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An Experimental Evaluation of CORM-2 and Ifenprodil in Reducing Pain via Thalamic NMDAR-2B and BDNF Suppression in Chronic Polyarthritis Rats

(Penilaian Eksperimen Terhadap Kesan Ubat CORM-2 dan Ifenprodil dalam Mengurangkan Kesakitan Melalui Penyekatan NMDAR-2B dan BDNF dalam Talamus Otak Tikus Poliartritis Kronik)

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Abstract

Rheumatoid arthritis (RA) pain is a debilitating symptom often persisting despite reduced inflammation, possibly due to dysregulated pain pathways. The study aimed to determine the effects of CORM-2 and Ifenprodil on nociceptive mechanisms involving NMDAR-2B and BDNF in thalamus of chronic polyarthritis rat mimicking RA. Eighty rats were randomly assigned into five groups: non-arthritic(N), arthritic control(A), arthritic groups treated with either CORM-2 (A+CORM-2), Ifenprodil (A+Ifenprodil), or Diclofenac (A+Diclofenac). Chronic polyarthritis was induced by injecting complete Freund's adjuvant (10mg/mL) in right footpad of the rats and intrathecal treatments were given for 7 days (Day-15 to Day-22). Pain behaviour tests evaluating tactile allodynia and thermal hyperalgesia development were conducted on Day-0 (baseline), Day-14 (pre-treatment), and Day-23 (post-treatment). Thalamus was collected for further molecular analyses. There was significant development of tactile allodynia and thermal hyperalgesia detected in arthritic rats ($p<0.05$) which were significantly reversed by CORM-2 and Ifenprodil treatments ($p<0.05$). Significant upregulation of mRNA level with increased BDNF and NMDAR-2B protein expression was detected in bilateral thalamic VPL regions ($p<0.05$). These proteins expression and mRNA levels were significantly reduced and downregulated with the treatments of CORM-2 and Ifenprodil ($p<0.05$).CORM-2 and Ifenprodil exert anti-nociceptive effects possibly by modulating thalamic nociceptive mechanisms through NMDAR-2B/BDNF signalling inhibition in chronic polyarthritis rat. This study provides insights into understanding possible mechanisms leading to RA pain for future therapeutic development and chronic pain management.

Keywords: N-methyl-D-aspartate-2B receptor, brain-derived neurotrophic factor, CORM-2, Ifenprodil

Abstrak

Kesakitan daripada Artritis Reumatoid (RA) merupakan gejala yang melemahkan dan selalunya berterusan walaupun setelah keradangan berkurang, berkemungkinan disebabkan oleh perubahan pada mekanisme kesakitan yang tidak terkawal. Kajian ini bertujuan untuk menentukan kesan CORM-2 dan Ifenprodil terhadap mekanisme kesakitan melibatkan NMDAR-2B dan BDNF dalam talamus tikus poliartritis kronik seperti RA. Sebanyak lapan puluh ekor tikus dibahagikan secara rawak kepada lima kumpulan: kumpulan bukan artritis (N), artritis kawalan (A), serta kumpulan artritis yang dirawat dengan CORM-2 (A+CORM-2), Ifenprodil (A+Ifenprodil), atau Diclofenak (A+Diclofenac). Induksi poliartritis kronik dilakukan melalui suntikan adjuvan pelengkap Freund (CFA; 10mg/mL) pada kaki kanan tikus dan rawatan secara intratekal diberikan selama tujuh hari (Hari ke-15 hingga -22). Ujian tingkah laku kesakitan seperti alodinia sentuhan dan hiperalgesia haba dijalankan pada hari ke-0 (nilai asas), -14 (pra-rawatan), dan -23 (pasca-rawatan). Sampel talamus kemudiannya diambil

untuk penganalisaan molekul. Peningkatan ketara pada alodinia sentuhan dan hiperalgesia haba dikesan dalam tikus artritis yang berjaya dikurangkan dengan ketara oleh rawatan CORM-2 dan Ifenprodil ($p<0.05$). Tahap mRNA yang meningkat dengan ekspresi protein BDNF dan NMDAR-2B yang lebih tinggi dikesan pada kedua-dua kawasan VPL dalam talamus ($p<0.05$). Ekspresi protein dan kadar mRNA penanda ini juga mengalami pengurangan yang ketara selepas rawatan diberikan. Kesan pengurangan kesakitan dengan rawatan CORM-2 dan Ifenprodil berkemungkinan disebabkan oleh modulasi mekanisma kesakitan di talamus melalui perencatan NMDAR-2B dan BDNF dalam tikus poliartritis kronik. Kajian ini memberikan pemahaman yang lebih mendalam mengenai mekanisma kesakitan RA yang berpotensi membantu dalam pengurusan kesakitan kronik pada masa hadapan.

Kata Kunci: Reseptor N-methyl-D-aspartate-2B, faktor neurotropik berasal dari otak, CORM-2, Ifenprodil

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease primarily affecting bilateral synovial joints leading to pain, inflammation, and progressive structural damage. RA is a progressive musculoskeletal disorder which is often characterised by periods of flare-ups and remission. According to global estimates, approximately 17.6 million individuals were affected by RA in 2020 with projections suggesting this number may rise to 31.7 million. This represents a 14.1% increase in prevalence since 1990 (Black et al. 2021), highlighting the urgency for improved therapeutic strategies to delay disease progression and alleviate symptoms.

Pain is among the most debilitating symptoms of RA with many patients experiencing moderate to severe pain (Ibrahim et al. 2022; Cox et al. 2025). Current clinical management primarily focuses on controlling disease activity through anti-rheumatic drugs and inflammatory markers. However, pain assessment often remains secondary despite being a major concern for patients and significantly impacting daily functioning. While international guidelines advocate for pain control via disease remission or low disease activity, the relationship between pain and inflammation is not always linear. Notably, some patients report persistent pain even when inflammation is well-controlled (Sandström et al.2022), suggesting alternative mechanisms may be involved. This underscores the importance of investigating the underlying pathophysiological processes that contribute to chronic RA pain.

Chronic pain involves complex interactions between neurons, non-neuronal cells, and peripheral tissues leading to pathological changes in both peripheral and central nervous systems. Among non-neuronal cells, microglia play a pivotal role in maintaining central nervous system homeostasis and responding to inflammatory or neuropathic insults (Qin et al. 2023). Upon activation, microglia undergo morphological transformation and release a range of pro-nociceptive, oxidative, apoptotic, and inflammatory mediators. One key activation pathway involves purinergic P2X and P2Y receptors, particularly the P2X4 receptor (P2X4R) which responds to ATP released from damaged cells. P2X4R activation leads to the release of brain-derived neurotrophic factor (BDNF), a potent modulator of synaptic plasticity and pain transmission as demonstrated in various neuropathic pain models (Qin et al. 2023; Fiore et al. 2022). BDNF has been shown to potentiate N-methyl-D-aspartate receptor subtype 2B (NMDAR-2B) in dorsal root ganglion neuronal cell cultures as evidenced by the increased Tyr-1472 phosphorylation and elevated NMDAR currents (Chen et al. 2014). This suggests a potential mechanism by which microglia-derived BDNF may potentiate NMDAR-mediated signalling in chronic pain. Meanwhile, NMDAR-2B activation has been widely implicated in neuropathic pain and inflammatory pain models (Ismail et al. 2021; Deng et al. 2019). Enhanced NMDAR-2B activity as driven by glutamate has been associated with hyperalgesia and allodynia whilst pharmacological inhibition of this receptor alleviates pain (Ismail et al. 2021). In chronic monoarthritic pain models, increased NMDAR-2B expression facilitates calcium (Ca^{2+}) influx leading to mitochondrial overload, dendritic damage, apoptosis, and synaptic dysfunction (Gong et al. 2017). Additionally, NMDAR-2B activation promotes recruitment of astrocytes and microglia, further amplifying nociceptive signalling and inflammatory cascades (Ismail et al., 2021). In RA, elevated BDNF and inflammatory mediators in synovial fluid may exacerbate NMDAR-2B signalling, contributing to joint degradation and disease progression (Sochal et al. 2022). Although NMDAR-2B is minimally expressed in normal cartilage, its upregulation in autoimmune arthritis underscores its potential as a therapeutic target in arthritic pain.

The use of nociceptive and inflammatory inhibitors has shown promise in mitigating pain hypersensitivity in chronic models. Ifenprodil, a selective NMDAR-2B antagonist, exhibits over 200-fold selectivity for the NR1/NR2B complex compared to NR1/NR2A. Its anti-nociceptive effects have been demonstrated in various models including sciatic nerve injury and diabetic neuropathy where it attenuated tactile allodynia potentially via

suppression of microglial activation and the BDNF/DREAM signalling pathway (Kim et al. 2012; Ismail et al. 2021). Tricarbonyldichlororuthenium (II) dimer (CORM-2) is a lipid-soluble carbon monoxide-releasing molecule (CORM-2) that possesses potent anti-oxidative and neuroprotective properties. Unlike inhaled carbon monoxide (CO), CORM-2 delivers CO directly to target tissues with minimal carboxyhaemoglobin production, suggesting a safer therapeutic profile. Previous studies have shown that CORM-2 can inhibit oxidative stress, reduce pro-apoptotic signalling, and protect neuronal structures (Joshi et al. 2020). Notably, CORM-2 also exerts anti-inflammatory and anti-nociceptive effects potentially through non-competitive antagonism of P2X4R and inhibition of microglial activation in both *in vitro* and *in vivo* models.

Despite these promising findings, the efficacy and mechanism of CORM-2 and Ifenprodil in RA-associated chronic pain remain unexplored. Therefore, the study aimed to evaluate the effects of CORM-2 and Ifenprodil on nociceptive mechanisms involving thalamic NMDAR-2B and BDNF in a rat model of chronic polyarthritis. We hypothesise that both chemicals exert anti-nociceptive effects through inhibition of the central nociceptive signalling pathway, offering therapeutic targets for RA-related chronic pain.

MATERIALS AND METHODS

Animals

The animal experimentation has strictly followed the guidelines approved by USM Institutional Animal Care and Use Committee (USM IACUC) (USM/IACUC/2019/(120)(1027). Eighty Sprague-Dawley male rats (275-300 g, 8-10 weeks old) were housed in polypropylene cages (3 rats per cage) maintained at standard 12-hours light-dark cycle with 27 ± 2◦C and 35-50% humidity. The rats were allowed free access to water and Atromin food pellets (ad libitum) and allowed for four days acclimatisation prior to the experimentation.

Experimental design and groups allocation

Prior to the research, a sample size calculation was performed using PS Software version 3.12 (Dupont and Plummer, 1998) taking into accounts the Type-1 error (α) of 0.05, power of study of 80%, and ratio of control to test (m) of 1 with 20% drop-out rate. The healthy rats with no prominent physical abnormalities were selected for this research. Based on the calculation, eighty rats were randomly assigned into five groups (n=16) comprising of (1) non-arthritic control (N), (2) arthritic control (A), arthritic groups treated with either CORM-2 (20 µg/day; A + CORM-2), Ifenprodil (0.5 µg/day; A + Ifenprodil), or Diclofenac (6 µg/day; A + Diclofenac). The experimentation took place 24 days. Rats were induced into inflammatory arthritic state on day-0 (D0) and allowed to progress into chronic condition for 2 weeks (D14). The treatments were intrathecally administered for seven days (D15 to D22) and behavioural tests were conducted on D0 (baseline), D14 (pre-treatment), and D23 (post-treatment). The rats were sacrificed on D24, and brain thalamus was collected for further molecular analyses. The experimental timeline is illustrated in Figure 1.

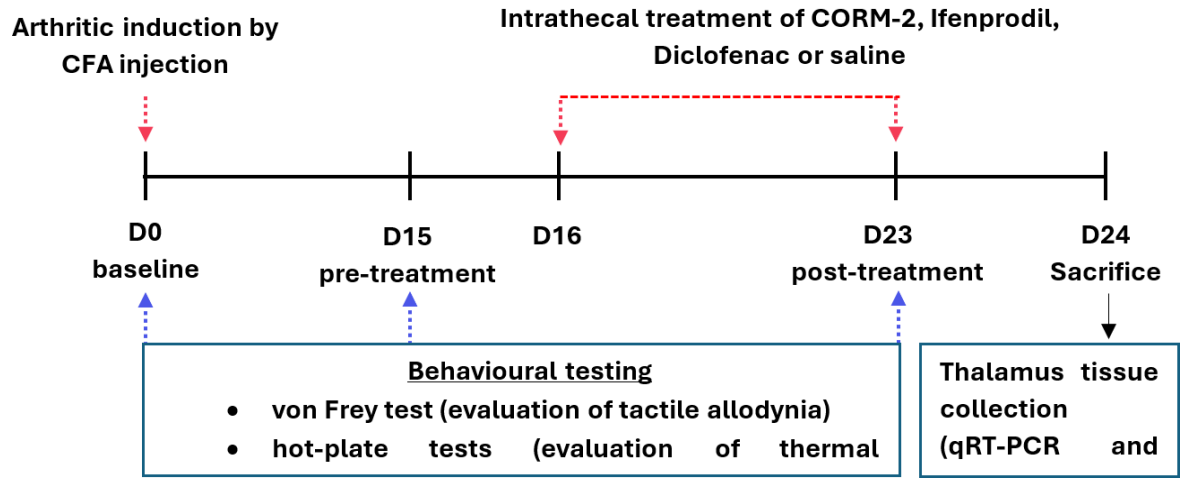


Figure 1. Schematic diagram of the study timeline.

Induction of chronic polyarthritis in rat

The induction of experimental immunological arthritis followed Mahdi *et al*., (2018). The rat was anaesthetised with 4% isoflurane-oxygen mixture to minimise the painful effect and physical struggle due to the adjuvant injection in the rats. Following the absence of pinch reflex, the right hind limb was initially sterilised by 70% (v/v) alcohol, and 0.1 mL of CFA containing *Mycobacterium butyricum* (heat-killed pathogen) at 10 mg/mL was injected into the subplantar region of the right foot pad. The time of adjuvant inoculation was referred to as day 0 (D0; baseline). After the procedure, the rat was allowed to be fully conscious within 2 minutes and transferred to the cage. The rats were observed and monitored on alternate days for the onset, progression, and severity of the arthritis manifest by oedema and erythema of the hind paws and ankle joints. For this purpose, body weight was measured by taking the percentage difference of body weight gain within 24 day-experimentation period in all groups.

Direct drug delivery by intrathecal injection

The procedure for direct drug delivery followed Lu and Schmidtko (2013) where the rats were initially anaesthetised by isoflurane inhalation until the absence of pinch reflex was detected. Space between L5 and L6 spinous processes representing cauda equina was identified and used as the main puncture site. A reflexive tail flick or S-shape formation by the tail was regarded as a reliable puncture of the dura mater. The confirmation of the puncture site indicated by the complete loss of all motor responses and temporary paralysis (~7 minutes) was confirmed by the 2% lidocaine injection. The rats received intrathecal administration of either vehicle (0.9% saline) (for control groups) or selected drugs (for treatment groups) for seven consecutive days standardised at 0800 – 0900 on day-15 to day-22 post-arthritic induction.

PAIN BEHAVIOURAL ASSESSMENTS

Tactile allodynia

Von-Frey test was carried out following Zulazmi et al. (2015) to assess the development of tactile allodynia in the rats. The experimentation was conducted in a quiet room to avoid any environmental-induced emotional disturbances. The rats were placed on a wire mesh floor separated by compartments and adapted for 30 minutes before the experimentation. A gradual increase of force was applied to the midplantar region of the rat's hind paw. The pressure that evoked withdrawal of the paw represents maximal noxious threshold and expressed in gram (g) (Deuis et al. 2017). The procedure was performed initially on the CFA-injected hind paw (ipsilateral side) followed by the non-CFA injected hind paw (contralateral side). The tactile stimulus was given three times, and the mean value of the paw withdrawal threshold was recorded (Zulazmi et al. 2015). 10-minute interval was allocated between the stimulations to hinder the development of sensitisation and adaptation to the stimuli.

Thermal hyperalgesia

Hot-plate analgesia meter was used to evaluate the development of thermal hyperalgesia in the rats. This test was performed after the von-Frey test. Before the experimentation, the animals were adapted to the behaviour assessment room for 10 minutes. Several indicators were applied to denote the rat's thermal threshold: the hind paw lick, back paw flick, rapid fanning or hop from the beginning of the thermal stimulation (Yam et al. 2020). The surface of analgesia meter plate was constantly heated at 52.5 ± 0.5˚C and cut-off time was set at 18 s to hinder any tissue injury in the absence of response (Mahdi et al. 2018). The rats were immediately removed from the apparatus after the test.

ANIMAL TERMINATION

The rats were deeply anaesthetised by an overdose injection of sodium pentobarbitone (100 mg/kg body weight) on day-24 post-arthritic induction. The rats were subjected to either fresh dissection for RT-qPCR purpose or cardiac perfusion followed by decapitation for immunohistochemistry purpose.

BRAIN THALAMIC MRNA TRANSCRIPT LEVELS OF BDNF AND NMDAR-2B

RNA extraction, quality, and purity determination

The thalamus tissues were thawed and removed from RNAprotect tissue reagent (Qiagen, USA). The RNA was extracted from each tissue (70-80 mg) using RNeasy Plus Universal Mini Kit (Qiagen, USA) following the manufacturer's protocols. The concentration and purity of the extracted RNA was determined using a BioPhotometer apparatus. The RNA concentration and RNA purity were determined and recorded based on the A260/280 ratio in which the purity was kept within the 1.8 to 2.0 range. The RNA integrity was evaluated by conducting gel electrophoresis. An aliquot of RNA sample was run

on a denaturing 1% w/v agarose gel stained with a highly sensitive stain for visualisation.

Real-time Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The qRT-PCR was performed using PCR Biosystem 2x qPCRBIO SyGreen (PCR Biosystem Ltd., United Kingdom) and Step One, Applied Biosystem (Thermo Fischer Scientific, USA). The qPCR mixture was prepared in PCR strips and the samples were assigned in the following sequence; GOI, internal reference, no template control (NTC) and negative RT (-RT). A summary on the volume of reagents placed into each well are listed in Table 2.8. All samples were prepared and run in triplicate. The 2-step cycling was conducted with cDNA polymerase activation for 2 minutes at 95˚C; PCR amplification for 40 cycles consisting of denaturation step for 5 seconds at 95˚C, annealing and extension steps for 30 seconds at 60˚C. The step was followed with melt curve stage. The relative mRNA expression level was normalised to GAPDH using the 2-ΔΔCt method (Livak and Schmittgen 2001).

BRAIN THALAMIC BDNF AND NMDAR-2B PROTEINS EXPRESSION

Perfusion-fixation procedure

After the euthanisation by an overdose injection of sodium pentobarbitone, a thoracotomy was performed on the rat. A 21 G branula attached to an automated pressure pump was inserted into the left ventricle of the heart while a snip was made to the right atrium as an outlet. Perfusion was conducted with an initial pressure of 35 mmHg to resemble physiological pressure before it was increased up to 55 mmHg to obtain the best possible preservation of the brain thalamus (Gage et al., 2012). 1X PBS solution was initially infused until clear fluid was flushed out followed by 500 mL of ice-cold 4% paraformaldehyde in 0.1 M PB solution (pH 7.4). The brain thalamus tissue was collected and fixed in 4% cold paraformaldehyde in 0.1M PB solution for 4 hours at 4 °C. Next, the tissue was cryopreserved overnight in 20% sucrose in 0.1M PB solution at 4 °C until the tissue sank to the bottom of the container.

Preparation of tissue sections

Approximately 0.2 cm of the anterior and posterior brain tissue was carefully cut to obtain ‘level 3’ area approximating at the Bregma -4.44 mm to -2.28 mm (Rao et al. 2011). The tissue was made into frozen blocks and sectioned at 7 µm by a cryostat. 2-3 brain tissue sections were gently transferred and aligned onto poly-L-lysine microscopic glass slides to prevent the peeling of the tissue sections. The slides were air-dried at room temperature for 30 min before the immunohistochemistry staining.

Immunohistochemistry staining

The sections were rinsed twice with tris-buffered saline (TBS) for 5 min each. A circle was drawn around each tissue using a hydrophobic peroxidase-anti-peroxidase (PAP) pen. 200 µL of diluted primary antibody was added onto the circle followed by an overnight incubation at 4 °C. On the next day, the tissue sections were rinsed with Tris/Triton-X (TBS-Tx) triple times for 5 min each followed by the incubation of biotinylated goat anti-rabbit IgG (1:200 dilution in mixed buffer solution; mixture of TBS with 2% normal goat serum and 0.2% Triton-X) for 1 hour at room temperature. Subsequently, the TBS/Tx rinse steps were repeated, and 200 µL of ABC reagent diluted at 1:50 in TBS solution was added onto the brain tissue slides and incubated for 1 hour at room temperature. Next, the tissue sections were rinsed before 200 µL of DAB reagent (1:10 dilution with 1X stable peroxidase substrate buffer) was added until brown colouration appeared. The reaction was then stopped by triple times rinsing with TBS solution (5 min each), followed by a quick rinse of distilled water. The tissue was air-dried, counterstained with haematoxylin solution for 20 s, and dehydrated in absolute ethanol for 15 min before mounted with Cytoseal mounting medium and cover slipped.

IHS scoring and analysis

Positive and negative controls from other tissues with high expressions of NMDAR-2B and BDNF staining (i.e. L4-L5 lumbar region of spinal cord) and elimination of primary or secondary incubation were run to validate the immunohistochemical interpretations. Three sections of the brain thalamus from each animal were examined in which the images of four non-overlapping areas in the region of interest, ventral posterolateral (VPL) nucleus was obtained using a light microscope connected to an image analyser at 40X magnification. The immune-positive cells regarded as dark brown staining of all targeted protein markers were counted using ImageJ Software version 1.8.0 (National of Health, USA) and were determined using combinative semiquantitative scoring schemes for BDNF and NMDAR-2B adapted from Zhan et al. (2020). The scoring was further validated by a pathologist and two independent observers blinded to the experimental groups. The counting rules of exclusion and inclusion

line were applied for the quantification process. A reference picture was then captured for each score and each measure to serve as a guide for all the experimental scores. The percentage of immune-positive cells was counted over the total cells in the four selected areas and scored. Tissue sections with high background staining were excluded to avoid misinterpretation. Next, the tissue morphology properties of IHC expression; staining intensity was described as no staining, weakly positive, positive, and strongly positive. Each property was also scored by multiplying the percentage of positive cells score by staining intensity score, as below:

Immunohistochemical scoring (IHS) = % positive cells score × staining intensity score

STATISTICAL ANALYSIS

The data obtained from this study was analyzed using GraphPad Prism version 8.0.2 software (GraphPad, San Diego, CA). The normality test and variance of the data was performed using a Shapiro-Wilk and D'Agostino-Pearson Omnibus to obtain data distribution. Two-way repeated measures ANOVA with post-hoc Tukey's test was employed to analyse von-Frey and hot-plate tests whilst one-way ANOVA with post-hoc Bonferroni test was used to analyse mRNA expression and IHS score of BDNF and NMDAR-2B markers at both ipsilateral and contralateral sides of brain thalamus. The level of significance was set at $p < 0.05$ and the results were presented as mean ± standard error of mean.

RESULTS

No change in percentage of body weight gain

Generally, a significant difference in the percentage of body weight gain was detected between the groups ($F_{4, 55} = 5.824$, $p < 0.001$). Further analysis by post-hoc Tukey's test reported a marked decreased percentage of the body weight gain in the arthritic groups compared to the non-arthritic control group ($p < 0.05$). No significant difference was detected in the body weight between the arthritic-treated groups and the A group (Figure 2). Additionally, the general evaluation revealed no prominent changes observed on the physical well-being in the arthritic rats compared to the N group pertaining to the skin, fur, eye colour, and nasal discharge. Despite the significant change in the body weight, the arthritic rats were alert to their surroundings.

Tactile allodynia development was inhibited by CORM-2 and Ifenprodil treatments

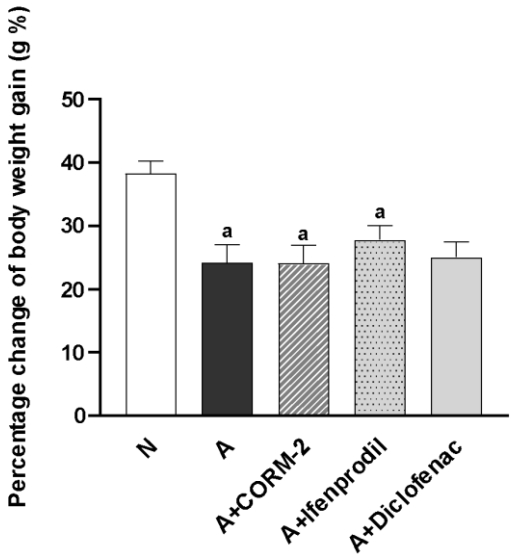
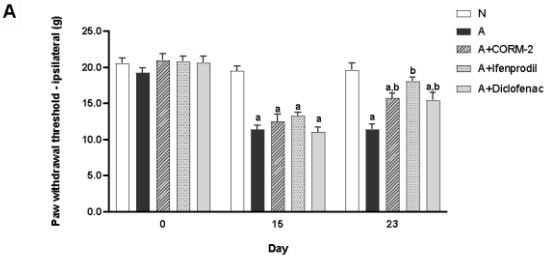


Figure 2. Percentage of body weight gain in the experimental groups. Values are expressed as mean ± S.E. M. (n = 16). ap < 0.05 significant comparison with nonarthritic (N) group.

Two-way ANOVA revealed significant main effects in the treatment groups ($F_{4, 55} = 17.48$, $p < 0.001$) and time ($F_{2, 108.7} = 131.6$, $p < 0.001$) for paw withdrawal threshold (PWT) at the ipsilateral side of the rat's hind paw. Similarly, the significant main effects in the treatment groups ($F_{4, 55} = 5.70$, $p < 0.05$) and time ($F_{2, 106.8} = 19.07$, $p < 0.001$) were also shown in the PWT of the contralateral hind paw of the rat. Further post-hoc analysis demonstrated a marked decrease in the ipsilateral and contralateral PWT of the A group compared to the N group ($p < 0.05$), indicating the development of tactile allodynia. Meanwhile, the 7-days intrathecal treatments of CORM-2 significantly increased the PWT at both sides of the hind paw, especially ipsilateral hind paw compared to A group ($p < 0.05$). The reversal of tactile allodynia development by these treatments was found to be as efficient as Diclofenac ($p < 0.05$). Meanwhile, the statistical analysis also reported the marked interactions between the treatment groups and time implying the effect of treatments on the bilateral development of tactile allodynia ($F_{8, 110} = 8.492$, $p < 0.001$ for ipsilateral and $F_{8, 108} = 5.914$, $p < 0.001$ for contralateral hind paws) may differ over the treatment days (Figure 3).



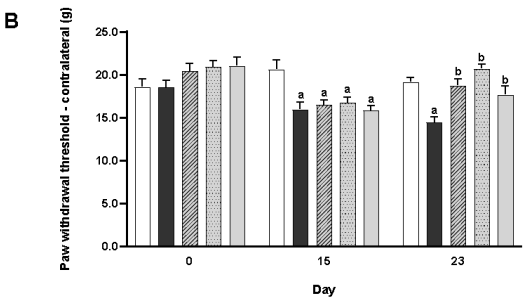


Figure 3. Paw withdrawal threshold representing development of tactile allodynia at (A) ipsilateral and (B) contralateral hind paws in the experimental groups using von Frey test on D0 (baseline), D15 (pre-intervention), and D23 (post-intervention). Values are expressed as mean ± S. E. M. (n = 16). $^{a}p < 0.05$ vs N group, $^{b}p < 0.05$ vs A group as reported by two-way ANOVA followed by Post-hoc Tukey test.

Thermal hyperalgesia development was attenuated by CORM-2 and Ifenprodil treatments

A significant main effect in the treatment groups ($F_{4, 54} = 14.31$, $p < 0.001$) and time ($F_{2, 104.6} = 40.38$, $p < 0.001$) was detected for thermal withdrawal threshold (TWT) in the arts. Post-hoc Tukey's test demonstrated constant reduction in the TWT throughout the experimentation in the A group compared to the N group ($p < 0.05$). The treatment with CORM-2 strongly increased the TWT in the arthritis rats compared to the A group ($p < 0.05$). The effect of CORM-2 was found to be comparable to Diclofenac, indicating its potent role in reversing the development of thermal hyperalgesia. Although there was no statistical difference, the TWT was significantly increased following the Ifenprodil treatment in the rats compared to the A group ($p < 0.05$). The statistical analysis also showed the prominent interactions between the treatment group and time ($F_{8, 108} = 2.243$, $p < 0.05$), suggesting the effectiveness of the treatments on thermal hyperalgesia vary over different experimental days (Figure 4).

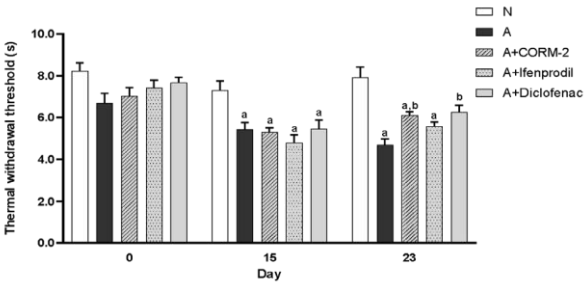


Figure 4. Thermal withdrawal threshold representing thermal hyperalgesia development by hot-plate test in the groups on D0 (baseline), D15 (pre-intervention), and D23 (post-intervention) (n = 16). The values are expressed in mean ± S. E. M. $^{a}p < 0.05$ vs N group, $^{b}p < 0.05$ vs A group as reported by two-way ANOVA followed by post-hoc Tukey test.

Mean relative NMDAR-2B (*Grin2B*) mRNA expression

The RT-qPCR data demonstrated a significant upregulation of the *Grin2b* expression in the A group compared to the N group at the bilateral regions of the thalamus ($p < 0.05$). This upregulation was successfully reversed upon the treatments of CORM-2, Ifenprodil and Diclofenac at both thalamic regions compared to the A group ($p < 0.05$). Further comparison made on the thalamic *Grin2b* mRNA expression showed significant differences between the groups at the ipsilateral ($F_{4, 20} = 7.892$, $p < 0.05$) and contralateral regions ($F_{4, 20} = 7.363$, $p < 0.05$), which showed that treatment with Ifenprodil expressed the lowest mean relative *Grin2b* level on the ipsilateral side (Figure 5).

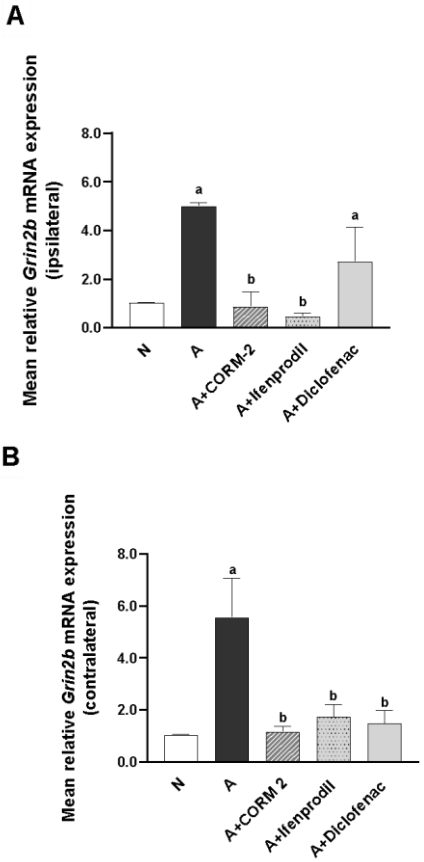


Figure 5. Thalamic mRNA expression for *Grin2b* (NMDAR-2B) in all groups at (A) ipsilateral and (B) contralateral sides. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group.Effects of CORM-2 and Ifenprodil on mean relative *BDNF* mRNA expression of thalamus

No significant effect of *BDNF* mRNA expression was detected at the ipsilateral side of the thalamus ($F_{4, 20} = 2.113$, $p > 0.05$) (Figure 6A). Conversely, a significant difference in this parameter was detected at the contralateral region of the rat's thalamus ($F_{4, 20}$

= 6.728, $p < 0.05$) (Figure 6B). Further analysis by post-hoc Tukey's test reported a marked upregulation of the *BDNF* mRNA level in the A group compared to the N group. The treatments by CORM-2 and Ifenprodil significantly downregulated this *BDNF* mRNA expression compared to the A group, and this effect was comparable to that of Diclofenac treatment ($p < 0.05$).

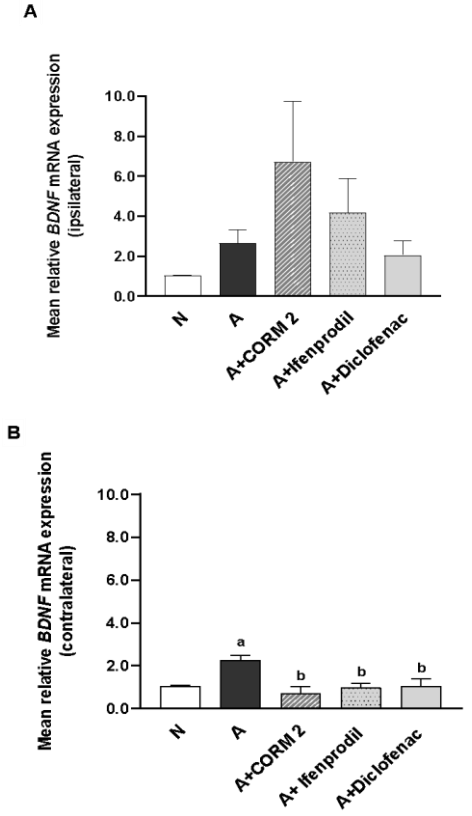


Figure 6. Mean relative *BDNF* mRNA expression in rat's thalamus in all groups at (A) ipsilateral and (B) contralateral sides. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group.

Attenuation of thalamic NMDAR-2B protein expression by CORM-2 and Ifenprodil treatments

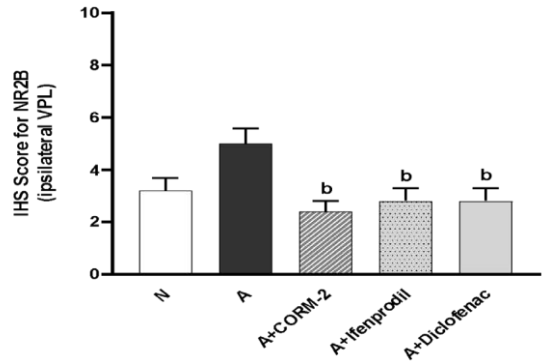


Figure 7. NMDAR-2B IHS score and protein expression at ipsilateral thalamic VPL in experimental groups. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group.

There was a marked difference in the NMDAR-2B protein expression between the groups at the ipsilateral ($F_{4, 20} = 3.900$, $p < 0.05$) and contralateral ($F_{4, 20} = 5.073$, $p < 0.05$) regions of the thalamic VPL. In specific, the semi-quantitative evaluation at the ipsilateral side revealed that the NMDAR-2B IHS scores were increased in the A group compared to the N group although it was not statistically significant. Compared to the A group, the NMDAR-2B IHS score was prominently lower in the rats treated with CORM-2, Ifenprodil, and Diclofenac ($p < 0.05$) (Figure 7). At the contralateral VPL of thalamus, a similar increase of IHS score of NMDAR-2B was shown in the A group compared to the N group ($p < 0.05$). Remarkably, the treatments of CORM-2 and Ifenprodil significantly decreased the NMDAR-2B IHS score compared to the A group, as effective as Diclofenac treatment ($p < 0.05$) (Figure 8).

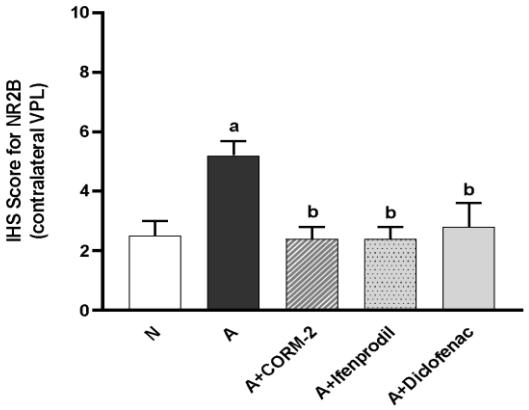


Figure 8. The IHS score and protein expression of NMDAR-2B at contralateral thalamic VPL in the experimental groups. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group. Arrows indicate NMDAR-2B-immunoreactive cell expression in the tissues.Suppression of thalamic BDNF protein expression by CORM-2 and Ifenprodil treatments

There was a significant difference in the BDNF protein expression between the groups at the ipsilateral region of the thalamic VPL by the analysis of one-way ANOVA ($F_{4, 20} = 5.994$, $p < 0.05$). A significant difference in the BDNF protein expression was similarly demonstrated at the contralateral thalamic VPL between the groups ($F_{4, 20} = 6.438$, $p < 0.05$).

Further post-hoc analysis revealed a prominent increase in the IHS score indicating BDNF positive cells expression in the A group compared to the N group ($p < 0.05$) at the ipsilateral VPL. The administration of CORM-2 and Ifenprodil significantly decreased the BDNF IHS score compared to the A group ($p < 0.05$). A similar pattern of reduction was also observed in the Diclofenac-treated group although it was not statistically significant (Figure 9).

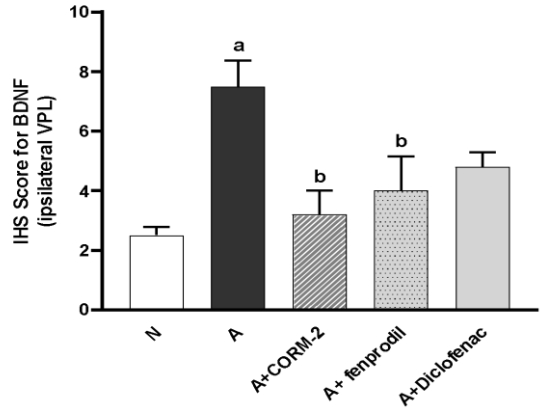


Figure 9. The IHS score and protein expression of BDNF at ipsilateral thalamic VPL in the experimental groups. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group. Arrows indicate BDNF-immunoreactive cell expression.

Correspondingly, the BDNF IHS score at the contralateral side of the thalamic VPL was significantly increased in the A group compared to the N group ($p < 0.05$). Similar to the ipsilateral region, the immunostaining showed a marked decrease in the BDNF IHS score after the administration of CORM-2, Ifenprodil, and Diclofenac at this region (Figure 10).

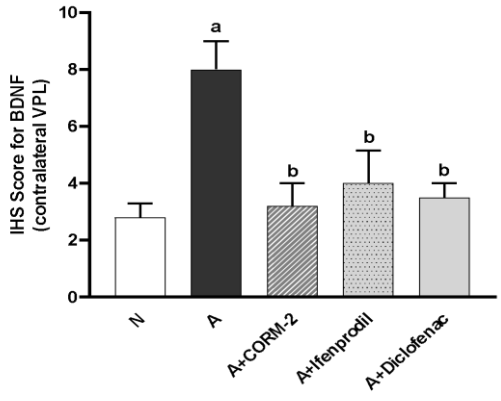


Figure 10. The IHS score and protein expression of BDNF at contralateral thalamic VPL in the experimental groups. Values are expressed as mean ± S.E.M (n = 5). $^{a}p < 0.05$ significant comparison with N group, $^{b}p < 0.05$ significant comparison with A group. Arrows indicate BDNF-immunoreactive cell expression in the tissues.

DISCUSSION

The present study investigated the possible involvement of thalamic NMDAR-2B and BDNF in increased pain behavioural responses via the development of tactile allodynia and thermal hyperalgesia in the chronic polyarthritis rat through the antagonizing effects of CORM-2 and Ifenprodil. The well-being of the arthritic rat was also monitored through changes in body weight and physical health.

Chronic polyarthritis rats displayed decreased paw withdrawal threshold at both ipsilateral and contralateral hind paws indicating the development of tactile allodynia. Similar allodynic symptoms have been reported in the previous studies following the arthritic induction by CFA in other rat models (Yang et al. 2023; Berke et al. 2022). Importantly, the development of allodynia as measured by the von Frey test is likely categorised as primary and secondary allodynia since the tested location is remote to the primary injury. It is generally agreed that the mechanisms mediating these reactions are different. The primary reaction is mainly attributed to the occurrence of peripheral sensitisation (at the injured site) and possible development of central sensitisation. Meanwhile, the secondary reaction is mainly caused by the changes in the sensory signals processing that results in the development of central sensitisation. The previous studies using different RA models also demonstrated that the development of allodynia is possibly due to the central changes mediated by activated microglia-driven mechanisms (Nieto et al. 2016). Hervera and co-workers (2012) proposed that the development of allodynia and hyperalgesia is possibly associated with the overexpression of nitric oxide synthase (i.e. NOS-1 and NOS-2) and microglia activation as shown in the spinal cord of NOS-2-knock-out mice with sciatic nerve injury. Moreover, it is also believed that the development of these neuropathic pain symptoms is contributed by the activation of the microglial P2X4 receptor in the CNS as reported by Jurga and colleagues (2017). Based on these findings, it can be postulated that the occurrence of tactile allodynia and thermal hyperalgesia in the chronic polyarthritis rats may results from the altered central plasticity of neuronal nociceptive pathways. Interestingly, the treatment with CORM-2 has markedly increased the tactile threshold and thermal hyperalgesia comparable to the standard drug Diclofenac as primarily observed in the ipsilateral hind paws of the arthritic rats. It is postulated that CORM-2 strongly inhibited peripheral and central synthesis of NO via the reduction of NOS-1/NOS-2 overexpression (Hervera et al. 2012). CORM-2 was also believed to exert anti-allodynic and anti-hyperalgesic effects by activating the haeme oxygenase-1/carbon monoxide (HO-1/CO) signalling activation, inhibited mitogen-activated protein kinase (MAPK) phosphorylation that further attenuates microglial activation in the spinal cord and brain regions of chronic constriction injury rat. Other than that, the attenuation of tactile allodynia by CORM-2 could be due to its inhibition against P2X4 receptor as previously reported in several chronic and neuropathic pain models (Jurga et al. 2017; Hervera et al. 2012). The inhibition of this mechanism by CORM-2 may indirectly suppress the development of central sensitisation by

microglia activation and glia-neuronal crosstalk.

Similarly, the intrathecal treatment of Ifenprodil markedly reduced the bilateral development of tactile allodynia although insignificantly reduced the development of thermal hyperalgesia in the arthritis rat, as similarly reported in PDN rat model (Ismail et al. 2019). The previous studies reported that the tyrosine phosphorylation of NMDAR-2B in the spinal cord was enhanced in the *in vivo* rat model of behavioural hyperalgesia and allodynia (Ismail et al. 2019; Guo et al. 2002). Although very limited study investigated the expression of thalamic NMDAR-2B during chronic pain state, the previous *in situ* hybridisation study demonstrated that NMDAR-2B is highly expressed in thalamus (Wu et al. 2009). The present study also showed the enhanced expression of NMDAR-2B in the bilateral thalamic VPL in the arthritis rat. It is possible that Ifenprodil non-competitively inhibited the activation of NMDAR-2B especially in the CNS, leading to the suppression of central sensitisation and pain hyper-responsiveness.

In the present study, the mRNA expression was investigated at the bilateral regions of thalamic VPL to determine the effect of CORM-2 and Ifenprodil on the associated nociceptive mRNA regulation during the pathogenesis of chronic polyarthritis. Although the *Grin2b* gene expression in the thalamus is scarcely reported, its similar upregulation has been reported in other regions including spinal cord following the CFA injection (Lv et al. 2020). Meanwhile, the increased NMDAR-2B protein expression at the bilateral thalamic VPL was observed in the present study where the results were contradicted by the insignificant changes of NMDAR-2B currents in the electrophysiological findings by Wu and colleagues (2005) in their CFA-induced inflammatory mice model. The increased NMDAR-2B mRNA expression could be an adaptive process to strengthen the synapses and lead to the development of central sensitisation (Torsney 2019). These modifications could be implicated by the protracted inflammatory processes or associated to persistent pathological changes during chronic polyarthritis. According to the previous literature, the activity of extracellular signal-regulated kinase ½ (ERK ½) and MAPK signaling pathway mediates the increased NMDAR-2B phosphorylation and activation (Deng et al. 2019).

As the NMDAR-2B is predominantly distributed in several nociceptive-relevant structures including brain thalamus (Wu et al. 2009; Kolhekar et al. 1997), the upregulation of NMDAR-2B mRNA and protein expression may result in heightened pain perception and increased pain behavioural responses in the rat. The excessive glutamate production may bind and activate ionotropic receptors including NMDARs in the thalamic region resulting in enhanced mechanical and thermal hypersensitivity (Rychlik et al. 2023). This is shown by Kolhekar and colleagues (1997) where the blockade of the NMDARs or decreasing these receptors' expression has yielded in the suppression of acute thermal and mechanical hyperalgesia in carrageenan-induced inflammatory rat model. The crucial involvement of NMDARs, specifically NMDAR-2B subtype, in the development of chronic pain is also supported by other previous findings. In a spared nerve injury animal model, the administration of NMDARs antagonist MK-801 and Dynorphin A (1-17) has reversed morphine-induced excessive glutamate release which further attenuated abnormal increase of primary afferent activity in the spinal cord and the occurrence of hyperalgesia (Ma et al. 2020). Since ACC neurons also receive inputs from various thalamic nuclei (Rychlik et al. 2023), it is possible that the increased NMDAR-2B protein expression in this brain region also indicates its possible increased expression in the thalamic region of the rat.

As hypothesised, the upregulated *Grin2b* mRNA and increased NMDAR-2B protein expressions in both thalamic VPL regions of the arthritic rat have been successfully reversed by the administration of Ifenprodil and CORM-2. The similar findings were also reported by Mirante and colleagues (2014) in mice brain of long-term depression (LTD) mechanisms concerning the thalamic pathway. Ifenprodil probably attenuated the related intracellular signalling pathways leading to the phosphorylation of the NMDAR-2B. Since NMDAR-2B may allow Ca^{2+} influx during the nociceptive signals transmission, the inhibition of Ca^{2+} into the neuron may inhibit Calcium/Calmodulin-dependent protein kinase II (CaMKII) pathway activation, thereby inhibiting further phosphorylation of various mediators and the increased synaptic strength and aberrant neuronal plasticity. While the effect of CORM-2 on NMDAR-2B is scarcely reported, it is postulated that CORM-2 inhibited the NMDAR-2B transcription and expression via its anti-oxidative action that indirectly implicates the HO-1/CO-mediated pathway. The increased HO-1 level may further inhibit the signalling activation pathways leading to the NMDAR-2B activation and consequently attenuated the NMDAR-2B-induced glutamate toxicity and synaptic hyperactivity (Lal et al. 2021). Besides, CORM-2 potentially disrupted the activation of MAPK signalling pathways including p38 MAPK and JNK ½ which are the crucial pathways to neuroinflammation and aberrant synaptic hyperactivity. In turn, the suppression of pro-inflammatory mediators' release including IL-1β and TNF-α by CORM-2 may subsequently decrease the NMDAR-2B activity (Yan et al. 2021; Wei et al. 2010). Notably, similar downregulation

of NMDAR-2B mRNA and decreased protein expression at the bilateral sides of thalamic VPL in the chronic polyarthritic rat of the present study also proves its significant role although further investigation on its mechanisms of action is required.

Meanwhile, the strong increase of thalamic BDNF protein expression was also observed in the chronic polyarthritis rat despite no statistical difference in its mRNA expression at the ipsilateral thalamic VPL region. A slight discrepancy was observed as a marked mRNA upregulation of BDNF was identified at the contralateral thalamic VPL region. This discrepancy suggests a post-transcriptional mechanism or cellular localisation as various regulatory mechanism can affect the translation of a protein. Furthermore, the mRNA and the protein could be localised differently in a cell such as nucleus and peripheral microglial processes (PeMPS). However, further investigation into this mechanism during arthritic state is required. It is also postulated that the increased BDNF protein transcription and translation is closely linked with the increased activation of P2X4 receptor of the activated microglia. Following the P2X4 receptor activation, p38 MAPK is phosphorylated and consequently results in the BDNF release from the activated microglia. Later, BDNF may activate tyrosine kinase-B (TrkB) receptor expressed on the neurons, downregulates potassium-chloride co-transporter-2 (KCC2) activity, and phosphorylates the NMDARs including NMDAR-2B that consequently results in aberrant neuronal excitation. This microglia-neuronal crosstalk mediated by BDNF is one of the major contributions to the development of central sensitisation and pain hyperresponsiveness (Ma et al. 2020; Nijs et al. 2015) as shown by the development of tactile allodynia and thermal hyperalgesia in the arthritic rats of the present study. It is also supported by Nijs and colleagues (2015) that the upregulated NMDAR with reduced KCC2 activity in spinal cord lamina I neurons and in spinothalamic neurons has contributed to the occurrence of central sensitisation and possibly to the development of secondary hyperalgesia and allodynia.

Interestingly, CORM-2 and Ifenprodil treatments has significantly downregulated the *BDNF* mRNA contralaterally but not ipsilaterally, while strongly suppressed the BDNF protein expressions in both regions of thalamic VPL of the arthritic rats. It is postulated that CORM-2 exerted this effect by attenuating the P2X4 receptor activation and its downstream signalling cascades including p38 MAPK and ERK ½ in the CFA-induced inflammatory pain rat (Jurga et al. 2017; Jurga et al. 2016). Meanwhile, the effect of Ifenprodil on thalamic BDNF level was scarcely reported in the previous study. However, the similar suppression by Ifenprodil was demonstrated in the spinal cord of PDN rats (Ismail et al. 2021). The thalamic BDNF suppression by Ifenprodil could be associated with the significant reduction of pain behaviour responses in the chronic polyarthritis rat as BDNF was reported to stimulate NMDAR-2B-mediated long-term potentiation development (Jurga et al. 2017; Jurga et al. 2016). Notably, BDNF acts as an autocrine signalling factor which may indirectly enhance the neuronal activity in CNS (Prowse et al. 2021). Ifenprodil possibly attenuate the downstream pathway to BDNF activation that consequently implicate the nociceptive transmission and pain behaviour responses during the pathogenesis of chronic polyarthritis.

CONCLUSION

The present study demonstrates that thalamic NMDAR-2B and BDNF play significant roles in the development of tactile allodynia and thermal hyperalgesia in the chronic polyarthritis rats, possibly via mechanisms involving neuronal-non-neuronal crosstalk. The treatments with CORM-2 and Ifenprodil were effectively alleviated the pain-related behaviours, possibly through their strong mechanisms involving selective P2X4 receptor and NMDAR-2B antagonisms. The disruption of these critical signalling mechanisms contributing to the suppression of central sensitisation development may offer potential therapeutic benefit of CORM-2 and Ifenprodil in the future management of chronic pain. In overall, the findings underscore the therapeutic potential of targeting thalamic NMDAR-2B and BDNF pathways to mitigate neuropathic pain and central sensitisation in chronic inflammatory conditions like RA.

ABBREVIATIONS

ATP : adenosine triphosphate
BDNF : brain-derived neurotrophic factor
Ca^{2+} : calcium ion
CaMKII : calcium/calmodulin-dependent protein kinase II
CFA : complete Freund's adjuvant
CNS : central nervous system
CO : carbon monoxide
CORMs : carbon monoxide-releasing molecules
CORM-2 : tricarbonyldichlororuthenium (II) dimer
DAB : 3, 3'-diaminobenzidine
DREAM : downstream regulatory element antagonist modulator
DRG : dorsal root ganglion
ERK : extracellular signal-regulated kinase
GABA : γ-amino butyric acid
GPCRs : G-protein coupled receptors

HO-1 : haeme oxygenase-1
IHS : immunohistochemical staining
IL-1β : interleukin-1β
IL-18 : interleukin-18
JNK : c-Jun N-terminal kinase
KCC2 : potassium-chloride co-transporter-2
LDP : long-term depression
LTP : long-term potentiation
MAPK : mitogen-activated protein kinase
mRNA : messenger ribonucleic acid
NF-kβ : nuclear factor kappa-light-chain-enhancer of activated B cells
NMDAR-2B : N-methyl-D-aspartate-2B receptor
NOS : nitric oxide synthase
PAP : peroxidase-anti-peroxidase
PB : phosphate buffer
PBS : phosphate buffered saline
PeMPS : peripheral microglial processes
PKC : protein kinase C
PYK2 : proline-rich tyrosine kinase 2
RA : rheumatoid arthritis
RT-qPCR : quantitative reverse transcription polymerase chain reaction
STAT3 : signal transduce and activator of transcription-3
TBS : tris-buffered saline
TBS/Tx : tris-buffered saline/Triton X
TNF-α : tumour necrosis factor-α
VPL : ventral posterolateral

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DECLARATIONS

Author contributions

Khir NAM: Conceptualisation, Investigation, Writing-original draft, Noh ASM: Investigation, Zin AAM: Validation, Writing-review & editing, Long I: Writing-review & editing, Yusop N: Writing-review & editing, Ismail CAN: Conceptualisation, Writing-review & editing, Supervision

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

This study was approved by the USM Institutional Animal Care and Use Committee (USM IACUC) (USM/IACUC/2019/(120)(1027).

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