THE RELATIONSHIP BETWEEN PROTEIN AND MRNA EXPRESSION OF TRANSCRIPTION REPRESSOR *DREAM*, C-FOS AND PRODYNORPHIN IN MODULATING PAIN RESPONSES INDUCED BY FORMALIN IN THE RAT SPINAL CORD

by

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AMPA	: 2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
AP-1	: activator protein-1 complex
ATP	: adenosine triphosphate
AD	: Alzheimer's disease
APS	: ammonium persulphate
ANOVA	: analysis of variance
ACG	: anterior cingular cortex
AA-NAT	: arylalkylamine N-acetyltransferase
ABC	: avidin-biotin peroxidase method
BCA	: bicinchoninic acid protein assay
BSA	: bovine serum albumin
CGRP	: calcitonin gene-related peptide
CaM kinase	: calcium-calmodulin dependent kinases
CRE	: calcium-cyclic adenosine monophosphate response element
CS	: calsenilin protein
αCREM	: cAMP response-element modulator alpha
cDNA	: complementary DNA
CREB	: cyclic adenosine monophosphate response element binding protein
CBP	: CREB-binding protein
cAMP	: cyclic adenosine monophosphate
CER II	: cytoplasmic extraction reagent II
DAB	: diaminobenzidine

DRASIC : acid sensing ion channel subtype

DEPC : diethylpyrocarbonate

DLPT : dorsolateral pontomesencephalic tegmentum

DRE : downstream regulatory element

DREAM : downstream regulatory element antagonistic modulator protein

EtBr : ethidium bromide

EDTA : ethylenediamine-tetra acetic acid

EAAs : excitatory amino acids

EPSPs : excitatory postsynaptic potentials

FLI : fos like immunoreactive

GABA : gamma-aminobutyric acid

IEG : immediate early gene

IDV : integrated density values

IL-3 : interleukin-3

IASP : international association for the study of pain

KA : kainate

KchIP3 : potassium channel α subunit interacting protein

LSD : least significance different test

LARUSM : Laboratory Animal Research Unit, Universiti Sains Malaysia

LCDs : leucine charged domains

mRNA : messenger of ribonucleic acid

MDEG : acid sensing ion channel subtype of the degenerin family

MGluRs : metabotrophic glutamate receptors

MAP kinase : mitogen activated kinases

TEMED : N, N, N’N’-Tetramethylenediamine

NGF : nerve growth factor

NKA : neurokinin A

NKB : neurokinin B

NK-1 : neurokinin-1

NK-2 : neurokinin-2

NK-3 : neurokinin-3

NMDA : N-methyl-D-aspartate receptor

NS : nociceptive specific neurons

NFQ : nonfluorescent quencher

NorBNI : norbinaltorphimine

NER : nuclear extraction reagent

NGCα : nucleus gigantocellularis pars α

NRM : nucleus raphe magnus

NRG : nucleus reticularis gigantocellularis

PFA : paraformaldehye

PB : phosphate buffer

PBS : phosphate-buffered saline

PI3K : phosphatidylinositol 3-kinase

PDGF : platelet-derived growth factor

PAF : primary afferent fibres

PLI : prodynorphin-like immunoreactivity

PN : projecting neurons

PKA : protein kinase A

PKC : protein kinase C

PSD-95 : postsynaptic density protein

VRL-1 : receptor-like channel receptors

RVM : rostral ventromedial medulla

SERCA : sarco/endoplasmic reticulum calcium ATPase

SRE : serum response element

SIE : sis-inducible element

SDS : sodium dodecyl sulphate

SDS-PAGE : sodium dodecyl sulphate polyacrylamide gel

SMT : spinomesencephalic tract

SRT : spinoreticular tract

STT : spinothalamic tract

SPSS : statistical package of social sciences software

SP : substance P

TBE : tris base boric acid ethylenediaminetetracetic acid

TBS : tris base saline

TBST : tris buffer saline

TBS-T20 : tris buffer saline-tween 20

TBS/TX : tris-triton

TNF : tumour necrosis factor

UNG : uracyl-N-glycosylase enzyme

VR1 : vanilloid receptor

VLF : ventrolateral funiculus

WDR : wide dynamic range neurons

PERKAITAN DI ANTARA EKSPRESI PROTEIN DAN MRNA BAGI PENINDAS TRANSKRIPSI *DREAM*, *C-FOS* DAN PRODYNORPHIN DALAM MEMINDA TINDAK BALAS KESAKITAN YANG DICETUSKAN OLEH FORMALIN DALAM KORDA SPINA TIKUS

ABSTRAK

Protein Downstream Regulatory Element Antagonist Modulator (DREAM) bertindak sebagai penindas transkripsi bagi gen *c-Fos* dan prodynorphin yang terlibat dalam proses kesakitan. Kajian ini dijalankan untuk mengesan perkaitan di antara ekspresi protein dan mRNA bagi DREAM, *c-Fos* dan prodynorphin di dalam meminda tindak balas kesakitan yang dicetuskan oleh formalin melalui reseptor opioid dan bukan-opioid dalam korda spina tikus. Tikus jantan Sprague Dawley seberat 250-350 g diagihkan kepada 5 kumpulan besar. Kumpulan 1 mengandungi tikus yang dirawat dengan pemberian ketamin pra-emptif (drug anestetik) (5 mg/kg berat badan) secara intraperitoneum dan selepas 30 minit diberikan suntikan formalin (Kumpulan K+F) (n=36). Kumpulan 2 mengandungi tikus yang dirawat dengan pemberian norbinaltorphimine dihydrochloride (norBNI) pra-emptif (perencat reseptor kappa opioid) (2 mg/kg berat badan) secara intraperitoneum dan selepas 24 jam diberikan suntikan formalin (Kumpulan N+F) (n=36). Tikus dalam kumpulan 3 dirawat dengan pemberian ketamin dan norBNI pra-emptif, secara intraperitoneum dan diberikan suntikan formalin (Kumpulan NK+F) (n=36). Tikus dalam kumpulan 4 hanya dirawat dengan pemberian salin normal dan diberikan suntikan formalin (Kumpulan F) (n=36) manakala tikus dalam kumpulan 5 tidak diberikan sebarang rawatan dan dianggap sebagai kumpulan kawalan (Kumpulan C) (n=18). Setiap kumpulan kecuali kumpulan kawalan diagihkan kepada subkumpulan tikus (n=6) yang dibunuh pada 2 dan 4 jam selepas suntikan formalin. Lima peratus formalin

(1.85% larutan formaldehyde) dengan isipadu 50 µl disuntik secara subkutaneus pada permukaan plantar kaki belakang sebelah kiri tikus sebagai rangsangan kesakitan. Seterusnya, kelakuan tindak balas kesakitan direkodkan selama 1 jam untuk kumpulan F, K+F, N+F dan NK+F. Dua dan 4 jam selepas suntikan formalin, tikus dibunuh dan korda spina di bahagian segmen lumbar L4/L5 dikeluarkan untuk analisis immunohistokimia, Western blot dan Real-Time PCR. Rangsangan kesakitan (suntikan formalin) dalam kajian ini meningkatkan jumlah bilangan neuron bagi FLI dan PLI selari dengan peningkatan aras relatif mRNA bagi *c-Fos* dan prodynorphin 2 jam selepas suntikan formalin di sisi ipsilateral. Purata aras relatif protein daripada ekstrak nukleus dan aras relatif mRNA bagi DREAM turut meningkat pada masa ini. Kesan ini berkemungkinan menyumbang kepada peningkatan kelakuan tindak balas kesakitan semasa fasa tonik ujian formalin. Walau bagaimanapun pemberian ketamin pra-emptif menghalang peningkatan jumlah bilangan neuron bagi FLI dan PLI serta aras relatif mRNA bagi *c-Fos* dan prodynorphin 2 jam selepas suntikan formalin di sisi ipsilateral. Kajian ini mendapati aras relatif mRNA bagi DREAM turut mengurang manakala aras purata relatif protein bagi DREAM daripada ekstrak nukleus meningkat pada masa yang sama. Kesan ini berkemungkinan menyumbang kepada pengurangan kelakuan tindak balas kesakitan semasa fasa tonik ujian formalin dalam kumpulan ini. Pemberian norBNI pra-emptif (perencat reseptor kappa opioid) meningkatkan jumlah bilangan neuron bagi FLI dan PLI selari dengan peningkatan aras relatif mRNA bagi *c-Fos* dan prodynorphin 2 jam selepas suntikan formalin di sisi ipsilateral. Kesan ini mengakibatkan pengurangan aras purata relatif protein bagi DREAM daripada ekstrak nukleus dan aras relatif mRNA bagi DREAM pada masa ini. Selanjutnya, pemberian norBNI pra-emptif

menggalakkan kelakuan tindak balas kesakitan semasa fasa tonik dalam ujian formalin. Pemberian ketamin dan norBNI pra-emptif menghilangkan sebahagian kesan pemberian pra-emptif bersendirian ketamin dan norBNI. Penemuan ini mencadangkan bahawa kedua-dua reseptor bagi NMDA dan kappa opioid terlibat dalam meminda proses kesakitan yang akut dalam kajian ini. Walau bagaimanapun perubahan ke atas protein dan mRNA bagi *c-Fos* dan DREAM tidak berpanjangan dan berbalik semula ke asal selepas 4 jam kecuali perubahan ke atas ekspresi protein dan mRNA bagi prodynorphin. Penemuan ini membuktikan bahawa ekspresi protein dan mRNA bagi prodynorphin adalah penting untuk mekanisma kesakitan yang berpanjangan. Aras purata relatif protein bagi DREAM daripada ekstrak sitoplasma tidak terjejas dalam setiap kumpulan eksperimen dalam kajian ini pada 2 dan 4 jam selepas suntikan formalin. Sebagai kesimpulan, kajian ini mencadangkan bahawa protein DREAM bertindak sebagai perencat transkripsi gen bagi *c-Fos* dan prodynorphin dan boleh dipinda oleh tindakan reseptor bagi NMDA dan kappa opioid semasa kesakitan akut. Ini berkemungkinan dicapai melalui perubahan lokasi protein DREAM di dalam nukleus atau sitoplasma sel neuron. Proses penting ini membenarkan peningkatan atau pengurangan transkripsi gen *c-Fos* dan prodynorphin serta ekspresi proteinnya dalam korda spina tikus semasa proses kesakitan yang akut.

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ABSTRACT

Downstream Regulatory Element Antagonist Modulator (DREAM) protein acts as a transcription repressor for *c-Fos* and prodynorphin gene which is involved in modulating pain processes. This study was conducted to investigate the relationship between DREAM, *c-Fos*, prodynorphin protein and mRNA expression through opioid and non-opioid receptors in the modulation of pain responses induced by formalin in the rat spinal cord. Male Sprague Dawley rats weighing between 250-300 g were divided into five major groups. Group 1 consisted of rats treated with pre-emptive administration of ketamine (anaesthetic drug) (5 mg/kg body weight) intraperitoneally (i.p) and given formalin injection (K+F group) (n =36), 30 minutes later. Group 2 consisted of rats treated with pre-emptive administration of norbinaltorphimine dihydrochloride (norBNI) (kappa opioid receptor antagonist) (2 mg/kg body weight) i.p and given formalin injection (N+F group) (n = 36) after 24 hours. Rats in group 3 were treated with pre-emptive administration of both ketamine and norBNI i.p and given formalin injection (NK+F group) (n=36). Rats in group 4 were only treated with normal saline and given formalin injection (F group) (n= 36) while rats in group 5 were not given any treatment and considered as a control group (C group) (n =18). Each experimental group except the control group was further divided into subgroups (n = 6) consisting of rats that were sacrificed at 2 and 4 hours after formalin injection. Fifty µl of formalin 5% (1.85% formaldehyde solution), was subcutaneously injected into the plantar surface of the left hind paw of the rat.

The pain behaviour responses were recorded for one hour for rats in the C, F, K+F, N+F and NK+F groups. The rats were then sacrificed 2 and 4 hours after formalin injection and the lumbar L4/L5 segments of spinal cords were removed for immunohistochemistry, Western blot and Real-Time PCR analysis. Noxious stimuli (formalin injection) in this study increased the total number of FLI and PLI neurons, consistent with an increase in relative *c-Fos* and prodynorphin mRNA levels at 2 hours after formalin injection on the ipsilateral side. The mean relative DREAM protein in the nuclear extract and relative DREAM mRNA levels were also increased at the same time. These effects probably contributed to increases in pain behaviour responses during the tonic phase of the formalin test. However, pre-emptive administration of ketamine prevented the increase of total number of FLI and PLI neurons and relative *c-Fos* and prodynorphin mRNA level at 2 hours after formalin injection on the ipsilateral side. At the same time, this study found that the relative DREAM mRNA level was decreased but the mean relative DREAM protein level in the nuclear extract was increased at this time. These effects may reduce pain behavior responses during the tonic phase of the formalin test in this group. Pre-emptive administration of norBNI (kappa opioid receptor antagonist) increased the total number of FLI and PLI neurons, consistent with the relative *c-Fos* and prodynorphin mRNA level at 2 hours after formalin injection on the ipsilateral side. These effects resulted in decreased mean relative DREAM protein level in the nuclear extract and relative DREAM mRNA level at this time. Furthermore, pre-emptive administration of norBNI enhanced pain behaviour responses during the tonic phase of the formalin test. Pre-emptive administration of both ketamine and norBNI eliminated some of the effects of pre-emptive administration of ketamine and norBNI. These findings

suggest that both NMDA and kappa opioid receptors are involved in modulating acute pain responses in this study. However, the changes in *c-Fos* and DREAM mRNA and protein expression at 2 hours are not prolonged and reverse to the basal state at 4 hours except for the changes in prodynorphin mRNA and protein expression. These findings suggest that prodynorphin mRNA and protein expression are important in persistent pain mechanisms. In addition, the mean relative DREAM protein in the cytoplasm extract was unaffected in each of the experimental groups in this study after 2 and 4 hours following formalin injection. In conclusion, this study shows that DREAM protein acts as a transcription repressor for *c-Fos* and prodynorphin gene and can be modulated by NMDA and kappa opioid receptor action during acute pain. The modulation is achieved through changes in the localization of DREAM protein in the nucleus or cytoplasm of neurons. This is an important mechanism which permits the upregulation and downregulation of *c-Fos* and prodynorphin gene and its protein expression in the rat spinal cord during acute pain processes.

CHAPTER 1

INTRODUCTION

1.1 PAIN

Pain is a universal phenomenon that affects millions of people around the world. Pain is a highly disagreeable sensation which results from a highly complex and interactive series of mechanisms integrated at all levels of the neuroaxis, from the periphery, via the dorsal horn to the higher cerebral structures (Millan, 1999). The International Association for the Study of Pain (IASP) defines pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Loeser and Melzack, 1999).

Pain consists of two separate phenomena: physiological pain and pathological pain. Under normal circumstances, physiological pain occurs as a result of intense or potentially damaging noxious stimuli, which activates the nociceptors. It is highly localized and transient with the main purpose of informing the body of potential danger. On the other hand, pathological pain arises as a result of tissue or nerve damage. It persists long after healing of the damaged peripheral tissue has occurred and can lead to chronic pain. Pathological pain in its acute form is usually associated with tissue damage and inflammation (inflammatory pain) and has a protective function. In its chronic form, pathological pain is usually associated with nerve damage (neuropathic pain). In this case, the pain no longer has any adaptive function at all (Loeser and Melzack, 1999).

1.2 PRIMARY AFFERENT FIBRES

Pain is initiated by specialized primary afferent fibres (PAF) called sensory nociceptor fibres which innervate peripheral tissues in all regions of the head and body. These nociceptor fibres are activated only by noxious stimuli (painful) and have characteristic thresholds or sensitivities that distinguish them from other sensory nerve fibres. In general, PAF are categorized into three main groups based on their structure, diameter and conduction velocity (Julius and Basbaum, 2001, Bessou *et al*., 1971).

1) Polymodal nociceptors, consisting of C unmyelinated axons, thin, 0.4 ± 1.2mm in diameter and slowly conducting at rates of 0.5 ± 2.0 m/s.
2) High threshold mechanoreceptors, consisting of myelinated Aδ axons, 2 ± 6 mm in diameter and conduct at rates of 12 ± 30 m/s.
3) Aβ fibres, >10mm in diameter, myelinated and fast conducting at rates of 30 ± 100 m/s.

The Aß-fibres detect innocuous stimuli (non-painful) and thus are not involved in pain processing (Djouhri *et al.*, 1998). However, noxious stimuli from various kinds of stimuli (mechanical, thermal and chemical) are processed by thinly myelinated (Aδ-fibres) and unmyelinated (C-fibres) afferent fibres. The nociceptive transducer receptor/ion channel complexes that are present in the terminals of these nociceptive fibres become activated and convert the noxious stimuli into depolarizing currents (Woolf and Salter, 2000). Examples of transducer proteins that respond to extrinsic or intrinsic irritant chemical stimuli include vanilloid receptor (VR1), acid sensing ion channel subtype (DRASIC), G-protein coupled adenosine triphosphate (ATP) receptors and ATP-gated ion

channel (P2Y and P2X) receptors (Caterina *et al.*, 1997). Transducers responsible for moderate thermal nociception are characterized by VR1 receptors and for high threshold heat responses are mediated by vanilloid receptor-like channel (VRL-1) receptors (Caterina *et al.*, 1999). A transducer for noxious mechanical stimuli has not been identified, although studies suggest that it may belong to the acid sensing ion channel subtype of the degenerin family (MDEG) (Waldmann and Lazdunski, 1998).

Noxious stimuli initiate receptor potentials, which then lead to the initiation of a depolarizing current. When the depolarizing current is sufficient enough to reach threshold potential, an action potential is initiated. The activated thin myelinated Aδ or unmyelinated C-fibre then transmits this action potential from peripheral tissues to the spinal dorsal horn and then projects the action potential to the cerebral cortex to elicit the pain response (Julius and Basbaum, 2001). Stimulation of nociceptors will also activate the withdrawal reflex with an increase in arousal as well as emotional, autonomic and neurohumoral responses (Woolf and Salter, 2000).

1.3 PERIPHERAL SENSITIZATION

Under normal circumstances, nociceptor-mediated pain only occurs in response to high intensity noxious stimuli. Following peripheral tissue injury, the inflammatory reaction generates a complex set of chemical signals that alters the transduction properties of nociceptors such that they can be activated by low intensity stimuli. This phenomenon is known as peripheral sensitization. This mechanism is achieved partly from the production and release of chemical

mediators from primary sensory terminals and from non-neural cells (for example, fibroblasts, mast cells, neutrophils and platelets) (Figure 1.1) (Woolf, 1991). Numerous chemicals that mediate or facilitate the pain process include bradykinin, prostaglandin, leukotriene, serotonin, histamine, substance P (SP), thromboxane, platelet-activating factor, adenosine and ATP, protons and free radicals. Cytokines, such as interleukins and tumour necrosis factor (TNF) and neurotrophins especially nerve growth factor (NGF), are also generated during pain processing (Meyer *et al*., 2006). Some of these agents can directly activate nociceptors, while others act indirectly through second messenger signalling cascades (Woolf and Salter, 2000). In addition, nociceptors that release SP and calcitonin gene-related peptide (CGRP) also induce vasodilation and plasma extravasations (leakage of protein and fluid from post-capillary venules) as well as activation of many non-neuronal cells, including mast cells and neutrophils. The variety of chemical mediators released during inflammation can have a synergistic effect in the potentiation of nociceptor responses (Meyer *et al.,* 2006).

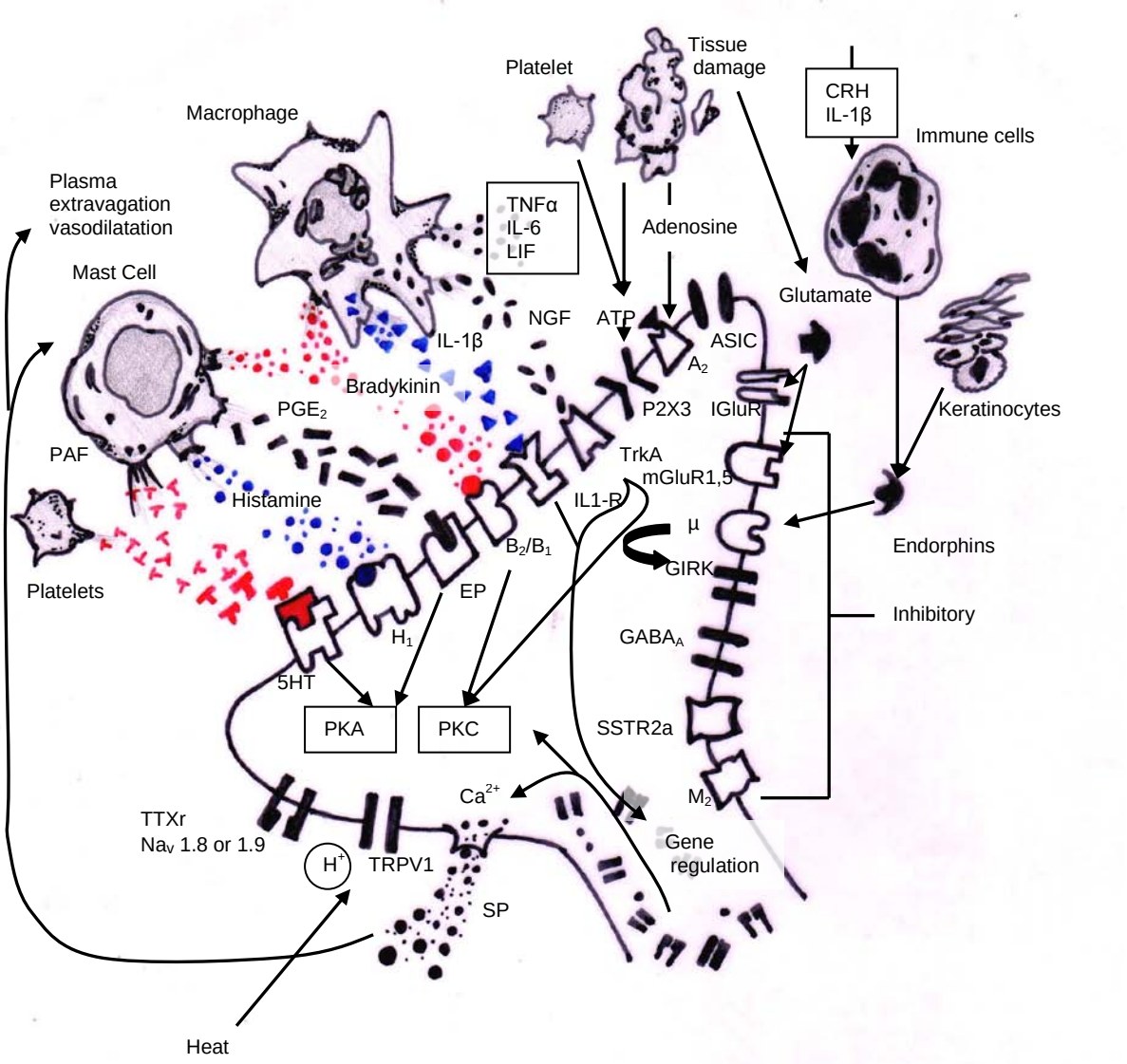

Figure 1.1: Potential peripheral mediators of peripheral sensitization after inflammation. Adapted from Woolf and Costigan, (1999).

1.4 PAIN PROCESSES IN THE SPINAL CORD

1.4.1 Types of neurons in the spinal cord

The dorsal horn of the spinal cord is the major receiving zone for PAF that transmits information from sensory receptors in the skin, viscera, joints and muscles of the trunk and limbs to the central nervous system. In the dorsal horn, PAF enter and ascend 1 or 2 segments in Lissauer's tract, and terminate in the grey matter of the dorsal horn. Fibre terminations are segregated in laminae according to their functional properties (Cross, 1994). High threshold C and Aδ-fibres terminate predominantly in laminae I, II and V, while low-threshold Aß mechanoreceptors terminate in deeper laminae (Willis, 2007).

Neurons in the dorsal horn of the spinal cord can be divided into three general classes: projecting neurons, propriospinal neurons and local interneurons (Willis and Coggeshall, 1991). The projecting neurons are the second order nociceptive neurons with cell bodies in the dorsal horn and axons that terminate in the thalamus. There are generally two types of projecting neurons: nociceptive specific (NS) neurons which respond specifically to noxious stimuli and wide dynamic range (WDR) neurons which respond to both noxious and innocuous stimuli (Riedel and Neeck, 2001). NS neurons are mainly located in the superficial aspects of the dorsal horn, outer aspect of lamina II and in lamina V and VI while WDR neurons are located in the deep dorsal horn (lamina IV-V) (Willis, 2007). The axons of both types of neurons cross the midline within 1 or 2 segments and ascend in the spinothalamic tract (STT) which is one of the major tracts involved in conveying nociceptive information to the higher centres.

The role of propriospinal neurons in nociception is poorly understood. However, it is known that these neurons are able to act as a multisynaptic pathway that transfers information to the brain. They have a major role in controlling locomotion and in organizing coordinated reflex responses while the majority of intrinsic dorsal horn neurons are local interneurons which send their axons for only a short distance within the spinal cord and comprise both excitatory and inhibitory interneurons (Powell and Todd, 1992).

1.4.2 Central sensitization

The activity-dependent alteration or plasticity in the dorsal horn neurons is termed central sensitization. Figure 1.2 outlines the mechanism of central sensitization where nociceptors respond in an abnormal or exaggerated way to afferent input (Ji *et al.*, 2003, Woolf, 1991). When dorsal horn excitability is increased, their response to sensory input is facilitated or sensitized, producing a state of hypersensibility (Ji *et al.*, 2003, Woolf and Salter, 2000). A low-intensity stimulus acting via low threshold afferents can now generate pain via a phenomenon known as allodynia, and noxious inputs result in a pain response that is augmented in amplitude (hyperalgesia) and duration (hyperpathia) (Koltzenburg *et al.*, 1994).

1.4.3 Synaptic transmission in the dorsal horn

Several neurotransmitters are released for nociceptive transmission in the neuron at the dorsal horn of the spinal cord (Figure 1.2). There are excitatory amino acids (EAAs), e.g. glutamate and aspartate that are released from the terminals of primary afferents, intrinsic interneurons and projection fibres (Gilron

and Coderre, 1997). The effects of the EAA actions are mediated via both metabotrophic glutamate receptors (mGluRs) and ionotropic receptors on the postsynaptic neurons of the dorsal horn of the spinal cord (Kawasaki *et al.*, 2004). There are three types of ionotropic receptors: 2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors (Dickenson and Sullivan, 1991). There are also a number of peptides involved in nociception, e.g. tachykinins (neurokinin A (NKA) and neurokinin B (NKB)), CGRP, somatostatin, gamma-aminobutyric acid (GABA), neurotensin, avian pancreatic peptide, cholecystokinin and neuropeptide Y (Cross, 1994). Postsynaptic receptors, which bind tachykinins, are neurokinin-1 (NK-1), neurokinin-2 (NK-2) and neurokinin-3 (NK-3) (Gilron and Coderre, 1997).

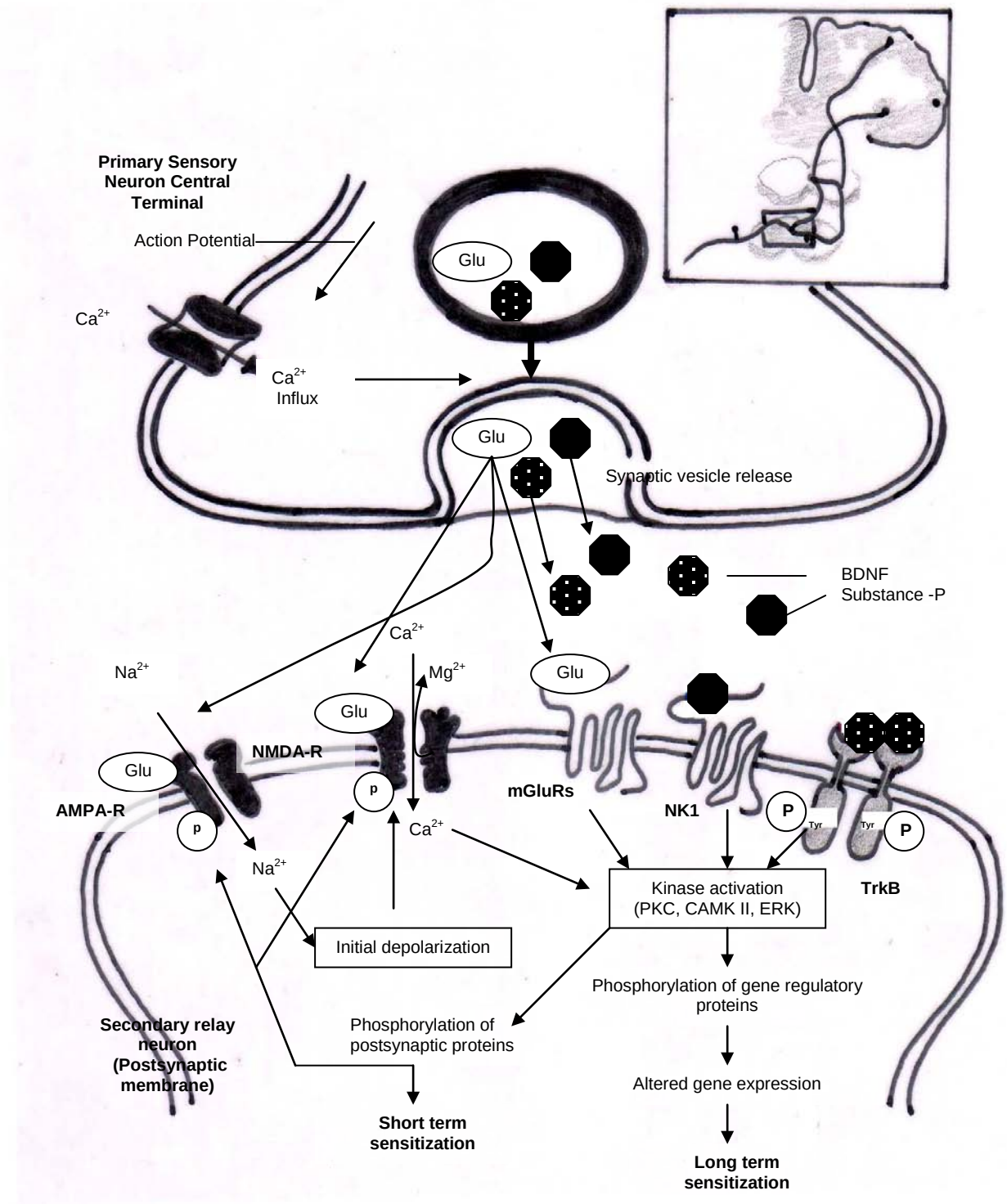

Figure 1.2: Central sensitization and synaptic transmission in the dorsal horn of the spinal cord. Adapted from Griffin and Woolf, (2005).

1.4.4 Ascending pathway

The ascending pain pathway transmits nociceptive information from peripheral tissue to the cerebral cortex for interpretation. The classic ascending pain pathway is the spinothalamic tract (STT). The STT consists of a three neuron chain that transmits pain information form the periphery to the cerebral cortex via the thalamus (Figure 1.3) (Renn and Dorsey, 2005, Cross, 1994). The first order neuron has its cell body in the dorsal root ganglion with two axons, one extending distally to the tissue it innervates while the other extends proximally to the dorsal horn of the spinal cord. In the dorsal horn, this axon synapses with the second order neuron that in turn will cross the spinal cord through the anterior white commissure and ascends through the spinothalamic tract to the thalamus. The majority of the projection neurons that travel in the STT originate in the superficial laminae I and II and deeper laminae V of the spinal dorsal horn (Apkarian and Hodge, 1989). In the thalamus, the second order neuron synapses with the third order neuron, which ascends through the internal capsule and corona radiata to the postcentral gyrus of the cerebral cortex. This arrangement is call somatotopy (Windhorst, 1996).

A second prominent ascending pathway is the spinomesencephalic tract (SMT), which originates in laminae I, II and V of the spinal dorsal horn, and travels in the ventrolateral funiculus (VLF) to the midbrain. Within the midbrain, the neurons in the SMT terminate in several areas, such as the periaqueductal grey (PAG) and nucleus cuneiformis (NCF) (Figure 1.3). A third tract that has also been shown to convey nociceptive in formation is the spinoreticular tract

(SRT) which terminates in the reticular formation of the medulla (Figure 1.3) (Renn and Dorsey, 2005).

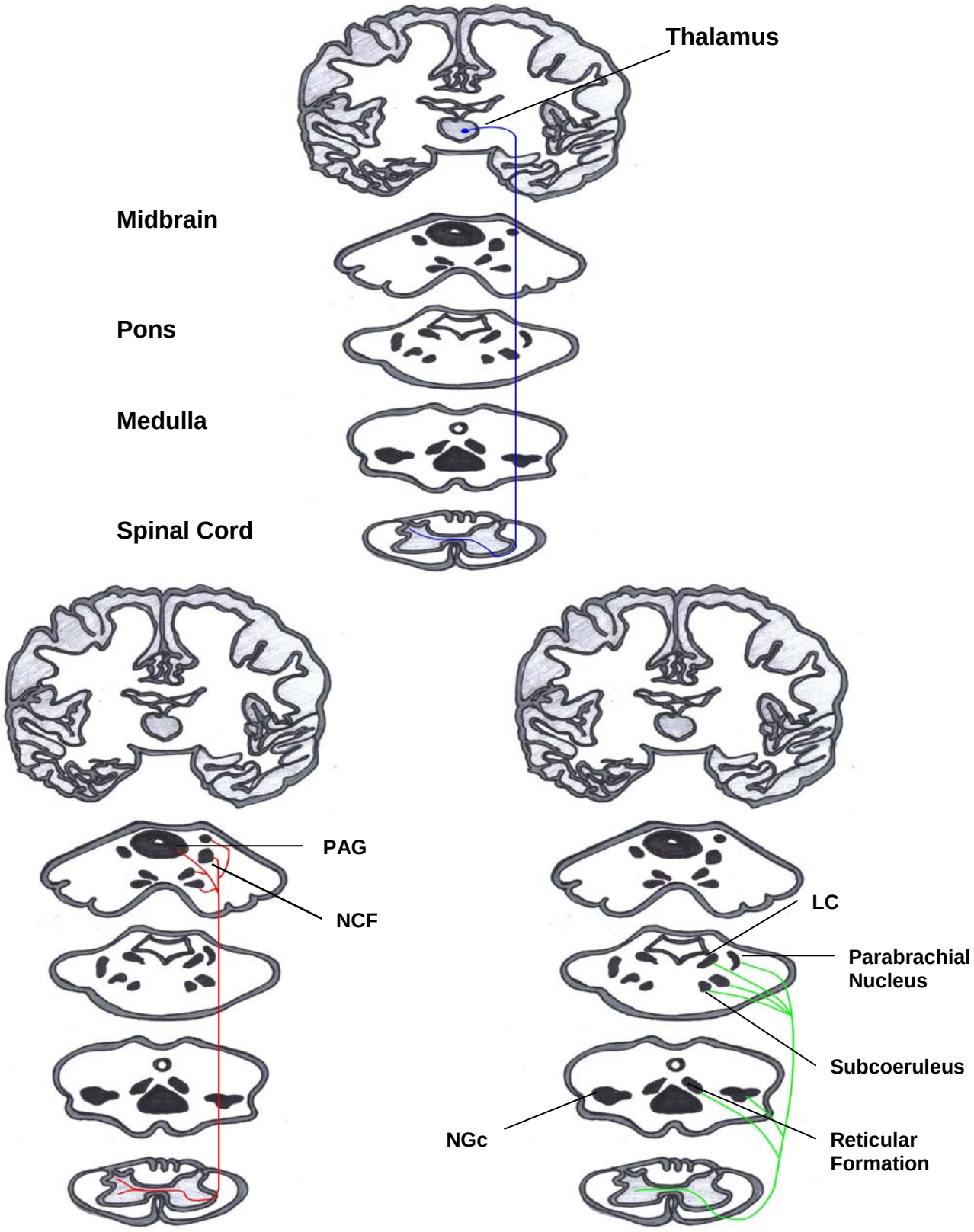

Figure 1.3: Ascending pain pathways. The spinothalamic (STT, top), spinomesencephalic (SMT, bottom left) and spinoreticular (SRT, bottom right) tracts. Adapted from Dubner and Ren, (1999).

1.4.5 Descending pathway

At the spinal cord, pain stimuli can be influenced by various endogenous mechanisms within the central nervous system both peripherally and centrally. These mechanisms can either be inhibitory or facilitatory (Millan, 2002, Dubner and Ren, 1999).

The attenuation of afferent sensory nerve activity at peripheral nerve terminals can be achieved by a neuroimmune mechanism. Inflammation of peripheral tissues will result in the upregulation of opioid receptors at peripheral sensory nerves (Stein, 1995). This is due to the release of endogenous substances such as corticotrophin releasing hormone and cytokines, which cause the expression of opioid peptides by resident immune cells in inflamed peripheral tissue. Binding of opioids to opioid receptors brings about an increase in potassium currents and a decrease in Ca^{2+} currents in cell bodies of sensory neurons causing inhibition of neuronal firing and transmitter release. Opioids also inhibit Ca^{2+} dependent release of excitatory, proinflammatory compounds such as SP from peripheral sensory nerve endings (Stein, 1995).

Central pain transmission is modulated by supraspinal sites via descending pathways and by segmental interneurons in the spinal cord (Millan, 2002). The descending pathways originate from the cortex, thalamus and brain stem (Cross, 1994, Basbaum and Fields, 1984). Forebrain structures including the medial preoptic area, the amygdala, the insular cortex and the anterior cingular cortex (ACG) modulate activity at the brainstem level which in turn project to the dorsal horn of the spinal cord (Dubner and Ren, 1999). The brain

stem analgesic system and its descending projections to the dorsal horn constitute a powerful negative feedback system that is activated by nociceptive stimulation (through the spinoreticular, spinothalamic and spinocortical pathways). This in turn, produces inhibition of the spinal transmission of nociceptive signals (Cross, 1994). The brain stem structures involved in pain modulation include the PAG, the rostral ventromedial medulla (RVM) and the dorsolateral pontomesencephalic tegmentum (DLPT) (Figure 1.4) (Dubner and Ren, 1999). These structures receive inputs or afferents from the cortex, hypothalamus, amygdala and other brainstem areas including reticular formation, NCF, locus coeruleus, and spinal cord (Millan, 2002).

The transmitters involved in the descending modulation of pain are serotonin, adrenaline, noradrenaline and the opioids (Millan, 2002). Opioids act at various levels of the descending control system to inhibit spinal nociceptive transmission. Serotonin acting at the serotonergic receptor in the nucleus raphe magnus (NRM) has an inhibitory effect on dorsal horn nociceptive neurons while noradrenaline acting at alpha2-adrenergic receptors found at the locus coeruleus inhibits dorsal horn nociceptive transmission (Li and Zhuo, 2001).

Segmental modulation occurs particularly at the spinal cord. The PAF (C, Aδ and Aß fibres) which terminate in the spinal cord can either directly stimulate projecting neurons (PN) which relay their messages to the brain, or indirectly engage PN to interneurons, which consist of excitatory and inhibitory interneurons. Descending pathways may then, modulate nociception via an interaction with several neuronal elements in the spinal cord dorsal horn such as

the terminal endings of PAF, PN, excitatory and inhibitory interneurons and terminal endings of other descending pathways (Millan, 2002).

Most inhibitory interneurons contain gamma-aminobutyric acid (GABA) and/or glycine as neurotransmitters. They synapse both presynaptically on PAF endings and postsynaptically on dorsal horn neurons (Powell and Todd, 1992). GABAergic cells make up approximately 25-30 % of the neurons in laminae I and II, and around 40% of those in lamina III (Todd and Sullivan, 1990). The inhibitory effects of GABA are mediated by two distinct classes of receptor, $GABA_A$ and $GABA_B$ (Desarmenien *et al.*, 1983). GABA mediates presynaptic inhibition by reducing the release of excitatory neurotransmitter, e.g. glutamate, SP, CGRP which are involved in nociceptive transmission in the spinal cord (Malcangio and Bowery, 1994). This indirectly inhibits the activity of Aδ and C-fibres by activating the inhibitory interneurons or inhibiting the excitatory interneurons. This in turn decreases the level of nociceptive input to higher brain areas (Loeser and Melzack, 1999). The action of GABA at post-synaptic receptors produces hyperpolarization (Malcangio and Bowery, 1996). Loss of tonic GABAergic inhibition at the level of the spinal cord is one of the factors that lead to enhanced pain sensitivity observed in models of neuropathic pain states (Miletic *et al.*, 2003) while administration of GABA agonists reverse the mechanical allodynia observed in neuropathic rats (Malan *et al.*, 2002).

In addition to these descending inhibitory pathways, there are also descending facilitatory pathways which arise from the anterior cingulated cortex (ACC) and rostoventral medial medulla (RVM) (Robinson *et al.*, 2004). ACC

activates the endogenous pain modulatory system due to its projecting connections to the PAG in the midbrain. Activation of ACC by electrical stimulation or chemical microinjection into ACC produces facilitation of spinal nociceptive reflexes (Calejesan *et al.*, 2000). The descending facilitatory modulation from ACC is most likely to be conveyed through the RVM because blockade of activity in the RVM attenuates or abolishes the facilitation. The RVM contains the raphe nuclei, adjacent complex of reticular nuclei including nuclei raphe magnus (NRM), nucleus reticularis gigantocellularis (NRG), and nucleus gigantocellularis pars α (NGCα). Stimulation of different populations of neurons within the RVM can either activate or inhibit nociceptive transmission at the level of the dorsal horn (Zhuo and Gebhart, 1991). Lesions of the NGC inhibit secondary hyperalgesia produced by application of mustard oil, suggesting that this region is involved in facilitation during inflammatory hyperalgesia (Urban *et al.*, 1996).

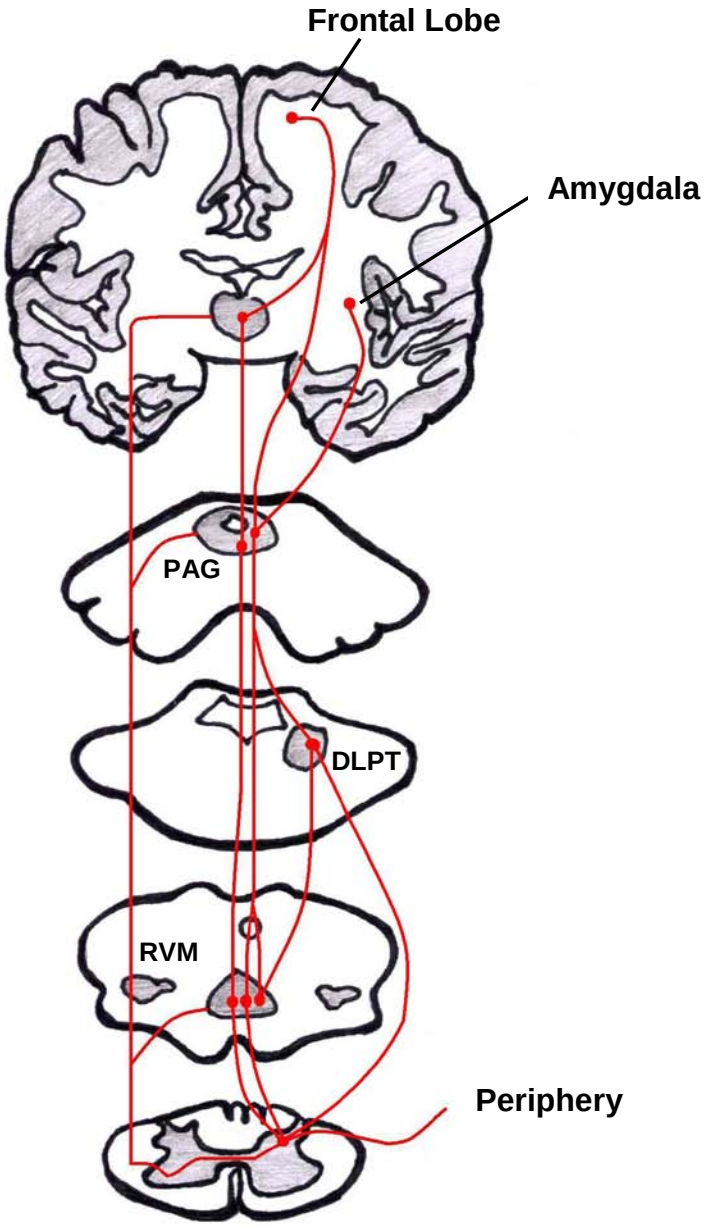

Figure 1.4: Descending pain pathways. Adapted from Dubner and Ren, (1999).

1.5 FORMALIN TEST

There are many animal models of pain that have been constructed to enable the study of the different mechanisms and forms of pain. The formalin test involves the injection of dilute formalin either in one of the forepaws (Dubuisson and Dennis, 1977) or in one of the hindpaws (Abbadie *et al.*, 1997) to elicit a nociceptive response. The hindpaw is usually chosen because it is rarely used during normal grooming behaviour, and thus may be more nociceptive-specific than the forepaw (Coderre *et al.*, 1993).

The formalin test is different from most models of pain. In this model, it is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissue (Tjolsen *et al.*, 1992). Due to this connection to tissue injury, it is believed that the formalin test provides a more valid model for clinical pain as its tonic nature makes it resemble human postoperative pain more closely (Chen *et al.*, 1989) than the tests with phasic mechanical or thermal stimuli.

Pain behaviour after formalin injection can be separated into two distinct phases. The first phase starts immediately after injection of formalin and lasts for 3-5 minutes. It is due to direct chemical stimulation of nociceptors (Dubuisson and Dennis, 1977) and predominantly evokes activity in C and not in Aδ fibres (Tjolsen *et al.*, 1992). The second phase starts approximately 15-20 minutes after formalin injection and lasts for 20-40 minutes (Tjolsen *et al.*, 1992). The injection of formalin into the hindpaw of rats increases Fos protein expression at L4/L5 segmental levels of the spinal cord (Bon *et al.*, 2002, Presley *et al.*, 1990). Fos