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RESEARCH PAPER

An evaluation of the anti-hyperalgesic effects of cannabidiolic acid-methyl ester in a preclinical model of peripheral neuropathic pain

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Background and Purpose: Chronic neuropathic pain (NEP) is associated with growing therapeutic cannabis use. To promote quality of life without psychotropic effects, cannabinoids other than Δ9-tetrahydrocannabidiol, including cannabidiol and its precursor cannabidiolic acid (CBDA), are being evaluated. Due to its instability, CBDA has been understudied, particularly as an anti-nociceptive agent. Adding a methyl ester group (CBDA-ME) significantly enhances its stability, facilitating analyses of its analgesic effects in vivo. This study examines early treatment efficacy of CBDA-ME in a rat model of peripherally induced NEP and evaluates sex as a biological variable.

Experimental Approach: After 14 consecutive days of intraperitoneal CBDA-ME administration at 0.01, 0.1 and 1 μg·kg^{-1}, commencing 1 day after surgically implanting a sciatic nerve-constricting cuff to induce NEP, the anti-nociceptive efficacy of this cannabinoid was assessed in male and female Sprague–Dawley rats relative to vehicle-treated counterparts. In females, 2 and 4 μg·kg^{-1} daily doses of CBDA-ME were also evaluated. Behavioural tests were performed for hind paw mechanical and thermal withdrawal thresholds once a week for 8 weeks. At endpoint, in vivo electrophysiological recordings were obtained to characterize soma threshold changes in primary sensory neurons.

Key Results: In males, CBDA-ME elicited a significant concentration-dependent chronic anti-hyperalgesic effect, also influencing both nociceptive and non-nociceptive mechanoreceptors, which were not observed in females at any of the concentrations tested.

Conclusion and Implications: Initiating treatment of a peripheral nerve injury with CBDA-ME at an early stage post-surgery provides anti-nociception in males, warranting further investigation into potential sexual dimorphisms underlying this response.

Abbreviations: CBDA-ME, cannabidiolic acid-methyl ester; CUT, cutaneous; CV, conduction velocity; DRG, dorsal root ganglia; HTM, high threshold mechanoreceptor; LTM, low threshold mechanoreceptor; MS, muscle spindle; NEP, neuropathic pain; PNS, peripheral nervous system; TRPV1, transient receptor potential vanilloid 1.

1 | INTRODUCTION

Chronic neuropathic pain (NEP) is a debilitating condition associated with various diseases or injuries involving the CNS and peripheral nervous system (PNS; Campbell & Meyer, 2006) that poses an economic burden due to increased health care costs and long-term disability. Given that current therapeutics, including opioids, are associated with significant undesirable side effects, new and effective evidence-based treatments are needed.

A growing body of evidence supports that cannabinoids may be efficacious in managing chronic neuropathic pain. Anti-nociception in response to cannabinoid administration has been established in preclinical models (Fine & Rosenfeld, 2014) and clinical trials have demonstrated potential analgesic effects (Ashton & Milligan, 2008; Rahn & Hohmann, 2009, Canadian Agency for Drugs and Technology, 2016). Characterization of endogenous cannabinoid receptors and ligands that play physiological roles in modulating neuroimmune crosstalk has generated interest in phytocannabinoids and synthetic derivatives as therapeutic agents (Manzanares, Julian, & Carrascosa, 2006). It will be clinically relevant to define spatial–temporal parameters associated with their effects, such as the site(s) of action and the timing of treatment initiation, as well as the biological factors that may affect their mechanisms of actions, such as sex and age.

Initiating treatment at an early stage post-nerve injury has been shown to limit the development of hyperalgesia in a rat model of peripheral neuropathic pain (Dableh & Henry, 2011). Investigations into whether anti-nociception imparted by early cannabinoid administration (i.e. shortly after an insult known to initiate chronic neuropathic pain, such as a surgery) is sustained weeks after treatment cessation are lacking. The majority of therapeutic modalities recommended for managing peripheral neuropathic pain act by reducing ectopic neural discharge-induced firing (Devor, 1991). Nociceptors may initially be sensitized by inflammation due to nerve damage and peripheral nerve fibres consequently develop patterns of ectopic neuronal discharge. A complex response is then evoked in the spinal cord, with neurons becoming hyper-excited (Devor, 1991; Schaible, 2007). Therefore, initiating cannabinoid treatment at an early stage post-insult to limit the development of chronic hypersensitivity may be a means to block the propagation of peripheral ectopic impulses into the CNS, curbing pain chronification.

Different cannabinoids may produce mechanistically distinct neuropathic pain-relieving effects (Manzanares et al., 2006). In contrast to Δ9-tetrahydrocannabidiol and cannabidiol (CBD), limited studies have explored the anti-nociceptive effects of their respective upstream acid precursors. The precursor of CBD, cannabidiolic acid (**CBDA**), was recently evaluated as an anti-hyperalgesic agent in an inflammatory rodent model of pain (Rock, Limebeer, & Parker, 2018). However, CBDA is known to be unstable, undergoing decarboxylation into CBD. The semi-synthetic analogue CBDA-methyl ester (**CBDA-ME**; HU-580) is significantly more stable than CBDA, facilitating in vivo studies to evaluate its anti-emetic properties (Pertwee et al., 2018), which is desirable for prolonged treatment regimens.

What is already known

- CBDA-methyl ester produces 5-HT_{1A} receptor-mediated suppression of nausea and anxiety in rats.
- Acute oral CBDA-methyl ester reduces depression-like behaviour in two genetic animal models of depression.

What this study adds

- tAt selected doses, CBDA-ME ameliorates tactile allodynia only in male rats without affecting heat-sensing response.
- Soma excitability of nociceptive and non-nociceptive mechanoreceptors in CBDA-ME-treated male rats was significantly affected.

What is the clinical significance

- Sex should be considered in evaluating the analgesic potential of cannabinoids for neuropathic pain management.

There are known sex differences in pain perception and neuroimmune responses (Sorge & Totsch, 2017), as well as sex-dependent cannabinoid-mediated effects (Cooper & Craft, 2018; Craft, Marusich, & Wiley, 2013). Therefore, potential sexual dimorphisms that may underlie the treatment efficacy of different cannabinoids in the context of neuropathic pain need to be addressed (Cooper & Haney, 2016), especially as these agents are being clinically applied with mixed outcomes, which could be due to a patient's sex, as well as being influenced by gender roles. The aim of this investigation was to assess the early treatment efficacy of CBDA-ME in a preclinical sciatic nerve cuff model of neuropathic pain (Mosconi & Kruger, 1996) in both males and females. Rats of each sex were administered vehicle or CBDA-ME at various different concentrations starting 1 day after cuff implantation, with treatments continuing for 14 consecutive days. Effects on hypersensitivity were behaviourally evaluated on a weekly basis for 8 weeks. Intracellular electrophysiological recordings obtained at endpoint were used to characterize long-term threshold changes in the soma of primary sensory neurons corresponding to relevant dorsal root ganglia (DRG) cell bodies. We report that the efficacy of early treatment to manage post-surgical neuropathic pain is dependent on the concentration of CBDA-ME, as well as sex differences.

2 | METHODS

All experimental procedures adhered to the Guide for the Care and Use of Experimental Animals, Volumes 1 and 2, of the Canadian Council on Animal Care, and all protocols were reviewed and approved by the McMaster University Animal Research Ethics Board. Male and female Sprague–Dawley rats (Charles River, QC) were housed in pairs in different rooms and were maintained on a 12-hr light cycle, with ad libitum access to food and water. In all cases,

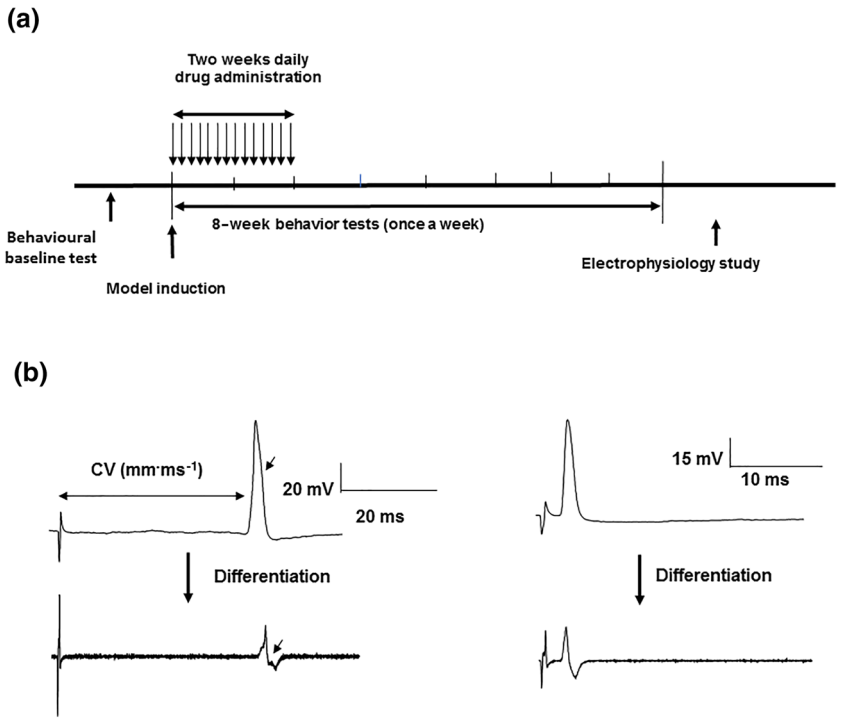

FIGURE 1 Experimental timeline and action potential parameters. (a) Timeline of experimental procedures. Model induction of peripheral neuropathic pain (NEP) occurred on Day 0. Before model induction, behavioural baseline tests were performed, with weekly testing carried out for 8 weeks. One day after model induction, daily drug administration was initiated and continued for 2 weeks. At the 8-week experimental endpoint, electrophysiological procedures were performed. (b) Action potential (AP) configuration analysis. Representative intracellular somatic action potentials of sensory neurons were evoked by electrical stimulation of the dorsal root. Upper left: an evoked AP classified as a C-fibre on the basis of conduction velocity, which was 0.35 mm·ms^{-1}. Upper right: an Aß-fibre classified based on conduction velocity, which was 12.65 mm·ms^{-1}. Lower left: differentiated derivative trace of a C-fibre AP. A plateau was identified on the repolarisation branch of the AP in the differentiated recording, which is indicative of a nociceptive neuron. Lower right: differentiated derivative trace of an Aß-fibre, without inflection on the falling phase in the differentiated recording, which is considered a non-nociceptive neuron

behavioural and electrophysiological tests were performed according to the timeline outlined in Figure 1a.

2.1 | Neuropathic pain model induction

Sprague–Dawley rats of both sexes, initially weighing 170–200 g, were used for this experiment. A peripheral nerve injury was induced using the "sciatic cuff model" according to methods previously described in detail (Mosconi & Kruger, 1996; Pitcher & Henry, 2002, 2004, 2008; Zhu & Henry, 2012; Zhu, Wu, & Henry, 2012). Animals were anesthetized with an IP-administered mixture of ketamine (Narketan; 5 mg/100 g; Vetoquinol N.-A. Inc.), xylazine (Rompun; 0.5 mg/100 g; Bayer Inc.) and acepromazine (Atravet; 0.1 mg/100 g; Ayerst Veterinary Laboratories), and the right sciatic nerve was exposed mid-thigh. A single cuff composed of 0.5-mm polyethylene (PE 90) tubing (Intramedic PE-90, Fisher Scientific Ltd.) was inserted around the exposed nerve. The muscle and skin were sutured separately, antibiotic ointment (Furacin; nitrofurazone 0.2%; Vetoquinol N.-A. Inc.) was applied to the closed wound, and 0.01 ml/100 g of antibacterial injectable solution (Baytril; Bayer Inc.) was subcutaneously administered. The sham model was induced using the same procedure except that no cuff was inserted around the sciatic nerve.

2.2 | Reconstitution and administration of CBDA-ME

Three different concentrations of methyl ester-stabilized CBDA (CBDA-ME; HU-580) were tested in both males and females, with an additional two higher concentrations tested only in females. CBDA-ME was supplied by Dr. Mechoulam and reconstituted for in vivo studies according to a previously described protocol (Pertwee et al., 2018). Briefly, lyophilized HU-580 was resuspended in 1 ml of ethanol in a glass tube, with the addition of 1 ml of Tween-80 (Sigma). Under a nitrogen stream, the ethanol was evaporated, followed by the addition of 9 ml of saline to the remaining solution. Rat body weight-adjusted working concentrations of CBDA-ME were freshly prepared each day in medium-chain triglycerides (vehicle; CannTrust). Each concentration of CBDA-ME or vehicle was administered IP during the onset phase of model induction, referred to as "early" treatment, at the same time each day at approximately 10–11 a.m. starting 1 day after cuff implantation surgery and continuing once daily for 14 consecutive days.

2.3 | Experimental groups

To define whether the sham surgery had an effect on the sciatic cuff model, rats of each sex were randomly divided into sham (n = 5 per sex; surgery, no cuff), naïve (n = 5 per sex; no surgery), and neuropathic pain (n = 20 per sex; surgery, with cuff) groups. The neuropathic pain male and female groups were then divided further into treatment groups (n = 5 rats per treatment), which included CBDA-ME at 0.01, 0.1, and 1 μg·kg^{-1}, as well as vehicle only (n = 5) in parallel to drug-tested animals. In follow-up experiments, higher concentrations of CBDA-ME at 2 and 4 μg·kg^{-1} were tested in female neuropathic pain rats (n = 5 per concentration). Group size selection for the CBDA-ME dose–response experiment was based on an ANOVA analysis method (Charan & Kantharia, 2013), which incorporates the

principle that any sample size that maintains *E* between 10 and 20 is considered adequate for an exploratory animal study. *E* is assessed using the formula: *E* = total number of animals − total number of groups. In the current investigation, there were four treatment groups for each sex, with *n* = 5 rats per group, corresponding to an *E* value of (4 * 5) − 4 = 16, which is within the acceptable limit and hence was considered to be an acceptable sample size. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.4 | von Frey test of mechanical paw withdrawal threshold

Rats were intermittently handled by dedicated investigators prior to initiation of the experiments. Each animal was introduced to a transparent Plexiglas chamber with 0.5-cm diameter holes spaced 1.5 cm apart on the floor to allow full access to the paw (Pitcher & Henry, 2002; Wu & Henry, 2010; Zhu et al., 2012) for approximately 15 min prior to behavioural testing to account for normal exploration and major grooming activities. Baseline von Frey tests were performed 1 week after arrival at the animal facility. Following sciatic nerve cuff surgeries, behavioural tests were carried out once a week for a total of 8 weeks to quantify the development of tactile hypersensitivity, a well-established characteristic of neuropathic pain. von Frey filaments (Stoelting Co.) were applied to the plantar surface of the right hind paw (corresponding to the limb associated with the sciatic nerve cuff) to determine mechanical withdrawal thresholds using the up-down method (Dixon, 1980). Each filament was applied five times for 3–4 s each at 3-s intervals to a different spot on the plantar surface of the injury-bearing hind paw. Filaments of different weight were applied in ascending order of force until a clear withdrawal response was observed. When this occurred, the next lightest filament was applied, and the process continued until a 50% withdrawal response threshold was achieved (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Brisk foot withdrawal in response to the mechanical stimulus was interpreted as a valid response.

2.5 | Hargreaves' test of thermal paw withdrawal threshold

A Hargreaves' apparatus (Plantar Analgesia Meter, IITC Life Science Inc.) was used to measure thermal nociception and to assess heat hyperalgesia, as previously described in detail in a well-established protocol (Moriarty, Roche, McGuire, & Finn, 2012). Animals were placed in the apparatus on top of a glass panel heated to 30°C and allowed to habituate for 20 min. A focused beam of radiant light was used to heat the plantar surface of the hind paw, and the latency to flinch, lick, or withdraw the hind paw was recorded. To prevent tissue damage, a cut-off parameter of 20 s was used. If no response occurred during this time, the cut-off time of 20 s was recorded as the latency. Each animal was subjected to five trials in total, with at least a 3-min interval between testing. The lowest and the highest values were discounted as outliers as described by others (Moriarty et al., 2012). The remaining trial values were used to calculate the average reaction time for each rat.

2.6 | Intracellular in vivo DRG recordings

Details of acute intracellular electrophysiological recording techniques have been reported previously in preclinical models of neuropathic pain (Wu & Henry, 2010; Zhu et al., 2012; Zhu & Henry, 2012). Briefly, each rat was initially anesthetized with i.p. delivery of a mixture of ketamine, xylazine and acepromazine. The right jugular vein was cannulated for intravenous drug infusion and the rat was fixed in a stereotaxic frame with the vertebral column rigidly clamped at lumbar (L) 2 and L6. The L4 DRG was selected for study, as it contains large numbers of hind leg afferent soma. A laminectomy was performed to expose the ipsilateral L4 DRG, corresponding to the region innervated by the sciatic cuff-bearing hind limb. The L4 dorsal root was sectioned close to the spinal cord and placed on a bipolar electrode (FHC) used for stimulation. The exposed spinal cord and DRG were covered with paraffin oil at 37°C to prevent drying. Rectal temperature was measured and maintained at 37°C using a temperature-controlled IR heating lamp.

During recordings, rats were maintained at a surgical level of anaesthesia using sodium pentobarbital (20 $mg \cdot kg^{-1}$; Ceva Sante Animal) and mechanically ventilated via a tracheal cannula using a Harvard Ventilator (Model 683, Harvard Apparatus). The ventilation parameters were adjusted so that the end-tidal CO_2 concentration was maintained at 40–50 mmHg, as measured using a CapStar-100 End-Tidal CO_2 analyzer (CWE). Immediately prior to initiating the recordings, a 1 $mg \cdot kg^{-1}$ dose of **pancuronium** (Sandoz) was administered to eliminate muscle tone. The effects of pancuronium were allowed to wear off periodically to confirm a surgical level of anaesthesia, which was monitored by observing pupil diameter and response to a noxious pinch of a forepaw. Supplemental sodium pentobarbital and pancuronium were administered at 1/3 of the previous doses of each agent, approximately every hour, via the jugular cannula.

Intracellular recordings from soma in the exposed DRG were made with borosilicate glass micropipettes (1.2 mm outside diameter, 0.68 mm inside diameter; Harvard Apparatus). Electrodes were pulled using a Brown-Flaming pipette puller (model P-87; Sutter Instrument Co.) and filled with 3-M KCl (DC resistance 50–70 MΩ). Signals were recorded with a Multiclamp 700B amplifier (Molecular Devices) and digitized online via the Digidata 1322A interface (Molecular Devices) with pClamp 9.2 software (Molecular Devices; RRID:SCR_011323). The microelectrode was advanced using an EXFO IW-800 micromanipulator (EXFO) in 2-μm steps until an abrupt hyperpolarization of at least 40 mV appeared. Once a stable membrane potential was confirmed, a single stimulus was applied to the dorsal root to provoke an action potential (AP). The "Protocol

Editor" function in the pClamp 9.2 software was used to evoke a somatic action potential through stimulation with a single rectangular intracellular depolarizing voltage pulse.

2.7 | DRG neuron classification

Recorded neurons were classified based on their action potential configuration, conduction velocity (CV) and receptive field properties defined using hand-held mechanical stimulators (Djouhri, Bleazard, & Lawson, 1998; Lawson, Crepps, & Perl, 1997; Zhu et al., 2012). Neurons were divided into three groups on the basis of dorsal root CV. The CV range for C-fibre neurons was ≤0.8 and 1.5–6.5 $m \cdot s^{-1}$ for Aδ-fibre neurons and >6.5 $m \cdot s^{-1}$ for Aβ-fibre neurons, as defined elsewhere (Djouhri et al., 1998; Wu & Henry, 2010; Zhu et al., 2012). The differentiation of high threshold mechanoreceptor (HTM) compared to low threshold mechanoreceptor (LTM) neurons was based on specific sensory properties identified during receptive field searching. HTM neurons responded to noxious stimuli including noxious pressure, pinch and probing with fine forceps, a sharp needle, coarse-toothed forceps or coarse flat forceps, whereas LTM neurons responded to innocuous stimuli such as a moving brush, light pressure with a blunt object, a light manual tap or vibration. The threshold of activation, the rate of adaptation, and the tissue location of the receptive field were used to further classify Aβ-fibre LTM neurons as cutaneous (CUT) or muscle spindle (MS) neurons. Considering that the sensory neuron threshold may be changed in response to treatment, Aβ-fibre nociceptive neurons were further identified based on their prominent inflection in the repolarization phase of the action potential in differentiated recordings, which is considered to be a feature unique to nociceptors (Caffrey, Eng, Black, Waxman, & Kocsis, 1992; Ritter & Mendell, 1992; Figure 1b).

2.8 | Excitability of DRG neurons

Excitability was measured by evoking action potentials in the soma of the DRG neurons using stimulation by direct injection of depolarizing current (Zhu & Henry, 2012). To quantify soma excitability, with the aid of the "Protocol Editor" function in the pClamp 9.2 software program (Molecular Devices), the threshold of depolarizing current pulses injected into the soma was determined. This was achieved by applying current injections of 100 ms each, delivered with an amplitude of 0.5 to 4 nA with increments of 0.5 nA (with the exception of muscle spindle neurons: 0.1 to 0.4 nA in increments of 0.05 nA).

2.9 | Statistical analyses

Statistical analyses (GraphPad Prism Software, Inc.; RRID: SCR_002798) were undertaken only for experiments in which the group size was $n = 5$, with n reflecting the indicated number of individual animals, and statistical tests carried out using these independent rat numbers. $P < .05$ was considered to indicate a minimum significant difference. No normalization or transformation of the data was carried out and any outliers were included in data analysis and presentation. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

Behavioural data for males and females, including baseline measurements and data from the 8-week time course, is presented separately as the mean ± SEM. First, the CBDA-ME concentration that was most effective in the neuropathic pain model for each sex was determined. The difference between each concentration relative to vehicle across the time course was analysed using a mixed two-way ANOVA, with a Bonferroni post hoc test. Sex effects were then analysed between males and females for the time course for vehicle and each concentration of CBDA-ME using a mixed two-way ANOVA with a Bonferroni post hoc test. Of note, in the multi-group behavioural studies with parametric variables, post hoc tests were conducted only if F in ANOVA achieved the necessary level of statistical significance and there was no significant variance in homogeneity. Electrophysiological data at endpoint for males and females are presented as the mean ± SEM. For each sex, the difference between vehicle and each concentration of CBDE-ME was first analysed by Mann–Whitney analysis. A mixed two-way ANOVA with a Dunn's post hoc test was further applied for statistical comparisons between sex with vehicle and CBDA-ME treatment.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Behaviour

Measurements collected up until 4 weeks post-surgery showed that both sexes experienced a significant decrease in mechanical withdrawal threshold only in the neuropathic pain model ($n = 5$ for each sex; Figure 2). Post hoc comparisons showed that the significant difference in both sexes occurred between the naive and neuropathic pain groups, with no significant differences between naïve and sham animals. The neuropathic pain group exhibited a significantly lower withdrawal threshold compared to naïve rats starting 1 week post-surgery, an effect that was sustained for the 4 weeks during which behavioural testing was carried out. These results confirmed that the neuropathic pain model based on implantation of a sciatic nerve cuff was robustly established within the first week post-surgery, with no significant differences occurring between sham and naïve rats in both the male and female groups.

Baseline levels established for the von Frey behavioural test in male and female neuropathic pain rats were 8.36 ± 1.01 and

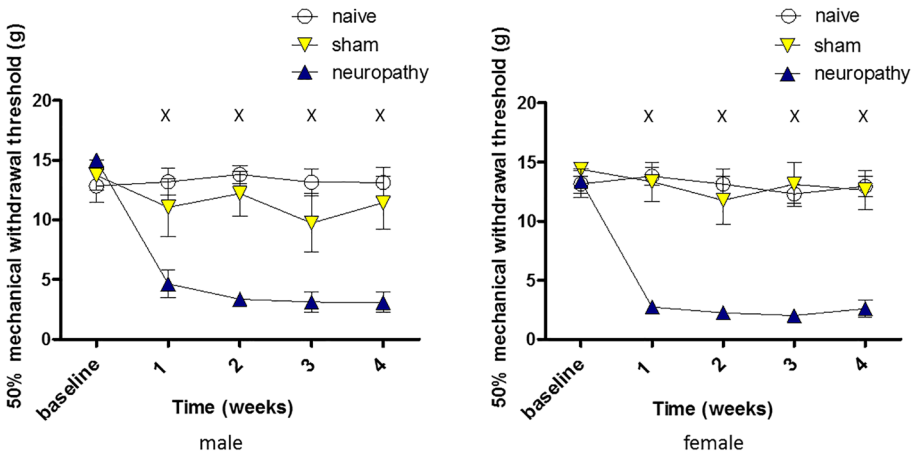

FIGURE 2 A comparison of mechanical withdrawal thresholds between naïve, sham, and male and female rats with a sciatic nerve cuff (neuropathy) using the von Frey behavioural test. Mechanical withdrawal thresholds were evaluated by von Frey testing once a week for a total of 4 weeks. Data are expressed as the mean ± SEM for different groups. Two-way ANOVA with a Bonferroni post hoc test was used for comparisons. A symbol (X) above the graphs indicates significant differences between the naïve and neuropathy groups for each sex. No significant differences were obtained between the naïve and sham groups

9.36 ± 1.02 g respectively. After sciatic nerve cuff surgery, rats were randomly assigned to different treatment groups according to sex. Table 1 lists the scores for withdrawal latency with mechanical stimuli, which were evaluated once a week for 8 weeks. Figure 3 illustrates the changes in withdrawal thresholds after sciatic nerve cuff implantation and different treatments relative to vehicle in males and females. Measurements collected during the first week showed that all groups for both sexes experienced a significant decrease in mechanical withdrawal threshold, consistent with the emergence of neuropathic pain. Starting during week 2 until the eighth week, CBDA-ME treatments in males resulted in continuous threshold increases. The concentration-dependent effect in male rats was significant. Post hoc comparisons showed that the CBDA-ME-1 group exhibited a significantly higher withdrawal threshold compared to animals treated with vehicle during the third, fourth, fifth, sixth and eighth week. However, no differences in thresholds were observed between female rats treated with vehicle and any of the tested concentrations of CBDA-ME. Post hoc comparisons showed that there were no differences between any of the CBDA-ME concentrations and vehicle tested over the 8-week period. Two higher concentrations of CBDA-ME were therefore tested in females, with neither CBDA-ME-2 nor CBDA-ME-4 eliciting significant anti-nociceptive changes in paw withdrawal thresholds.

Sex effects were then analysed between males and females receiving the highest concentration of CBDA-ME-1, which was the concentration that elicited the most pronounced anti-nociceptive effect in males. The sex difference with CBDA-ME-1 was significant . Post hoc comparisons showed that male rats exhibited a significant threshold increase relative to females during Weeks 4 and 8 (Figure 3).

The scores for withdrawal latency to a thermal stimulus were determined by Hargreaves' testing (Table 1 and Figure 4). The baseline level in male rats was 14.19 ± 1.45 s, and in females, it corresponded to 15.02 ± 1.26 s. All treatment groups were evaluated on a weekly basis for 8 weeks following treatment initiation. There were no significant differences in paw withdrawal thresholds between vehicle and any of the CBDA-ME concentrations that were tested within the time course, and there were no significant differences in withdrawal latency between males and females for any of the CBDA-ME treatments over time.

3.2 | Electrophysiology

The action potential responses to intracellular depolarizing current pulse injection were tested to determine whether there was a difference in soma excitability at the 8-week endpoint induced by CBDA-ME-1 treatment during weeks 1 and 2 in males and females. Figure 5 illustrates the threshold currents that elicited action potentials for the different treatment groups compared to vehicle in both sexes. In males, the activation threshold of both high (HTM) and low threshold (LTM) C-fibre neurons, assessed at the 8-week experimental endpoint (6 week after daily treatment with CBDA-ME ceased), was significantly increased by the initial 2-week CBDA-ME-1 administration regimen relative to vehicle. In C-fibre HTM neurons (the threshold was 1.4-fold higher in response to CBDA-ME (2.00 ± 0.45 nA [n = 6] in the vehicle group compared to 2.83 ± 0.68 nA [n = 6] in the CBDA-ME-1 group) and in C-fibre LTM neurons, it was up by 1.6-fold (0.67 ± 0.26 nA [n = 6] in the vehicle group compared to 1.08 ± 0.20 nA [n = 6] in the CBDA-ME-1 group). Similarly, the activation threshold of Aβ-fibre HTM neurons was increased by 1.5-fold in response to CBDA-ME-1 in males relative to vehicle-treated counterparts (1.92 ± 0.38 nA [n = 6] in the vehicle group compared to 2.92 ± 0.80 nA [n = 6] in the CBDA-ME-1 group). In muscle spindle and cutaneous neurons, which also represent Aβ-fibre neurons, the thresholds were increased by 2.1- and 1.7-fold respectively (muscle spindle: 0.19 ± 0.06 nA in the vehicle group [n = 7] and 0.40 ± 0.10 g in the CBDA-ME group [n = 8]; cutaneous: 1.00 ± 0.44 nA in the vehicle group [n = 5] and 1.66 ± 0.41 nA in CBDA-ME [n = 5]).

In marked contrast, in females, none of the activation thresholds in early CBDA-ME-1-treated rats measured at the 8-week endpoint differed significantly from animals treated with vehicle. The activation threshold of both C-fibre HTM and C-fibre LTM neurons remained unchanged in response to treatment with CBDA-ME-1 (C-fibre HTM:

TABLE 1 Mechanical and thermal scores measured over 8 weeks

Week	Vehicle	CBDA-ME-1	CBDA-ME-0.1	CBDA-ME-0.01
Male Mechanical Threshold (g)				
Baseline: 8.36 ± 1.01				
1	3.42 ± 0.84	3.18 ± 0.94	3.03 ± 0.47	2.02 ± 0.57
2	3.21 ± 0.68	5.70 ± 1.89	9.73 ± 2.40	1.45 ± 0.29
3	3.86 ± 0.34	10.86 ± 2.63	8.32 ± 2.20	4.80 ± 0.78
4	4.22 ± 0.77	13.05 ± 1.95	11.06 ± 2.57	5.89 ± 1.85
5	4.70 ± 1.40	12.27 ± 1.95	12.20 ± 2.02	12.17 ± 1.72
6	4.48 ± 0.46	12.26 ± 1.75	12.67 ± 2.34	10.13 ± 2.51
7	6.88 ± 1.58	10.17 ± 2.32	9.76 ± 2.42	8.98 ± 2.00
8	3.53 ± 0.28	12.56 ± 1.55	11.72 ± 1.94	9.51 ± 1.66
Female Mechanical Threshold (g)				
Baseline: 9.36 ± 1.02				
1	3.68 ± 0.21	2.71 ± 1.01	1.91 ± 0.61	2.77 ± 0.59
2	4.64 ± 0.60	6.49 ± 2.77	1.86 ± 0.76	4.48 ± 2.28
3	4.29 ± 0.62	7.16 ± 1.73	8.46 ± 2.94	6.51 ± 2.79
4	4.11 ± 0.42	6.99 ± 2.64	2.41 ± 0.79	8.93 ± 2.72
5	4.33 ± 0.93	8.81 ± 2.29	8.86 ± 2.37	5.06 ± 0.99
6	5.78 ± 1.09	9.40 ± 2.59	5.55 ± 2.07	2.98 ± 0.99
7	5.27 ± 0.84	6.51 ± 2.32	6.05 ± 1.98	3.74 ± 0.87
8	5.26 ± 0.86	6.20 ± 2.30	5.77 ± 1.94	3.27 ± 0.82
Male Thermal Threshold (s)				
Baseline: 14.19 ± 1.45				
1	7.97 ± 0.25	7.41 ± 1.24	11.87 ± 2.05	7.49 ± 0.18
2	9.75 ± 0.94	9.51 ± 0.97	9.09 ± 0.98	10.05 ± 0.82
3	9.17 ± 0.96	11.22 ± 1.97	8.14 ± 0.58	8.63 ± 0.55
4	6.81 ± 0.93	7.88 ± 0.77	10.28 ± 1.87	9.26 ± 1.12
5	9.07 ± 0.54	7.10 ± 0.99	8.64 ± 0.54	6.98 ± 0.56
6	11.82 ± 1.47	9.18 ± 0.94	12.48 ± 1.61	9.67 ± 1.53
7	11.30 ± 0.77	9.47 ± 1.28	8.76 ± 0.86	8.30 ± 1.37
8	9.23 ± 1.00	7.71 ± 0.83	10.21 ± 0.81	8.68 ± 0.68
Female Thermal Threshold (s)				
Baseline: 15.02 ± 1.26 s				
1	11.15 ± 1.52	8.92 ± 0.76	10.41 ± 2.12	5.99 ± 0.36
2	9.09 ± 0.32	7.62 ± 0.40	10.76 ± 2.18	8.11 ± 0.51
3	7.04 ± 0.26	10.73 ± 1.83	8.59 ± 1.07	12.50 ± 1.96
4	10.21 ± 0.60	7.72 ± 0.62	11.21 ± 1.23	10.92 ± 0.87
5	11.82 ± 1.47	11.29 ± 1.26	10.67 ± 1.08	11.79 ± 1.52
6	12.02 ± 1.25	9.78 ± 1.11	10.44 ± 1.81	11.30 ± 1.57
7	13.19 ± 0.74	8.39 ± 0.49	11.61 ± 1.47	11.64 ± 0.94
8	13.11 ± 1.25	8.99 ± 1.03	10.44 ± 1.30	11.21 ± 1.34

Note. CBDA-ME-1, CBDA-ME-0.1 and CBDA-ME-0.01 represent different concentrations, corresponding to 1, 0.1 and 0.01 $\mu g \cdot kg^{-1}$ respectively.

1.80 ± 0.27 nA [n = 5] in the vehicle group compared to 1.90 ± 0.22 nA [n = 5] in CBDA-ME-1 group; C-fibre LTM: 0.58 ± 0.20 nA [n = 6] in the vehicle group compared to 0.67 ± 0.26 nA [n = 6] in CBDA-ME-1 group). For Aβ-fibre HTM and muscle spindle neurons, a similar lack of response was obtained (Aβ-fibre HTM 1.70 ± 0.27 nA [n = 5] in the vehicle group and 1.80 ± 0.27 nA [n = 5] in the CBDA-ME group; muscle spindle: 0.16 ± 0.09 nA in the vehicle group [n = 6] and 0.20 ± 0.07 nA in the CBDA-ME group [n = 6]). The activation thresholds of cutaneous Aβ-fibre neurons, corresponding to 0.58 ± 0.20 nA in the vehicle group (n = 6) and 0.75 ± 0.27 nA in the CBDA-ME group (n = 6),

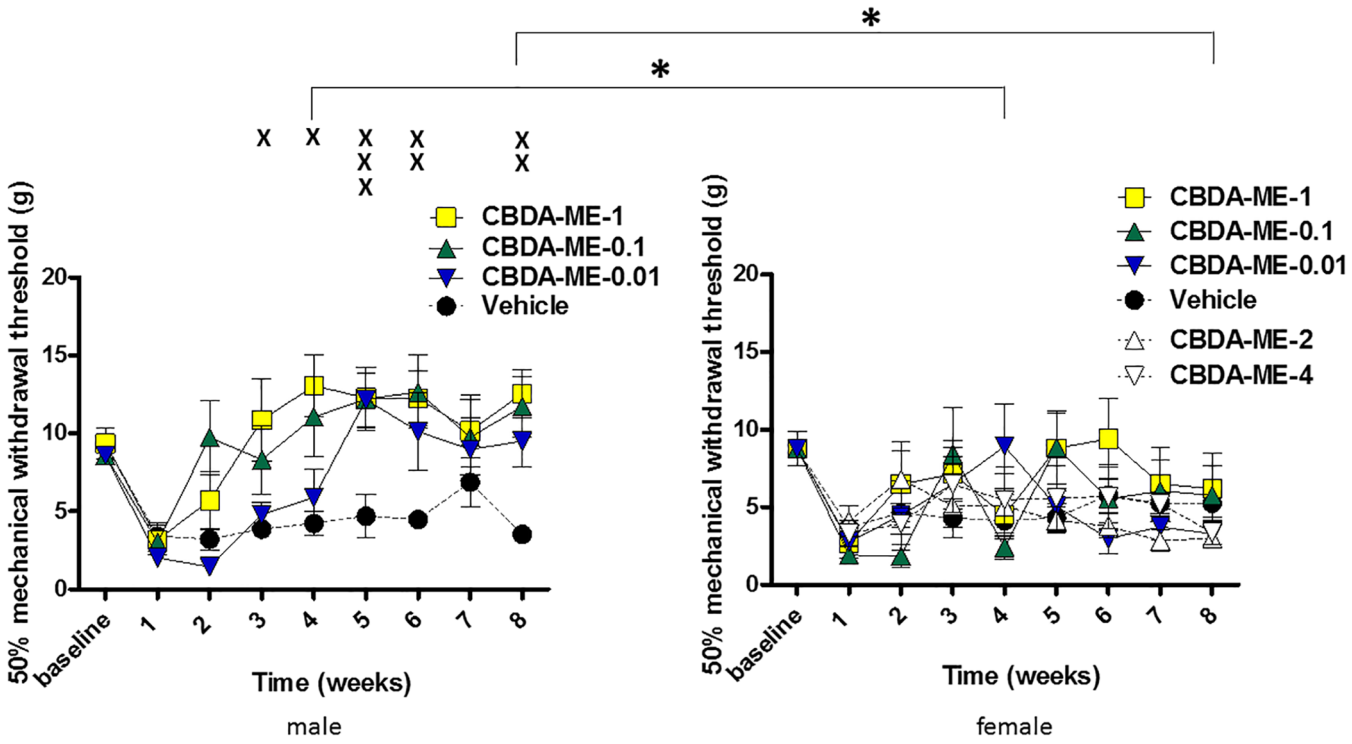

FIGURE 3 A comparison of mechanical withdrawal thresholds in the behavioural study between vehicle and CBDA-ME-treated male and female rats. Mechanical withdrawal thresholds of sciatic cuff-implanted rats were evaluated by von Frey testing once a week to determine the effect of CBDA-ME treatment, which was assessed via dose–response analyses. Data are expressed as the mean ± SEM for each treatment group. Two-way ANOVA with a Bonferroni post hoc test was used for comparisons. A symbol (X) above the left-hand graph indicates significant differences between vehicle and CBDA-ME-1, CBDA-ME-0.1 and CBDA-ME-0.01, represented by an upper, middle, or lower row of this symbol, respectively, during the fourth fifth, sixth, and eighth weeks. Asterisks (*) across the two graphs indicate significant differences between males and females receiving the CBDA-ME-1 treatment, with these differences occurring during weeks 4 and 8. CBDA-ME-1, CBDA-ME-0.1, and CBDA-ME-0.01 represent different concentrations, corresponding to 1, 0.1 and 0.01 $\mu g \cdot kg^{-1}$ respectively. In addition, in females, two higher concentrations, CBDA-ME-2 and CBDA-ME-4, were tested, corresponding to 2 and 4 $\mu g \cdot kg^{-1}$, respectively

reflected a 1.3-fold increase, although this change was also not statistically significant.

Both nociceptive and non-nociceptive mechanoreceptors in CBDA-ME-1-treated rats showed a significant difference between males and females. Sex differences in thresholds of CHTM neurons was significant in response to CBDA-ME-1. Similarly, significant sex differences in thresholds were observed in C-fibre LTM neurons, Aβ-fibre HTM neurons, Aβ-fibre muscle spindle neurons and Aβ-fibre cutaneous neurons. Post hoc comparisons for all classes of neurons showed that males displayed significantly higher withdrawal thresholds than females in response to CBDE-ME-1 treatment.

A sex-based analysis between vehicle-treated rats showed no significant differences, although the activation threshold in females across all types of neurons that were classified in the current investigation was always slightly lower than in males. The most noticeable difference occurred in Aβ-fibre cutaneous neurons, which exhibited a 1.7-fold lower activation threshold in females compared to males treated with vehicle.

Representative recordings for male and female CBDA-ME-1 treatment groups relative to their respective vehicle groups are shown in Figure 6.

4 | DISCUSSION

Cannabinoids are known to have analgesic properties (Iversen & Chapman, 2002), with evidence supporting an impact on normal inhibitory pathways and pathophysiological processes that influence nociception in various pain states (Fine & Rosenfeld, 2014). Agonist-activated cannabinoid receptors are able to modulate nociceptive thresholds, inhibit the release of pro-inflammatory molecules, and synergize with other systems that influence analgesia (Manzanares et al., 2006). For example, the understudied phytocannabininoid CBDA elicits anti-hyperalgesic responses in an inflammatory pain model (Rock et al., 2018). However, its instability makes it a challenging compound to work with experimentally, especially in the context of in vivo studies that may require repeated treatment administration over a prolonged period of time. This limitation prompted the development of CBDA-ME, which has been shown to elicit more potent suppression of anxiety, depression, and nausea in rats than CBDA (Hen-Shoval et al., 2018; Pertwee et al., 2018). The established base of evidence provided a rationale to examine whether CBDA-ME limits or delays the development of chronic hypersensitivity in a preclinical model of surgically induced peripheral neuropathic pain.

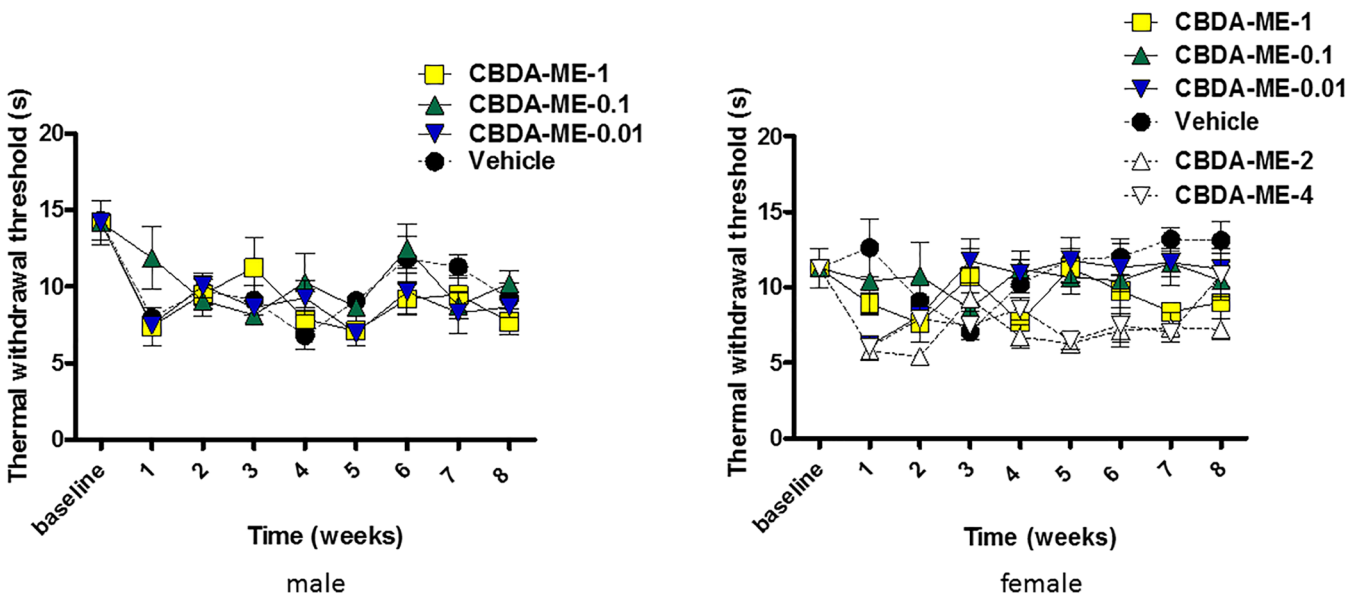

FIGURE 4 Comparison of thermal withdrawal thresholds in the behavioural study between vehicle and CBDA-ME-treated male and female rats. Thermal withdrawal thresholds of sciatic cuff-implanted rats were measured using the Hargreaves' test once a week to determine the effect of CBDA-ME treatment, assessed via a dose–response analysis. Data are expressed as the mean ± SEM for each treatment group. Two-way ANOVA with a Bonferroni post hoc test was used for comparisons. The absence of a symbol or an asterisk indicates lack of a statistically significant difference. CBDA-ME-1, CBDA-ME-0.1 and CBDA-ME-0.01 represent different concentrations, corresponding to 1, 0.1, and 0.01 $\mu g \cdot kg^{-1}$ respectively. In addition, in females, two higher concentrations, CBDA-ME-2 and CBDA-ME-4, were tested, corresponding to 2 and 4 $\mu g \cdot kg^{-1}$, respectively

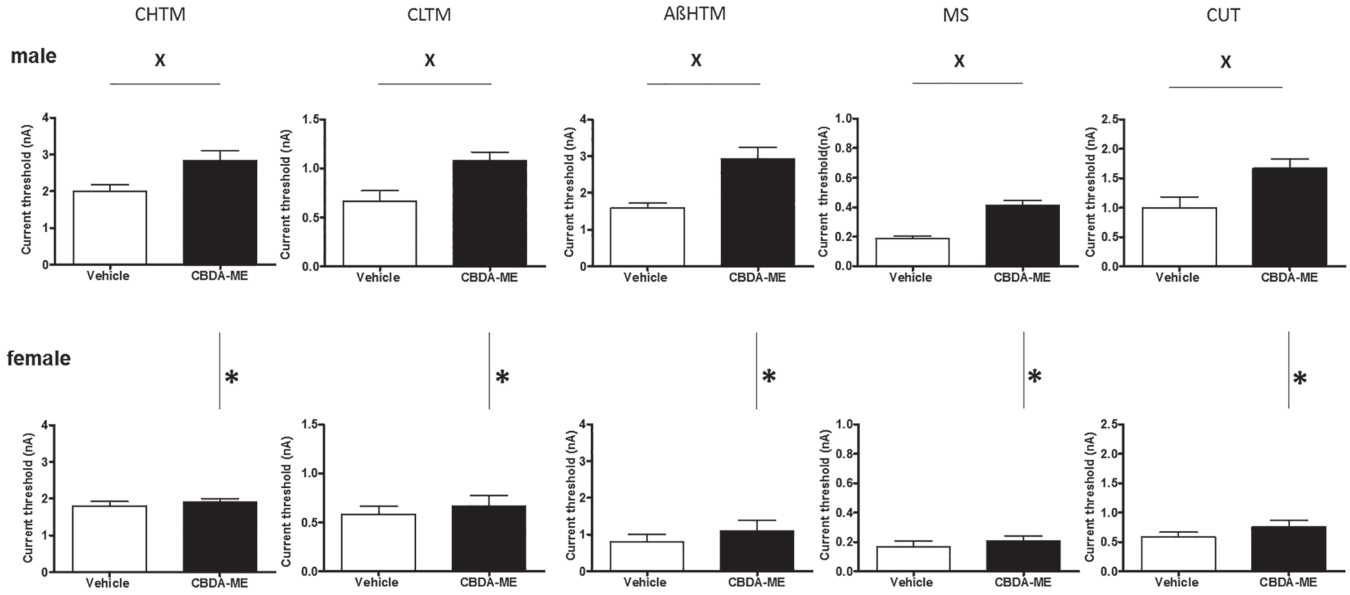

FIGURE 5 Comparison of the activation threshold of mechanical sensory neurons in response to intracellular current injection in vehicle and CBDA-ME-treated male and female rats. The current threshold was defined as the minimum current required to evoke an action potential (AP) by intracellular current injection. Data are expressed as the mean ± SEM for each treatment group. The comparison between vehicle and CBDE-ME-1 treatment, corresponding to 1 $\mu g \cdot kg^{-1}$ for each sex, was analysed by Mann–Whitney analysis. A symbol (X) above the graphs indicates significant differences between the vehicle and CBDA-ME-1 treatment. Two-way ANOVA with a Dunn's post hoc test was then applied for statistical comparisons between sexes with vehicle and CBDA-ME-1 treatment. Asterisks (*) between the graphs indicate a significant difference between males and females in the vehicle and CBDA-ME-1 treatment groups. AβHTM, Aβ-fibre high threshold mechanoreceptor; cutaneous (CUT), Aβ-fibre cutaneous neuron; CHTM, C-fibre high threshold mechanoreceptor; CLTM, C-fibre low threshold mechanoreceptor; MS, Aβ-fibre muscle spindle neuron

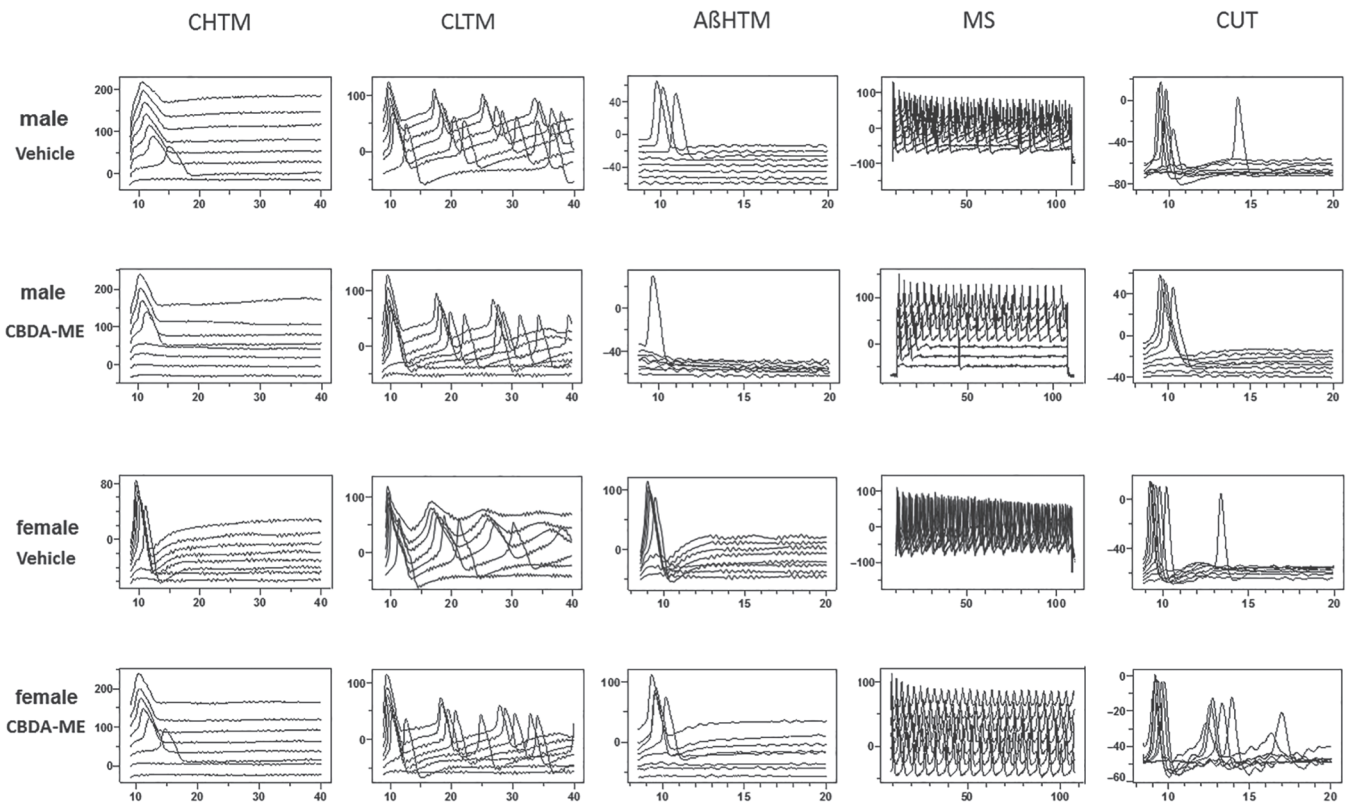

FIGURE 6 Representative raw recordings in response to intracellular current injection in vehicle and CBDA-ME-treated male and female rats. Discharge was evoked by injecting a series of depolarizing current pulses into DRG soma through the recording electrode [X-axis: time (ms); Y-axis: voltage (mV)]. Representative raw recordings show the threshold and repetitive charges of action potentials (APs) evoked by intracellular current injection in different types of mechanoreceptor neurons. APs were evoked by current pulses of 0.5 to 4 nA, in increments of 0.5 nA. Neurons from males and females treated with either CBDA-ME at the 1 $\mu g \cdot kg^{-1}$ concentration or vehicle were tested. AβHTM: Aβ-fibre high threshold mechanoreceptor; CHTM, C-fibre high threshold mechanoreceptor; CLTM, C-fibre low threshold mechanoreceptor; MS, Aβ-fibre muscle spindle neuron; CUT: Aβ-fibre cutaneous neuron

Upon confirming that robust mechanical hyperalgesia is induced in male and female rats surgically implanted with a sciatic nerve cuff relative to sham-operated and naïve counterparts, we established, for the first time, that daily administration of CBDA-ME during the early phase post-nerve constriction imparts significant anti-nociception in males. Male rats received 0.01, 0.1 or 1 $\mu g \cdot kg^{-1}$ of CBDA-ME, with the highest dose significantly inducing long-term recovery. In contrast, even higher concentrations of 2 and 4 $\mu g \cdot kg^{-1}$ failed to provide anti-nociceptive effects in females. These findings reaffirm the in vivo potential of CBDA-ME established by others, extending its application to include treatment of pain.

To assess changes in nociception imparted by CBDA-ME, classic von Frey filaments to measure mechanical hyperalgesia and radiant light applied to the affected paw to evaluate heat-associated responses were used. Both behavioural tests were carried out on a weekly basis spanning an 8-week post-surgical time course, which extended 6 weeks beyond the point at which CBDA-ME administration ceased. In addition, in vivo electrophysiological data were obtained at endpoint to assess CBDA-ME-mediated changes on action potential activity, given that we previously demonstrated the threshold of mechanoreceptors to be significantly lower in rats with sciatic nerve cuff-induced neuropathic pain compared to recordings obtained from naive counterparts (Zhu & Henry, 2012). Our von Frey data clearly show that early intervention in the form of repeated daily CBDA-ME administration in males returns the mechanical threshold to baseline. This finding is supported by electrophysiological measurements in the soma of male L4 DRGs, demonstrating that the activity of all relevant classes of neurons that were assessed in this study, including C-fibre HTM and LTM, Aβ-fibre HTMs and Aβ-fibre muscle spindle and cutaneous neurons, were chronically responsive to early CBDA-ME administration. While no statistically significant sex differences in the activation thresholds of these neurons at the level of the DRG were observed between vehicle-treated male and female rats, females did exhibit consistently lower thresholds than males. These small differences will be examined more closely in future experiments.

In the peripheral nervous system (PNS), heat-sensing receptors are thought to be represented by unmyelinated C-fibres (slow CV), while those responding to cold include C-fibres as well as thinly myelinated Aδ fibres (faster CV; Darian-Smith et al., 1979). Thermal nociceptive input is then regulated by CNS-associated ascending and descending pathways (Baron, Binder, & Wasner, 2010; Kuner,

2010). In the current study, we did not electrophysiologically examine changes in action potential activity in DRG soma in response to heat, given that the 2-week daily intraperitoneal CBDA-ME injection regimen had no statistically significant effects on heat-induced paw withdrawal latency in males or females over the experimental time course. Of note, a foundational basic and clinical science study linking the endocannabinoid system, cannabinoids and their therapeutic role in the management of chronic pain stated that "when cannabinoids lead to a reported reduction in pain, it remains unclear where the effects are triggered, or which aspect of the pain experience is most affected and under what circumstances" (Fine and Rosenfeld, 2013). Evidence suggests that crosstalk between the endocannabinoid system and thermal neuronal activity may depend on the site of pain modulation. For example, it has been proposed that cannabinoids could suppress heat-evoked activity in a wide range of neurons in the rat lumbar dorsal horn, based on effects observed after injecting a **CB_1 receptor** agonist into the cerebral ventricle (Hohmann, Tsou, & Walker, 1999). Interestingly, when the same CB_1 agonist was intravenously administered to animals that had undergone a spinal nerve transection, heat-evoked activity of neurons in the lumbar dorsal horn was not suppressed (Manzanares et al., 2006). These particular studies highlight the complexity that underlies cannabinoid-mediated changes in thermal stimulus-induced effects, providing important insights into the design of future studies aimed at examining why the selected doses of CBDA-ME did not significantly alter heat-induced allodynia evoked by sciatic nerve constriction. Interestingly, in a paxclitaxel-induced rat model of neuropathy, both low and high doses (0.032 and 3.2 $mg \cdot kg^{-1} \cdot day^{-1}$) of subcutaneously administered **AM1710**, a **CB_2**-selective agonist, suppressed the development of mechanical hyperalgesia, with only the high dose suppressing cold allodynia (Rahn et al., 2014). Future work will therefore also examine the effects of CBDA-ME on cold sensing in the neuropathic pain model, given that there are known differences in heat and cold pain pathways. In addition, it will also be of interest to examine differences in modulating nociception peripherally versus centrally and to test whether acute CBDA-ME administration produces different outcomes than its chronic peripheral delivery.

The administration of CBDA-ME did not elicit significant behavioural or electrophysiological effects in females at any of the selected concentrations. The present findings diverge from preclinical reports demonstrating that female rodents are consistently more sensitive to reward-related and anti-nociceptive effects of cannabinoids relative to males (Craft et al., 2013). However, a recent study showed that anti-nociception is restricted to acute cannabinoid administration in females (Manzanares et al., 2006), which differs from our 2-week injection regimen. Furthermore, there may be significant differences in male and female responses depending on the type of cannabinoid that is tested (i.e. whether it induces psychotropic or locomotor effects, such as reported for Δ9-tetrahydrocannabidiol). The effects in males and females observed in response to CBDA-ME suggest that there are potential mechanistic differences, including in vivo potency, which could be affected by drug metabolism. However, given that a fourfold increase in the concentration of CBDA-ME failed to elicit anti-nociceptive responses in females, other factors appear to be playing a role.

In addition to dosing parameters, substantial evidence supports that sex differences in nociception that are associated with nerve damage may involve distinct immune system interactions with the PNS and CNS. In this context, it is possible that CBDA-ME ameliorates the development of neuropathic pain during the early hyperinflammatory course accompanying a peripheral nerve injury, as CBDA has been shown to elicit anti-inflammatory effects in a rodent model of hyperalgesia induced by carrageenan-induced inflammation (Rock et al., 2018). After peripheral nerve injury, microglial-neuronal signalling in the spinal cord appears to mediate hypersensitivity in male mice, while in females, adaptive immune cells contribute to this response (Mapplebeck, Beggs, & Salter, 2016). Given that microglia are present in the CNS, these cells may be one of the sexually dimorphic components influencing the firing rates of peripheral nociceptors, which occurs more prominently in males. It is therefore possible that early CBDA-ME treatment prevents the generation of ectopic neuronal activity in peripheral sensory nerves by also preventing microglial reactivity in males, which could explain their recovery during the later chronic phase of neuropathic pain. In contrast, females reportedly do not rely on microglial responses for the chronicity of nociception. Rather, peripheral immune cells play a more pronounced role in female, influencing nociceptive responses. We speculate that adaptive immune cell activity may not be strongly regulated by CBDA-ME, which remains to be experimentally determined. The treatment-associated sex differences observed here highlight the importance of including both males and females in preclinical neuropathic pain models to assess the effects of therapeutically relevant cannabinoids.

While an exploration of the mechanism(s) underlying the anti-nociceptive effects of CBDA-ME was beyond the scope of the current investigation, it has been shown by others that this modified cannabinoid produces more potent **$5\text{-}HT_{1A}$ receptor**-mediated suppression of nausea, anxiety and depression in rats than CBDA (Hen-Shoval et al., 2018; Pertwee et al., 2018). In both the peripheral and central nervous system, 5-HT has long been considered to play an important role in modulating nociception. Behavioural and clinical studies have shown that $5\text{-}HT_{1A}$ agonists may be promising therapeutic agents for the treatment of pain (Bardin, 2011). The cell bodies of sensory neurons representing peripheral nociceptors are located in DRG along the spinal cord, and some of these sensory neurons show high levels of 5-HT receptor expression, with many of these same neurons co-expressing a known nociceptor, transient receptor potential vanilloid 1 (**TRPV1**; Loyd, Henry, & Hargreaves, 2013). Interestingly, CBDA also targets the TRP family of ion channels (De Petrocellis et al., 2008), including TRPV1 (Ligresti et al., 2006). TRP channels detect physical and chemical stimuli and promote painful sensations via nociceptor activation (Marwaha et al., 2016) and the stimulating effects of cannabinoids at TRPV1 may lead to desensitization of this channel (Morita et al., 2006; Ursu, Knopp, Beattie, Liu, & Sher, 2010). Furthermore, CBDA has been shown, by computational modelling and cell culture-

based experiments, to act as a dual **PPAR-α** and **PPAR-γ** agonist (D'Aniello et al., 2019). A recent study demonstrated a link between dual PPARα/γ activation and TRPV1 in two different models of rat neuropathic pain (Alsalem et al., 2019). It is therefore possible that CBDA-ME may elicit anti-nociceptive effects directly through 5-HT receptors, TRP channels and/or its interactions with PPARα/γ, although a systematic mechanistic evaluation comparing CBDA and CBDA-ME with specific receptor antagonists will need to be carried out in future experiments.

In conclusion, we show for the first time that the anti-nociceptive effects of CBDA-ME, a stable derivative of CBDA, promote recovery of tactile hypersensitivity in response to treatment initiated during the early phase of neuropathic pain development, resulting in altered nociceptor firing patterns several weeks after drug administration ceases. These effects occur in males but not in females, and sex may be an important factor that should be accounted for in examining the analgesic potential of natural cannabinoids, as well as synthetic derivatives of phytocannabinoids, to effectively manage neuropathic pain in the clinic.

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AUTHOR CONTRIBUTIONS

Yong Fang Zhu designed the experiment, performed the electrophysiological experiments, analysed the data, and wrote the first draft of the manuscript. Katja Linher-Melville provided critical feedback on the experimental results and co-wrote and edited the manuscript. Javad Niazmand, Manu Sharma, Ayesha Shahid, and Kan Lun Zhu administered the compound and performed von Frey and Hargreaves' tests. Yong Fang Zhu, Ayesha Shahid, Natalka Parzei, and Jesse Sidhu induced the neuropathic pain model. Christeene Haj synthesized CBDA-ME. All authors reviewed successive drafts of the manuscript. Gurmit Singh provided feedback on the design of the experiment, supervised the project and provided funding for the study. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

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DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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