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Effect of cannabidiol, cannabinol and tetrahydrocannabivarin in managing inflammatory pain

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Current medications used to treat the inflammatory pain either have limited effectiveness or may be associated with serious side effects. Non-psychoactive phytocannabinoids may have a better safety profile and could be used as an alternative approach to treat this condition. The aim of this study was to test the potential of three phytocannabinoids, cannabinol (CBN), tetrahydrocannabivarin (THCV) and cannabidiol (CBD), in treating inflammatory pain. The inflammatory pain was triggered in male rats by a single intra-articular knee injection of the complete Freund's adjuvant (CFA). One week later, rats were given four daily administrations of ibuprofen, CBN, THCV or CBD. Our data demonstrated that CFA injection triggered an inflammatory response expressed as damage of the synovial tissue, reduced locomotor activity, increased mechanical and, to a lesser extent, thermal pain sensitivity, and loss of body weight. All phytocannabinoids reduced mechanical hyperalgesia and had no or a minor effect on locomotor activity. Treatment with CBN also lowered thermal hypersensitivity. Treatment with CBN and THCV recovered the body weight of CFA-injected rats. However, administration of CBD reduced body weight and elevated blood monocyte and granulocyte levels above those of the CFA-injected control animal group. We conclude that CBN and THCV may have potential in managing inflammatory pain.

Keywords Inflammatory pain, Rats, Cannabidiol, Cannabinol, Tetrahydrocannabivarin, Ibuprofen

Infections (viral, bacterial) can trigger inflammatory processes in the body (e.g., irritable bowel syndrome, cystitis, myositis, arthropathy, peripheral neuropathy) that are typically accompanied by the development of chronic pain persisting even after successful treatment of the infection[1]. In these situations, pain may be caused by several contributing factors, including an abnormal immune response, persistently elevated levels of inflammatory enzymes, and sensitization of both the peripheral and central nervous systems[1]. Opioids are the most effective analgesics to date; however, they are not only ineffective in treating inflammatory pain-related conditions[2] but can also increase an organism's susceptibility to infections[3]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, relieve inflammation and nociceptive pain through inhibition of cyclooxygenase (COX) enzymes. However, these drugs may increase the severity of established infections and reduce antibiotic efficacy[4], and their long-term use may be associated with a risk of gastrointestinal and cardiovascular adverse events[5,6].

The endogenous cannabinoid system is one of the most abundant systems in the brain, modulating nearly all physiological processes, including pain perception, arousal, and functions of the immune system[7–9]. It has also been demonstrated that this system may be involved in many known pathological conditions, including the state of chronic inflammation and pain[8]. In recent years, research has focused on modulating the endocannabinoid system functions by use of non-psychoactive compounds of the cannabis plant[10].

Cannabis plants have long been used both medicinally and recreationally, mainly due to the psychoactive compound delta-9-tetrahydrocannabinol (THC). However, the health benefits of these plants may be attributable to over a hundred other non-psychoactive compounds or their metabolites, collectively termed phytocannabinoids[11]. While studies on the major phytocannabinoid cannabidiol (CBD) for treating inflammatory pain have yielded contradictory results, the compound has demonstrated efficacy in reducing

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neuropathic pain in several animal models[12–14], suggesting that CBD has the potential to treat pain-related conditions, either as a monotherapy or in combination with other drugs[15]. CBD has multiple molecular targets, behaving as an antagonist of the CB1 and CB2 receptors and as an agonist of several TRP ion channels, all of which are essential for transmission of pain signals and regulation of anti-inflammatory responses[10,16–18]. Additionally, CBD enhances effects of the endocannabinoid anandamide (AEA) via several mechanisms[19,20]. The antinociceptive effect of CBD may also involve several other peripheral and central targets (e.g. opioid, 5-HT, glycine, adenosine receptors)[17].

In the present study we explored the impact of three phytocannabinoids – CBD, cannabinol (CBN) and tetrahydrocannabivarin (THCV) – on inflammatory pain in male Wistar rats. It has been demonstrated that CBN acts as a low potency partial agonist of the CB1 receptor and as an agonist of both the CB2 receptor and TRPA1 channels[16,21]. THCV is a neutral antagonist of the CB1 receptor; it also acts as a partial agonist of the CB2 receptor and an agonist of TRPV1/TRPV2 channels[16,22,23]. Because of their mechanism of action, it has been suggested that both CBN and THCV may have anti-inflammatory and analgesic properties[16,24].

To achieve the study goals, inflammatory pain was induced by a single intra-articular knee injection of the complete Freund's adjuvant (CFA). CFA contains heat-killed mycobacteria, causing localized hyperalgesia associated with immune cell recruitment and inflammatory changes in the cartilage and synovium of the joint[25–28]. Ibuprofen was used as a positive control, as it has already been demonstrated that this treatment is effective in reducing both inflammation and hyperalgesia in this animal model [e.g.[29]]. To verify induction of chronic inflammation in the CFA-injected knee, the synovial tissue damage was evaluated three weeks after the injection procedure by scoring synovial hyperplasia and inflammatory cell infiltration. To test the effectiveness of ibuprofen, CBD, CBN and THCV on rat behaviour, three behavioural tests were employed: the locomotor activity was measured in the open field test; mechanical and thermal pain thresholds of the intact and injured legs were assessed using the von Frey and Hargreaves tests. Blood samples were collected 24 h following the last administration of the test compounds to monitor the general health status of rats.

Materials and methods

Animals

One hundred forty-three two-month-old outbred male Wistar rats (from our own breeding colony at Vilnius University, Lithuania) were used. Animals were housed in groups throughout the experimental period in standard rat cages under a 12-hour light/12-hour dark cycle (lights on at 7:00 a.m.). Room temperature was kept constant (temperature: 22 ± 1 °C). Standard laboratory rat food (Altromin, Germany) and tap water were provided *ad libitum* throughout the experimental period. All experimental procedures were approved by the State Food and Veterinary Service of the Republic of Lithuania and were carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

Drugs

Ibuprofen (Sigma-Aldrich, Germany) and CBD (THC Pharm GmbH, Germany) were suspended in 0.9% saline containing 0.5% methylcellulose (Sigma-Aldrich, Germany) and injected as a volume of 3 ml/kg intraperitoneally (IP). CBN and THCV (generously provided by Sanobiotec, Lithuania) were dissolved in Tween (Sigma-Aldrich, Germany) and then diluted with 0.9% saline to a final Tween concentration of 4%, and injected as a volume of 2 ml/kg IP. Control animals received an equal volume of respective vehicles.

Experimental design

Experiment 1: Model establishment

In order to establish a CFA-induced inflammatory pain model in the lab, 2 groups of rats ($n = 8$ per group) were used (animals were randomly assigned to experimental groups). One group of rats was given a single unilateral injection of 100 µl of CFA (Sigma-Aldrich, Germany) into the knee joint of one hind leg using a 27G needle under brief isoflurane anaesthesia. Another group received the same volume of 0.9% saline injected into the corresponding knee joint. All rats were tested three times – once before and twice after administration of either saline or CFA (at the end of the first and third post-injection weeks). Locomotor activity, mechanical and thermal (heat) pain thresholds of both intact and CFA-injected hind legs were assessed in three behavioural tests: open field, von Frey and Hargreaves. One day after completing the behavioural tests, rats were sacrificed by exposing them to CO2, and the knee joints of both saline- and CFA-injected rats ($n = 4$ per group) were collected for histological assessment.

Experiment 2: Pharmacological study

Although inflammatory markers in the knee joint were identified three weeks after the CFA injection, findings of Experiment 1 showed that changes in rat behaviour were not as pronounced at this time point as they were one week after the CFA injection. Hence, in order to test the effect of ibuprofen and phytocannabinoids, animals underwent behavioural testing twice, the first time three days (T1) and the second time ten days (T2) following an injection of either saline or CFA into one knee joint. For the ibuprofen and phytocannabinoid testing, four independent experiments were performed (Fig. 1). In each experiment, CFA-injected rats ($n = 24$) were divided into three groups based on their behaviour at T1 (i.e., distance travelled in the open field test, mechanical and thermal pain sensitivity in the von Frey and Hargreaves tests) but maintaining grouping randomisation within each cage ($n = 8$ per treatment condition). Starting on the 7th CFA post-injection day, rats were subjected to four once daily administrations of either vehicle (see "Drugs", CFA-Veh reference control group), ibuprofen (15 kg/kg and 60 mg/kg), CBD (10 mg/kg and 20 mg/kg), CBN (5 mg/kg and 10 mg/kg) or THCV (1 mg/kg and 5 mg/kg). Please note that the dosing of test compounds and treatment regimen was based on our pilot studies and published reports[29–34]. Prior to this study, we established that a single administration of all phytocannabinoid

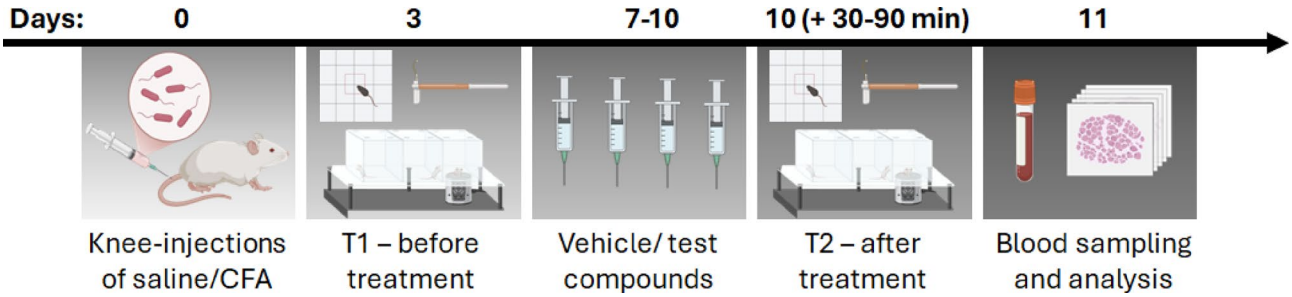

Fig. 1. Experimental time-line of the pharmacological study. On day 0, rats were injected with either saline or CFA into the knee joint of one hind leg, and underwent behavioural testing twice: three days (T1) and ten days (T2) following the injection. CFA-injected rats were divided into groups based on their behaviour in T1. Starting on the 7th post-injection day, CFA-injected rats were subjected to four once daily administrations of vehicle (CFA-Veh control rats), ibuprofen, CBD, CBN or THCV. Saline-injected rats received four vehicle administrations (SAL-Veh healthy control rats). On the last treatment day, behavioural assessments were performed approximately between 30–90 min post-injection. Blood samples were collected and analysed on the following day.

doses used in this study did not alter locomotor activity in healthy rats in the open field test. The rationale for repeated drug administration was to increase the translatability of our findings, since medications for inflammatory pain are typically taken over long periods, which can lead to drug tolerance and the development of side effects. Saline-injected rats received four vehicle administrations (see "Drugs") and served as the healthy control group (SAL-Veh, $n = 7–8$ per experiment). On the last treatment day, behavioural assessments were performed between approximately 30 and 90 min post-injection (T2), starting with the open field test, followed by von Frey and Hargreaves tests. Blood samples were collected the following day to assess the effect of treatment on the general health status of the rats. Body weights of rats were measured prior to saline/CFA injection and on the last day of behavioural tests. The experimenters responsible for animal handling, behavioural testing, and data analysis were blind to the experimental design.

Histological assessment of the knee joint

Tissue preparation

Knee joints were dissected, fixed in 10% buffered formalin and decalcified in formic acid and hydrochloric acid solutions for 48 h. After that, the knee joints were cut along the sagittal plane and embedded in paraffin (Milestone Tissue Processors, Milestone Medical, Italy). Then, specimens were cut into 3 µm sagittal sections using a microtome (LEICA RM 2145, Leica Biosystems, Germany) and stained with the haematoxylin and eosin (H&E) stain (LEICA HistoCore SPECTRA Workstation, Leica Biosystems, Germany). Stained sections were scanned using the Leica Aperio AT2 scanner (Leica Biosystems, Germany) and analysed using the open-source QuPath-0.4.3 software for digital bioimage analysis.

Histopathological assessment of synovitis

Histopathological assessment of synovitis was performed according to the method described by Kaneguchi et al.[35]. Hyperplasia of the synovial lining was scored as 0 = no changes (< 2 cell layers thick), 1 = mild lining hyperplasia (3–5 cell layers thick), 2 = moderate lining hyperplasia (6–9 cell layers thick), and 3 = marked lining hyperplasia (≥ 10 cell layers thick). Inflammatory cell infiltration of the synovial lining and synovial stroma was scored as 0 = no infiltration, 1 = minimal infiltration, 2 = moderate infiltration (diffuse and/or presence of occasional cell aggregates), and 3 = marked infiltration (diffuse and/or presence of many aggregates). All assessments were performed in the posterior (near the posterior horn of the meniscus) and anterior (near the patella) region of the knee joint.

Behavioural tests

Open field test

To measure locomotor activity, anxiety levels, and willingness to explore a novel environment, an open field apparatus was used. The open field was a dark plexiglass box (floor: 48 × 48 cm, height: 50 cm). The light was adjusted to 80 lx (measured in the centre of a square). A centre zone (16 × 16 cm) was defined in the middle of each box (the time spent in the centre of the arena was not affected by any of the procedures and thus was omitted from the result description). All animals were habituated to the experimental room one day prior to the test. For the test, the animal was placed in the centre of the box, and the experimenter then left the room. A camera above the apparatus recorded the animal's movements for a period of 30 min. The program "Viewer" (Biobserve GmbH, Germany) was used to analyse the distance travelled by the animal (horizontal activity) and the centre visits. The number of rearings (vertical activity) was manually scored by a trained observer blind to the experimental design.

von Frey test
To evaluate mechanical pain threshold, the von Frey apparatus was used. The apparatus consisted of several rat-holding clear acrylic chambers (floor: 20 × 10 cm, height: 16 cm) placed on an elevated wire mesh, and a series of von Frey monofilaments (ranging from 0.6 to 26 gram-force; Bioseb, France). Prior to the test, animals underwent three 5-minute habituation sessions (separated by 2–3 days) to the test chambers. In order to test the mechanical pain threshold, monofilaments were applied perpendicularly to the plantar surface of both hind paws with sufficient force to cause slight bending. Each monofilament was applied at least twice in succession, and a paw withdrawal response was recorded as a positive reaction. The up-down method[36] was used to determine the mechanical pain threshold, defined as ≥ 50% paw withdrawal, in response to mechanical stimulation (gram-force).

Hargreaves test
IITC Plantar Analgesia Meter Model 400 Base apparatus (IITC Life Science, USA) was used to evaluate thermal nociceptive sensitivity of hind paws[37]. The apparatus consisted of several rat-holding clear acrylic chambers (floor: 20 × 10 cm, height: 16 cm) placed on an elevated glass platform. A concentrated infrared laser beam was guided by visible light to assist in directing the beam to the centre of the paw. The paw withdrawal delay was measured by a built-in timer (operated by a push-button), which displayed the reaction time in 0.01-second increments. To avoid damaging the paw tissue, a 20-second cut-off time was used. Prior to the test, animals underwent three 5-minute habituation sessions (separated by 2–3 days) to the test chambers. Each paw withdrawal was attempted at least three times, with a minimum of five minutes between each trial.

Blood micro sampling procedure and analysis
To minimize stress and ensure animal welfare, a blood micro sampling procedure was employed, whereby blood samples were collected into a capillary tube from the gently punctured tail tip (1–2 mm from the end) using a sterile needle. The blood was immediately transferred to an automated analyser (Exigo EOS; Boule Medical, Sweden) for haematological analysis. The complete blood count values were measured, including lymphocytes (LYM), monocytes (MONO), granulocytes (GRAN), haemoglobin (HGB), haematocrit (HCT), red blood cells (RBC), platelets (PLT), and others.

Statistics and data analysis
Experiment 1: Model establishment
Data obtained from the open field, von Frey and Hargreaves tests were analysed using a two-way ANOVA with repeated measures [factors were: group (saline vs. CFA) and time (weeks)]. Whenever significant differences were found, post hoc Student-Newman-Keuls tests were performed. Histological data and body weight changes were analysed using an independent two-tailed t-test [factor: group (saline vs. CFA)]. The chosen level of significance was $p < 0.05$.

Experiment 2: Pharmacological study
A linear mixed-effects model was used to evaluate treatment effects on rat behaviour, haematological parameters and treatment-induced body weight changes, while accounting for variability across independent experiments, each of which included a matched control group and identical treatment conditions. Treatment effects were therefore estimated relative to each batch's baseline (CFA-Veh), allowing aggregation across batches and comparison of the treatments while controlling for inter-batch variability. Prior to model fitting, variables that did not satisfy normality assumptions were transformed using a Box–Cox procedure. The model included 'treatment' as a fixed factor and 'experiment batch' as a random intercept to capture baseline differences between experiments, thereby acknowledging the hierarchical data structure and avoiding the biased estimates that would result from a simpler fixed-effects model. Random slopes were not included because the design did not support slope variation across experiments. Models were fitted using Restricted Maximum Likelihood (REML), chosen for its superior performance in estimating variance components when the number of random-effect levels is limited. Degrees of freedom were estimated using the Satterthwaite approximation. Post hoc comparisons were performed using estimated marginal means (emmeans) to compare treatment effects, and the p-values were adjusted for multiple comparisons using the false discovery rate (FDR) method. Degrees of freedom were estimated using the Kenward-Roger method to account for uncertainty in variance component estimates. The chosen level of significance was $p < 0.05$.

Results
Experiment 1: Model establishment
The effect of intra-articular CFA injection on rat behaviour and body weight
Open field data analysis using a two-way ANOVA with repeated measures revealed that one week after the saline/CFA injection into the knee joint of one hind leg, locomotor activity of rats injected with CFA was significantly lower than that of saline injected rats [factor group × time interaction: $F(2,47) = 6.09$, $p < 0.01$ and $F(2,47) = 10.69$, $p < 0.001$ for horizontal and vertical activity, respectively] (Fig. 2A and B). Post hoc analysis demonstrated that the difference in locomotor activity between the saline and CFA groups was measurable by the end of the first post-injection week; however, the groups were no longer significantly different by the third post-injection week (Fig. 2A and B).

Mechanical pain threshold, assessed by the von Frey test, was significantly lower in the CFA-injected hind leg compared to the saline-injected leg of the healthy control group [factor group × time interaction: $F(2,47) = 4.61$, $p < 0.05$] (Fig. 2C). Interestingly, the pain threshold also tended to be lower in the intact leg [factor group × time interaction: $p = 0.12$] (Fig. 2D). This effect remained for the whole 3-week testing period demonstrating

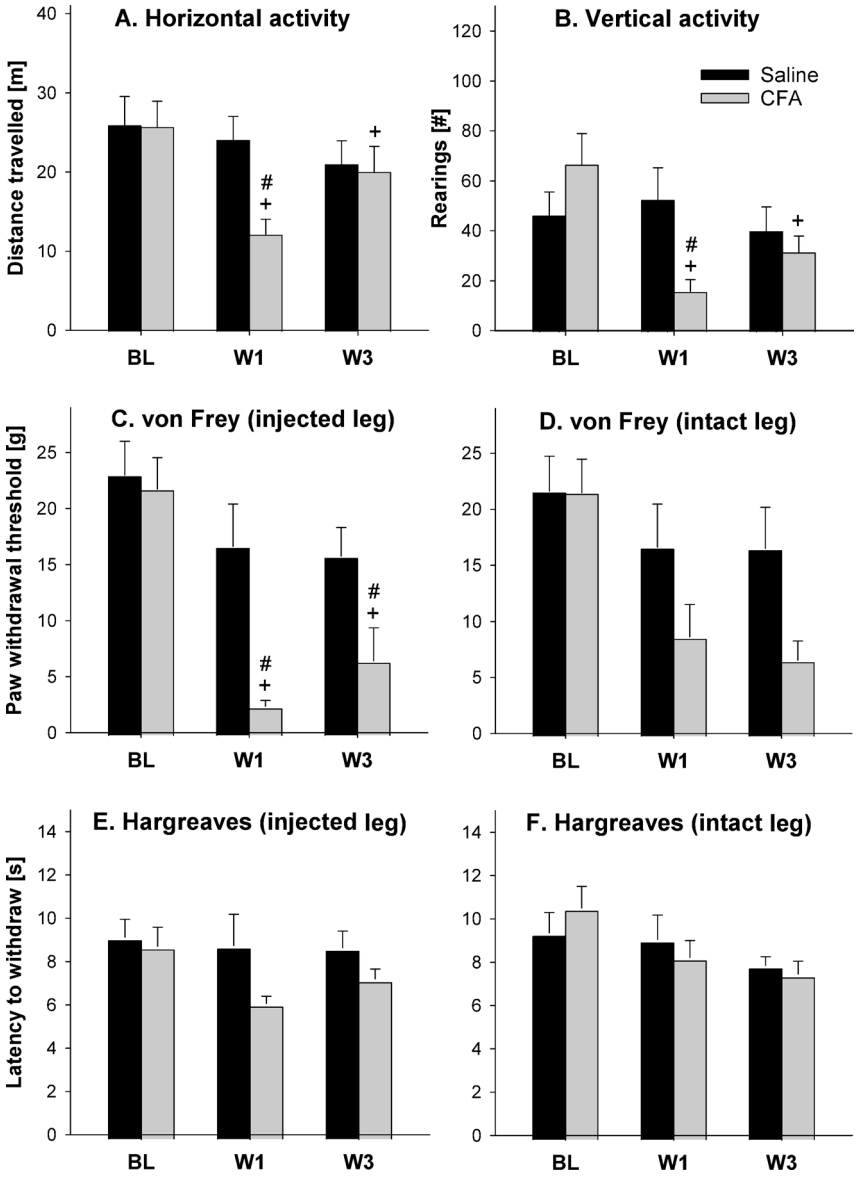

Fig. 2. Model establishment. Behaviour of rats one week before (baseline, BL), one week after (W1), and three weeks after (W3) injection of either 100 µl of saline or CFA ($n = 8$ per group) into one knee joint, measured as (A) horizontal activity (distance travelled, m) and (B) vertical activity (number of rearings) in a 30-min open field test; (C, D) resistance of Von Frey monofilaments (g) required to induce withdrawal reflex when pressed against the paw of (C) intra-articular knee saline/CFA injected and (D) intact hind legs; and (E, F) latency (s) to withdraw (E) saline/CFA injected and (F) intact hind legs from a noxious thermal (heat) stimulus in the Hargreaves test. Data are presented as means ± S.E.M. # indicates significant differences from the saline control group, + indicates significant differences from the baseline (BL) within the group, $p < 0.05$.

that CFA injection may cause long-lasting and generalised increase in pain sensitivity similar to that seen in the hyperalgesia condition.

Evaluation of the thermal nociception threshold using the Hargreaves test showed only a short-lasting tendency to a higher heat sensitivity in the CFA-injected leg compared to the saline-injected leg [factor group × time interaction: $p = 0.28$] (Fig. 2E and F).

Furthermore, an independent two-tailed t-test revealed that the CFA injection caused small but statistically significant weight loss (approximately − 0.7%) compared to saline injection (approximately + 2.0%) [$t(14) = 3.21$, $p < 0.01$], suggesting that these experimental procedures caused a general reduction in animals' well-being.

The effect of intra-articular CFA injection on the synovial lining and stroma of the knee joint

In the intra-articular knee saline-injected healthy control group, a thin synovial lining layer (made of one to two layers of the synovial lining cells) was observed in the posterior and anterior regions of the knee joint capsule. Inflammatory cells in the synovial lining and stroma of the saline-injected rats were almost completely

absent (Fig. 3A). In the CFA-injected group, mild or moderate synovial lining hyperplasia with minimal inflammatory cell infiltration was found (Fig. 3B). In this group, synovial stroma was in an active inflammatory state characterized by hyperplasia and moderate or marked infiltration. Diffuse infiltration with occasional to numerous aggregates of inflammatory cells were found around dilated blood vessels inside enlarged folds of the synovium, especially apparent in the posterior region of the knee joint. Enlarged joint spaces were found in both posterior and anterior articular regions of the CFA-injected rats (Fig. 3B). Analysis of the synovitis scoring results using an independent two-tailed t-test revealed significantly higher hyperplasia and inflammatory cell infiltration in the synovial lining of the CFA-injected rat group compared to the saline-injected rat group [factor group: $t(6)=-14.14$, $p<0.001$ and $t(6)=-5.66$, $p<0.01$ for hyperplasia and infiltration, respectively] (Fig. 3C). The inflammatory cell infiltration score in the synovial stroma was also higher in CFA-injected rats [factor group: $t(6)=-6.43$, $p<0.001$].

Experiment 2: Pharmacological study

Treatment effect on the locomotor activity in the open field test

A linear mixed-effects model revealed that before drug treatment (T1 testing phase), distance travelled in the open field test was significantly higher in saline-injected healthy control rats (SAL-Veh) relative to the CFA-injected (CFA-Veh) control rats [estimate = 3.20 ± 0.72, $t(114.08)=4.46$, $p<0.001$] (Fig. 4A). Analysis of the T2 data showed that, compared to the CFA-Veh reference group, treatment with 10 mg/kg of CBD, 10 mg/kg of CBN and 5 mg/kg of THCV caused a slight but significant sedation in rats [estimate=−18.99 ± 8.54, $t(114.92)=-2.22$, $p<0.05$, estimate=−19.62 ± 8.51, $t(115.45)=-2.31$, $p<0.05$ and estimate=−20.97 ± 8.51, $t(115.45)=-2.47$, $p<0.05$ for CBD, CBN and THCV, respectively] (Fig. 4B).

Treatment effect on the mechanical pain sensitivity in the von Frey test

Analysis of the mechanical pain sensitivity data obtained from the von Frey test demonstrated that intra-articular CFA injection induced hypersensitivity to mechanical stimuli in the affected leg. A linear mixed-effects model revealed that before drug treatment (T1), healthy control SAL-Veh rats had a significantly higher pain threshold compared to the CFA-Veh control rats [estimate = 0.82 ± 0.05, $t(117.00)=16.42$, $p<0.001$] (Fig. 5A). The CFA

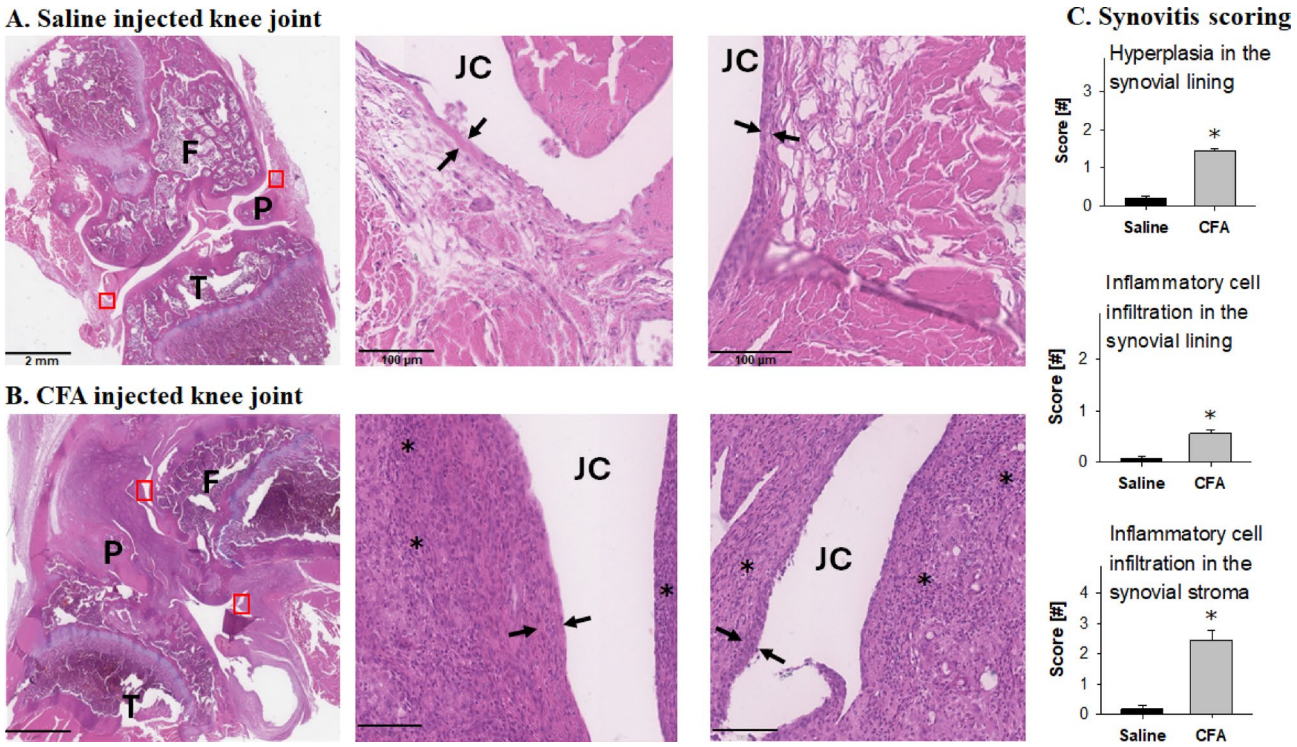

Fig. 3. The synovial lining and stroma of the knee joint. Representative histological samples of (A) saline injected and (B) CFA injected knee joints stained with hematoxylin and eosin shown as the general view (left scans, scale bars = 2 mm) and the magnified fields (red squares, left scans) in the posterior (middle scans) and anterior (right scans) regions of the knee joint (scale bars = 100 µm). Synovial lining (black arrows) and stroma below the lining were thickened and infiltrated in the CFA-injected joint. There were abundant diffuse inflammatory mononuclear cells and inflammatory cell aggregates (*) in the whole tissue of the synovial lining and stroma. The knee joints were collected three weeks after injection of either 100 µl of saline or CFA ($n=4$ per group). Histopathological assessment of the synovitis was scored (C) as follows: hyperplasia and inflammatory cell infiltration in the synovial lining, and inflammatory cell infiltration in the synovial stroma. F, femur; T, tibia; P, patella, JC, joint cavity.

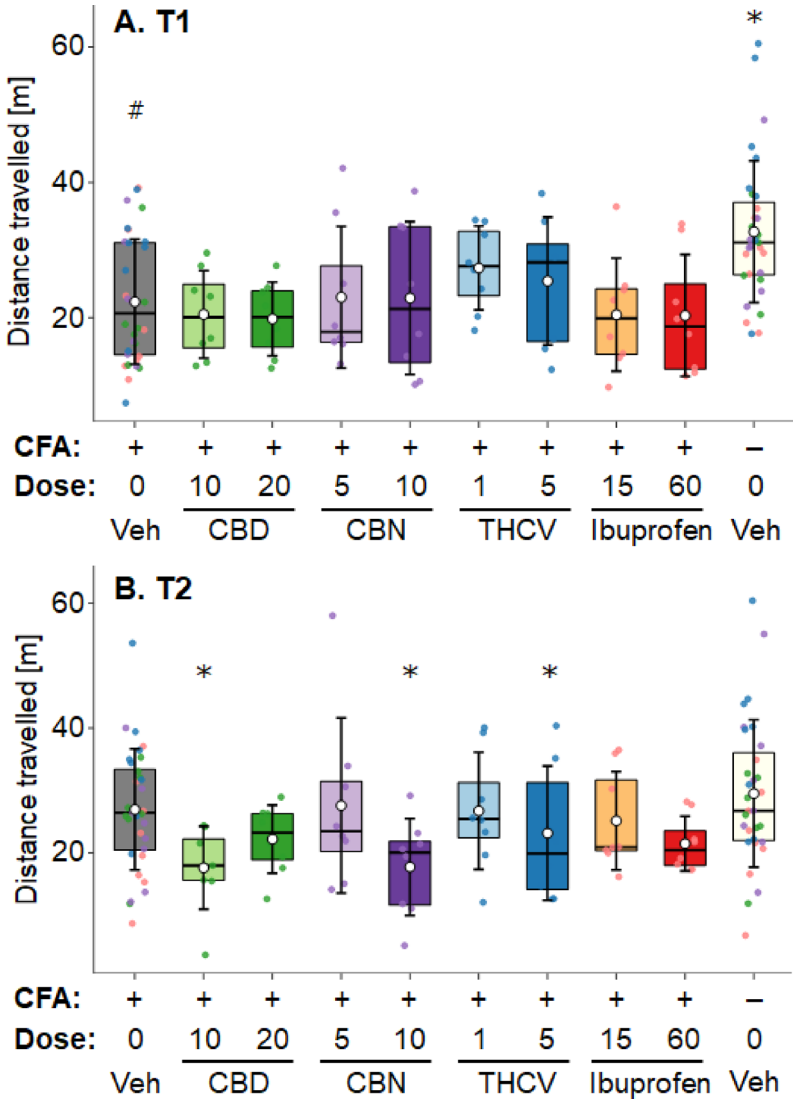

Fig. 4. The locomotor activity in the open field test. Locomotor activity in the 30-minute open field test was measured as the distance travelled (m) by male rats three (T1, A) and ten (T2, B) days following injection of 100 µl saline (–) or CFA (+) into one knee joint ($n = 7–8$ per treatment condition). Rats did not receive any treatment prior to behavioural tests performed three days after saline/CFA injection (T1, A). Four once daily administrations of vehicle (Veh), CBD (10 mg/kg and 20 mg/kg), CBN (5 mg/kg and 10 mg/kg), THCV (1 mg/kg and 5 mg/kg), or ibuprofen (15 mg/kg and 60 mg/kg) started on the 7th day after the intra-articular saline/CFA injection and ended 30 min prior to the open field test on the 10th day (T2, B). Data are presented as box-plots (medians are shown as horizontal lines, means ± SD are shown as white circles and whiskers) and as individual data points (CBD is marked green, CBN – violet, THCV – blue, and ibuprofen – red). # indicates significant difference from the intra-articular saline-injected vehicle control group, * indicates significant difference from the intra-articular CFA-injected vehicle control group, $p < 0.05$.

administration procedure had either minimal or no effect on the mechanical pain sensitivity in the intact leg (data not shown).

Analysis of the T2 data revealed that, relative to the CFA-Veh reference group, the pain threshold was increased in all drug treatment groups [i.e., 10 mg/kg CBD (estimate = 0.875 ± 0.119, t(112.95) = 7.34, $p < 0.001$), 20 mg/kg CBD (estimate = 0.86 ± 0.12, t(112.95) = 7.25, $p < 0.001$), 5 mg/kg CBN (estimate = 0.48 ± 0.12, t(113.78) = 4.04, $p < 0.001$), 10 mg/kg CBN (estimate = 1.02 ± 0.12, t(113.78) = 8.60, $p < 0.001$), 1 mg/kg THCV (estimate = 0.71 ± 0.12, t(113.78) = 5.96, $p < 0.001$), 5 mg/kg THCV (estimate = 0.76 ± 0.12, t(113.78) = 6.44, $p < 0.001$), 15 mg/kg ibuprofen (estimate = 0.96 ± 0.12, t(113.78) = 8.09, $p < 0.001$) and 60 mg/kg ibuprofen (estimate = 0.74 ± 0.12, t(113.78) = 6.21, $p < 0.001$)] (Fig. 5B). At T2, sensitivity to mechanical stimuli in SAL-Veh rats was still lower than that in CFA-Veh rats [estimate = 1.33 ± 0.07, t(114.04) = 19.52, $p < 0.001$]. Even though all drug treatments increased the mechanical pain threshold of the CFA-injected leg, they differed in their effectiveness. Hence, a post hoc analysis showed that treatment with 10 mg/kg CBD, 10 mg/kg CBN, and 15 mg/kg ibuprofen was significantly more effective than treatment with 5 mg/kg CBN. However, mechanical

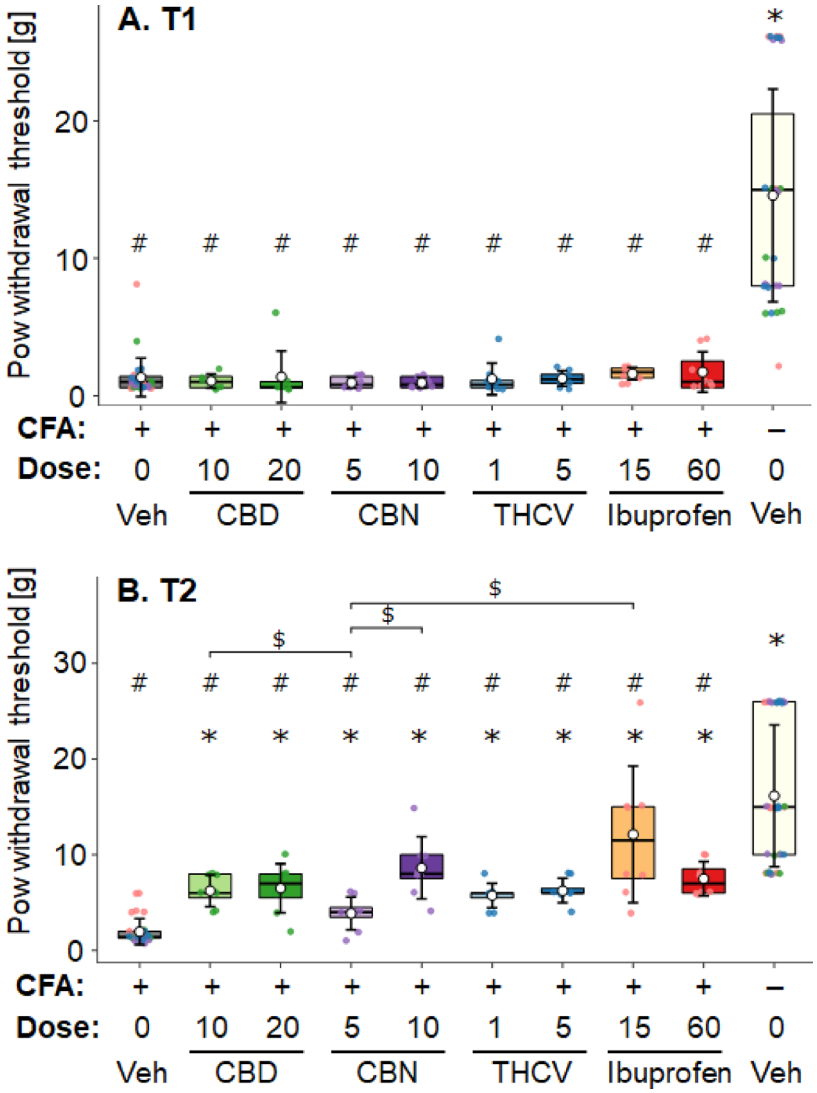

Fig. 5. The mechanical pain sensitivity in the von Frey test. Resistance of Von Frey monofilaments (g) required to induce withdrawal reflex when pressed against the hind paw of male rats three (T1, A) and ten (T2, B) days following injection of 100 µl saline (–) or CFA (+) into the knee joint ($n = 7–8$ per group). Rats received no treatment prior to behavioural tests performed three days after saline/CFA injection (T1, A). Four once daily administrations of vehicle (Veh), CBD (10 mg/kg and 20 mg/kg), CBN (5 mg/kg and 10 mg/kg), THCV (1 mg/kg and 5 mg/kg), or ibuprofen (15 mg/kg and 60 mg/kg) started on the 7th day after the intra-articular saline/CFA injection and ended 65 min prior to the Von Frey test on the 10th day (T2, B). Data are presented as box-plots (medians are shown as horizontal lines, means ± SD are shown as white circles and whiskers) and as individual data points (CBD is marked green, CBN – violet, THCV – blue, and ibuprofen – red). # indicates significant difference from the intra-articular saline-injected vehicle control group, * indicates significant difference from the intra-articular CFA-injected vehicle control group, $p < 0.05$. $ indicates a significant difference between drug treatment groups, $p < 0.05$.

pain sensitivity remained significantly higher in all treatment groups compared to the healthy control SAL-Veh group (Fig. 5B).

Treatment effect on the thermal pain sensitivity in the Hargreaves test

Intra-articular CFA injection induced thermal hyperalgesia in the hind leg, measured in the Hargreaves test, before the drug treatment procedure (T1). Data analysis using a linear mixed-effects model revealed that saline-injected healthy SAL-Veh control rats had a significantly higher pain threshold compared to the CFA-Veh control rats [estimate = 0.07 ± 0.02, $t(114.04) = 3.29$, $p < 0.01$] (Fig. 6A). Intra-articular CFA injection had no effect on the intact hind leg (data not shown).

A linear mixed-effects model revealed that drug treatment had a limited effect on the thermal hyperalgesia in the T2 testing phase. Specifically, relative to the CFA-Veh reference group, an increase in latency to withdraw the CFA-injected hind leg was observed only following repeated administration of 10 mg/kg CBN

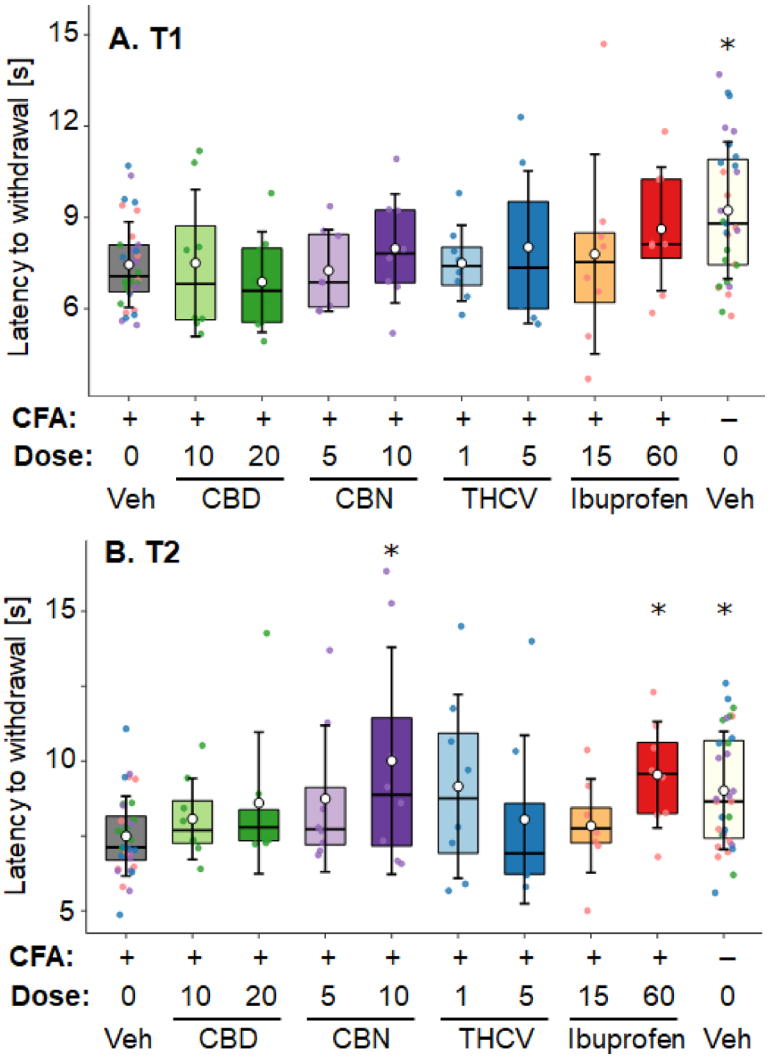

Fig. 6. The thermal pain sensitivity in the Hargreaves test. Latency (s) to withdraw hind leg from a noxious thermal (heat) stimulus in the Hargreaves test in male rats three (T1, A) and ten (T2, B) days following injection of either 100 µl of saline (–) or CFA (+) into the knee joint ($n = 7–8$ per group). Rats did not receive any treatment prior to behavioural tests performed three days after saline/CFA injection (T1, A). Four once daily administrations of vehicle (Veh), CBD (10 mg/kg and 20 mg/kg), CBN (5 mg/kg and 10 mg/kg), THCV (1 mg/kg and 5 mg/kg), or ibuprofen (15 mg/kg and 60 mg/kg) started on the 7th day after the intra-articular saline/CFA injection and ended approximately 80 min prior to the Hargreaves test on the 10th day (T2, B). Data are presented as box-plots (medians are shown as horizontal lines, means ± SD are shown as white circles and whiskers) and as individual data points (CBD is marked green, CBN – violet, THCV – blue, and ibuprofen – red). * indicates significant difference from the intra-articular CFA-injected vehicle control group, $p < 0.05$.

[estimate = 0.02 ± 0.01, t(117.00) = 2.47, $p < 0.05$] and 60 mg/kg ibuprofen [estimate = 0.02 ± 0.01, t(117.00) = 2.71, $p < 0.01$]. The T2 data analysis revealed that vehicle-treated SAL-Veh rats maintained higher thermal threshold relative to CFA-Veh rats during this testing phase [estimate = 0.01 ± 0.005, t(117.00) = 3.09, $p < 0.01$] (Fig. 6B).

Treatment effect on the haematological parameters and the body weight

Analysis of the blood cell count data demonstrated that CFA administration induced marked activation of the innate immune system. Specifically, relative to the CFA-Veh reference group, healthy control SAL-Veh rats had lower levels of GRAN (by approximately 43%) [estimate=−0.17 ± 0.05, t(114.08)=−3.66, $p < 0.001$] (Fig. 7 A) and MONO (by approximately 45%) [estimate=−0.15 ± 0.05, t(114.02)=−3.17, $p < 0.01$] (Fig. 7B). The CFA administration procedure had no effect on LYM levels [$p = 0.65$] and had either minimal or no effect on other haematological parameters (data not shown).

Interestingly, a linear mixed-effects model revealed that treatment with 20 mg/kg CBD even further increased GRAN and MONO levels compared to CFA-Veh treatment [estimate = 0.17 ± 0.08, t(80.06) = 2.16, $p < 0.05$ and estimate = 0.29 ± 0.08, t(116.40) = 3.49, $p < 0.001$ for GRAN and MONO, respectively], whereas for treatment with 10 mg/kg CBD only an increase in MONO levels was observed [estimate = 0.22 ± 0.08, t(116.40) = 2.70, $p < 0.01$] (Fig. 7A and B). Repeated administration of both doses of CBN lowered CFA-induced activation of

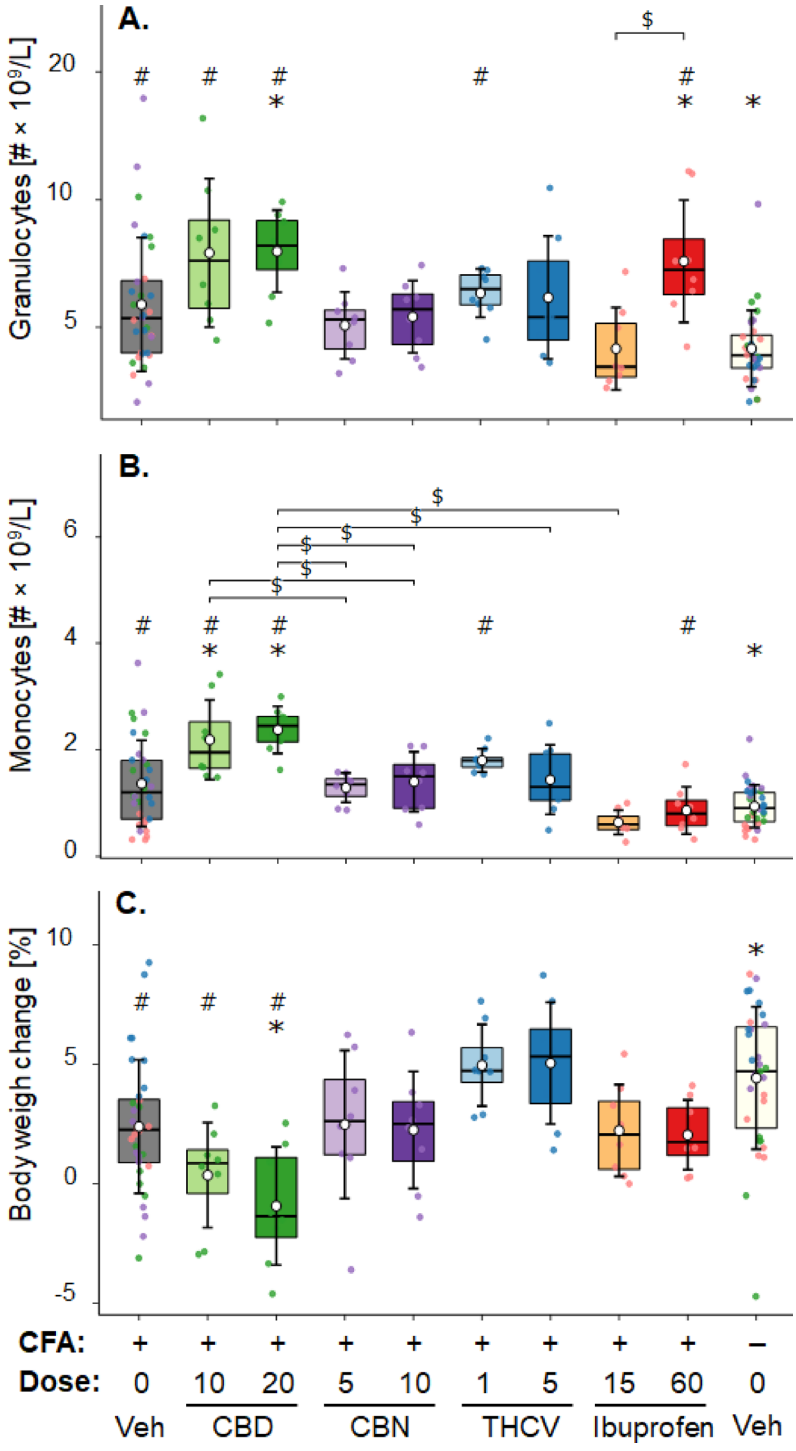

Fig. 7. The haematological parameters and the body weight. Blood levels of (A) granulocytes and (B) monocytes eleven days following injection of 100 µl saline (–) or CFA (+) into one knee joint, and (C) body weight change during the experimental period (calculated as percentage change before and after all experimental procedures) ($n = 7–8$ per group). Blood samples were collected the next day after the T2 testing phase. Four once daily administrations of vehicle (Veh), CBD (10 mg/kg and 20 mg/kg), CBN (5 mg/kg and 10 mg/kg), THCV (1 mg/kg and 5 mg/kg), or ibuprofen (15 mg/kg and 60 mg/kg) started on the 7th day after the intra-articular saline/CFA injection and ended on the 10th day. Data are presented as box-plots (medians are shown as horizontal lines, means ± SD are shown as white circles and whiskers) and as individual data points (CBD is marked green, CBN – violet, THCV – blue, and ibuprofen – red). # indicates significant difference from the intra-articular saline-injected vehicle control group, * indicates significant difference from the intra-articular CFA-injected vehicle control group, $p < 0.05$. $ indicates significant difference between drug treatment groups, $p < 0.05$.

the innate immune system, as neither GRAN nor MONO levels were different from that in healthy control SAL-Veh rats. Treatment with THCV did not cause a further increase in any of the white blood cells relative to the CFA-Veh treatment group; however, GRAN and MONO levels were still higher in 1 mg/kg THCV-treated rats than those measured in the healthy control SAL-Veh group. Similarly to the CBD experiment, treatment of CFA-injected rats with 60 mg/kg ibuprofen caused a further increase in GRAN levels above those measured in the CFA-Veh treated rats [estimate = 0.20 ± 0.08, t(82.64) = 2.25, $p < 0.05$] (Fig. 7A and B).

A linear mixed-effects model revealed that body weight gain during the experimental period was significantly higher in healthy control SAL-Veh rats than in CFA-Veh animals [estimate = 2.42 ± 0.53, t(109.10) = 4.60, $p < 0.001$] (Fig. 7C). Treatment with CBN, THCV, and ibuprofen for four consecutive days reduced the effect of CFA-injection on body weight so that they were not different from the SAL-Veh group. This suggests that these compounds were well-tolerated, did not impair food intake or metabolism, and may have improved the overall well-being of the animals. However, treatment of CFA-injected rats with CBD induced a significant loss in body weight compared to both healthy SAL-Veh rats [estimate=−2.96 ± 0.95, t(111.85)=−3.11, $p < 0.05$ and estimate=−4.25 ± 0.95, t(111.85)=−4.46, $p < 0.001$ for 10 mg/kg CBD and 20 mg/kg CBD, respectively] and CFA-Veh rats [estimate=−1.83 ± 0.92, t(111.96)=−1.98, $p < 0.05$ for 20 mg/kg CBD] (Fig. 7C).

Discussion

Our study demonstrated that an acute intra-articular knee injection of CFA in male Wistar rats triggered an inflammatory response and hyperalgesia, as shown by synovial tissue damage in the affected joint, decreased body weight, reduced locomotor activity, and increased mechanical and, to a lesser extent, thermal pain sensitivity. Pharmacological interventions differed in their effectiveness. Mechanical hyperalgesia was significantly reduced by all treatments; however, thermal hypersensitivity was reduced only by higher doses of ibuprofen and CBN. Neither dose of ibuprofen affected the locomotor activity; however, all phytocannabinoids exhibited mild sedative properties. We found an increase in blood monocyte and granulocyte levels in CFA-injected control animals compared to healthy saline-injected control rats, demonstrating activation of the innate immune system. Both doses of CBN and the higher dose of THCV were effective in lowering the elevated immune response triggered by the CFA injection; however, 20 mg/kg CBD and 60 mg/kg ibuprofen increased the number of these cells above levels measured in untreated CFA-injected rats. Finally, treatment with CBN, THCV, and ibuprofen restored the body weight of the rats, so that by the last experimental day, it did not differ from that of healthy, intra-articular saline-injected control rats. These findings suggest that the compounds (at the dosages used in the study) were not only well-tolerated but may also have had a positive impact on the overall well-being of the animals. However, repeated administration of CBD did not restore the body weight of the rats, and 20 mg/kg CBD lowered the body weight below that of the CFA-injected control group.

Our results confirmed previous studies, which have shown that the intra-articular administration of CFA in rats can activate the immune system and induce an inflammatory state in the synovial tissue [e.g.[28]]. Synovial tissue damage may cause persistent pain in individuals with osteoarthritis[38]. The present study demonstrated that inflammatory changes in the synovial tissue were indeed associated with increased pain sensitivity measured even three weeks after CFA injection. This procedure also affected body weight gain and reduced locomotor activity, demonstrating a general reduction in the well-being of the animals. Interestingly, clear tendency toward increased mechanical pain sensitivity was observed in the contralateral intact leg, demonstrating that CFA administration can cause a generalized increase in pain sensitivity similar to chronic, widespread hyperalgesic conditions[39,40]. This finding supports earlier studies showing that following the CFA administration procedure, mechanical hypersensitivity may occur beyond the injury site[41]. Altogether, these data suggest that intra-articular CFA administration in male rats caused a long-lasting inflammation and hyperalgesia similar to that seen in the arthritis-like chronic pain condition in humans.

The pharmacological study demonstrated that treatment with either ibuprofen or phytocannabinoids CBD, CBN, and THCV was similarly effective in alleviating mechanical hyperalgesia of the intra-articular CFA-injected leg. However, only higher doses of ibuprofen and CBN were effective in reducing the thermal hypersensitivity. Findings with ibuprofen are in agreement with multiple earlier studies demonstrating that NSAIDs are effective in lowering hyperalgesia in animals [e.g.[29,30,42,43]]. Treatment with CBD and THCV yielded similar results with respect to their analgesic effect. Both compounds reduced the mechanical pain sensitivity of the CFA-injected leg but neither CBD nor THCV affected the thermal hyperalgesia measured in the Hargreaves test. The discrepancy between mechanical and thermal pain may be explained by differences in the underlying neurophysiological mechanisms of these modalities. Thermal pain is primarily mediated by heat-sensitive TRPV1- and TRPV2-expressing nociceptors, and both phytocannabinoids are known to increase the activity of these channels[16], which may lower their effectiveness in reducing thermal pain sensitivity. Pharmacodynamic characteristics of CBD and THCV also partly overlap with respect to the CB1 receptor. As mentioned above, CBD is a negative allosteric modulator and THCV is a neutral antagonist of the CB1 receptor[23]. This may be a disadvantageous mode of action for an antinociceptive medication that generally aims to enhance the activity of the endocannabinoid system[9]. Indeed, while several previous studies demonstrated that administration of CBD increased the mechanical pain threshold in several animal pain models [e.g.[13,44,45]], others reported either minimal or no effect of CBD in alleviating pain hypersensitivity and some even noted an increase in thermal sensitivity mediated by the activation of TRP ion channels[46–48]. Interestingly, results from clinical trials examining the effect of treatment with CBD on pain reduction in patients with arthritis are also contradictory [e.g.[49–51]]. For instance, a study by Vela and colleagues[49] showed that pain intensity did not improve in patients receiving daily CBD for 12 weeks compared to placebo.

The results of the present study showed that CBN was effective in lowering both mechanical and thermal hypersensitivity. There are multiple studies showing that the psychoactive compound THC, a partial agonist of the CB1 receptor, is effective in producing antinociception[52]. As mentioned above, CBN is also a partial

agonist of the CB1 receptor and activates CB1 receptors, but with much lower potency than THC[21]. Previous reports have also demonstrated that CBN was effective in increasing sleep duration and quality in both rats and humans[31,53]. Poor sleep quality may be caused by the chronic pain condition[54] and, in turn, worsen the hyperalgesic state[30]. Sleep quality-related effects may be an additional beneficial effect of treatment with CBN that could have contributed to recovery of the body weight.

As mentioned above, besides their direct action on the cannabinoidergic receptors, all three phytocannabinoids interact with several other receptors and ion channels that may also contribute to the treatment of inflammatory pain and have other additional beneficial therapeutic effects. Indeed, the anti-inflammatory and analgetic effects of many phytocannabinoids have been attributed to their interactions with TRP channels[10,16]. Multiple other targets have been identified for CBD that may contribute to or even mediate its analgesic effect, including 5-HT1A, adenosine, and glycine receptors[10,55,56]. For instance, preclinical studies have demonstrated that the anti-allodynic effect of CBD may be independent of CB1 or CB2 receptors, but rather on the activation of 5-HT1A receptors[12,13]. In addition, CBD has been shown to exert antidepressant and anxiolytic effects, which may be important for managing generalized increases in pain sensitivity[57,58]. The anti-nociceptive effect of THCV seems to involve both CB1 and CB2 receptors[33]. However, more research is needed to fully identify the pharmacological targets of CBN and THCV[59].

The increase in blood granulocyte levels following treatment with 60 mg/kg ibuprofen is consistent with previous concerns that NSAIDs may be associated with increased severity of bacterial infection[4]. Previous findings showed that CBD, THCV and CBN may have an immunosuppressive effect[15,18,24]; however, only CBN and the higher dose of THCV were effective in lowering the increased blood monocyte- and granulocyte-levels triggered by the CFA injection. Treatment with CBD appeared to be disadvantageous as treatment during an active immune response, given that both monocyte and granulocyte levels were increased above that of CFA-injected control rats. This may have been the reason why CBD treatment was associated with loss of body weight. Taking these data into consideration, we conclude that the effect of phytocannabinoids on the peripheral and/or central pain transmission mechanisms may provide effective pain relief, even when not reducing the inflammatory response.

To the best of our knowledge, this is the first study to compare the effect of ibuprofen, CBD, THCV and CBN in the CFA-induced inflammatory pain model. Our findings on THCV and CBN are in agreement with earlier reports suggesting that phytocannabinoids can offer a multi-target approach to treat inflammatory pain. In the present study CBN, a CB1 receptor partial agonist and a CB2 receptor agonist, was the most effective phytocannabinoid in reducing inflammatory pain. This compound, not only reduced both mechanical and thermal pain sensitivity, but also recovered the loss of body weight.

Despite these promising results, the study has a few limitations. First, findings on the effect of phytocannabinoid treatment in managing inflammatory pain in male Wistar rats might not apply to female rats [e.g.[41]]. Then, even though previous studies have suggested that CBD, CBN, and THCV may possess anti-inflammatory properties[10,15,24], synovitis was not assessed after treatment with test compounds. Finally, the future research could explore if phytocannabinoids could be used in treating pain sensitization by testing their effectiveness in reducing hyperalgesia occurring outside the site of injury.

Data availability

The data supporting the conclusions of this article will be made available by the authors upon reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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