EFFECTS OF IFENPRODIL TREATMENT ON PAIN BEHAVIOUR, INFLAMMATION, AND NOCICEPTIVE RESPONSES IN THE SPINAL CORD OF COMPLETE FREUND'S ADJUVANT-INDUCED CHRONIC POLYARTHRITIS RAT

AIN' SABREENA BINTI MOHD NOH

UNIVERSITI SAINS MALAYSIA

2024

EFFECTS OF IFENPRODIL TREATMENT ON PAIN BEHAVIOUR, INFLAMMATION, AND NOCICEPTIVE RESPONSES IN THE SPINAL CORD OF COMPLETE FREUND'S ADJUVANT-INDUCED CHRONIC POLYARTHRITIS RAT

by

AIN' SABREENA BINTI MOHD NOH

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2024

ACKNOWLEDGEMENT

I am sincerely grateful to Allah Almighty for His guidance and blessings throughout my academic journey.

I am deeply indebted to my esteemed supervisor, Dr. Che Aishah Nazariah Ismail, Assoc. Prof. Dr. Rahimah Zakaria, Assoc. Prof. Dr. Anani Aila Mat Zin, Assoc. Prof. Dr. Idris Long, and Assoc. Prof. Dr. Che Badariah Ab Aziz, for their invaluable mentorship and support. I extend my sincerest thanks to the dedicated teams at the Physiology Laboratory, Neuroscience Laboratory, Pathology Laboratory, ARASC, and Central Research Laboratory for their unwavering support and technical expertise.

I am profoundly grateful to Universiti Sains Malaysia and the Ministry of Higher Education, Malaysia, for their generous support through the Short-Term Grant (304/PPSP/6315333) and Fundamental Research Grant Scheme (FRGS/1/2019/SKK08/USM/03/13), respectively. Additionally, I am deeply indebted to Universiti Sains Malaysia for awarding me the MSc scholarship (GRA-Assist 2020-2021), which has been instrumental in my academic progress from master's to doctorate which significantly shaped my academic pursuit and fueled my aspirations toward a doctorate in philosophy.

Lastly, I would like to express my gratitude to my esteemed colleagues, particularly Dr. Nurul Ajilah Mohamed Khir, Dr. Sameer Badri Hadi Al-Mhanna, Dr. Zaida Zakaria, Nurul Ashykin Zamri, Siti Hajar Zabri, Michelle Tiong Hui Ling, and Nur Azmina Hassan. Lastly, but most importantly, to my beloved parents and cherished siblings: your prayers and unwavering presence have been my greatest source of strength. Thank you for standing by me and helping me realise this lifelong aspiration.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

Page

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACPA	Anti-Citrullinated Peptide Antibody
AIA	Adjuvant-induced arthritis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	β-cell lymphoma-2
BDNF	Brain-derived neurotrophic factor
Ca2+	Calcium ion
CAKβ	Cell adhesion kinase β
CaM kinase	Calcium-calmodulin dependent kinases
cDNA	Complementary DNA
CFA	Complete Freund's-Adjuvant
CGRP	Calcitonin gene-related peptide
COX	Cyclooxygenase
CREB	Cyclic adenosine monophosphate response element binding protein
DMARDs	Disease-modifying anti-rheumatic drugs
DRG	Dorsal root ganglion
ELISA	Enzyme-Linked Immunosorbent Assay
EphB	Ephrin B
ERK	Extracellular signal-regulated kinase
FasL	Fas ligand
GABA	γ-aminobutyric acid
GIRK	G protein-activated inwardly rectifying potassium
GPCR	G protein-coupled receptor
H+	Hydrogen ion
IL	Interleukin
JNK	c-Jun N-terminal kinase
LTP	Long-term potentiation

Mg2+	Magnesium ion
mGluRs	Metabotrophic glutamate receptors
NF-κβ	Nuclear factor-kappa β
NGF/TrkA	Nerve growth factor-tyrosine kinase A
NMDA	N-methyl-D-aspartate
NMDAR-2B	N-methyl-D-aspartate receptor-2B
NO	Nitric oxide
NORT	Novel object recognition test
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
P2X	Purinergic receptor
P2X4R	P2X4 receptor
PAG	Periaqueductal gray
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PI3Kcb	Phosphoinositide 3-kinase-cb
PKB	Protein kinase B
pNMDAR-2B	Phosphorylated NMDAR-2B
RA	Rheumatoid arthritis
RACK1	Receptor for activated C kinase-1
RNA	Ribonucleic acid
RT-qPCR	Real-time reverse transcriptase-quantitative polymerase chain reaction
RVM	Rostral ventromedial medulla
SCI	Spinal cord injury
SEM	Standard error of mean
SFK	Serine family kinases
TBS/TX	Tris-triton
TMB	3,3’,5,5’ tetramethyl-benzidine
TNF-α	Tumour necrosis factor-α
tNMDAR-2B	Total NMDAR-2B
TRP	Transient receptor potential

LIST OF APPENDICES

KESAN RAWATAN IFENPRODIL KE ATAS TINGKAH LAKU KESAKITAN, KERADANGAN, DAN RESPON NOSISEPTIF DALAM SARAF TUNJANG TIKUS POLIARTRITIS KRONIK YANG DIARUHKAN OLEH PEMBANTU LENGKAP FREUND

ABSTRAK

Artritis reumatoid (RA) adalah penyakit keradangan kronik di mana kesakitan adalah gejala yang sering dilaporkan. Ifenprodil iaitu sejenis perencat terpilih bagi reseptor N-methyl-D-aspartate-2B (NMDAR-2B) telah menunjukkan kesan anti-kesakitan yang ketara dalam model kesakitan kronik. Kajian ini bertujuan untuk menyiasat kesan Ifenprodil sebagai perencat NMDAR-2B ke atas tingkah laku kesakitan, keradangan, dan respon nosiseptif dalam saraf tunjang tikus poliartritis yang diaruhkan oleh pembantu lengkap Freund dan kemungkinan kesan sampingannya terhadap fungsi ingatan. Tikus artritis menerima rawatan intratekal sama ada Ifenprodil (0.5 atau 1.0 μg/μL) atau Diclofenac (6 μg) (kawalan positif) manakala tikus-tikus kawalan artritis (A) dan bukan artritis (C) diberikan air garam 0.9% selama 7 hari (hari ke-16 hingga -22 selepas diaruhkan). Diameter dan lilitan sendi buku lali, ujian tingkah laku kesakitan termasuk ujian-ujian von-Frey dan plat panas, skor pergerakan serta ujian ingatan telah dijalankan pada hari ke-0 (nilai asas), -15 (sebelum rawatan) dan -23 (selepas rawatan). Pemeriksaan histopatologi dilakukan pada sendi buku lali ipsilateral manakala kawasan lumbar saraf tunjang (L4-L5) diambil untuk penganalisaan ELISA, imunohistokimia dan RT-qPCR. Kumpulan yang menerima Ifenprodil (0.5 μg/μL) menunjukkan peningkatan berat badan yang tidak signifikan dan tiada perubahan dalam jumlah pengambilan makanan, pengurangan hyperalgesia haba dan alodinia sentuhan dengan penambahbaikan pergerakan serta peningkatan ketara dalam struktur tisu kaki belakang dan sendi buku lali ipsilateral berbanding dengan tikus

artritis yang menerima Ifenprodil pada dos 1.0 μg/μL. Sementara itu, Ifenprodil telah menurunkan tahap protein NMDAR-2B, bahan P, BDNF, dan TNF-α disokong dengan perencatan terhadap mRNA dan ekspresi protein untuk keseluruhan dan fosforilasi reseptor NMDAR-2B, BDNF, mikroglia teraktif (Iba-1) dan reseptor P2X4 dalam tikus poliartritis kronik. Selain itu, penanda pro-apoptosis iaitu 'caspase-3', 'caspase-8', PKB, dan PI3Kcb telah menurun manakala tiada perubahan pada tahap anti-apoptosis Bcl-2 dalam saraf tunjang selepas rawatan Ifenprodil dibandingkan dengan kumpulan kawalan artritis. Peningkatan ketara dalam indeks diskriminatif ingatan yang dilihat pada tikus dengan poliartritis kronik terutamanya pada dos 0.5 μg/μL menunjukkan pengaruh Ifenprodil yang positif pada fungsi ingatan. Oleh itu, Ifenprodil terutamanya pada dos 0.5 μg/μL menunjukkan kesan anti-kesakitan dan anti-keradangan yang ketara setanding dengan kesan Diclofenac. Keputusan ini menunjukkan potensi penglibatan NMDAR-2B yang diaktifkan dalam patogenesis kesakitan artritis kronik.

EFFECTS OF IFENPRODIL TREATMENT ON PAIN BEHAVIOUR, INFLAMMATION, AND NOCICEPTIVE RESPONSES IN THE SPINAL CORD OF COMPLETE FREUND'S ADJUVANT-INDUCED CHRONIC POLYARTHRITIS RAT

ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory condition characterised by frequent reports of pain. Ifenprodil, a selective N-methyl-D-aspartate receptor-2B (NMDAR-2B) antagonist, has demonstrated a significant anti-nociceptive effect in chronic pain models. This study aimed to investigate the effect of Ifenprodil as a selective NMDAR-2B antagonist on pain behaviour, inflammation, and nociceptive responses in the spinal cord of CFA-induced polyarthritis, as well as its possible side effect on memory function. Arthritic rats received intrathecal treatment of either Ifenprodil (0.5 or 1.0 μg/μL) or Sodium Diclofenac (6 μg) (positive control) whereas arthritic (A) and non-arthritic (C) control groups were administered with 0.9% normal saline for 7 days (day-16 to -22 post-arthritic induction). Ankle joint diameter and circumference, pain behaviour assessments including von-Frey and hot-plate tests, mobility scoring, and memory test were conducted on day-0 (baseline), day-15 (pre-intervention) and day-23 (post-intervention). Histopathological examination was performed on the ipsilateral ankle joint while the lumbar region of the spinal cord (L4-L5) was collected for ELISA, immunohistochemistry and RT-qPCR analyses. The group receiving Ifenprodil (0.5 μg/μL) showed a non-significant trend of increased body weight with no change in total food intake, attenuation of thermal hyperalgesia, tactile allodynia and improved mobility and significant improvement in the morphology of the ipsilateral hind paws and ankle joints when compared to the arthritic rats receiving Ifenprodil at 1.0 μg/μL. Meanwhile, Ifenprodil has significantly decreased the level of

NMDAR-2B, substance P, BDNF and TNF-α proteins with attenuation on the total and phosphorylated NMDAR-2B, BDNF, activated microglia (Iba-1) and P2X4 receptor (P2X4R) mRNA and proteins expression in the polyarthritis rats. Furthermore, pro-apoptotic caspase-3, caspase-8, PKB, and PI3Kcb markers were decreased with no change in the anti-apoptotic Bcl-2 level in the spinal cord compared to the arthritis control group. A significant improvement in the memory discriminative index observed in the polyarthritis rats, especially at the concentration of 0.5 µg/µL implies a beneficial influence of Ifenprodil on memory enhancement. Thus, Ifenprodil, particularly at 0.5 µg/µL demonstrated significant anti-nociceptive and anti-inflammatory effects comparable to those of Sodium Diclofenac. These results underscore the potential involvement of NMDAR-2B activation in the pathogenesis of chronic arthritic pain.

CHAPTER 1

INTRODUCTION

1.1 Pain

Individuals may encounter pain in their life which frequently arises from diseases, trauma, or other conditions. It is one of the major factors of a person seeking analgesic medications and a major cause of work disability. According to the latest definition by the International Association for the Study of Pain (IASP), pain is defined as ‘an unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage’ (Raja et al., 2021). It is affected by physical, psychological, social, and cultural factors which contribute to a multidimensional framework for the biopsychosocial experience (Love-Jones, 2019). The prevalence of chronic pain is a predominant concern worldwide. In the United States, more than 100 million people are living with chronic pain which hugely affects their work productivity and fewer hours of work (Dahlhamer et al., 2016). Meanwhile, a study conducted in primary healthcare clinics in other countries reported that pain affects the patient’s quality of life and functioning, severely reduces sleep and walking abilities, daily house chores, mood instability, interpersonal relationships, and enjoyment of life (Rauf et al., 2003; Pandelani et al., 2023).

Although pain taxonomy is multidimensional and complex, it can most generally be categorised into two temporally distinguished categories: acute and chronic (Cioffi et al., 2017). Acute nociceptive pain is caused by transient or acute noxious mechanical, chemical, and/or thermal elicitation of peripheral receptors which present for less than three months. As withdrawal reflexes are triggered, acute pain protects the tissue from additional harm (Derderian et al., 2023). Acute inflammatory pain results from tissue injury and the related pain sensations generally rise in conjunction with the

resolution of inflammation and healing of the injured tissue (Cioffi, 2017). Meanwhile, chronic pain is defined as the pain that lasts or recurs over three months. In contrast to acute pain, chronic pain does not confer a protective biological role as it can be decoupled from noxious stimuli and persist beyond tissue repair and healing. For that reason, chronic pain is considered a pathological process or disease state in itself (Cioffi, 2017).

1.2 Chronic pain

Chronic pain is among the most common symptoms complained about in an outpatient clinic. It is a cost burden to a country where the pain-related expenses were reported to exceed those for the costs of diabetes, cancer, and heart-related diseases (Dydyk and Conermann, 2023). In chronic pathological pain, the pain experienced can be heightened and protracted in response to a noxious stimulus (hyperalgesia), may occur spontaneously, or may arise from a normally innocuous stimulus (allodynia) (Cioffi, 2017). According to Dydyk and Conermann (2023), chronic pain can be categorised into several types:

(1) Neuropathic pain is caused by a nerve injury which could rise either peripherally or centrally. This type of pain develops due to the development and maintenance of central sensitisation and is accompanied by hyperalgesia and allodynia. Neuropathic pain results from several pathological processes, including axotomy or nerve damage (including spinal cord injury, post-mastectomy pain, post-operative hernia repair pain, and other types of post-surgical pain), carpal tunnel syndrome, central pain syndrome (including multiple sclerosis and stroke), degenerative disc disease (including 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) (Finnerup et al., 2021).

(2) Nociceptive pain results when the tissues are inflamed or damaged which also includes the occurrence of spontaneous pain[1], hyperalgesia and/or allodynia (Schug et al., 2011).

(3) Musculoskeletal pain is either acute or chronic pain that affects muscles, bones, ligaments, tendons and even nerves. This type of chronic pain comprises distinguished pain syndromes ranging from local pain to neuropathic pain (El-Tallawy et al., 2021). The example includes back pain and myofascial pain (Dydyk and Conermann, 2023).

(4) Psychogenic pain is contributed by psychogenic factors such as headaches or abdominal pain due to emotional, psychological or behavioural factors (Dydyk and Conermann, 2023).

(5) Mechanical pain is defined as pain that arises intrinsically from the spine, intervertebral discs, or surrounding soft tissues due to an acute traumatic event or trauma (Will et al., 2018).

(6) Inflammatory pain refers to the augmented hypersensitivity of perception and emotional response to pain stimuli that arises from inflammatory insults associated with tissue damage. The increased production of inflammatory mediators acts on pain-sensitive nerve endings, decreases the thresholds of neuronal hyperexcitability and increases the sensitivity of firing rates leading to

[1] Pain resulting in the absence of any stimulation.

the development of peripheral and central sensitisation (Zhang et al., 2023). Inflammatory pain derived from the occurrence of infections and autoimmune disorders such as rheumatoid arthritis (RA), Crohn's disease, Hashimoto Grave disease due to thyroid dysregulation, ulcerative colitis, osteoarthritis, and many more (Dydyk and Conermann, 2023).

Given the focus of the present study on RA, the following subsections delve into the pathomechanisms leading to inflammatory pain specifically RA pain conditions including the changes in the peripheral and central nervous system (PNS and CNS, respectively) leading to the occurrence of peripheral and central sensitisation.

1.3 Rheumatoid Arthritis (RA)

Arthritis is derived from a Greek phrase known as "disease of the joints" (Yamanaka, 2020). Several types of arthritis reported but the most occurring forms are osteoarthritis, rheumatoid arthritis, and gout (Senthelal et al., 2023). These three diseases represent chronic states of progressive and destructive injury to soft tissues, joints, and the spinal column (Dray, 2008). Specifically, RA is a chronic inflammatory and systemic autoimmune disease characterised by hyperplasia of the synovial membrane, degeneration of cartilage and erosion of bones in diarthrodial joints (Kemble and Croft, 2021). RA extensively affects joints, causing aberrant inflammatory reactions, discomfort, and a high risk of permanent joint damage if left untreated. In the long term, patients with RA may eventually develop bilateral symmetrical impairments in their extremities (Noh et al., 2020). The United States Health Interview Survey (2013–2015) estimated that 22.7 percent of people had a doctor-diagnosed case of arthritis each year. The prevalence was higher in women (23.5%) than in males (18.1

percent) (Barbour et al., 2017). According to the Global Burden of Disease Study 2019, 18 million people worldwide are living with RA and approximately 70% of this population are women. The causative factor to the development of RA is yet unknown. However, it is believed to be associated with hereditary, environmental, and behavioural factors. Infections, smoking, exposure to silica dust, and stochastic factors (i.e., patients with positive anti-citrullinated protein antibody (ACPA) test) may enhance the development of RA in individuals (Guo et al., 2018). Since it is associated with an auto-immune disorder, RA is suggested to be a T-cell mediated disease where the elevated number of T-cells causes the increase of macrophages and fibroblasts that leads to destructive changes in the joints of the patients.

Pain is the major complaint in the RA patients. Like other chronic pain symptoms, RA pain is characterised by a complex integration of sensory, affective, and cognitive processes that involve a variety of abnormal cellular mechanisms at the peripheral (joints) and central (spinal, supraspinal, and descending system) of the nervous system (Dray et al., 2008, Zhang and Lee, 2019). In clinical research, Leffler and colleagues (2002) found that individuals with RA experiencing symptoms for more than five years suffered from hyperalgesia to benign cold, increased sensitivity to light touch, and generalised allodynia to pressure particularly on the thigh area. Although other factors might be at play, inflammation is thought to be one of the reasons for pain flares in the patients. The extensive inflammation causes changes to the peripheral and central nervous systems that lead to pain sensitisation during RA (Figure 1.1).

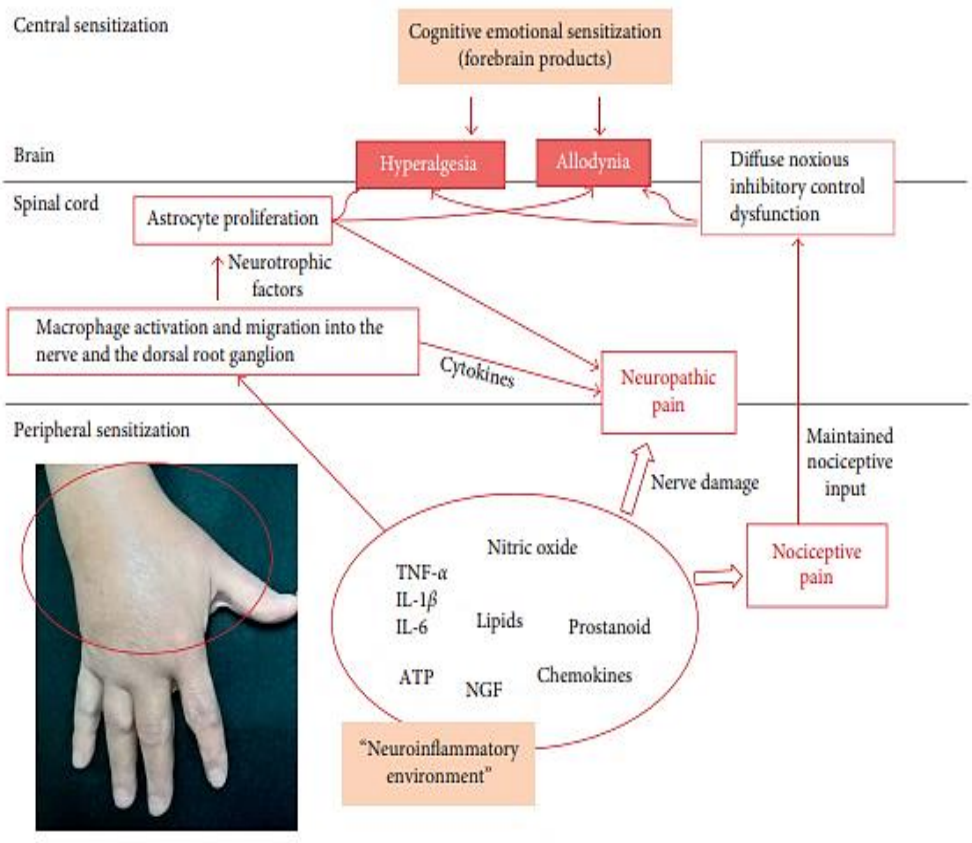

Figure 1.1 Mechanisms leading to peripheral and central sensitisation in inflammatory arthritis. The inflammation in the synovium can stimulate the release of mediators responsible for both nociception and neuropathic pain (via the injury of the nerves and recruitment of macrophages in the nerves themselves and dorsal root ganglion). The repeated nociceptive inputs may alter the role of diffuse noxious inhibitory control (DNIC) consequently resulting in aberrant pain responses. Inflammatory cells may induce the proliferation of glial cells at the level of the central nervous system which in turn provoke the neuronal modifications that are responsible for hyperalgesia and allodynia. Adapted from Salaffi et al. (2018).

1.3.1 Peripheral mechanism of pain sensitisation

Articular changes in RA may induce or sensitise primary afferent nerves, leading to pain in the extremities. The synovium and capsule of the joints are heavily innervated by postganglionic sympathetic nerve fibres and peripheral afferents of the dorsal root ganglion (DRG). These areas are innervated by primary sensory neurons including mechanoreceptors (i.e., Aα- and Aß-fibres) and nociceptors (i.e., Aδ- and C-fibres). In RA patients, the autoimmune reactions in the joints are primarily attributed to the presence of ACPA released by B cells which include peptides such as collagen, histones, α-enolase, fibrin, vimentin, fibronectin, and Epstein-Barr nuclear antigen I (EBNA-1) (Guo et al., 2018). The presence of peptidyl arginine deiminase (PAD) enzyme in the affected joints undergoes catalysis and produces autoantibodies against these ACPA proteins. The increased production of ACPA proteins may disrupt immunological tolerance by potentially triggering MHC class II-dependent T cell activation through the citrullination of neoantigens. In turn, this condition facilitates B-cells to produce more ACPA proteins in the joints (Wu et al., 2021). α-enolase, one type of ACPA, enhances the activity of p38 mitogen-activated protein kinase/nuclear factor-kappa B (p38 MAPK/NF-kB) signalling pathway and binds to Grp78 on the surface of monocytes/macrophages. Consequently, this interaction results in the release of tumour necrosis factor-α (TNF-α), interleukin (IL)-1α/ß, interferon-γ (IFN-γ), and prostaglandin E2 (PGE2) (Bae et al., 2012; Cheng and Penninger, 2004; Zhang and Lee, 2018).

The binding of the inflammatory cytokines on their specific receptors (e.g., IL-1 to IL-1 receptor or Toll-like receptor superfamily) facilitates the activation of N-methyl-D-aspartate receptor (NMDARs) through the upregulation of tyrosine phosphorylation on its c-terminal. As a result, the increased calcium ion (Ca^{2+}) influx

into the nerve fibres increases the frequency of action potentials carrying the nociceptive signals to CNS (Trepanier et al., 2012). At the same time, migrating immune cells such as macrophages and monocytes may produce chemical mediators including histamine, bradykinin, serotonin, adenosine triphosphate (ATP), and hydrogen ions (H+). Simultaneously, the peripheral nociceptive fibres also release substance P, calcitonin-gene-related peptide (CGRP), and somatostatin which further facilitates the inflammatory process and pain transmission (Schaible et al., 2002). Substance P also induces plasma extravasation, mediates the recruitment and migration of immune cells to the inflammatory sites, T-cells release and mast cell degranulation. This neurotransmitter also stimulates cytokines production from the macrophages and promotes the proliferation of fibroblasts and endothelial cells (Mashaghi et al., 2016). These neuromodulators and inflammatory mediators synergistically sensitise the peripheral terminals of Aδ- and C-fibres to increase the generation of action potentials and facilitate pain transmission (Cheng and Penninger, 2004).

Besides that, the released inflammatory, chemical mediators and neuromodulators also act on several ion channels that play significant roles in transmitting noxious and innocuous inputs. These ion channels include transient receptor potential (TRP) channels (i.e., TRPV1-4, TRPM3 which detect noxious heat sensation, TRPC5 and TRPM8 which transmit noxious cold temperatures, TRPA1 and TRPV4 which sense noxious mechanical inputs, TRPA1, TRPV1, TRPV3, TRPM8 and TRPC3 which are sensitive to itch sensation) which are heavily expressed on the nociceptors (i.e., Aδ- and C-fibres) (Salzer et al., 2019). The wide range of signalling molecules causing aberrant inflammatory reactions may gradually lower the nociceptive threshold and/or enhance the magnitude of responsiveness at the peripheral

nerve terminals of the sensory fibres which contribute to a phenomenon known as peripheral sensitisation (Salaffi et al., 2018).

To date, the involvement of N-methyl-D-aspartate receptors (NMDARs) in the peripheral region during the arthritic process has not been extensively explored. Nevertheless, existing research has identified the expression of NMDARs in the rat model of visceral pain, particularly at dorsal root ganglion (DRG) neurons (McRoberts et al., 2001). In a pre-clinical investigation, Banerjee and co-workers (2009) have identified significant expression of the NMDAR-2B on small- and medium-sized diameter primary afferents. The activation of NMDAR-2B by the binding of glutamate mediates peripheral cell-to-cell interactions (e.g., upon release from the immune cells) or autocrine regulation (e.g., upon the release from sensory nerves following TRPV1-mediated Ca^{2+} influx (Gangadharan and Kuner, 2013). N- and T-type channels also undergo increased upregulation in the context of chronic pain (Feldman and Khanna, 2013). In essence, these altered molecular mechanisms contribute to the generation of spontaneous pathological activity and excessive spontaneous firings in the afferent neurons.

1.3.2 Central mechanism of pain sensitisation

The extent of tissue damage and inflammation at the peripheral articular joints is not only the predictive factor for the severity of RA pain. It is also associated with impaired pain regulatory mechanisms at the central level which may also influence the psychosocial factors of pain perception (Salaffi et al., 2018). Like peripheral sensitisation, dysregulation of specific pain pathways (e.g., inactivation or overactivation) may cause hyperalgesia and allodynia and an imbalance between the pain pathways that facilitate and inhibit pain may underlie the conditions linked with

chronic pain. These include the alteration of central pain regulatory mechanisms to (1) descending facilitatory pathways, (2) descending inhibitory pathways, and (3) central sensitisation (Zhang and Lee, 2018).

1.3.2(a) Alteration of descending facilitatory and inhibitory pathways

Descending pain inhibitory pathway originates from the brain, travels through the brainstem and reaches the spinal cord dorsal horn where the pain suppression system is activated. The main regulatory centres of the brainstem include the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM). PAG receives the nociceptive signals from the brain's frontal cortex, amygdala (limbic system), and hypothalamus triggered by-pain-associated psychosocial factors (i.e., stress and emotion). PAG signals the nociceptive inputs to the RVM to activate descending inhibitory pathways (Zhang and Lee, 2018). In fact, RVM has dual roles in pain where it is capable of inhibiting and facilitating pain transmission depending on the specific pathways activated. The activation of RVM ON cells leads to increased activity linked to nociceptive reflexes and enhances nociception. Conversely, the activation of RVM OFF cells exhibits a cessation of activity during nociceptive reflexes and produces anti-nociception (Peng et al., 2023). In normal conditions, the nociceptive signals are then relayed from the RVM to the spinal cord dorsal horn where the interneurons are activated at the synapse of first-order neurons. Consequently, the release of glutamate and substance P neurotransmitters is reduced while the release of norepinephrine and endogenous opioids is increased and results in decreased transmission of nociceptive signals to the second-order neurons. Additionally, the neurotransmitter uptake into the second-order neurons is also attenuated and results in both pre-synaptic and post-synaptic inhibition of nociceptive inputs (Cioffi et al., 2017; Zhang and Lee, 2018) (Figure 1.2). However,

during prolonged occurrence of pain, the disruption of this facilitatory-inhibitory balance in the RVM cells favours the facilitation of nociception. This condition further results in the augmentation of nociceptive transmission rather than nociceptive inhibition in the spinal cord dorsal horn and contributes to the development of central sensitisation (Zhang and Lee, 2018).

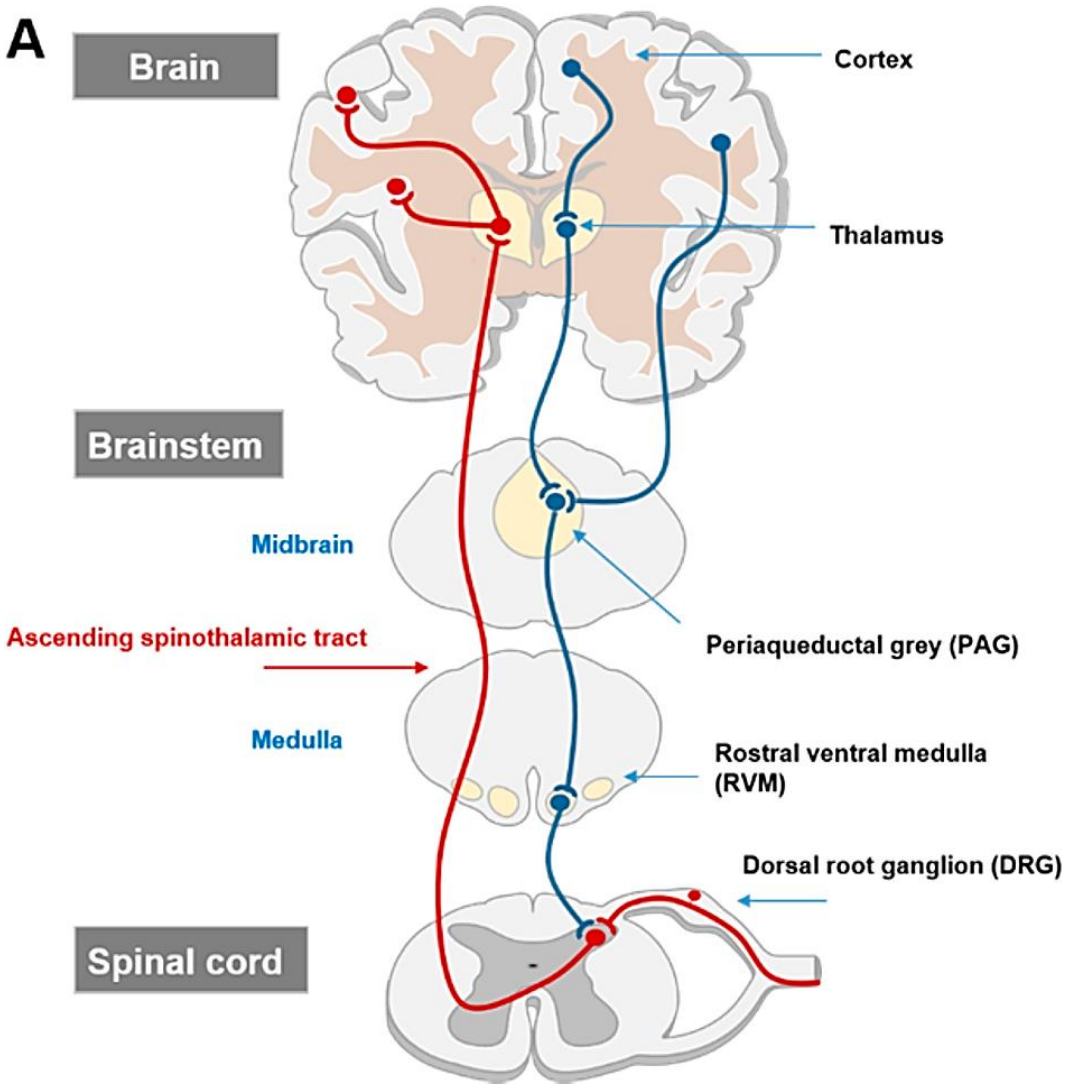

Figure 1.2 Activation of descending pathway activation during pain transmission. Descending pathways project from periaqueductal gray (PAG) in the midbrain and rostral ventromedial medulla (RVM) to the spinal cord dorsal horn for the modulation of pain transmission (activation of pain inhibitory system). Adapted from Cioffi (2017).

1.3.2(b) Central sensitisation

Intense, recurrent, and persistent afferent bombardment from the nociceptors in response to peripheral tissue or nerve damage stimulates neuroplastic alterations within the spinal cord dorsal horn that result in central sensitisation. Central sensitisation is a form of activity-dependent long-term potentiation (LTP) that advances from an early-onset phase to a late-onset phase (Cioffi, 2017). There are two primary phases of central sensitisation: (1) an acute phase involving the activation of NMDARs by glutamate, and (2) a chronic phase characterised by the transcription of pain-regulatory peptides and activation of spinal microglia (neuronal-microglia interaction) (Zhang and Lee, 2018; Zhang et al., 2023).

The early onset (acute) phase begins as an adaptive and reversible process which plays a protective role by promoting the activity that aids in the recovery of an injured area of the body until the tissue repair and healing are completed. This phase involves increased responsiveness of spinal postsynaptic secondary-order projection neurons to intense glutamate release from afferent nociceptors. In fact, the initiation and maintenance of central sensitisation relies upon the activation of postsynaptic NMDARs. The activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate (mGluR) receptors aids prolonged membrane depolarization of the postsynaptic neuron where it is a crucial step in removing magnesium ion (Mg^{2+}) block from the NMDARs channel pore before its activation (Sandkühler, 2017).

Once fully activated, NMDARs lead to increased intracellular Ca^{2+} influx into the neurons, triggering secondary messenger systems such as protein kinase C (PKC), phospholipase C (PLC), calcium/calmodulin-dependent protein kinase II (Cam-KII), phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and

Src-family kinases (SFKs) that concomitantly initiate and subsequently strengthen the occurrence of LTP (Cioffi, 2017; Zhuo, 2017). These mechanisms include phosphorylation of NMDARs, AMPARs, and other ion channel receptors that lower their activation threshold and kinetics as well as increased trafficking of AMPA and NMDARs to the synapse. Moreover, the release of additional neuromodulators from the afferent fibres such as CGRP, bradykinin and BDNF may also contribute to the activation of intracellular cascades leading to membrane depolarisation and maintenance of LTP (Cioffi, 2017). Furthermore, the NMDARs-mediated release of retrograde neurotransmitters such as nitric oxide enhances the LTP by diffusing to the presynaptic terminal and promoting the enhanced release of synaptic glutamate, substance P, and CGRP. The concurrent action of these mediators reduces the threshold required for nociceptive signalling activation and facilitates the hyperexcitability of suprathreshold excitatory inputs. During chronic pain, the nociceptive signals transmitted from the free nerve terminals and thermoreceptors cause the release of substance P (SP) and glutamate at the presynaptic axon terminal into the synaptic cleft of the spinal cord dorsal horn. SP aids to central sensitisation through its binding to NK3 receptors which is coupled to phospholipase C and the formation of intracellular messengers which downstream effects include depolarising the neuronal membrane and aiding the functions of NMDARs and AMPARs. Meanwhile, the binding of SP to the NK1 receptor leads to the increased production of prostaglandins whilst the activation of the NK3 receptor also leads to the release of NO (Gao et al., 2022). These neuro-inflammatory mediators act as retrograde messengers across the synapses and aid nociceptive signalling in the spinal cord. At the level of the spinal cord, the cellular effects of CGRP and SP contribute to the amplification of synaptic strength between the nociceptors and neurons involved in pain pathways. Additionally, distinct

intracellular signalling pathways activate different transcription factors which consequently initiate alterations in gene expression leading to prolonged neuronal hyperexcitability and perpetuation of hyperalgesia (Seybold, 2009).

1.4 Pain responses

The physiological function of nociception allows and enforces withdrawal or avoidance behavioural response to protect the body or tissue from further damage. However, the susceptibility of the affected tissue during the state of injury is increased. In order to cope with this enhanced susceptibility to guarantee adequate protection of the affected tissue, the physiological nociceptive system acts by locally reducing the nociceptive thresholds and facilitating nocifensive responses. The behavioural responses that correlate to these adaptations are known as allodynia and hyperalgesia (Deuis et al., 2017). These responses are not signs of an inadequate response but link more to an appropriate shift in pain threshold to avoid further tissue damage. The development of thermal hyperalgesia and tactile allodynia is attributed to different pain pathways (Jang et al., 2020). However, some factors like the location, duration or magnitude of pain, hyperalgesia and/or allodynia have become maladaptive rather than protective, therefore the pain is no longer a homeostatic factor but rather a disease by its definition.

1.4.1 Tactile allodynia

'Allodynia' is the term used to describe heightened nociceptive sensitivity when the stimuli that were previously not painful are perceived as painful (Zhang and Lee, 2018). Tactile allodynia is mediated by the large diameter A-beta fibres (Aβ-fibres)

which are responsible for touch sensation, and it is not affected by the modulation of spinal opioids (Zhang et al., 2007). The Aβ-fibres are the low threshold and only a few of these types of fibres have the potential to be nociceptive (Niimi et al., 2023). Therefore, under normal circumstances, selective stimulation of Aβ-fibres, such as electrical nerve stimulation, typically does not cause pain. However, impulses delivered via Aβ-fibres may cause pain after nerve injury or inflammation (not under normal conditions), as some of these fibres change their phenotypic and begin to release SP (Zieglgänsberger, 2019). Upon activation, the increased release of SP enhances the responsiveness of spinal nociceptive neurons and facilitates spinal nociception (Latremoliere and Woolf, 2009). Specifically, interneurons in the deeper lamina (laminae III or IV) of the spinal cord dorsal horn are evoked by the Aβ-afferent fibres resulting in touch-evoked pain or allodynia (Nelson, 2019). Most of the non-nociceptive (laminae III) or convergent input (laminae VI/VI) is transmitted by the neurons of the deeper lamina (III/IV) (Schaible, 2007).

In the neuropathic pain rat model, there was a significant increase in c-Fos expression (a marker of neuronal activity) in the spinal cord lamina II after repeated touch stimuli (Bojovic et al., 2016). This suggests that Aβ-fibres may play crucial roles in allodynia either by directly or indirectly stimulating the lamina II neurons that are specific to nociception (Yam et al., 2018). This phenomenon is also evidently shown in neuropathic pain models involving sciatic nerve transection or chronic constriction injury where the noxious inputs transmitted by Aβ-fibres to the spinal cord dorsal horn exhibited a substantial increase in response (Campbell and Meyer, 2006). Additionally, Browne and colleagues (2020) suggested that peripheral nerve injury prompts Aβ-fibres to extend beyond their usual termination site in the deeper dorsal horn and form synapse-like structures with lamina II neurons. It also demonstrated that Aβ-fibres

facilitate nociceptive inputs to the lamina II region by activating pre-existing polysynaptic pathways in the spinal dorsal horn (Zeilhofer et al., 2021). These modifications in the synaptic transmission following the nerve injury contribute to the augmented excitability of the second-order neurons where it is typically responsive to high-threshold sensory inputs to receiving the inputs from the Aβ-fibres (Yam et al., 2018). This misinterpretation of inputs in the spinal cord causes low-threshold sensory inputs to be perceived as nociceptive which explains the occurrence of allodynia in chronic neuropathic pain.

1.4.2 Thermal hyperalgesia

'Hyperalgesia' is the term used to explain heightened nociceptive sensitivity where the noxious signals that were previously perceived as mildly pain are now perceived as more painful (Zhang and Lee, 2018; Schug et al., 2011). It is noteworthy that hyperalgesia may manifest not only in the vicinity of tissue damage or lesions but also in the skin distant from the affected inner organ or muscle. The occurrence of hyperalgesia is associated with altered central mechanisms of neuronal excitation and inhibition (Bodnar and Heinricher, 2022).

Thermal or chemical hyperalgesia is mediated by the unmyelinated C-fibres and is almost sensitive to the inhibition by spinal opioids (Basbaum et al., 2009). Following the nerve lesion, the thermal nociceptive signals are transmitted by the peripheral sensory neurons into the spinal cord dorsal horn and subsequently conveyed to the supraspinal site at the structures above the T8 level (Dubin and Patapoutian, 2010). The discharge of nerve injury-induced impulses and ectopic nerve lesion potentials, aberrant sympathetic effects and/or sensitisation of ion channels associated with NMDAR activation directly contribute to the development of hyperalgesia

(Finnerup et al., 2021; Hansen et al., 2017). Additionally, continuous excessive stimulation of excitatory amino acids (EAA) receptors in the spinal cord along with subsequent intracellular processes such as PKC translocation or activation following inflammation or tissue damage also contribute to the development of central sensitisation and eventually hyperalgesia (Lai et al., 2014). Apart from that, the sprouting of a subpopulation of nociceptive primary afferents that release substance P and CGRP in the spinal cord dorsal horn is also believed to not only develop allodynia but also hyperalgesia (Iyengar et al., 2017). Besides, the dysregulation of the descending inhibitory circuit may also result in hyperalgesia. Studies have shown that excitatory amino acid-induced PKC translation or activation and nitric oxide (NO) production may lead to disinhibitory processes associated with excitotoxic effects including neuronal death within the CNS (Fakhri et al., 2021). This mechanism is facilitated through the inhibition of GABA and glycine system in the spinal cord, thereby intensifying thermal hyperalgesia as shown in a rat model of sciatic nerve injury (Tadokoro et al., 2022). Figure 1.3 demonstrates the generation of allodynia and hyperalgesia as adapted from Cervero and Laird (1996).

Pain is one of the major unresolved symptoms suffered by most of the RA patients. Unfortunately, this symptom persists to present in the patients although inflammation is successfully controlled by the prescription of anti-rheumatic drugs. Addressing RA-related concerns and linking them to RA pain management is a critical component of delivering comprehensive treatment to people living with this condition.

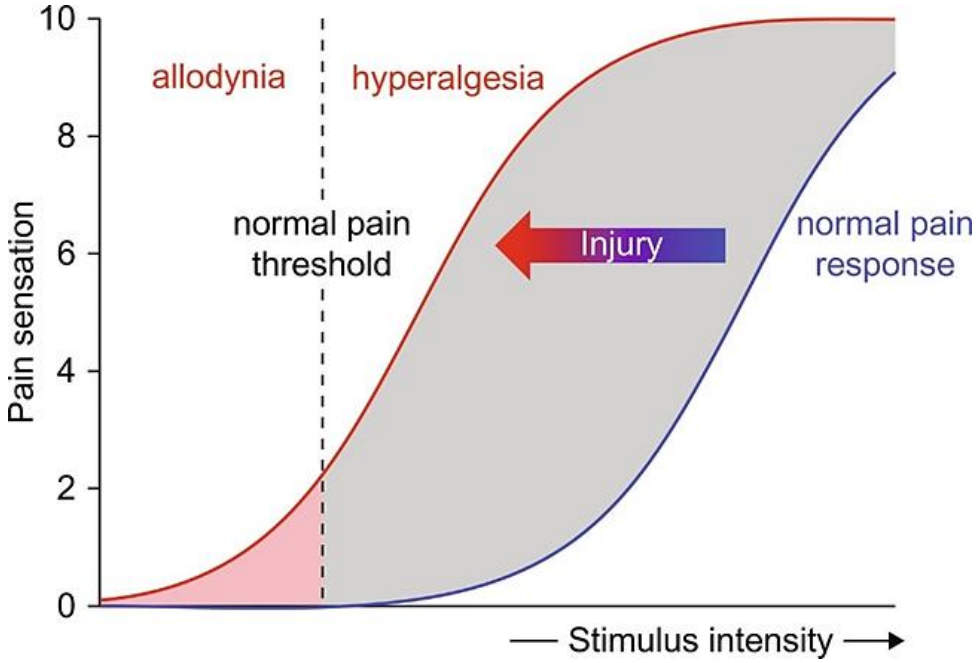

Figure 1.3 Hyperalgesia and allodynia. Tissue damage heightens the nociceptor's sensitivity to peripheral stimuli. Normally, mild or non-threatening inputs do not evoke a pain response. During tissue damage, responses to stimuli become sensitised causing a leftward shift in the response curve. Consequently, a moderately painful stimulus is interpreted as more intense (resulting in hyperalgesia) and a formerly innocuous stimulus can evoke pain perception (leading to allodynia). Adapted from Cervero and Laird (1996).

1.5 Role of N-methyl-D-aspartate receptor-2B (NMDAR-2B) in RA

In general, NMDARs consist of several subtypes: 1) NR1 (contains eight spice variants; a basic channel-forming subunit), (2) NR2 (A-D subunits), and (3) NR3 (A and B subunits) (Noh and Ismail, 2020). Amongst the NR2 subtypes, NMDAR-2B, also known as NR2B subunits, is highly expressed in the spinal cord dorsal horn, and extensively studied especially related to the pathogenesis of chronic pain (Wu and Zhuo, 2009). NMDAR-2B is predominantly located at extra-synaptic sites (i.e., post-synaptic density) (Meng and Shen, 2022) and highly expressed in the cerebral cortex, hippocampus, and spinal cord regions (Noh and Ismail, 2020; Zhu et al., 2015). NMDAR-2B possesses large intracellular C-terminal tails (approximately 650 amino acids) where each tail comprises 25 tyrosine residues. Among the important tyrosine residues phosphorylated by the serine family kinases (SFKs) are Y_{1252}, Y_{1336}, and Y_{1472}. The phosphorylation of these tyrosine residues results in the recruitment of downstream signalling proteins (Nakazawa et al., 2001; Noh and Ismail, 2020).

Remarkably, after inflammation and other pathological situations, the development of long-term potentiation (LTP) may drive a higher influx of Na^+ and Ca^{2+} into the neurons and result in further depolarisation to induce phosphorylation of SFKs to the tyrosine residues of NMDAR-2B causing its activation. The SFKs are activated and the actions of NMDAR-2B are upregulated by the activation of GPCR through PKC and cell adhesion kinase β (CAKβ). Apart from that, GPCR may also project via PKA to alleviate the inhibition of Fyn by the receptor for activated C kinase-1 (RACK1), which also causes an increase in the NMDAR-2B activation. The integrin receptor, cytokine receptor, and ephrin B (EphB) receptor pathways are additional mechanisms that may also trigger SFKs phosphorylation and thereby increase the NMDAR-2B activation (Noh and Ismail, 2020) leading to neuronal hypersensitivity (Figure 1.4).

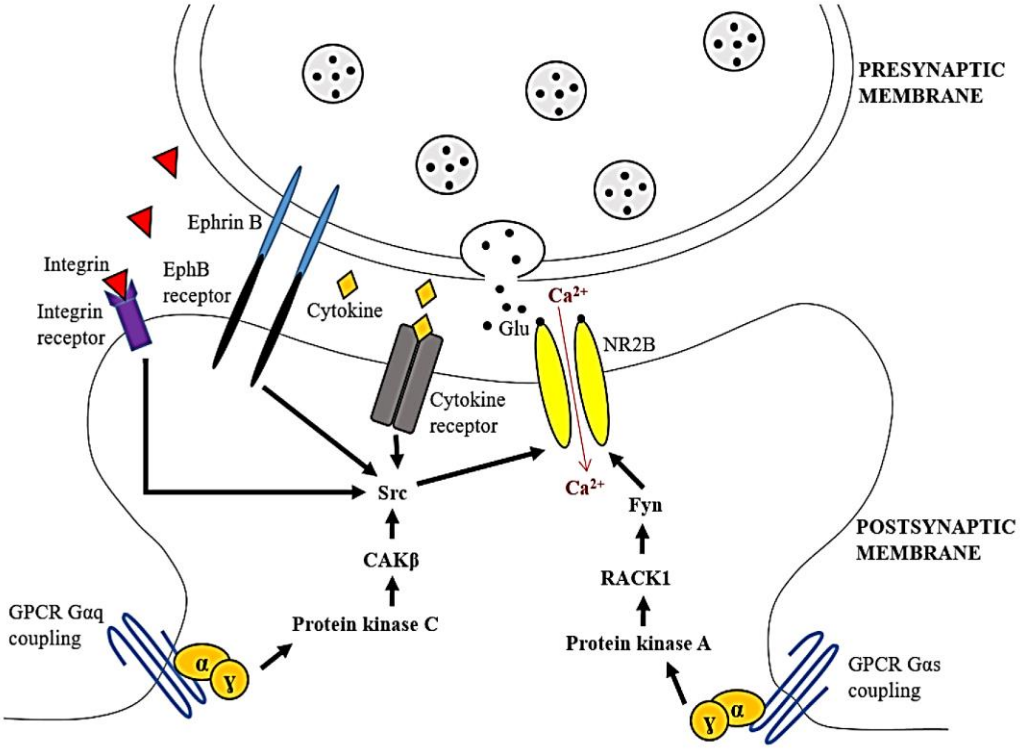

Figure 1.4 Mechanisms leading to N-methyl-D-aspartate-2B receptor (NMDAR-2B) activation. The pathways driving the increased activity of NMDAR-2B involve several activations of GPCRs, cytokine receptors, integrin receptors and EphB receptors. GPCRs transmit signals via PKC and CAKβ leading to phosphorylation of Src kinases and subsequent increase of NMDAR-2B activity. The activation of this receptor subtype also occurs through Gαs-coupled GPCRs/PKA/RACK1 signalling pathway, integrin and EphB receptor activation. The release of Mg^{2+} from NMDAR-2B following the simultaneous actions from AMPARs and mGluR activation facilitates its activation and allows the Ca^{2+} and Na^{+} influx into the neurons causing neuronal hyperresponsiveness. Adapted from Noh and Ismail (2020).

(GPCRs = G-protein coupled receptors, EphB = Ephrin B, PKC = protein kinase C, CAKβ = cell adhesion kinase β, PKA = protein kinase A, RACK1 = receptor for activated C kinase-1, AMPARs = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and mGluR = metabotropic glutamate receptor).

1.6 Neuron-microglia crosstalk during chronic pain

During the development and maintenance of chronic pain, neuronal hypersensitivity produces augmented neuronal responses to stimuli and manifests itself as pain hypersensitivity. Activated glial cells are shown to regulate this activity-dependent plasticity along the ascending pain circuit at the spinal cord dorsal horn and several brain regions. Abundant distribution of spinal microglia activation following the nerve damage, and gain or loss of function of these non-neuronal cells may act as a gatekeeper for regulating acute-to-chronic pain transition (Ho et al., 2020).

1.6.1 Microglia activation

Microglia is a specialised immune cell in the CNS which is essential in maintaining central homeostasis and responding to a range of pathological situations. In the laboratory, specific established protein markers to identify microglia activation include Iba-1, CD68, CD206, CD45 and OX-42. It is also can be found under other names such as allograft inflammation factor-1 (Aif-1), microglia response factor (MRF-1) or daintain (Jurga et al., 2020). Microglia activation leads to the production of various signalling substances, such as prostaglandins, growth hormones, cytokines, complement factors, lipid mediators, extracellular matrix components, enzymes, free radicals, neurotoxins, and NO. Microglia is beneficial for tissue regeneration and repair at optimal activation, however, when excessively activated, it leads to neuronal degeneration (Kempuraj et al., 2016). Ji and Xu (2021) demonstrated that microglia contribute to the development of hyperalgesia due to its release of neuromodulators including cyclooxygenase and NO upon activation.

During chronic pain, microglia activation occurs through various pathways. It is asserted that the neurotransmitters including CGRP, substance P, glutamate, γ-aminobutyric acid (GABA), glycine, serotonin, and noradrenaline may not signal rapid electrical or chemotactic responses in the resting (inactivated) microglia (Chen et al., 2018). However, microglia activation may occur through other mechanisms including ATP and its purinergic receptors (P2X and P2Y receptors), fractalkine, CX3 chemokine receptor 1 (CX3CR1), MCP-1, and CCR2. Amongst these signalling pathways, the spinal microglia exhibit rapid chemotaxis in response to ATP via their purinergic signalling pathway, P2X and P2Y receptors (Crespo-Castrillo and Arevalo, 2020, Zhang et al., 2023). Both ionotropic receptors (i.e., P2X4 and P2X7 receptors) and metabotropic receptors (i.e., P2Y6 and P2Y12) are abundantly expressed on the surface of this non-neuronal cell (Illes et al., 2020). The activation of the P2X4 receptor (P2X4R) following the binding of ATP on microglia induces brain-derived neurotrophic factor (BDNF) release (Long et al., 2020) which explains the relation of microglia activation by P2X4R with the release of BDNF for during chronic pain.

1.6.2 Activated microglia-induced brain-derived neurotrophic factor (BDNF) release

The P2 purinergic receptor family on the surface of microglia is the key to microglia activation. This receptor includes ion channel P2X receptors (consisting of P2X1-7 subtypes) and metabotropic G protein-coupled P2Y receptors (consisting of P2Y1, 2, 6, 11, 12, 13, and 14 subtypes) (Zhang et al., 2023). Among these receptor subtypes, P2X4 and P2X7 receptors allow the organic macromolecules to pass between the cells and contribute to hyperalgesia. The P2X receptor family is abundantly

expressed in many tissues and is essential for several roles including synaptic transmission, muscle contraction, inflammation, platelet aggregation, and inflammatory and neuropathic pain (Bernier et al., 2018; Zhang et al., 2023). One of the most sensitive P2X families, P2X4R is widely expressed in the PNS and CNS as well as microglia where it is critically involved in mediating neuropathic pain and maintaining hyperalgesia (Zhang et al., 2023).

Brain-derived neurotrophic factor (BDNF) is a critical signalling protein in the nervous system and has been implicated in the pathophysiology of chronic pain. It plays a significant role in the initial development of the nervous system, synaptogenesis, and maintenance of existing neurons (Reis et al., 2022). BDNF is generally known as a key player in the pathogenesis of neuropathic pain including pain of peripheral and central origin (Yamada et al., 2021). Importantly, BDNF can suppress the spinal neuronal activity while unmasking a subpopulation of "silent" neurons leading to increased neuronal excitability (Alles et al., 2021). Additionally, it is recognised as a potent modulator of synapses, thereby influencing the effectiveness of synaptic transmission (Rauti et al., 2020). This endogenous neuromodulator may be produced from primary afferent terminals within the spinal cord in a manner dependent on activity and functions to regulate the transmission of nociceptive signals (Geng et al., 2010).

1.6.3 Activated microglia-induced tumour necrosis factor-α (TNF-α) release

Microglia is also activated via mitogen-activated protein kinases (MAPKs). MAPK family is generally classified into three main members: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Zhang et al., 2023). Among these three MAPK members, p38 phosphorylation of microglia is the