatory element antagonist modulator (DREAM) in the neuron-glia crosstalk (Zhang et al., 2007, Ren and Dubner, 2008, Tsuda, 2016). It is possible that the pathogenesis of PDN is also similar to the other models of neuropathic pain in which the signalling neuromodulators may play the similar roles for the neuron-glia interactions. If so, inhibiting these signalling molecules leading to the activation of NR2B subunit of NMDA receptor as well as microglia may perhaps bring some hope to combat the development of PDN.

1.2 Problem statements

It is already known worldwide that neuropathic pain, specifically PDN, is not easy to be treated. The mechanisms underlying PDN are still unclear. However, the previous studies of neuropathic pain revealed strong neuronal and non-neuronal interactions leading to the development and maintenance of neuropathic pain (Hossain et al., 2017, Ji et al., 2017). The neuronal mechanisms involving the spinal NMDA receptor activation especially NR2B subunit activation has been revealed to play a vital role in initiating and maintaining the neuropathic pain (Qu et al., 2009) as the persistent NR2B subunit of NMDA receptor activation causes the stimulation of non-neuronal cells (i.e microglial activation) through the cascades of immune system activation (Hossain et al., 2017, Ji et al., 2017). These interactions are also succeeded by the aid of signalling neuromodulators such as BDNF and DREAM proteins, free radicals and pro-inflammatory cytokines released by these neuronal and non-neuronal cells to interact with each other in the pathogenesis of neuropathic pain (Geng et al., 2010, Old and Malcangio, 2012).

By targeting neuronal and non-neuronal interactions, it is believed that the pathogenesis of PDN can be combated. Therefore, the drugs targeting these neuronal and non-neuronal pathways is needed to inhibit the development of painful diabetic neuropathy. Ifenprodil has been demonstrated in the previous study of neuropathic pain model to possess a greater selectivity for NR2B subunit of NMDA receptor activation compared to other similar antagonists (Boyce et al., 1999) to non-competitively inhibit this NMDA receptor activation. Meanwhile, minocycline has been shown to strongly inhibit the microglial activation in the animal models of neuropathic pain (Pabreja et al., 2011, Taves and Ji, 2016). However, the effects of these drugs in inhibiting the development of PDN is not clear. It is also unknown whether these drugs may have some effect on the signalling neuromodulators such as DREAM and BDNF proteins expressions, pro-inflammatory cytokines and oxidative stress markers to interrupt the interactions between neuronal and non-neuronal mechanisms, therefore, may possibly combat the development of PDN.

1.3 Objectives of the study

1.3.1 Main objective

Thus, the main objective of this study was to explore the roles of protein biomarkers (NR2B subunit of NMDA receptor phosphorylation and activation, microglial activation, BDNF and DREAM proteins), oxidative stress (malondialdehyde, superoxide dismutase and catalase enzymes activities) and pro-inflammatory markers (interleukin-1β and tumour necrosis factor- α). This study also aimed to explore the anti-nociceptive, anti-inflammatory and anti-oxidant effects of ifenprodil and minocycline and to determine whether the signalling pathways of

BDNF and DREAM protein expressions, pro-inflammatory cytokines and oxidative stress were involved in the spinal cord of STZ-induced PDN rat.

1.3.2 Specific objectives

1. To compare the effects of ifenprodil and minocycline administered intrathecally on tactile allodynia, thermal hyperalgesia and formalin-induced inflammatory nociceptive behaviour in the STZ-induced PDN rats.
2. To compare the effects of ifenprodil and minocycline administered intrathecally on the microglial cell marker expression (OX-42), total expression of NR2B subunit of NMDA receptors, phosphorylation of NR2B subunit of NMDA receptors, BDNF and DREAM proteins expression by immunohistochemistry and western blot analyses in the spinal cord of STZ-induced PDN rat.
3. To compare the effects of ifenprodil and minocycline administered intrathecally on the pro-inflammatory markers level of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), oxidative stress markers (malondialdehyde (MDA) level and catalase and superoxide dismutase (SOD) enzymes activities).
4. To correlate the microglial cell marker (OX-42), total NR2B subunit of NMDA receptors, phosphorylation of NR2B subunit of NMDA receptors, BDNF and DREAM proteins expression, IL-1β and TNF-α levels, MDA level, catalase and SOD enzymes activities in the spinal cord with tactile allodynia, thermal hyperalgesia and formalin-induced inflammatory nociceptive behaviour in the STZ-induced PDN rat.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pain

Pain, whether originates physiologically or pathologically is an unpleasant sensory experience stimulated with the presence of noxious stimuli. The increasing prevalence of pain, particularly chronic pain, is seriously alarming and appears to increase with age in both men and women (Schim and Stang, 2004). It could be the most probable reason of why patients seek medical consultation. According to the International Association for the Study of Pain (IASP), pain is defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Williams and Craig, 2016).

2.1.1 Pain pathways

In general, pain pathways consist of pain signal transduction at the peripheral receptor site, pain signal conduction along the peripheral nerve, pain modulation at spinal cord level, pain perception at the supraspinal site and lastly, the related emotional feelings, sensation and affective state (Kitahata, 1993).

2.1.1(a) Pain signal transduction at the peripheral receptor site

The propagation of pain is actually elicited with the activation of nociceptors found abundantly in the skin (epidermis, dermis), dental pulp, mucosal membranes of the oral and nasal cavities of the respiratory, gastrointestinal, and urinary tracts, muscles, tendons, ligaments, joint capsules and bones. These nociceptors are naked and free nerve endings and respond only when a stimulus is sufficiently strong to cause injury. Three main categories of primary afferent fibres are Aβ-fibre, Aδ-fibre and C-

fibre. These fibres which are also the first-order neurons are classified depending on the diameter, structure and conductivity speed.

Myelinated Aβ-fibres are the greatest fibre diameter and the highest speed of conductivity compared to the other types of primary afferent fibres. The Aβ-fibres are responsible for the touch sensation. Meanwhile, myelinated Aδ-fibres carry mechanical and thermal stimuli (pain) from the peripheral nerve. This type of fibres promotes an immediate sensation of the first phase or acute pain and initiate withdrawal actions. Two distinctive types of Aδ fibres were discovered; Type I of Aδ fibres respond to fibres with high-threshold mechanoreceptors that mainly respond to the mechanical input of high intensity and weakly respond to thermal or chemical inputs. Meanwhile, another type of Aδ fibres, Type II is the fibres with mechanothermal receptors for strong heat (45-53 ºC) and some receptors for the extreme cold (-15 ºC) and sensitized to vigorous mechanical stimuli at non-noxious thresholds (Millan, 1999). Aδ fibres propagate the 'fast pain' in which they transmit the pain inputs fairly quickly. Meanwhile, C-fibres possesses several distinctive characteristics and response to thermal and mechanical stimuli. Majority of C-fibres with high-threshold receptors respond equivalently to thermal and mechanical stimuli or are sensitive to mechanical, thermal and chemical inputs (polymodal C-fibres). There is also another special type of polymodal C-fibres that react to high-intensity thermal stimuli and seems accountable for sudden brief burst of response after the tissue injury. Apart from that, there is also another type of C-fibres that are slowly conducted, insensitive to mechanical stimuli, mediated by histamine and possibly involved in the burning sensation. Last but not least is another type of C-fibres which do not respond to noxious input in general, but become merely activated in the presence of inflammation (silent C-fibres) (Willis and Westlund, 1997, Almeida et al.,

2004, Millan, 1999). The unmyelinated C-fibres mediate nociceptive inputs in a slower manner or 'slow pain', at times secondary to the action of Aδ fibres (Almeida et al., 2004).

2.1.1(b) Signal conduction of pain

In brief, the sensory pathway as shown in Figure 2.1 is classified into three sections which are:

1) First-order neurons that carry sensory inputs from the nociceptive receptor to the CNS.
2) Second-order neurons which transmit the nociceptive signal from CNS to the thalamus.
3) Third-order neurons that propagate the nociceptive input from the thalamus to the cerebral cortex.

First-order neurons propagate the nociceptive inputs from the peripheral nerve to the spinal cord and the brainstem via the cranial nerve pairs V, VII, IX and X. The first-order neurons will detach from the thicker fibres, organizing themselves in the ventrolateral bundle of roots and then form synapses with second-order neurons distributed along the dorsal horn of the spinal cord (Merskey, 1986).

The axons of second-order neurons form afferent bundles with anterolateral or posterior fascicle that project the pain impulse to brainstem and diencephalon structures including thalamus, periaqueductal substance, parabrachial region, reticular formation of the medulla, amygdaloid complex, septal nucleus and hypothalamus. The previous findings indicated that the axons of second-order neurons project either in the direct (spinothalamic) or indirect (spinoreticular) pathway of the anterolateral system, or as three sets of fibres which are the spinomesenphalicspinotectal or spinohypothala-

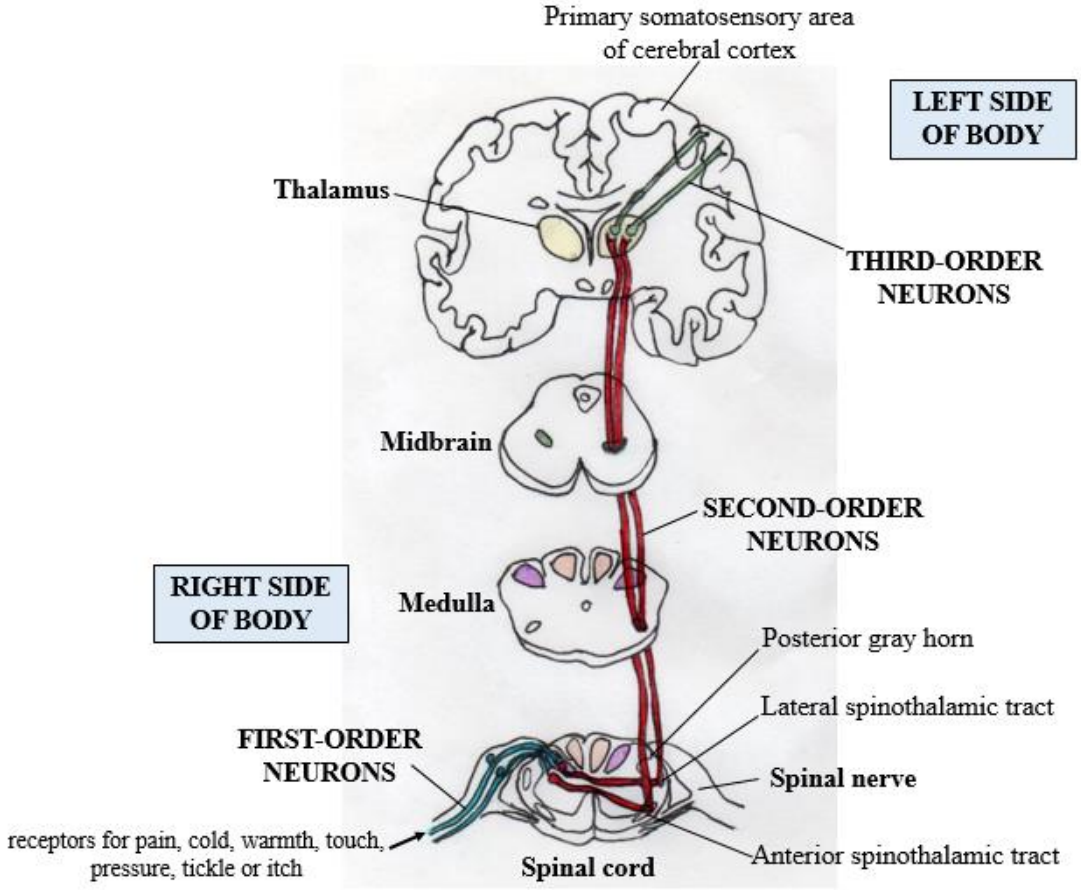

Figure 2.1 First-, second- and third-order neurons in pain pathways. First-order neurons carry the sensory inputs from nociceptor to the CNS whilst second-order neurons transmit the nociceptive inputs from the CNS to thalamus. Meanwhile, third-order neurons propagate the nociceptive signals from the thalamus to cerebral cortex. Adapted from Chan (2010).

mic fibres (the remaining components of the anterolateral system). These tracts are believed to propagate noxious, thermal and crude tactile signals to the higher brain centres. Almost 15% of the afferent fibres propagate directly to the thalamus whilst 85% of the afferent fibres project to the thalamus through a relay in the reticular formation (Millan, 1999).

Direct pathway of the anterolateral system

Aδ-fibres of the first-order neurons synapse mainly with second-order neurons in lamina I (posteromarginal nucleus) and lamina V (reticular nucleus) of the spinal cord. But most of these first-order neurons synapse with the spinal cord interneurons that are related to a reflex motor activity. The axons of the second-order neurons are then projected across the midline to the contralateral side of the spinal cord in the anterior white commissure to form the spinothalamic tract.

In brief, spinothalamic tract (neospinothalamic pathway) is comprised of the lateral spinothalamic tract (found in the lateral funiculus) and anterior spinothalamic tract (found in the anterior funiculus). This tract disseminates noxious, thermal and crude touch signals to the contralateral ventral posterior lateral nucleus of the thalamus. Apart from that, spinothalamic tract also transmits the information to the ventral posterior inferior and the intralaminar nuclei of the thalamus. Although the spinothalamic tract terminates in the thalamus, this tract also transmits collaterals to the reticular formation as if ascends via brainstem (Tortora and Derrickson, 2008).

Indirect pathway of the anterolateral system

Unmyelinated C-fibres of the first-order neurons end on the interneurons in lamina I (substantia gelatinosa) and lamina II of the dorsal horn. The axons of these

interneurons then synapse with second-order neurons in laminae V-VIII. Most of these axons ascend ipsilaterally but some project to the contralateral side of the spinal cord in the anterior white commissure. These axons form the more pronounced ipsilateral and smaller contralateral spinoreticular tracts (paleospinothalamic pathway). The spinoreticular tract propagates the noxious, thermal and crude touch inputs from the spinal cord to the thalamus in an indirect pathway by forming abundant synapses in the reticular formation before projecting to the thalamus (Abbracchio and Reggiani, 2013).

Third-order neurones are situated in the ventral posterior lateral nucleus of the thalamus and ascend in the posterior limb of the internal capsule and corona radiata to end in the primary somatosensory cortex of postcentral gyrus (SI). The primary somatosensory cortex is then projected to the secondary somatosensory cortex (SII) situated on the superior border of the lateral fissure. Some of the third-order neuron fibres from the thalamus may also directly terminate in the SII (Almeida et al., 2004, Perl, 2011).

2.1.1(c) Modulation at the spinal cord level

It is believed that the dorsal horn of the spinal cord is the first site of modulation for the pain signal arriving from the periphery to CNS (Kitahata, 1993). To understand the process of pain modulation in the spinal cord, Melzack and Wall (1967) have proposed the 'gate control theory' to understand the pain modulation process in a better way (Figure 2.2).

The pain impulse reaching the spinal cord is transmitted to three dominant spinal cord systems which are:

1) cells of the substantia gelatinosa (SG) in the dorsal horn

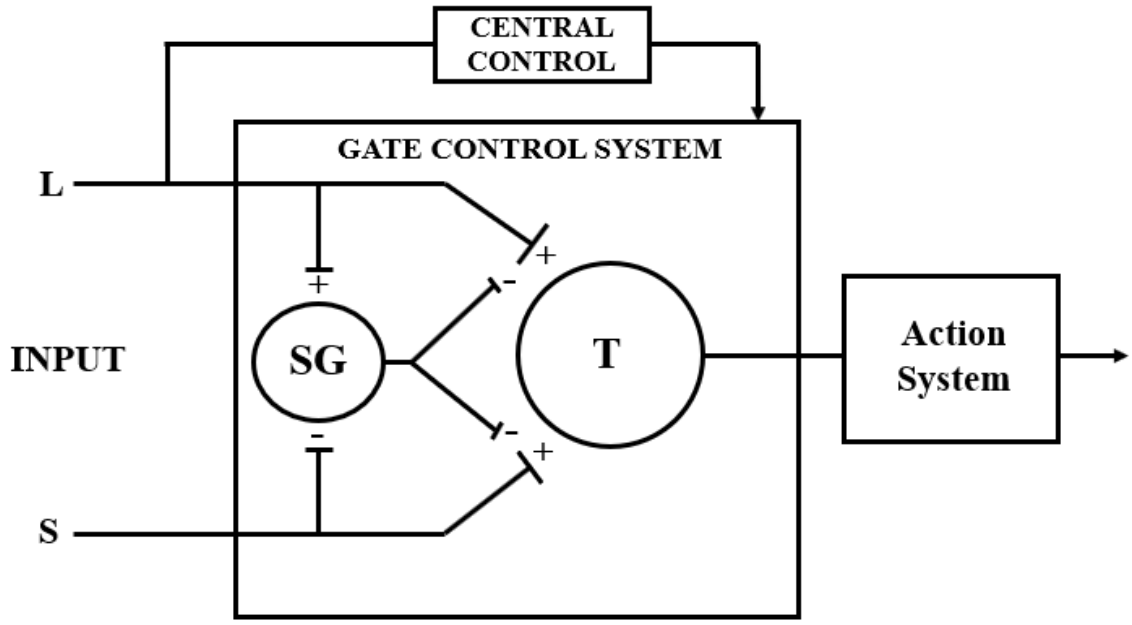

Figure 2.2 Schematic diagram of the gate control theory of pain mechanisms. L = large diameter Aβ-fibres, S = small diameter Aδ- and C-fibres, T = transmission cells, SG = cells of substantia gelatinosa, (+) = excitation and (—) = inhibition. The inhibitory effect employed by SG cells on the afferent fibre terminals is elevated by the activity in 'L' fibres and reduced by the activity in 'S' fibres. The line running from the large fibre system to the central control mechanisms represents 'central control', and these mechanisms in turn, propagate back to the gate control system. T cells propagate to the entry cells of the action system. Adapted from Melzack and Wall (1967).

2) dorsal-column fibres that project toward the brain (central control)
3) first central transmission (T) cells in the dorsal horn

It is proposed that SG acts as a gate control system that modulates the afferent patterns before they affect the T cells. Meanwhile, the afferent patterns in the dorsal column system functions as a central control trigger that may stimulate selective brain processes that affect the modulation properties of the gate control system. Furthermore, T cells may stimulate neural pathways which consist of the action system accountable for response and perceptions.

In gate control theory, the afferent fibres (Aβ-, Aδ- and C-fibres) that carry noxious signals project to SG and T cells. T cells propagate the noxious signals up to the brain whilst SG cells (inhibitory interneurons) prevent the pain signal transmission. Activities in C, Aδ- and Aβ-fibres influence activities in the T cells in which thin diameter Aδ- and C-fibres excite the SG cells (tend to allow T cells to fire) while large-diameter myelinated Aβ-fibres excite the inhibitory SG cells (tend to attenuate T cells activity). Briefly, the activation of non-noxious large diameter Aβ-fibres may disturb the signals from pain fibres (Aδ- and C-fibres), therefore inhibiting pain.

The laminae of the spinal cord which are involved in receiving pain inputs from Aδ- and C-fibres, also receive the signals from Aβ-fibres. Aβ-fibres indirectly attenuate the effects of Aδ- and C-fibres, ‘closing a gate’ to the propagation of their pain inputs. In addition, in other parts of the laminae, the Aδ- and C-fibres also suppress the effects of Aβ-fibres, thus ‘opening the gate’. This happens when the output of the T cells exceeds a critical level thus allowing the pain transmission to the brain.

2.1.1(d) Descending pain inhibition

Research has established that the stimulation of midbrain and medullary sites exerts dual control over nociception. Descending pain pathways (as indicated by red- and green-coloured pathway in Figure 2.3) communicate from the brain to the body which attenuates pain. There are a minimum of two major pathways that descend to the spinal cord to suppress the projection of pain. The most significant descending pathway starts in the periaqueductal gray (PAG). The neurons beginning in the PAG terminate on cells in the medulla including the serotonergic cell bodies of the raphe nuclei (Figure 2.3). The serotonergic neurons then descend the spinal cord to attenuate cell firing. Other cells in the PAG end next to the locus coeruleus in the brainstem. PAG functions to receive inputs from higher brain centres and is able to activate a strong analgesic effect without affecting the body's ability to detect temperature, pressure or touch (Zhuo, 2008, Ossipov et al., 2014). This is proven by early research that microinjection of opioids into PAG attenuates the neuropathic pain perceived by rats (Yaksh et al., 1976, Lewis and Gebhart, 1977). The microinjection of morphine into PAG after the application of peripheral pain stimuli also demonstrated a marked attenuation of dorsal horn second-order neurons activity (Bennett and Mayer, 1979).

Other than that, rostro ventromedial medulla (RVM) comprises the serotonergic nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis-pars alpha and nucleus paragiganto-cellularis lateralis (Vanegas and Schaible, 2004). RVM is capable of either facilitating or attenuating the pain signals and functions as a final relay in the control of descending pain facilitation (Ossipov et al., 2014).

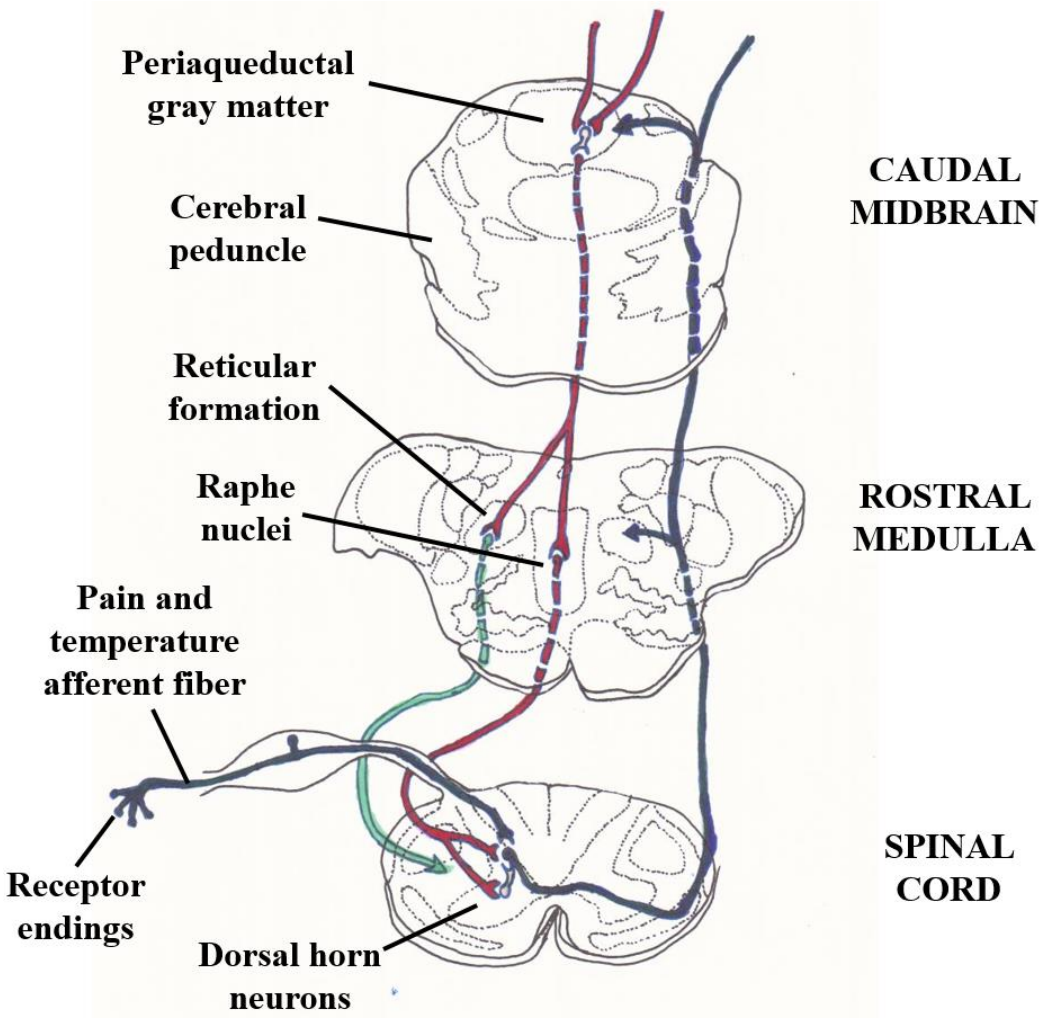

Figure 2.3 Descending pain pathway. Descending pain pathways (as indicated by red- and green-coloured pathway) communicate from the brain to the body which attenuates pain. There are at minimum of two major pathways that descend to the spinal cord to suppress the projection of pain. The most significant descending pathway starts in periaqueductal gray (PAG). The activation of PAG has been shown to result in analgesia, but exhibit no change in the ability to detect temperature, pressure or touch. The neurons begin in the PAG terminate on cells in the medulla including the serotonergic cell bodies of the raphe nuclei. The serotonergic neurons then descend into spinal cord to attenuate cell firing. Other cells in the PAG end next to the locus coeroleus in the brainstem. Adapted from Zhuo (2008).

In a neuropathic pain model of spinal nerve ligation, tactile allodynia that is developed after unilateral ligation of L_5 and L_6 spinal nerves was found to be attenuated by inactivation of the RVM by lidocaine injection (Pertovaara, 1998). PAG may affect the descending pain modulation mainly via its mutual connection with RVM. The trigger of PAG neurons may also trigger the activity of RVM neurons and is related to the attenuation of nocifensive reflexes in rats (Behbehani and Fields, 1979). Jensen and Yaksh (1986) through their research, demonstrated that morphine blocked the nocifensive input to thermal noxious stimuli when it is microinjected into RVM of the rat since RVM has neuronal communication with the PAG. Furthermore, similar to the 'gate control theory' proposed by Melzack and Wall (1967), the modulation of nociceptive responses by RVM is assisted by two distinguished populations of neurons: ON and OFF cells. ON cells are accountable for the facilitatory effect on the nociceptive processing via descending systems projecting to the spinal cord (Ossipov et al., 2000, Ossipov et al., 2014). Meanwhile, OFF cells are assumed to comprise a descending inhibitory system that block nociceptive input directly at the level of the spinal cord (Fields et al., 1991, Ossipov et al., 2000), for example, OFF cells attenuate firing immediately prior to the tail-flick in rats (Ossipov et al., 2014).

Although the descending pain facilitatory and inhibitory systems maintain a homeostatic state, the occurrence of illness, injury or inflammation can disturb this balance. Studies have revealed that persistent pain after tissue or nerve damage is related to the amplified activation of descending modulatory circuits (Vanegas and Schaible, 2004, Guo et al., 2006). Injury or inflammation may enhance RVM ON-cell activity. Moreover, the pharmacological, neurochemical and physical disturbance of descending facilitation from RVM diminishes the enhanced behavioural responses to the evoked stimuli without inhibiting the acute, protective nociceptive reflex (Ossipov

et al., 2014). This increased nett descending facilitatory drive contributes to the augmentation and spread of pain (Vanegas and Schaible, 2004, Guo et al., 2006).

Descending noradrenergic projections to the spinal dorsal horns communicate with RVM and PAG. Recent findings suggest that during the nerve injury, the activity of descending noradrenergic system is enhanced in an effort to compensate for the enhanced nociceptive signals. The injury causes the increased synthesis and production of noradrenaline along with an increased efficacy of spinal α_2-adrenergic receptors (Muto et al., 2012).

2.1.1(e) Perception at the supraspinal sites

Previous studies indicate that supraspinal structure involved in pain comprises the midbrain, brainstem, thalamus, hypothalamus, lentiform nucleus, somatosensory cortex, insular cortex and pre-frontal, anterior and parietal cingulate cortex (Almeida et al., 2004, Zhuo et al., 2011).

Thalamus is believed to be the primary relay structure for sensory inputs transmitted to the cortex and involved in the reception, integration and the transfer of nociceptive potential. The lateral nuclear complex of the thalamus is comprised of three types of nuclei; ventroposterolateral (VPL), ventroposteromedial (VPM) and ventroposterior (VPI) nuclei. These nuclei respond to thermal and mechanical stimuli and some also respond to freezing (Almeida et al., 2004). It is well-known that VPL and VPM are involved in the inhibitory interactions which form a modulatory system in the propagation of pain to superior centres, similar to the proposed ‘gate control theory’. The afferent fibres from the spinocervical tract, spinoparabrachial tract and spinoreticular tract are also projected to the lateral complex of the thalamus (Almeida et al., 2004).

Meanwhile, the posterior complex of the thalamus is comprised of the pulvinar oralis nucleus, posterior nucleus (PO) and the posterior division of the ventromedial nucleus (VmPO). PO and VmPO nuclei establish connections with the insular and cingulate cortex and contribute to affective cognitive aspects of pain (Treede et al., 1999, Almeida et al., 2004). In addition, these nuclei are believed to become the centres of integration for painful and thermal noxious inputs. The posterior complex of the thalamus receives the noxious signals from afferent fibres derived from the spinothalamic tract, spinohypothalamic tract, spinoparabarachial tract and postsynaptic pathway from the dorsal column (Almeida et al., 2004).

Furthermore, the medial complex of the thalamus is comprised of the ventral region of the dorsal medial nucleus (MDvc), lateral central nucleus (LC) and the centromedial nucleus (CM). These nuclei are projecting to the cingular cortex and it is proposed that they are involved in the motivational-affective aspects of pain. The nuclei of the medial complex of thalamus receive afferent fibres from laminae I and V of the spinothalamic tract and interconnect with striatum and cerebellum, possibly involved in the escape behaviour in the presence of a dangerous input (Millan, 1999).

Pain signals mediated by the lateral, posterior and medial systems are then projected to three important cortical regions which are SI, SII and anterior cingulate cortex (Millan, 1999, Almeida et al., 2004). The lateral system is involved directly in the sensory-discriminative attribution of pain and involves specific thalamic nuclei which propagate to spinal nerves and wide-dynamic-range neurons (WDR) of the SI and SII cortices. The SI and SII cortices are interconnected with the posteroparietal area and with the insula via a cortico-limbic somatosensory pathway which is related to learning and memory. On the other hand, the medial system has less defined projections from the medial complex of the thalamus to SI and SII, including the limbic

structures (e.g insula and anterior cingulate cortex). Thus, it is the reason why the medial region of thalamus contributes to the motivational-affective component of nociception although it may also take part in the sensory-discriminative circuitry (Treede et al., 1999).

The insula functions in receiving the nociceptive inputs from the lateral system and propagates to the limbic system, mainly amygdala and some regions of prefrontal cortex. These regions are responsible for the emotional and affective component and memory associated with the painful experience (Price, 2000). Meanwhile, the anterior cingulate cortex functions in bringing the attentional and emotional mechanisms to pain experience (Treede et al., 1999).

2.1.2 Types of pain

Pain experience according to Kitahata (1993) could be categorized into four distinguished types and stages which are:

1) Processing of acute pain signals (acute physiological nociceptive pain)
2) Sympathetically maintained pain (pathophysiological nociceptive pain)
3) Persistent pain stimulation secondary to peripheral tissue damage (chronic pain)
4) Neuropathic pain

Acute physiological nociceptive pain results from the brief or acute noxious mechanical, chemical and/or thermal elicitation of the peripheral receptors. This type of pain defends the tissue from further damage as the withdrawal reflexes are stimulated (Schaible and Richter, 2004). Furthermore, **pathophysiological nociceptive pain** results as the tissue is inflamed or damaged. The spontaneous pain (pain in the absence of any stimulation) and/or hyperalgesia and/or allodynia may

appear. **Chronic pain** may be followed by neuroendocrine dysregulation, fatigue, dysphoria and diminished physical and mental performance (Chapman and Gavrin, 1999). Chronic pain may also result from chronic diseases and possibly result from persistent pain processes. Last but not least, **neuropathic pain** is caused by the neuronal injury or disease in the peripheral or central nervous system. Neuropathic pain usually transmits abnormal signals felt as burning sensation which tends to be acute or prolonged. It may also be combined with allodynia and hyperalgesia. Several pathological processes may lead to neuropathic pain namely axotomy or nerve damage (e.g spinal cord injury, post-mastectomy pain, post-operative hernia repair pain and other types of post-surgical pain), carpal tunnel syndrome, central pain syndrome (e.g stroke and multiple sclerosis), degenerative disc disease (e.g. arthritis), diabetic neuropathy, phantom limb pain, postherpetic neuralgia (shingles), pudendal neuralgia, sciatica, trigeminal neuralgia, Guillain-Barre syndrome, cancer, kidney disorders, alcohol and human-immunodeficiency virus (HIV) (Jacques, 2017). Thus, in the present study, we focus in detail on neuropathic pain as the major pathological process resulting from DM.

2.2 Neuropathic pain

Neuropathic pain, according to Merskey (1986), is defined as 'pain initiated or caused by a primary lesion or dysfunction in the nervous system'. The pain is produced without any stimulation to the nociceptors or inappropriate response to the stimulation of nociceptors (Brannagan III, 2013). The person experiencing neuropathic pain would complain the feeling of burning, lancinating, stabbing, cramping and aching sensation (Bridges et al., 2001) which could be paroxysmal or continuous (Schaible and Richter, 2004). Individuals with neuropathic pain often exhibit hyperalgesia, allodynia or

hyperpathia (Ossipov et al., 2000, Brannagan III, 2013). Axotomy or nerve or plexus damage, metabolic diseases such as DM or herpes zoster are some of the pathological processes leading to neuropathic pain (Schaible and Richter, 2004). It is reported that neuropathic pain is amongst the most difficult types of chronic pain to be treated (Leung and Cahill, 2010) and impairs the quality of life.

There are several animal models developed to represent various types of neuropathic pain. Numerous studies have applied total nerve transaction and ligation to induce the clinical condition of amputation (Wall et al., 1979). Furthermore, partial nerve ligation (Seltzer et al., 1990) and spared nerve injury (Decosterd and Woolf, 2000) have been used to simulate the clinical form involving partial peripheral nerve injury. Meanwhile, spinal nerve ligation is developed to mimic the spinal root damage to a lumbar disc herniation (Kim and Chung, 1992). Immune or toxin-mediated demyelination induces demyelinating neuropathy (Wallace et al., 2003). Furthermore, polyneuropathy due to tumour chemotherapy is mimicked by applying vincristine, paclitaxel and cisplatin to the animal models (Polomano et al., 2001, Peltier and Russell, 2002, Quasthoff and Hartung, 2002). Last but not least, diabetic neuropathy is mimicked in animal models by damaging the rodent's pancreatic insulin-producing cells using STZ (Courteix et al., 1993, Rondon et al., 2010).

2.2.1 Mechanism of neuropathic pain

2.2.1(a) Spinal mechanism

Peripheral sensitization

In the normal physiological state, pain sensation is usually elicited by C- and Aδ-primary afferent neurons. These two fibres are normally silent in the absence of pain stimulation. After the occurrence of a peripheral lesion, however, the dramatic

molecular and cellular changes at the level of primary afferent nociceptor results in abnormal oversensitivity of these neurons. Thus, these changes lead to the development of pathological spontaneous activity (Baron, 2006) and a large increase in the level of spontaneous firing in afferent neurons associated with the site of nerve injury (Bridges et al., 2001).

The nerve lesion also causes changes in voltage-gated sodium channels that are selectively expressed in the nociceptive primary afferent. The damaged, degenerated axons, as well as the intact axons in the peripheral nerves as a result of the lesion triggers the expression of sodium channels on the damaged C-fibres (Figure 2.4) (Baron, 2006). The accumulation of sodium channel clusters occurs at the site of nerve lesion as well as within the intact dorsal root ganglion. This Na^+ accumulation leads to the occurrence of ectopic discharge (Matzner and Devor, 1994). This effect leads to the lowering of action potential threshold followed by the occurrence of hypersensitivity (Lai et al., 2003).

Apart from the changes in sodium channels, changes of calcium channels after peripheral nerve injury has also been demonstrated to affect the development of allodynia and hyperalgesia (Bridges et al., 2001). The diminution of high-voltage activated N-type calcium ion (Ca^{2+}) channels together with *de novo* synthesis of rapidly repriming III channels and down-regulation of tetradotoxin-resistant sodium ion (Na^+) channels after peripheral nerve injury amplifies the excitability of the neurons. These effects, in turn, may subsequently lead to an increase in firing susceptibility and frequency (indicated by the yellow stars in orange-coloured neurons in Figure 2.4) that may result in spontaneous pain and central sensitization (Bridges et al., 2001).

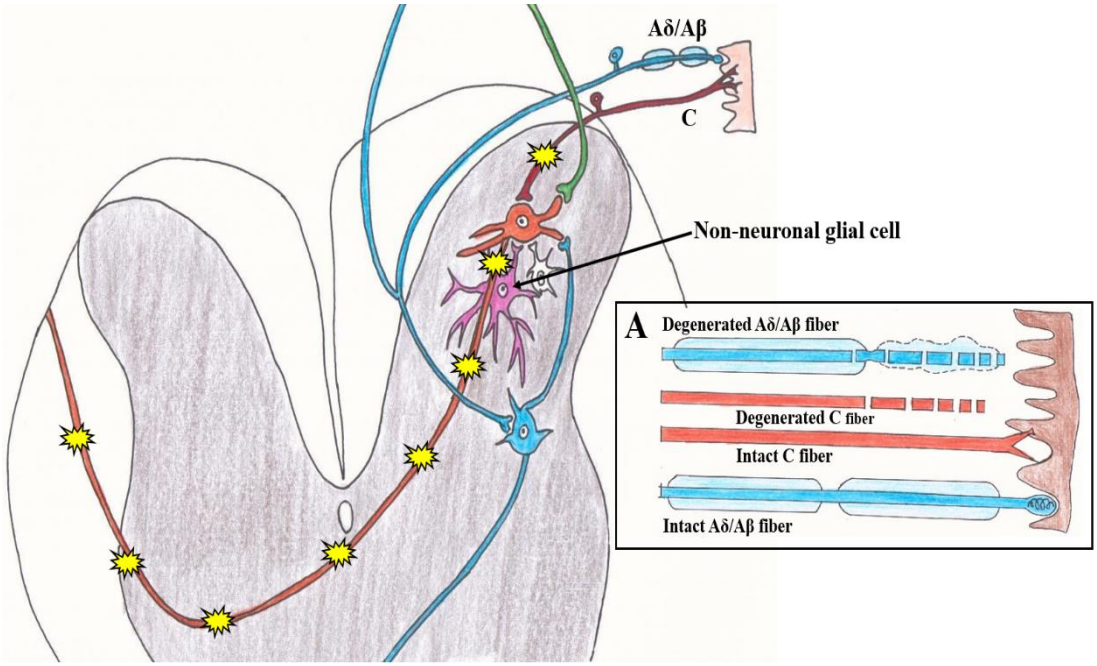

Figure 2.4 Mechanisms of peripheral and central sensitization in neuropathic pain. C-fibres (red) terminate at spinothalamic projection neurons in upper laminae (orange neuron), while non-nociceptive myelinated A-fibers (blue) project to the deeper laminae. The second-order projection neuron is of the wide dynamic range (WDR) type that receives direct synaptic signals from nociceptive terminals and also multi-sinaptic signal from myelinated A-fibers. γ-aminobutyric acid (GABA)-releasing interneurons (green) usually exert inhibitory synaptic input on the WDR neuron. Moreover, descending modulatory systems synapse at the WDR neuron. Spinal cord glial cells (pink) also communicate with the WDR neuron. Peripheral changes at primary afferent neurons after peripheral nerve damage leads to peripheral sensitization (A). Some axons are damaged and degenerated while others are still intact and connected with the peripheral end organ (skin). The lesion triggers the expression of sodium channels on damaged C-fibres. The spontaneous activity of C-fibres induce secondary changes in central sensory processing, contributing to spinal cord hyperexcitability (central sensitization of second-order WDR neurons (indicated by yellow stars in orange-coloured neuron). This causes signals from mechanoreceptor A fibres to be perceived as pain (mechanical allodynia). Inhibitory interneurons and descending modulatory control systems (green neuron) are dysfunctional after the nerve damage, thus leading to disinhibition or facilitation of spinal cord dorsal horn neurons and further central sensitization. The peripheral nerve injury also induces spinal cord non-neuronal glial cells (pink) which further augment excitability in WDR neurons by releasing cytokines and increasing glutamate levels. Adapted from Baron (2006).