ROLE OF P2X4 AND N-METHYL-D-ASPARTATE-2B RECEPTORS ON PAIN MODULATION IN THALAMUS OF COMPLETE FREUND'S ADJUVANT-INDUCED CHRONIC POLYARTHRITIS RAT UPON CORM-2 AND IFENPRODIL TREATMENTS

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by

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin complex
ACC	Anterior cingulate cortex
ACIA	Antigen-collagen-induced arthritis
ACPA	Anti-cyclic citrullinated peptide antibody
AE	Adverse events
AIA	Adjuvant-induced arthritis
AIF	Allograft inflammatory factor
AMPA	A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APCs	Antigen presenting cells
ATD	Amino-terminal domain
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
bDMARDs	Biologic disease-modifying anti-rheumatic drugs
Ca^{2+}	Calcium ion
CaMKII	Calcium-calmodulin-dependent protein kinase II
CCI	Chronic constriction injury
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO	Carbon monoxide
CORM-2	Carbon monoxide-releasing molecule
COX	Cyclooxygenase
CPG	Clinical practice guideline
CSI	Central sensitisation inventory
CTD	Carboxy-terminal domain
CX3CR1	Chemokine receptor 1
CXCL2	Chemokine ligand 2
DAB	Diaminobenzidine

dATP	Deoxyadenosine triphosphate
DISC	Death-inducing signalling complex
DMARDs	Disease-modifying anti-rheumatic drugs
DNIC	Diffuse noxious inhibitory controls
DREAM	Downstream regulatory element antagonist modulator
DRG	Dorsal root ganglion
EAAT3	Excitatory amino acid transporter-3
EBNA-1	Epstein-Barr nuclear antigen
ELISA	Enzyme-linked immunoabsorbent assay
EphBs	Ephrin-B families
EPSCs	Excitatory postsynaptic currents
EPSPs	Excitatory post-synaptic potentials
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
ETC	Electron transport chain
FADD	Fas-associated death domain
FasL	Fas-ligand
FLS	Fibroblast-like synoviocytes
GABA	Gamma-aminobutyric acid
GAPDH	Gyceraldehyde-3-phosphate dehydrogenase
GCs	Glucocorticoids
gDNA	Genomic deoxyribonucleic acid
GDNF	Glial cell-line derived neurotrophic factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
GPCR	G-protein-coupled receptor
GREs	Glucocorticoid response elements
HCI	Hydrochloric acid
HCQ	Hydroxychloroquine
HO-1	Haem oxygenase-1
HRP	Horseradish peroxidase
IASP	International Association for the Study of Pain
IBA-1	Ionized calcium-binding adapter molecule 1
ICD-11	International Classification of Diseases

IGF-1	Insulin-like growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IHS	Immunohistochemical scoring
IL-1	Interleukin-1
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	Jun N-terminal kinases
KCC2	K+/Cl- cotransporter-2
KCI	Potassium chloride
LBD	Ligand binding domain
LEF	Leflunomide
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR5	Metabotropic glutamate 5 receptor
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MOH	Ministry of Health
MRF-1	Microglia response factor-1
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NaCI	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-light-chain enhancer of activated B cells
NGF	Nerve growth factor
NK-1R	Neurokinin-1 receptor

NLRP3	Nod-like receptor protein 3
NMDA	N-methyl-d-aspartate
NMDAR-2B	N-methyl-D-aspartate-2B receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR2B	N-methyl-D-aspartate-2B receptor
NRM	Nucleus raphe magnus
NSAID	Non-steroidal anti-inflammatory drugs
NTC	No template control
NTD	N-terminal domain
O.D.	Optical density
P13K	Phosphoinositide 3-kinases
P2X4R	Purinergic receptor P2X, ligand-gated ion channel 4
P2X6	Purinergic receptor P2X, ligand-gated ion channel, 6
PAD	Peptidyl arginine deiminase
PAG	Periaqueductal grey
PAP	Peroxidase-antiperoxidase
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK-1	Phosphoinositide-dependent kinase-1
PeMPS	Peripheral microglial processes
pERK	Phosphorylated ERK
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
pMAPK	Phosphorylation of MAPK
PPADs	Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonoic acid
PTP	Permeability transition pore
PWT	Paw withdrawal threshold

PYK	Proline-rich tyrosine kinases
QoL	Quality of life
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RIP3	Receptor-interacting protein kinase 3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinases
RVM	Rostroventromedial medulla
SCI	Spinal cord injury
SLE	Systemic lupus erythematosus
SSZ	Sulfasalazine
T2T	Treat-to-target
TBS	Tris-buffered saline
TLR4	Toll-like receptor-4
TLRs	Toll-like receptors
TMD	Transmembrane domain
TNC	Trigeminal nucleus caudalis
TNF-α	Tumour necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
TRP	Transient receptor potential
TRPV-1	Transient receptor potential-1
TRPV-2	Transient receptor potential-2
TWT	Thermal withdrawal threshold
VPL	Ventral posterolateral
VPM	Ventral posteromedial
VTA	Ventral tegmental area
WDR	Wide dynamic range
WHO	World health organization

LIST OF APPENDICES

PERANAN RESEPTOR P2X4 DAN N-METHYL-D-ASPARTATE-2B TERHADAP MODULASI NOSISEPTIF DALAM TALAMUS TIKUS POLIARTRITIS KRONIK YANG DIARUHKAN OLEH PEMBANTU LENGKAP FREUND SELEPAS RAWATAN CORM-2 DAN IFENPRODIL

ABSTRAK

Artritis reumatoid (RA) adalah penyakit keradangan yang kronik dengan kesakitan sebagai simptom utama yang dilaporkan oleh pesakit RA. Simptom ini berterusan walaupun selepas keradangan reda, menunjukkan terdapat mekanisme yang berubah selain daripada keradangan periferi. CORM-2 dan Ifenprodil dipilih kerana keupayaannya memodulasi laluan nosiseptif pusat secara khusus melalui perencatan reseptor P2X4 dan NMDAR-2B yang terlibat dalam kesakitan kronik artritis reumatoid. Kajian ini bertujuan untuk menyiasat kesan rawatan CORM-2 (antagonis reseptor P2X4) atau Ifenprodil (antagonis NMDAR-2B yang tidak kompetitif) terhadap mekanisme nosiseptif dan apoptosis dalam talamus tikus Jantan Sprague-Dawley yang mengalami poliartritis kronik yang diaruhkan pembantu lengkap Freund (CFA). Sebanyak lapan puluh ekor tikus Jantan Sprague-Dawley dibahagikan secara rawak kepada lima kumpulan (n=16) terdiri daripada kumpulan kawalan bukan artritis (N), kawalan artritis (A), kumpulan artritis yang dirawat dengan CORM-2 (A+CORM-2), Ifenprodil (A+Ifenprodil), dan Diclofenac (A+Diclofenac) sebagai kawalan positif. Tikus artritis menerima suntikan intratekal sama ada CORM-2 (20μg), Ifenprodil (0.5μg/μL), atau Diclofenac (6μg) selama tujuh hari (hari ke-16 hingga ke-22 selepas induksi artritis). Sementara itu, kumpulan kawalan A dan N menerima suntikan larutan garam normal 0.9%. Diameter sendi buku lali dan respons tingkah laku terhadap

kesakitan (aktiviti tingkah laku spontan, ujian von-Frey dan plat-panas) direkodkan pada hari ke-0 (nilai asas), hari ke-15 (pra-rawatan) dan hari ke-23 (selepas rawatan). Tikus dikorbankan pada hari ke-24 dan sampel talamus disimpan untuk ujian ELISA, RT-qPCR, imunohistokimia, dan pemeriksaan histologi melalui pewarnaan Nissl. Tikus artritis menunjukkan peningkatan ketara pada diameter sendi kedua-dua belah buku lali beserta dengan pengurangan aktiviti tingkah laku spontan dan mengalami alodinia sentuhan dan hiperalgesia haba yang konsisten. Rawatan dengan CORM-2 dan Ifenprodil meningkatkan aktiviti tingkah laku spontan dan mengurangkan kejadian alodinia sentuhan dan hiperalgesia haba dengan ketara setanding dengan Diclofenac. Ifenprodil mengurangkan diameter sendi buku lali dengan ketara pada tikus artritis, manakala tiada perubahan diperhatikan dengan rawatan CORM-2. Peningkatan ketara dalam ekspresi mRNA dan protein NMDAR-2B, P2X4R, dan BDNF dapat diperhatikan pada kedua-dua talamus VPL dalam tikus artritis, yang dikurangkan dengan ketara oleh CORM-2 dan Ifenprodil. Peningkatan ketara dalam ekspresi mikroglia yang mengalami hipertrofi yang disokong oleh peningkatan tahap mRNA turut diperhatikan dalam kumpulan artritis dan parameter ini juga telah dihalang dengan ketara oleh kedua-dua rawatan tersebut. Peningkatan tahap penanda pro-apoptotik (protein kinase B; PKB, caspase-3 and caspase-8) meningkat secara signifikan tanpa perubahan ketara pada tahap protein anti-apoptotik Bcl-2 telah dikesan dalam kumpulan artritis dan parameter pro-apoptotik ini berkurang dengan rawatan CORM-2 dan Ifenprodil. Bilangan sel neuron positif Nissl menurun dalam VPL talamus kumpulan artritis dan peningkatan sel-sel ini dapat dilihat dalam kumpulan yang menerima rawatan CORM-2 dan Ifenprodil. Kesimpulannya, CORM-2 dan Ifenprodil menunjukkan kesan anti-kesakitan, anti-radang, dan anti-apoptotik terhadap tikus poliartritis kronik yang diaruhkan oleh CFA dan ia setanding dengan

kesan Diclofenac. Antagonis yang digunakan berpotensi untuk diterokai pada masa hadapan sebagai terapi tambahan yang khusus bagi menguruskan kesakitan kronik yang berterusan walaupun keradangan telah dikawal dengan baik, yang merupakan keperluan yang masih belum dipenuhi dalam pengurusan RA. Kajian ini juga memberikan pemahaman tentang peranan reseptor P2X4 dan NMDAR-2B dalam memodulasi mekanisme pusat ketika patogenesis kesakitan RA.

ROLE OF P2X4 AND N-METHYL-D-ASPARTATE-2B RECEPTORS ON PAIN MODULATION IN THALAMUS OF COMPLETE FREUND'S ADJUVANT-INDUCED CHRONIC POLYARTHRITIS RAT UPON CORM-2 AND IFENPRODIL TREATMENTS

ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease and pain being a major debilitating symptom reported by RA patients. This symptom frequently persists even after inflammation subsides, suggesting altered mechanisms beyond peripheral inflammation. CORM-2 and Ifenprodil were selected for their targeted modulation of central nociceptive pathways, specifically through inhibition of P2X4 and NMDAR-2B receptors implicated in chronic RA pain. This study aimed to investigate effects of CORM-2 (P2X4R antagonist) and Ifenprodil (non-competitive NMDAR-2B antagonist) treatments on nociceptive and apoptotic mechanisms in thalamus of complete Freund's adjuvant (CFA)-induced chronic polyarthritis rat. Eighty Sprague-Dawley male rats were randomly divided into five groups (n=16) consisting of non-arthritic control (N), arthritic control (A), arthritic groups treated with CORM-2 (A+CORM-2), Ifenprodil (A+Ifenprodil), and Diclofenac (A+Diclofenac) (positive control). The arthritic rats received intrathecal injection of either CORM-2 (20μg), Ifenprodil (0.5μg/μL), or Diclofenac (6μg) for seven days (day-16 to -22 post-arthritic induction). Meanwhile, A and N control groups were administered with normal saline (0.9%). Ankle joints' diameter and pain behaviour responses (spontaneous behaviour activities, von-Frey and hot-plate tests) were recorded on day-0 (baseline), day-15 (pre-treatment), and day-23 (post-treatment). The rats were sacrificed on day-24 and thalamus was collected for ELISA, RT-qPCR, immunohistochemistry, and

histological examination by Nissl staining. Arthritic rats demonstrated significant increase in bilateral ankle joints' diameter with reduced spontaneous behaviour activities, and persistent development of tactile allodynia and thermal hyperalgesia. Treatments with CORM-2 and Ifenprodil significantly improved spontaneous activities and attenuated tactile allodynia and thermal hyperalgesia development as comparable to Diclofenac. Ifenprodil significantly reduced ankle joints' diameter in arthritic rats, while no notable change was observed with CORM-2 treatment. Significant mRNA upregulation and increased proteins expression of NMDAR-2B, P2X4R, and BDNF were observed in bilateral thalamic VPL regions of arthritic rats which were strongly attenuated by CORM-2 and Ifenprodil. A marked increase in hypertrophied microglia expression supported by the mRNA upregulation were observed in arthritic groups and these parameters were significantly inhibited by both treatments. Significant increase in pro-apoptotic markers level (protein kinase B; PKB, caspase-3 and caspase-8) with no significant change observed in anti-apoptotic Bcl-2 was identified in arthritic groups and these proteins level were significantly reversed by CORM-2 and Ifenprodil treatments. Reduced Nissl-positive neuron cells were detected in thalamic VPL of arthritic groups and the cells were significantly increased in CORM-2- and Ifenprodil-treated groups. In conclusion, CORM-2 and Ifenprodil exerted their anti-nociceptive, anti-inflammatory, and anti-apoptotic effects on CFA-induced chronic polyarthritis rats which are comparable to Diclofenac. The antagonists could be explored in future as adjunct therapies aimed at specifically managing chronic pain that persists despite adequate control of inflammation, which is a major unmet need in RA management. This study also provides insights into the role of P2X4R and NMDAR-2B in modulating central nociceptive mechanisms during pathogenesis of RA pain.

CHAPTER 1

INTRODUCTION

1.1 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja et al., 2021; Anand and Craigb, 1996). Pain can also be referred to conscious, subjective experience or perception of feeling or sensation. Pain perception that reaches the central nervous system (CNS) comprises two distinct components which are the sensory discriminative aspect of pain (first pain) and the affective-motivational aspect of pain (second pain). These components are transmitted independently via different pain pathways (Ab Aziz and Ahmad, 2006).

Prior to the COVID-19 outbreak, the incidence of pain was on the rise, leading to adverse impacts on individuals' quality of life. Pain poses challenges not only to the economy but also to the healthcare system. It has been reported that on a global scale, one-third of the population suffers pain every day and surprisingly 1 in 5 adults under 30 years old are diagnosed with chronic pain each year. Moreover, in post-pandemic, there has been a notable escalation in the prevalence of pain conditions globally (Macchia et al., 2024). There are 85% of Malaysians were reported to experience pain, with body pain being among the most occurring types of chronic pain (Glaxo Smith Kline, 2020; Murugesan, 2023).

1.1.1 Pain transmission

Physiologically, pain signals or nociception that travel in the body from periphery to the cerebral cortex involves three important interconnecting pathways.

The first pathway starts with the first-order neuron, where nociceptors respond to different types of stimuli innervate the tissues, and are connected to axons that terminate in the spinal cord dorsal horn. In response to the stimulus, pain impulse is transmitted and transduced to its cell body within the dorsal root ganglion (DRG) (Steeds, 2016). Nociceptors are differentiated based on the conductive properties and myelination levels. Generally, nociceptors are divided into three classes: Aβ-, Aδ-, and C-fibres. Aβ-fibre is characterised by its large diameter axon (> 10 μm), myelinated with low mechanical threshold, and primarily transmits non-painful tactile as well as proprioceptive sensations. Aδ-fibre is smaller in axon diameter (> 2 - 6 μm) which transmits innocuous and noxious mechanical and thermal stimuli via Type I or Type II Aδ-fibres, respectively. Meanwhile, C-fibre is the most prevalent sensory neurons, possesses smaller axon diameter (0.2 - 1.4 μm), unmyelinated and thus results in slower conduction that responds to various noxious stimuli such as pruritogens, tissue damage, and temperature changes. C-fibres can be further categorised into peptidergic and non-peptidergic subtypes based on their response thresholds and types of neurotransmitters (Kuner and Kuner, 2021).

Communication between primary afferent terminals and second-order neurons in the spinal cord dorsal horn relies on the releasing neurotransmitters into the synaptic cleft via the exocytosis of vesicles from the pre-synaptic membrane. Glutamate, an excitatory neurotransmitter, is expressed by all primary afferent central terminals and is stored within the synaptic vesicles. Glutamate is released from the sensory neuron terminals upon membrane depolarisation and subsequent increase in intracellular calcium (Ca^{2+}) influx in the presynaptic axon. Subsequently, the neurotransmitter can activate glutamate receptors on the post-synaptic membrane, including α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and ionotropic N-methyl-D-aspartate

receptors (AMPARs and NMDARs, respectively). Noxious stimuli cause the primary nerve endings to activate AMPARs on the second-order dorsal horn neuron. This activation leads to the sodium ion (Na^+) influx and subsequent depolarisation of the neuron. Although NMDARs also respond to glutamate, their activity is normally inhibited by the binding of magnesium ion (Mg^{2+}) on its surface. However, prolonged membrane depolarisation removes this blockage, allowing glutamate-mediated activation of NMDARs and the influx of Ca^{2+}. The activation subsequently triggers a calcium/calmodulin-dependent intracellular signalling pathway in the post-synaptic neurons which further promotes the development and maintenance of sensitisation. Moreover, besides glutamate, neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) can be released from peptidergic C-fibres primary afferents upon the noxious stimulation. These neuropeptides primarily exert their effects via its receptors which present on the post-synaptic membrane surface of the second-order spinal cord dorsal horn neurons (Straub and Pongratz, 2013).

In general, the dorsal horn consists primarily 75% of excitatory interneurons and 25% of inhibitory interneurons. Excitatory interneuron releases glutamate following its stimulation whilst the inhibitory interneuron mainly secretes γ-amino butyric acid (GABA) and glycine following its activation. Within the dorsal horn, both primary afferent terminals and second-order projection neurons possess various receptors for excitatory and inhibitory neuromodulators. This allows neurotransmitter release from the primary afferents and the direct modulation of neuronal excitation (Marchand, 2008; Steeds, 2016). The primary afferent neurons synapse with the second-order neurons (spinal nerve) in lamina I and IV for Aδ-fibres and lamina I-II for C-fibres (Yam et al., 2018). There are three distinct types of second-order neurons which are nociceptive specific (NS) that respond to high threshold noxious stimuli,

wide dynamic range (WDR) that responds to sensory stimuli, and low threshold (LR) which responds to innocuous stimuli (Steeds, 2016). Subsequently, the second-order neurons propagating the nociceptive impulses will be decussated in the spinal cord via the anterior white commissure and carry the action potential up to the brain thalamus through the lateral and medial spinothalamic tracts. The lateral and anterior spinothalamic tract ascends to ventral posterolateral (VPL) nucleus of the thalamus. In the thalamus, the second-order neurons synapse with the third-order neurons and transmits the nociceptive impulses to the cerebral cortex which crucially capable to perceive pain (Ab Aziz and Ahmad, 2006; Lee and Neumeister, 2020). The overall pathway of the pain transmission is shown in Figure 1.1.

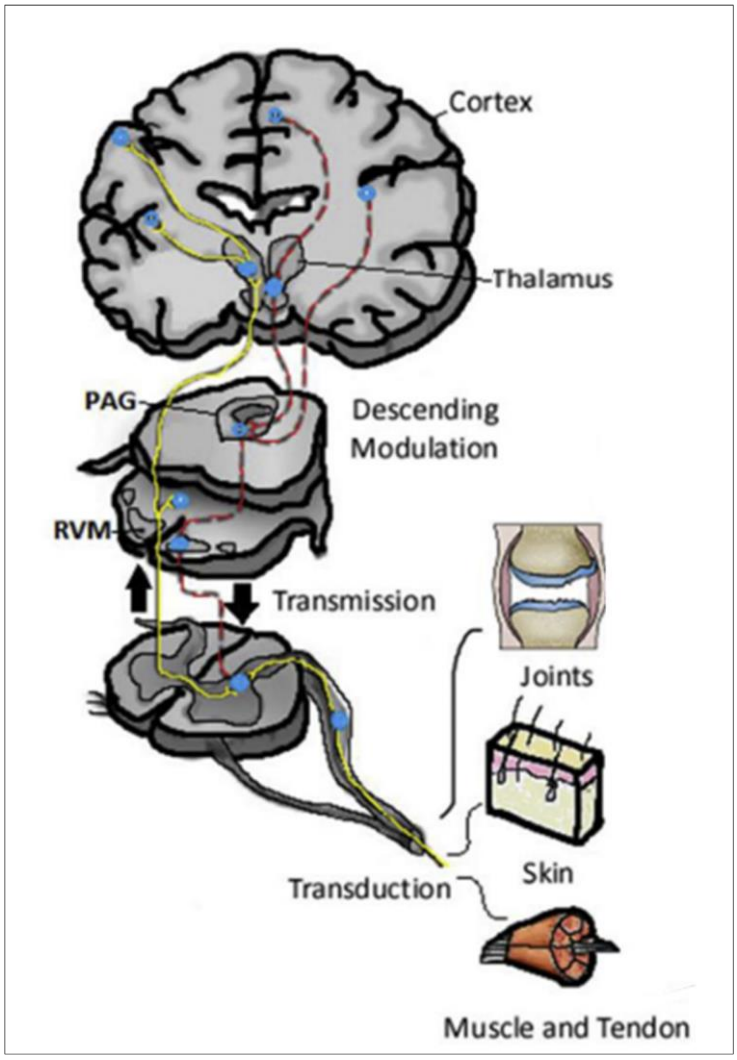

Figure 1.1 The intricate pathways of pain. The pain impulse from the nociceptive nerve endings in periphery including joints, muscles/tendons, and skin are conveyed to the spinal cord and ascends upwards to the cerebral cortex through several process encompassing transduction, transmission, and modulation of the pain signal. Adapted from Lee and Neumeister (2020).

1.1.1(a) Thalamus as a relay pain center

The thalamus is a brain region that receives nociceptive signals from several ascending pain pathways. It is located in diencephalon, closer to the brain centre and conducts multiple functions with establishing intricate connections with different brain region (Pires et al., 2024). Thalamus acts as a relay station and regarded as one of the supraspinal structures as it receives projections from various ascending pathways. The thalamocortical neurons are not only act as conduits for nociceptive signals transmission, but also involves in its processing before being relayed to the cerebral cortex (Pires et al., 2024). All the sensory inputs from the body's senses except smell are processed via thalamus before being relayed to various areas of the brain cerebral cortex. Thalamus is divided into three groups of the thalamic nuclei: (1) a lateral nuclear group, (2) a medial nuclear group, and (3) an anterior nuclear group (Figure 1.2). The significant thalamic nuclei involve anterior nuclei, dorsomedial nuclei, ventral posterolateral nuclei (VPL), ventral posteromedial (VPM) nuclei, ventral anterior and ventrolateral nuclei, medial and lateral geniculate nuclei, and reticular nuclei (Pires et al., 2024; Sola and Pulido, 2022).

The thalamic VPL nucleus is one of the various termination sites for the spinothalamic tract. The VPL neurons respond to noxious and innocuous mechanical stimuli, and some respond to visceral pain. Previous literature has demonstrated that VPL nociceptive neurons possess restricted receptive fields and accurately encode the intensity of the noxious stimuli; a function that is different from the nociceptive neurons in the ventromedial nucleus that have a larger receptive field and respond to noxious mechanical and thermal stimulation from several parts of the body (Ab Aziz and Ahmad, 2006). In fact, the VPL nucleus is regarded as a part of the ventrobasal complex of the thalamus other than VPM nucleus, which receives representative of

somatosensory projections, and VPM nucleus, which receive the projections of the stimuli from trigeminal area of the body (Sola and Pulido, 2022). The previous study demonstrated that the activation of the dorsal area of the ventrobasal complex produced non-painful tactile sensations in the awake humans (Tasker and Emmers, 1975). It is postulated that the VPL and VPM nuclei may involve in the localisation and detection of noxious and innocuous inputs, which are preferably transmitted via lemniscal pathway (Sola and Pulido, 2022).

Thalamic nuclei have participated in sensory discriminative (first pain) and affective-motivational components of the pain (second pain) (Ab Aziz and Ahmad, 2006). In the sensory discriminative aspect of the pain which involves quality, intensity, and location of the pain stimulus processing, the spinothalamic pathway carries the stimulus originating mainly from the neck of spinal cord dorsal horn neurons and terminating within the ventroposterior and ventrobasal thalamus before being projected to the primary somatosensory cortex. This pain circuit constitutes the lateral pain system which mainly functions in the discrimination of the nociceptive stimuli. Meanwhile, the affective-motivational aspect of the pain is more widespread where the stimulus derived primarily from lamina I neurons of the spinal cord dorsal horn expressing neurokinin-1 (NK1) receptor and terminates in the parabrachial area and periaqueductal grey (PAG). This circuit is mediated by medial pain pathway which include intralaminar thalamic nuclei and posterior region of ventromedial thalamic nuclei that propagates the stimuli to somatosensory cortex and limbic structures, representing the unpleasant nature of the pain perception. While spinal cord mechanisms and peripheral inflammation dominating RA pain research, the role of the thalamus is less frequently studied and thus under reported. Recent neuroimaging and neurophysiological data highlight the thalamus's potential contribution to RA pain,

advocating for more focused research on thalamic involvement (Fuggle et al., 2014; Zheng at al., 2024).

Thalamus also receives the nociceptive messages from deeper lamina V/VI from the spinal cord dorsal horn to the parabrachial internal lateral nuclei that further project the signals to paracentral nucleus or other intralaminar nuclei. The intralaminar nuclei project immensely into the brain including the anterior cingulate cortex subserving the motivational components of the pain. Due to its extensive innervation and projections from and to various pathways, thalamus is considered as parts of a neural network that participates in pain modulation and requires further research to understand the complexity of pain perception.

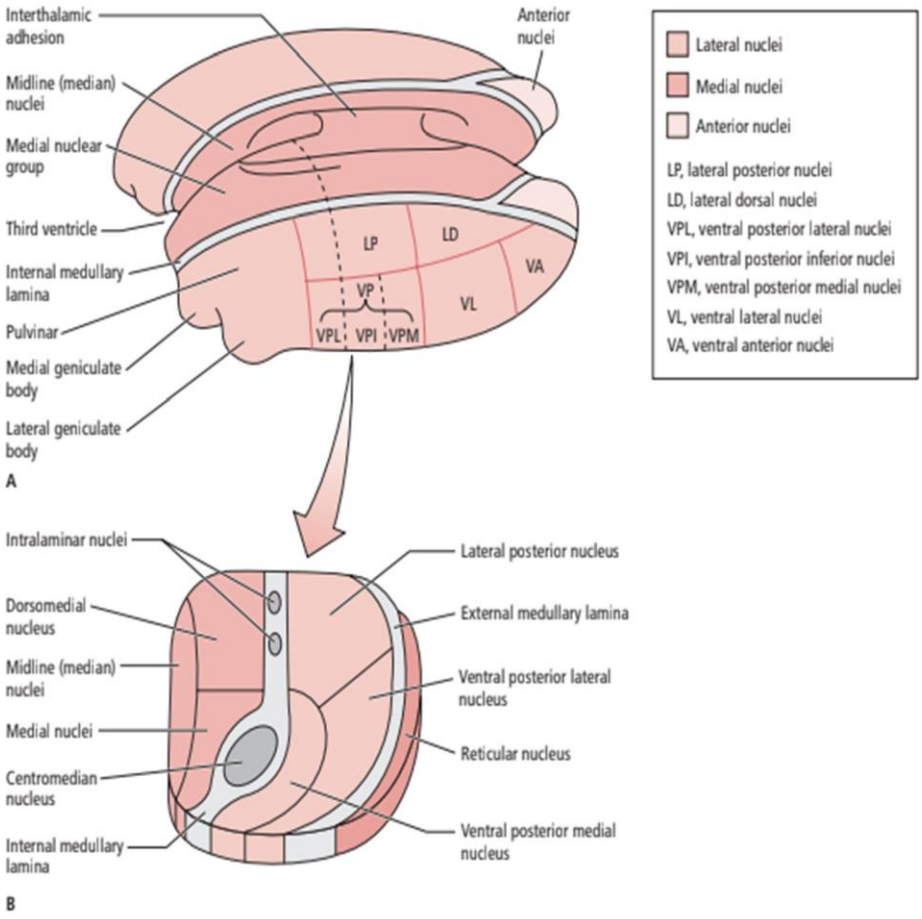

Figure 1.2 Various subdivisions of thalamus. Adopted from Patestas and Gartner (2016).

1.1.2 Pain modulation

The transmission of pain signals from the peripheral region to the spinal cord region is controlled by several mechanisms which modulate the nociceptive transmission by either enhancing or diminishing it. With appropriate treatment regimens, patients may experience reduced pain, allowing them to lead more productive routines. Previous literature has put numerous efforts to explain the ability of our body in modulating and modifying the pain. The first mechanism is descending inhibitory system which consists of the PAG and nucleus raphe magnus (NRM) regions in the brainstem that work in concert to inhibit the pain transmission. In the normal state, PAG receives the signals from higher brain centres and produces a strong analgesic action. The PAG further implicates the descending pain modulation mainly via its reciprocal connections with rostroventromedial medulla (RVM) (Fields, 2005). Meanwhile, the RVM contains on- and off-cells that are able to facilitate or inhibit the nociceptive signals, and acts as a final relay in this pain modulatory circuits. The descending pain facilitatory and inhibitory systems function in concert to sustain a baseline condition of the sensory processing. However, this balance can be perturbed by the occurrence of illness, injury, or inflammation where the heightened pain is evident if the facilitation is favoured. Whereas an increase in inhibition may conceal an underlying enhanced pain state, thereby preserving a homeostatic condition (Sola and Pulido, 2022).

Second mechanism is the activity in Aβ-fibre collaterals that commonly known as the ‘gate theory’, proposed by Melzack and Wall (1965)where the noxious stimuli can be modulated involving the A-, Aδ- and C-fibres. At the level of lamina II and III of the spinal cord dorsal horn (i.e. substantia gelatinosa), a dynamic communication between the stimuli can be observed through the large, myelinated A- fibres and that

of the thin (Aδ-fibres) and unmyelinated (C-) fibres. There are neurons that produce a presynaptic inhibition of both Aδ- and C-fibres at their synapses with T (transmitter) cells possibly located in lamina V of the spinal cord dorsal horn from which the spinothalamic fibres arise directly or indirectly. This modulation can be achieved when the large, myelinated Aβ-fibre, responsible for touch sensation (i.e. non-harmful stimuli), sends collaterals that stimulate the inhibitory interneurons of substantia gelatinosa in the spinal cord. This results in the increased inhibition of the T cells (the gate is closed and hinders the transmission of the pain signals). Meanwhile, the unmyelinated C-fibres transmit the inhibitory signals to the substantia gelatinosa cells and thus preventing the T cells' inhibition (leading to the gate opening and permitting the transmission of the pain inputs). These mechanisms eventually result in the suppression of pain signals transmission by the C-fibres (Sola and Pulido, 2022). This explains why rubbing the injured body part aids in reducing the pain sensation. The third mechanism involves spinal modulation by endogenous compounds like opioids, cannabinoid, and inhibitory amino acids. Modifying the neural activity along the pain pathway can leads to the suppression or inhibition of pain transmission (Lee and Neumeister, 2020).

1.1.3 Types of pain

There are three primary classes of pain based on the mechanism of injury. The most basic and primitive aspect of pain is nociceptive pain, or also known as acute pain. This type of pain is an early-warning physiological protective system which is essential to detect and minimise contact with damaging or noxious stimuli. It produces coordinated defence response including the withdrawal reflex and autonomic response (i.e. increased heart rate with hypothalamic-pituitary axis activation) while restricting

movement to promote tissue healing (Sola and Pulido, 2022). Acute pain refers to a transient experience lasting from a few seconds to three months which typically linked to actual or potential tissue injury (Bonica, 1973; Sola and Pulido, 2022). Some physicians further classified this physiological pain into subacute pain which occurs from 2 to 6 months from the onset. The patients with subacute pain experience distress and somatising pain where anxiety and irritability occur along with insomnia and social isolation. These persons are prescribed with sedatives and analgesics until the pain subsides (Hendler et al., 1982). According to the clinical opinion, acute pain is "normal" pain where individuals experiencing it respond well to the prescribed analgesics (Sola and Pulido, 2022; Hindler, 1982).

Meanwhile, pathological pain is the other types of pain where the pain becomes maladaptive rather than protective. This type of pain is not merely the symptoms disorders but a disease state of the nervous system (Woolf, 2010). This type of pain is known as chronic pain, occurred when the noxious inputs are persistent over time or by the modified mechanisms involved in the onset and resolution of the pain. Even after the underlying issue has been resolved, the pain continues to exist as an illness rather than as a symptom of the condition that gave rise to it. The transition of acute to chronic pain can be contributed by underlying factors such as genetics, environment, and psychology. Chronic pain encompasses persistent or recurring discomfort lasting beyond three months, often lasting for several years (Barke et al., 2022). This type of pain is in fact the transformation from the acute pain types to pathological state where the pain persists for an extended duration even in the absence of ongoing insults or after the primary tissue damage has been adequately repaired.

Clinically, the physicians categorise the pathological pain into two types: (1) chronic pain and (2) hyperchronic pain (Hindler, 1982). Individuals are indicated as

having chronic pain when the pain occurs from 6 months to 8 years with the symptoms of depression, anguish, and progressive hostile but remain dependent on their treatments. Chronic pain involves the affective-emotional component of pain via the activation of rostral and anterior dorsal surface of the corpus callosum, insula and nucleus accumbens, and dorsolateral prefrontal cortex (Galambos et al., 2019; Sola and Pulido, 2022; Vachon-Presseau et al., 2013). Meanwhile, hyperchronic pain occurs from 3 to 12 years from the onset of pain. Individuals suffering from this type of pain typically adapt to live with it, but they do not give up seeking relief. They recover their normal routines, getting back to their jobs, resuming their social and family life with the improvement in depression and sleeplessness as they adapt (Sola and Pulido, 2022; Hindler, 1982). Both chronic and hyperchronic pain involve the activation of paleospinothalamic and reticular substance pathways with the participation of the hypothalamus and limbic system (Leknes et al., 2013). Highlighting the significance of the chronic pain, Cohen et al. (2021) identified it as the primary cause of disability, impacting various aspects of a person's life. The present study will focus on delving deeper into chronic pain as its global prevalence is alarming and falls into one of the priorities by the World Health Organization (WHO) in improving the global health.

1.2 Chronic pain

There are physiological and anatomical changes during the development of chronic pain leading to peripheral and central sensitisation, formation of new neural connections, and specific pathological brain alterations (Cohen et al., 2021). Both peripheral and central sensitisation have been reported to contribute to the chronicity of the pain. Peripheral sensitisation occurs following the reduced nociceptive threshold

and the increased excitability of the peripheral nociceptors or primary afferent neurons driven by the influence of pro-nociceptive mediators. Following the tissue injury or inflammation, endogenous chemicals such as prostaglandins, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, bradykinin, substance P, extracellular adenosine triphosphate (ATP), and protein kinases are released from the injured or inflamed site. These mediators activate the voltage-gated ion channels or the ligand-gated ion channels to augment the neuronal depolarisation and enhance the action potentials that further result in a process known as sensitisation (Ohashi et al., 2023; Wei et al., 2019). These processes gradually reduce the threshold of the nociceptors for firing, amplify the responses to noxious stimuli, and stimulate firing in response to non-noxious stimuli (Steeds, 2016).

Meanwhile, central sensitisation arises from the sustained nociceptive input following the hyperexcitability of ascending neurons in the spinal cord dorsal horn and insufficient inhibitory descending signalling mechanisms (Ohashi et al., 2023). Moreover, the activation of postsynaptic NMDARs and AMPARs are important for the induction and maintenance of the central sensitisation (Ji et al., 2018). In this pathophysiological state, the affected individuals may experience spontaneous pain, perceive pain from non-noxious stimuli (allodynia), and exaggerated pain from painful stimuli (hyperalgesia) (Kohno and Tsuda, 2021; Volcheck et al., 2023).

1.2.1 Types of chronic pain

Based on the International Classification of Diseases (ICD-11), chronic pain has been categorised into several types (Treede et al., 2019):

(a) Chronic primary pain is the pain sensation seen as a disease itself. It is characterised by the persistent or recurring pain lasting over three months

affecting one or multiple anatomical areas. This pain is accompanied by notable emotional distress or functional impairment such as interference with daily activities and social participation. Chronic primary pain includes chronic widespread pain, complex regional pain syndrome, chronic primary headache and orofacial pain, chronic primary visceral pain, and chronic primary musculoskeletal pain (Barke et al., 2022).

(b) Chronic secondary pain is associated with other illnesses where the pain may initially be seen as a symptom. Even after successfully treating the initial cause, the chronic pain may persist, necessitating ongoing diagnosis and treatment such as in cancer, work accident, diabetic neuropathy, inflammatory bowel disease, or rheumatoid arthritis (Cohen et al., 2021; Treede et al., 2019).

1.2.1(a) Classification of arthritis

In general, arthritis can be classified either non-inflammatory or inflammatory arthritis depending on the location and pattern of the joint pain, presence or absence of joint oedema, rapidity of the disease onset, and the presence of signs and symptoms at the extra articular joints. The clinical features of inflammatory arthritis include degenerative ‘wear and tear’, slowly progressing, asymmetrical joint pain, and bony enlargement. The example of non-inflammatory arthritis disease includes osteoarthritis, hemathrosis, Charcot joint, hypertrophic, acromegaly, hemochromatosis, and episodic arthritis of the cystic fibrosis. The pain associated with non-inflammatory arthritis is typically worsened by the movement and weight bearing. Normally, the pain can be felt at one or both sides of the joints. However, the symptom becomes better with rest.

Meanwhile, the inflammatory arthritis causes joint stiffness with rest especially morning stiffness. Other clinical features involve auto-immune or inflammatory origin with sudden onset, relapsing, and thereafter remitting pain, symmetrical joint swelling, and increased inflammatory markers when further investigated. This type of arthritis is further categorised into either mono-, oligo-, or polyarticular types depending on the number of affected joints. Notably, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are amongst the examples of inflammatory arthritis affecting joints symmetrically or the same joints on both sides of the body (Maru and Mulla, 2020) (Figure 1.3). Given the focus of the current study on RA, the following subtopics offer important insights into the pathological mechanisms leading to RA pain, emphasising the intricate interplay between inflammatory mechanisms and pain pathways.

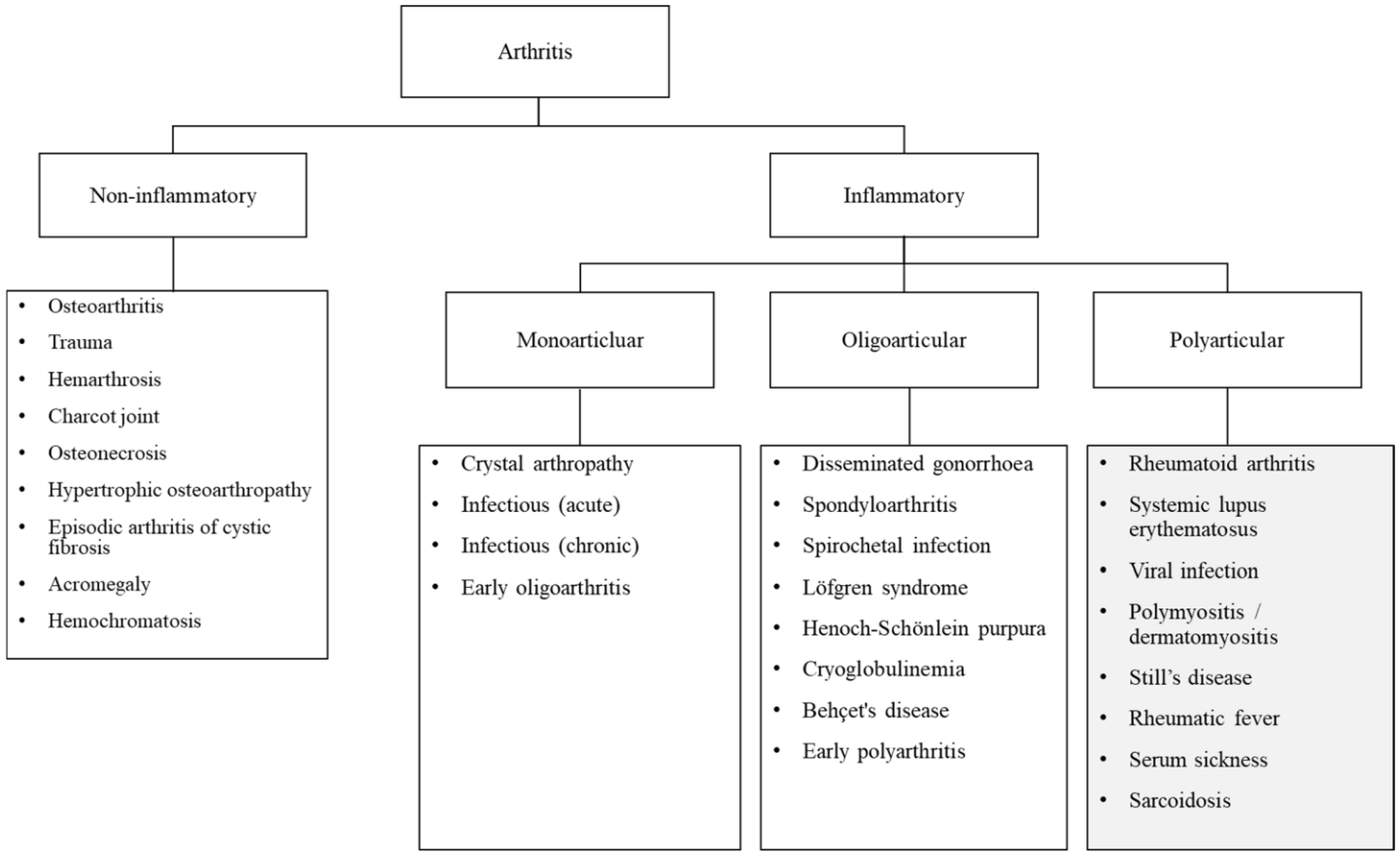

Figure 1.3 Classification of arthritis. Adapted from Maru and Mulla (2019)

1.3 Rheumatoid arthritis

Rheumatoid arthritis (RA) represents one of the most occurring chronic health problems worldwide and is a leading cause of disability (Barbour et al., 2017). A survey by Ministry of Health (MOH) shows that the prevalence of RA has increases to 74.5% with females dominantly occupies the chart and Indians are the major ethnic who suffers from RA (54.5%) followed by Malays (31.4%), Chinese (11.6%) and others (2.5%) (Shahrir et al., 2008). RA is a progressive autoimmune disease associated with a chronic inflammatory process resulting in joint and systemic inflammation. In most cases, RA manifests as symmetrical polyarthritis, although it can also exhibit non-specific symptoms such as fatigue, malaise, and mild fever. Delayed or inadequate treatment can lead to bone erosion, cartilage destruction, and joint stiffness as the progression of the disease results in severe disability (Barbour et al., 2017). RA does not only affect individuals physically and emotionally (Rahim and Cheng, 2018) but also imposes significant socioeconomic burdens on our country (Fazal et al., 2018). In 2020, the global statistics have shown the estimation of 17.6 million individuals suffered from RA with 14.1% increment since 1990 and it was estimated that 31.7% million individuals will be living with RA by 2050. Thus, reducing the global burden of RA is an urgent issue worldwide (Black et al., 2023).

Despite several biomolecular mechanisms being suggested, the exact aetiology of RA remains incompletely understood. One prevailing theory suggests that there are immunological changes due to epigenetic modification of genomic structures and environmental factors that occurs many years before the onset of RA, which regarded as pre-RA phase (Krock et al., 2018). This leads to modification of self-antigens of protein molecules such as fibrinogen, vimentin, α-enolase and collagen type II to undergo citrullination process, an irreversible post-translational modification of

arginine to citrulline residues mediated by enzymes called peptidyl arginine deiminases (PADs). In the case of RA, the immune system fails to recognise citrullinated proteins as self-antigens. As a result, antigen presenting cells (APCs) mistakenly uptake these antigens and trigger immune responses. Subsequently, the $CD4^+$ helper T cells will be activated resulting in sequential proliferation and differentiation of B cells into plasma cells that produce autoantibodies (Radu and Bungau, 2021; Volkov et al., 2020). Autoantibodies refers to proteins generated by immune system that loses its ability to differentiate between self-tissue and non-self, leading to unintended targeting of self-tissue and structures. Anti-citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF) are the most prominent autoantibodies and commonly used for RA diagnosis. These autoantibodies are significantly found in both early and established RA patients (Ministry of Health, 2019; Volkov et al., 2020). RF is a type of IgM antibody which can forms complex with IgG and complement protein following its migration to the synovial fluid to cause inflammation and joint damage.

The transition from autoimmunity to inflammation is facilitated by the changes in the inflammatory properties. Autoantibodies can trigger synovial macrophages to release TNF-a, IL-1, IL-6, stimulating fibroblast-like synoviocytes (FLS) and osteoclast cells (Yap et al., 2018). This heightened osteoclast activity may result in bone erosions. Additionally, the activated FLS generate matrix metalloproteinases (MMPs) leading to degradation of the joint cartilage. Furthermore, the presence of neutrophils in the synovial fluid has been observed following the activation of granulocyte-macrophage colony-stimulating factor (GM-CSF). This Janus kinase (JAK) pathway modulator produces protease and reactive oxygen species (ROS), exacerbating inflammation and contributing to bone and cartilage erosions (Phull et

al., 2018). Once the synovial lesions are established, a variety of both innate and adaptive immune cells are drawn to the site of injury through angiogenesis, a process that enhances vascular permeability (Wang et al., 2021).

The key symptoms of RA presented in the Clinical Practice Guideline (CPG) include joint pain, swelling and morning stiffness. In addition, RA manifests clinically through both articular and extra-articular symptoms. The articular pattern of RA is symmetrical polyarthritis affecting various joints such as metacarpophalangeal, proximal interphalangeal, interphalangeal joint of thumbs, wrist, elbows, and metatarsophalangeal. Meanwhile, the extra-articular symptoms may involve various organs such as the skin, eyes, and lungs, and result in non-systemic symptoms such as fever, malaise, and weight loss that may occur before joint symptoms (Ministry of Health, 2019; Radu and Bungau, 2021). Therefore, understanding the activity of numerous signalling molecules with pathophysiological relevance to RA is essential to modulate their mechanisms for therapeutic purposes.

1.3.1 RA pain management

Compared to other chronic diseases, pain arising from RA is multifaceted and complicated and with the pain manifesting distinguishable qualities, distribution, and intensity between the patients. A definite cure for RA remains elusive where approximately 90.4% of the RA patients refer to health professionals for severe pain (Sánchez-Flórez et al., 2022). A cross-sectional study by Azalan and colleagues (2021) reported pain had a significant association with functional difficulty in RA patients. Despite new lines of treatments and sophisticated medical technologies, they have a scarcity of analgesic options (Sánchez-Flórez et al., 2022). The available therapeutics suggested for RA pain usually rely on the duration and severity of the

symptoms. Integrated approaches to RA patient care have been employed over the years to enhance the patient's quality of life (QoL), including non-pharmacological and pharmacological approaches. The non-pharmacological approaches namely patient education and occupational therapy focus on prevention strategy as an important part of the general management of RA. Meanwhile, the pharmacological approach is based on the 'treat-to-target' (T2T) strategy involving the use of combined therapeutics to control RA pain (Ministry of Health, 2019; Smolen et al., 2010).

Various pharmacological medications are employed in the management of RA pain based on the symptoms. Disease-modifying anti-rheumatic drugs (DMARDs) are the fundamental treatment basis (Sánchez-Flórez et al., 2022) where these therapeutics are used as soon as the RA is diagnosed to slow down the disease progression (Ministry of Health, 2019). DMARDs are slow-acting drugs that may take up to eight weeks to exert their effects. Thus, these therapeutics are typically administered over the long term for the sustained effectiveness (Radu and Bungau, 2021). There are various types of DMARDs including conventional synthetic (csDMARDs), targeted synthetic (tsDMARDs), biologic (bDMARDs), and biosimilar bDMARDs. Amongst the DMARDs, csDMARDs are the first-line therapy includes methotrexate (MTX), sulfasalazine (SSZ), leflunomide (LEF) and hydroxychloroquine (HCQ). MTX serves as the key treatment in RA by inhibiting the synthesis of purine and cytokine production and potentiating adenosine receptors that contribute to its anti-inflammatory properties (Friedman and Cronstein, 2019). In the cases where the csDMARDs are ineffective or poorly tolerated by the RA patients, bDMARDs and tsDMARDs) will be prescribed.

Besides that, glucocorticoids (GCs) including hydrocortisone, prednisolone, dexamethasone, and cortisone are opted to treat aberrant inflammatory reactions. Low

doses of GCs are often used as adjunct therapy alongside (DMARDs) to achieve rapid symptomatic relief. Studies have shown that GCs can slow the progression of rheumatoid arthritis (RA) by up to 50% during the early stages of the disease (Hua et al., 2020). According to the RA clinical practice guidelines, the Ministry of Health (2019) recommends a daily dose of ≤10 mg daily of oral prednisolone for a duration of less than three months in patients with active RA. The therapeutics action of GCs is elicited through its interaction with the glucocorticoid receptor GRα and GRβ. The GC-receptor will then translocate to the nucleus and bind to the glucocorticoid response elements (GREs). This interaction regulates the genomic transcription of several genes involves in inflammation and immune response resulting in upregulation of anti-inflammatory proteins and suppression of pro-inflammatory cytokines (Hardy et al., 2020). The GCs may also result in reduction of immune cell activation including T cells and macrophages such as interleukin-1, interleukin-6 and TNF-α which are crucial in the pathogenesis of RA (Clarke and Kirwan, 2012). Furthermore, GCs may also involve in non-genomic mechanism by interacting with cell membrane-bound receptors and mediate transmembrane activity that contribute to its anti-inflammatory response (Strehl et al., 2017).

Despite its rapid action, GCs are typically prescribed for short-term use due to its potential side effects. However, GCs have a low incidence of short-term adverse events (AE), but the risk is mainly dose- and duration-dependent. The AEs of the prolonged use of GCs are commonly reported in almost all tissues and bodily system and these includes osteoporosis, glucose intolerance, obesity, muscle atrophy and increased risk of infections (Hardy et al., 2020; Hua et al., 2020). Patients usually supplemented with calcium and vitamin D and undergo frequent monitoring for infection (Ministry of Health, 2019). Long-term use of medium or high dose GCs has

been reported to increase total plasma cholesterol, triglycerides and cause redistribution of body fat (Berardicurti et al., 2020; Da Silva et al., 2006). Additionally, GCs are associated with hyperglycaemia via the alteration in insulin production. However, this condition can be reversed upon the discontinuation of GCs. Nevertheless, patients predisposed to diabetes due to the factors such as family history, aging, obesity, or a history of gestational diabetes may remain at a higher risk of developing the disease (Berardicurti et al., 2020). While GCs therapy represented a significant breakthrough for RA treatment in the 1950s, the focus today has shifted more towards its drawbacks than its advantages and the recent guidelines advocate its use as adjunct therapy with DMARDs and cannot be considered for monotherapy, as to maximise the benefits while minimising the risk.

While glucocorticoids rapidly modulate inflammation through genomic and non-genomic mechanisms, non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed to alleviate pain by mitigating cyclooxygenase (COX)-induced inflammation. These medications comprise non-selective NSAIDs (i.e. diclofenac, naproxen, and ibuprofen) and selective NSAIDs (i.e. meloxicam, celecoxib, and etoricoxib) which particularly suppress cyclooxygenase-2 (COX-2). Generally, NSAIDs effectively suppress the biosynthesis of prostaglandins at the level of the COX enzymes including cyclo-oxygenase-1 (COX-1) and COX-2. Indirectly, the control of inflammation by NSAIDs may also attenuate joint pain and peripheral tissue damage in arthritic patients (Salis and Sainsbury, 2024). It is also noteworthy that the analgesic effects of NSAIDs may also stem from their targeted actions on the CNS. Of note, the NSAIDs and GCs are judiciously prescribed at the lowest effective dose by physicians and become supplementary to the DMARDs for the management of RA pain.

In the present study, diclofenac was employed as a reference drug due to its frequent application in various anti-rheumatic research models. Diclofenac exhibits powerful suppression of mild-to-moderate pain via the strong inhibition of inflammatory processes. The administration of diclofenac through various treatment routes including oral, intramuscular, and intraperitoneal has consistently demonstrated its efficacy in lowering arthritic scores (Verhoeven et al., 2017), joint diameter (Amraoui et al., 2019; Cui et al., 2019), inflammatory cytokines levels (Al-Gareeb et al., 2019; Zhang et al., 2020), inhibiting inflammatory cells infiltration (Helmy et al., 2017), and preventing weight loss (Gonzal et al., 2020). Furthermore, the intrathecal administration of diclofenac has also been found to attenuate hyperalgesia in postoperative pain models (Vicente-Baz and Rivera-Arconada, 2020). Despite its primary target against COX, several studies have underscored diclofenac's effects on additional pathways such as transmitter systems (Fabiola Atzeni et al., 2018) and ion channels (Gwanyanya et al., 2012). Although several lines of RA medications are available, they are frequently associated with adverse side effects that are not easily tolerated by the patients related to gastrointestinal, cardiovascular, and renal systems.

The above-mentioned treatments for RA target biological components including cytokines (e.g. TNF-α and interleukins), intracellular signalling pathway (JAK pathway), and immune cells (e.g. B-lymphocyte cells) which are often co-administered with various types of DMARDs. In RA, pain is considered a generic inflammation feature and is expected to subside when the inflammation is successfully controlled. Unfortunately, the patients persistently complained of rising episodes of flare pain although the joint inflammation has been resolved (Simon et al., 2021; Zhang and Lee, 2018). This may imply that the pain is not solely caused by the inflammation but possibly due to the dysregulation of pain regulatory pathways. Like