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Research report

Antinociceptive action of cannabidiol on thermal sensitivity and post-operative pain in male and female rats

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ABSTRACT

This study investigated the antinociceptive potential of cannabidiol (CBD) in male and female Wistar rats. The assessment and analysis included tail withdrawal to thermal stimulation (tail flick test) and mechanical allodynia induced by plantar incision injury (von Frey test). CBD reduced acute thermal sensitivity in uninjured animals and post-operative mechanical allodynia in males and females. In the tail flick test, CBD 30 mg/kg i.p. was required to induce antinociception in males. During the proestrus phase, females did not show a statistically significant antinociceptive response to CBD treatment despite a noticeable trend. In contrast, in a separate group of rats tested during the late diestrus phase, antinociception varied with CBD dosage and time. In the post-operative pain model, CBD at 3 mg/kg decreased mechanical allodynia in males. Similarly, this dose reduced allodynia in females during proestrus. However, in females during late diestrus, the lower dose of CBD (0.3 mg/kg) reduced mechanical allodynia, although the latency to onset of the effect was slower (90 min). The effectiveness of a 10-fold lower dose of CBD during the late diestrus stage in females suggests that ovarian hormones can influence the action of CBD. While CBD has potential for alleviating pain in humans, personalized dosing regimens may need to be developed to treat pain in women.

1. Introduction

The increasing de-criminalization of cannabis and cannabinoid-related products by many countries has re-ignited interest in research into their therapeutic potential for pain relief. Even so, in 2021, due to the lack of high-quality clinical evidence, the International Association for the Study of Pain (IASP) task force felt unable to endorse the general use of cannabis and cannabinoids for pain relief [1]. Several recent review articles concur with this view [2–4]. However, a multicriteria decision analysis model that compares pharmacotherapy for chronic neuropathic pain showed the benefit-safety profiles for cannabinoids were higher than for other commonly used medications for chronic pain, mainly because cannabinoids contribute more to quality of life and have a more favorable side effect profile [5]. Nevertheless, all studies emphasize the pressing need for further preclinical and clinical studies to fill the research gap.

Within the field of pain research, preclinical studies are still strongly biased toward using male rodents, 91 % for rats and 81 % for mice [6]. However, clinical studies consistently report higher pain prevalence and severity among women than men [7,8 for recent reviews]. Moreover, evidence is emerging for robust differences in the genetic, molecular, cellular, and systems-level mechanisms of acute and chronic pain processing between males and females in both rodents and humans [7], which impacts the responsiveness to analgesic drugs. From the preclinical perspective, several recent rigorous meta-analyses support the antinociceptive effect of cannabinoids in a range of inflammatory and

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neuropathic pain models [2,6,9]. However, it should be acknowledged that the findings derived from predominantly male-biased literature may not directly translate to females. Therefore, it is imperative to include females in preclinical studies to inform and guide clinical practice effectively.

The two major bioactive constituents of *Cannabis sativa* are delta-9-tetrahydrocannabinol (Δ^9 -THC) and cannabidiol (CBD). Δ^9 -THC, the most extensively studied compound, has been demonstrated to have significant antinociceptive effects in models of both acute and neuropathic pain in female rats. Interestingly, it was found to be significantly more potent in females compared to males [10,11]. In contrast, CBD has received less attention even though, from the translational perspective, the absence of psychotomimetic effects of CBD [12] makes its therapeutic potential arguably more attractive than that of Δ^9 -THC and worthy of further investigation. We, therefore, designed a study to investigate the antinociceptive potential of CBD in phasic and post-operative pain models in male and female rats. In a recent study of the anxiolytic effects of CBD in females, we found that rats in the late diestrus phase of their estrous cycle were more sensitive than rats in the proestrus phase [13]. In the present study, we also explored the potential influence of the estrous cycle on drug responsiveness in females. Our findings revealed a significant increase in sensitivity to the antinociceptive effects of CBD in females during the late diestrus phase.

2. Methods - experimental procedures

2.1. Animals and housing

Male and female Wistar rats (220–240 g) from the animal facility of the University of São Paulo (USP) at Ribeirão Preto (RP) Campus were used They were housed under a 12:12-h light/dark cycle starting at 7:00 AM. All of the experiments received formal approval from the Committee on Animal Research and Ethics of the USP-RP and were performed in compliance with the recommendations of the Conselho Nacional de Controle de Experimentaç ão Animal - Ministério da Ciência e Tecnologia, Brazil. The animals had access to food and water ad libitum and were assigned randomly to experimental and control groups. The first cohort of rats was used to examine whether the tail flick response of male (n = 7) and female rats at different stages of the estrous cycle (n = 31) was differentially expressed in the acute pain model. These females during proestrus or late diestrus stages and males were also used to test the effect of the vehicle or CBD treatment in the tail flick test; additional males (n = 22) and females (n = 21) were used to complete the number of animals in each treatment group. The second cohort of naïve rats, females (n = 41) and males (n = 25) were used to investigate the effect of CBD in the incision pain model. The naïve rats in all the cohorts (males and females) were handled, and daily examination of vaginal smears was performed to determine the estrous cycle stage in females.

2.2. Determination of stage of estrous cycle

The histology of vaginal smears was used to determine the estrous cycle stage, as described by [13,14]. A vaginal smear was taken daily, starting 10 days before the behavioral tests, every morning between 9 h and 10 h to establish that the animals were cycling regularly. In brief, an inoculation loop was sterilized in a flame, dipped in sterile water, and then gently inserted into the vagina to gather cells, which were smeared onto a glass slide. The smears were stained with a 2 % methylene blue solution from Panótico LB Kit (Laborclin Produtos para Laboratórios Ltda, Pinhais, PR, Brazil). Proestrus was characterized by nucleated epithelial cells, estrus by cornified squamous cells, early diestrus by a preponderance of small nucleated leucocytes typically with lobed nuclei, and in late diestrus, fewer leukocytes were present with clumped and or disintegrating nuclei [15,16]. Experiments were performed on animals that had completed at least two regular cycles. Three observers confirmed the estrous stages. Handling stress associated with the collection of vaginal smears is a potential source of variability that could influence behavior in females [17]. Rats were handled, and smears were collected daily for at least 10 days prior to the experimental day. The reason for this was: firstly, to fully habituate the animals to the procedure and secondly, to establish that each rat was cycling normally, i.e., progressing through two full 4–5 day cycles. On the experimental day, vaginal smearing was done as usual, and cages were brought to the common staging area 1 h prior to testing in order to avoid generating stress in animals. An extra vaginal smear was taken following the completion of each experimental test to confirm the estrous cycle stage. Females that presented different stages before and after the behavioral testing were excluded from the analysis. Seven females were excluded from the study either because they failed to undergo at least two regular cycles or because estrous cycle stage had changed before and after the behavioral test.

2.3. Drug

Cannabidiol (99.6 % pure, kindly supplied by BSPG-Pharm, Sandwich, United Kingdom) was freshly dissolved in 2 % TWEEN 80 (Sigma–Aldrich, St. Louis, MO, United States) and saline (NaCl 0.9 %) for intraperitoneal (i.p.) injections as previously described [18–20]. In the first set of experiments using the acute pain model, we tested CBD 0.3–30 mg/kg. This dose range was chosen based on our previous finding in male rats [18]. Guided by our finding that female rats were sensitive to low doses of CBD in the acute pain model, and in order to minimize animal usage, for the second series of experiments using the post-operative pain model, we compared responsiveness to only two doses of CBD: 0.3 and 3 mg/kg.

2.4. Tail flick test

The tail flick test involves the application of a heat stimulus to a focused area on the tail of rodents, and the time taken for the tail to "flick" or twitch is recorded [21]. Since novelty can induce antinociception, rats were first familiarized to being loosely restrained by the experimenter. On the day of the experiment, they were acclimated to the procedure room for 1 h prior to testing to stabilize skin and ambient temperature. The experimenter loosely restrained each rat whilst the ventral surface of the animal's tail, between 4 and 6 cm from the tip, was exposed to the chrome steel spiral with an electrically-induced increasing temperature ramp [22]. The time required for the animal to remove its tail from the steel spiral was expressed as the tail flick latency.

The tail flick latency was measured at 5-minute intervals until a stable baseline was obtained on 3 consecutive tests. Only rats that presented stable basal latency in up to 6 tests were used in the experiment. Baseline values were in the range of 2.5–3.0 s, and a 6 s cut-off time was used to prevent tail skin tissue damage caused by excessive heating. Given the observed variation in baseline (vehicle) latencies among the groups, transforming individual latencies into a percentage of the maximum possible effect (%MPE) facilitated a more precise comparison of the CBD effect between groups. Antinociception was quantified according to the method of Harris and Pierson [23] as the %MPE, which was calculated as %MPE = [(test latency – control latency) / (6 – control latency)] × 100. The test latency after CBD treatment was assessed at 30, 60, 90, 120 and 150 min.

2.5. Post-incision pain model

Rats were anesthetized with ketamine/xylazine (100/7.5 mg/kg, i.p.), and a 1 cm longitudinal incision was made through the skin and fascia of the plantar surface of the right hind paw to expose the underlying muscle. The plantaris muscle was then elevated, stretched, and incised longitudinally, with the muscle origin and insertion remaining

intact [24]. The incised skin was stitched with two 5–0 nylon sutures. The rats were tested 24 h after surgery.

2.6. Algesimetric test

The threshold for mechanical stimulation was assessed with an electronic von Frey apparatus (IITC Electronic Equipment, United States), which consisted of a rigid plastic tip (tip area = 0.7 mm^2) connected to a hand-held probe unit. The rat was placed in an acrylic cage (12 cm × 10 cm × 17 cm) with a wire-grid floor for 30 min to allow behavioral acclimation to the environment. A tilted mirror below the grid provided a clear view of the animal's hind paw. Increasing upward pressure was applied with the plastic tip against the mid-plantar surface of each hind paw, bordering the incision wound near the heel. During this procedure, the applied force in grams (g) was continuously recorded by a main unit connected to the probe. The threshold was determined for removal of the paw followed by clear flinching movements. At this moment, the movement of the probe stopped, and the intensity of the pressure at the threshold was automatically determined [22].

2.7. Statistical Analysis

Statistical differences were determined by two-way repeated measure ANOVA using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). For the assessment of behavioral experiments, all data are expressed as mean ± standard error. Two-way repeated-measures ANOVA with Tukey's or Dunnett's multiple comparisons test were used to assess the effect of treatments and time separately for males and females (in proestrus or late diestrus). Selected post hoc statistical tests are specified in the results. In all cases, the threshold for significance was $p < 0.05$.

3. Results

3.1. Phasic pain

In initial experiments, we tested the tail flick latency in female rats at all four stages of the estrous cycle, as well as in males, in order to establish a baseline (Fig. 1). A one-way ANOVA demonstrated a significant difference between groups ($F_{4,33} = 11.84$; $p < 0.0001$). A Tukeys's post hoc test revealed that the baseline tail flick latencies were significantly longer during the late diestrus phase compared to other estrous phases and the male group. For subsequent experiments, we chose females in proestrus or late diestrus based on this baseline result and our previous finding [13] to test the effect of CBD on behavior in phasic and post-operative pain models.

3.2. Effects of cannabidiol on phasic pain

We examined the effect of a single injection of CBD in males (Fig. 2A) and females during proestrus (Fig. 2B), and late diestrus (Fig. 2C) on tail flick latencies expressed as the %MPE of antinociception. Although males and females were derived from the same cohort of animals, the experiments were not conducted simultaneously. Consequently, we analyzed the data separately for each group (sex/estrous cycle phase), normalizing tail flick latencies to %MPE. We performed two-way repeated-measures ANOVA, considering 'treatment' and 'time' separately for each group (sex/estrous cycle phase).

In males, two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{4,100} = 19.52$; $p < 0.0001$), treatment ($F_{3,25} = 5.84$; $p = 0.0036$), and interaction of factors ($F_{12,100} = 3.70$; $p = 0.0001$). A Dunnett's post hoc test revealed significant differences between treatment groups. Fig. 2A shows that CBD at a dose of 30 mg/kg produced maximum antinociception in male rats, which became apparent 60 min post injection ($p < 0.001$) and was relatively consistent

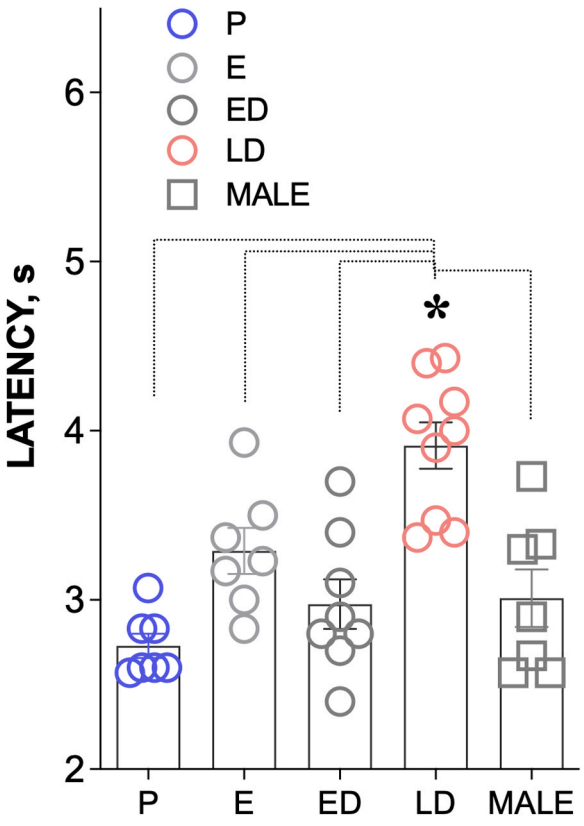

Fig. 1. Tail flick latency mean ± SEM was measured for females in proestrus (P), estrus (E), early diestrus (ED) and late diestrus (LD) and male rats. The results showed a significant difference of the late diestrus group compared to the other estrous phases and male group (* $p < 0.05$). One-way ANOVA with Tukey's post hoc test.

across all experimental sessions varying between 20 % and 40 % antinociception MPE. There was no statistically significant effect of lower doses (0.3 and 3 mg/kg) over time compared to the control group.

CBD also evoked an antinociceptive effect in female rats. In females during proestrus, two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{5,100} = 3.17$; $p = 0.0106$) without effect of treatment ($F_{3,20} = 1.86$ $p = 0.1695$) and no interaction ($F_{15,100} = 0.910$; $p = 0.5557$) between the factors (Fig. 2B). In females in late diestrus, two-way repeated-measures ANOVA revealed a significant effect of time ($F_{5,125} = 32.87$; $p < 0.0001$), treatment ($F_{3,25} = 6.23$; $p = 0.0031$) and interaction between the factors ($F_{15,125} = 4.66$; $p < 0.0001$). Female rats in late diestrus demonstrated sensitivity to CBD and, intriguingly, displayed a trend towards an inverted U-shaped dose-response relationship. Specifically, Dunnett's post hoc test revealed the significance of maximum antinociception achieved at different doses and time points. In groups administered 3 and 30 mg/kg of CBD, rats exhibited an antinociceptive response 30 min after drug injection ($p < 0.05$). The lower dose of CBD (0.3 mg/kg) also produced antinociception, but with a delayed onset, which did not become evident until 150 min post-injection (Fig. 2C).

3.3. Effects of cannabidiol on the post-operative pain model

Before surgery, there was no notable sex difference in paw withdrawal thresholds elicited by applying 40–55 g of force using the electronic von Frey apparatus in male and female rats. However, when retested 24 h following surgery, the mean withdrawal threshold for the incised paw had decreased significantly in males and in both groups of female rats, indicating the presence of mechanical allodynia (Fig. 3A-C). Administration of the vehicle did not have an impact on the magnitude

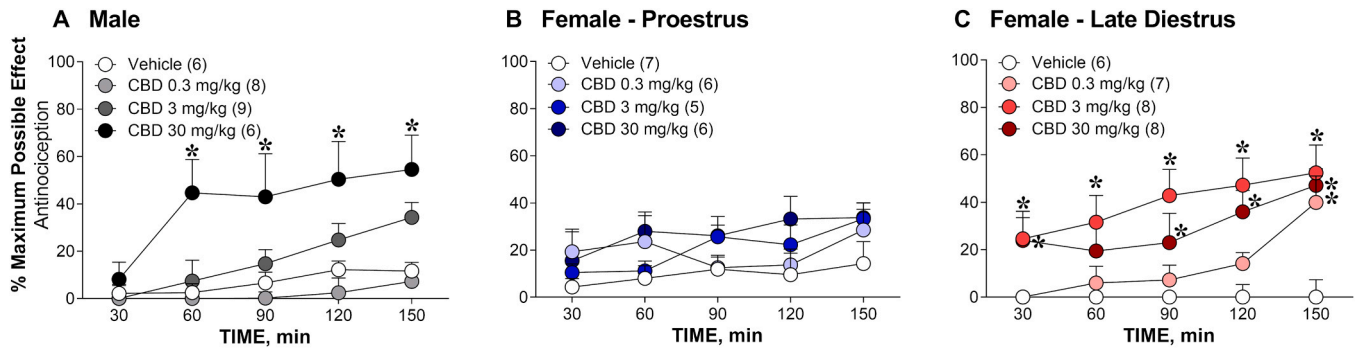

Fig. 2. Effect of CBD (0.3–30 mg/kg i.p.) on tail flick latency in male (A) rats and female rats in proestrus (B) or late diestrus (C). Results are expressed as the %MPE ± SEM in response to cannabidiol (CBD) treatment. The numbers in parentheses indicate the number of rats in each group. * indicates a significant difference from the vehicle-treated group at the same time point, with $p < 0.05$. The analysis was conducted using two-way ANOVA with Dunnett's post hoc test.

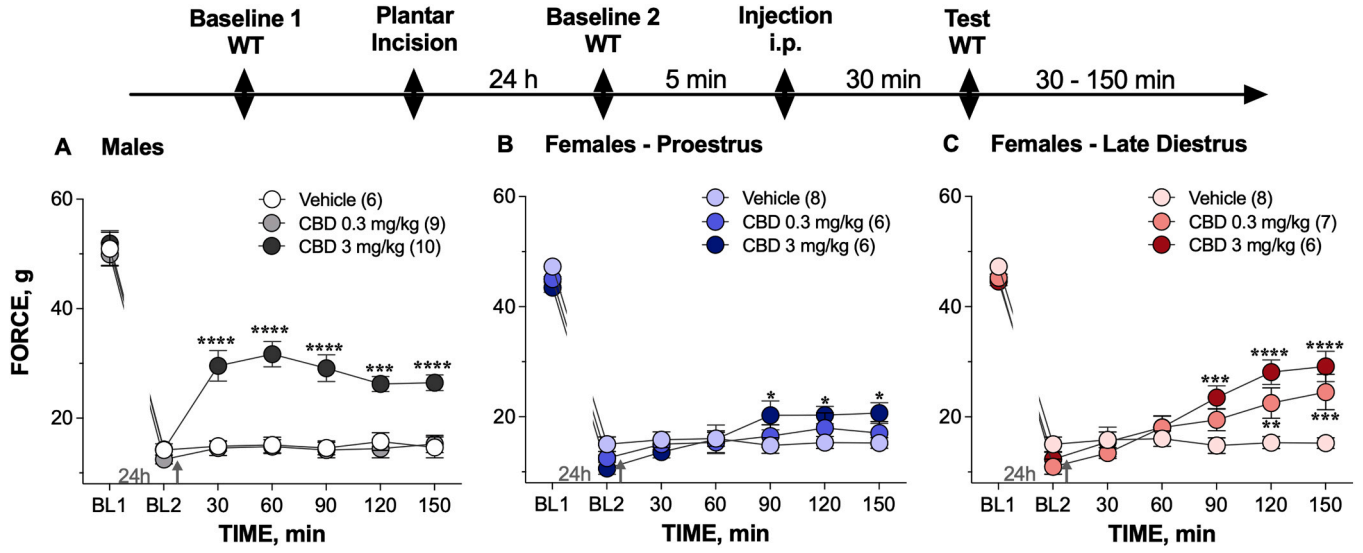

Fig. 3. Reduction of mechanical hypersensitivity by cannabidiol (CBD). The timeline depicts the experiment protocol, and Figure A, B and C illustrate the time-course of changes in withdrawal thresholds (WT) measured in the operated hind paws of males (A) and females rats in proestrus (B) or late diestrus (C). Baseline (BL) measurements were obtained preoperatively (BL1) and 24 h postoperatively (BL2). The numbers in parentheses indicate the number of rats in each group. Significantly different compared to the vehicle-treated group at the same time point are denoted by asterisks. Statistical analysis was performed using a two-way ANOVA with Dunnett's test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. The data are presented as means ± SEMs. Small SEMs are obscured by symbols at many data points.

or time course of mechanical allodynia in the incised paw across all groups (Fig. 3 A-C). A two-way repeated-measures ANOVA revealed a significant effect of time on the incised paw (before and after surgery; $F_{6,66} = 174.2$, $p < 0.0001$), but no significant effect of vehicle treatment between male and female rat groups ($F_{2,11} = 0.65$, $p < 0.54$) and no interaction between factors ($F_{12,66} = 0.97$, $p = 0.49$). The withdrawal threshold for the non-incised contralateral paw remained unaltered after surgery and following drug treatments. Two-way repeated-measures ANOVA showed no interaction between factors and no effect of time and treatment on the non-incised contralateral paw in males and females tested during proestrus or late diestrus (Supplementary material, Table 1).

In males, a two-way repeated-measures ANOVA revealed a significant effect of time ($F_{6,132} = 212.4$, $p < 0.0001$), treatment ($F_{2,22} = 22.89$, $p < 0.0001$), and interaction between factors ($F_{12,132} = 7.07$, $p < 0.0001$). Dunnett's post hoc test showed significant differences between treatment groups. Administering 3 mg/kg CBD to males resulted in a significant reduction in allodynia, which was evident within 30 min of drug administration and persisted over 150 min (Fig. 3A). However, the lower dose tested (0.3 mg/kg) did not have an effect.

CBD also reduced mechanical allodynia in females. In females in proestrus, a two-way repeated-measures ANOVA demonstrated a significant effect of time ($F_{6,102} = 308.9$, $p < 0.0001$) and an interaction between factors ($F_{12,102} = 4.31$, $p < 0.0001$), but no effect of treatment ($F_{2,17} = 0.128$, $p = 0.88$). Post hoc analysis revealed a significant effect in response to the 3 mg/kg dose of CBD, but not the lower dose (Fig. 3B). In contrast, females in late diestrus showed notably increased sensitivity to CBD, even at the lowest dose (0.3 mg/kg). A two-way repeated-measures ANOVA showed a significant effect of time ($F_{6,108} = 246.6$, $p < 0.0001$) and an interaction between factors ($F_{12,108} = 9.81$, $p < 0.0001$), but no effect of treatment ($F_{2,18} = 3.12$, $p = 0.06$) in females during late diestrus. Interestingly, antinociception in females in either the proestrus or late diestrus phases was not observed until 90 min post-injection, while a notable effect was evident in males within 30 min (Fig. 3A-C).

4. Discussion

In this study, we investigated the antinociceptive effects of acute administration of CBD in phasic and post-operative pain models using male and female Wistar rats. Our findings demonstrated that CBD exerted antinociceptive effects in both pain models. CBD appeared to

exhibit greater potency in attenuating post-operative pain compared to thermal sensitivity in uninjured animals. However, it is worth noting that drug potency in acute thermal tests is strongly dependent on stimulus intensity. It is possible that CBD may have appeared more potent if we had used a weaker heat stimulus to evoke a tail flick at a longer latency. Interestingly, in terms of acute pain (tail flick test) we found that the maximum antinociceptive effect of CBD in females during the late diestrus phase was attained at a dose 100 times lower than that observed in males. Although our experimental protocol precluded direct statistical comparison between the sexes, these findings point to potential sex differences in the responses to CBD and suggest a modulatory role of the estrous cycle in CBD-mediated antinociception. Further investigations employing simultaneous analysis of males and females, along with varying hormone dosages, are necessary to elucidate whether CBD effects are correlated with variations in potency, efficacy, peak time of effect, and duration of action, particularly associated with sex differences.

Our finding of CBD-induced antinociception in the tail flick test is consistent with a previous study conducted in male Wistar rats [25], but contrasts with recent studies that failed to detect antinociception using the tail flick test in male or female Sprague Dawley rats [26,27]. CBD was, however, reported to attenuate hyperalgesia in an acute inflammatory pain model in male Sprague Dawley rats [28]. These findings suggest that the sensitivity to CBD may be influenced by factors such as strain and the specific pain model or test employed. Indeed, in mice, strain differences, nociceptive test variations, and procedural factors are recognized to impact the responsiveness to classical analgesic drugs [29 for a review]; CBD is no exception to this [30–32].

In our study using female Wistar rats, we observed that the antinociceptive effect of CBD in the tail flick test was dependent on the estrous cycle. During the proestrus phase, although there was a noticeable trend, female rats did not exhibit a statistically significant antinociceptive response to CBD treatment. In contrast, in the late diestrus phase, females displayed antinociception at a dose 100-fold lower than that required in males. This dose-dependent effect of CBD in females followed a complex and nonlinear dose-response relationship, consistent with findings from other studies [13,33,34]. The optimal effects were achieved with doses of 3 and 30 mg/kg, with an onset latency of 30 min. A lower dose was ineffective at this time point, although antinociception did develop later, starting at 150 min post-injection.

Our finding that CBD attenuated mechanical allodynia following plantar incision injury is perhaps of more relevance to clinical medicine, as relieving persistent pain is a particular clinical challenge. Notably, the dose of CBD required to produce antiallodynic effects after a plantar incision injury was one-tenth of that needed to reduce thermal sensitivity in the tail flick test. Moreover, in females, the effect appeared estrous cycle sensitive, with rats in late diestrus responding to a much lower dose of CBD than in the proestrus phase. Previous studies have reported antiallodynic effects of acute CBD in different models for evoked and spontaneous pain in male Wistar rats [18,35–37] and in male mice [38]. On the other hand, in Sprague Dawley rats with chronic inflammatory pain, CBD produced only a modest antiallodynic effect and no sex differences were detected [34]. However, a more recent study demonstrated that CBD exhibited anti-allodynic and anti-hyperalgesic effects after chronic administration in a chronic inflammatory pain model in both male and female Sprague Dawley rats [33]. Interestingly, that study did not consider the estrous cycle stage in the females used, but it is worth noting that the peak anti-hyperalgesic effect of CBD occurred at a 10-fold lower dose in females compared to males [33], in line with the findings of our study (Fig. 3).

Chronic pain models induce a physical injury that triggers an inflammatory reaction, which leads to tissue hypersensitivity. Recent reviews have highlighted the involvement of immune cells in chronic pain pathways, both in the peripheral and central nervous systems [39–42]. CBD is known to display potent anti-inflammatory properties, which may contribute to its effectiveness in chronic pain models [43]. Several studies have shown CBD to be effective in reducing allodynia and objective measures of inflammation induced in chronic pain models in Wistar rats of both sexes [6,44] as well as in mice [45,46]. Interestingly, studies in Sprague Dawley rats have reported minimal anti-inflammatory effects of CBD, which may explain the weaker antinociceptive effect observed in neuropathic pain in this particular strain [34].

There is evidence to suggest that persistent inflammatory pain is associated with an increase in anxiety-like behaviors, which likely reflect the adverse emotional component of the pain experience [47]. Studies conducted in male rats with inflammatory pain have shown that CBD not only reduced allodynia but also decreased anxiety-like behaviors [18,44,48]. It could be argued that anxiolysis was secondary to a reduction in the physical components of pain. However, anxiolytic-like actions of CBD in uninjured pain-free rats of both sexes are well established [13,49–52]. This suggests that CBD may have multimodal effects on pain, influencing both the sensory and affective dimensions of pain. Indeed, it has been suggested that the antinociceptive effects of CBD in neuropathic pain might be more attributable to its effects on the affective-motivational dimension of pain rather than on somatosensory processing [18,53,54].

Several recent findings have highlighted sexual dimorphism in the signaling mechanisms underlying pain hypersensitivity [55–57]. Indeed, sexual dimorphism in the central neuronal mechanism of chronic pain is a consistent finding across species [58]. According to Carmichael and collaborators [59]. The inflammatory response induced by capsaicin or noxious heat is greater in females compared to males, suggesting possible sex-related changes in mechanisms mediated by the TRPV-1 receptor. Inflammatory pain is also modulated by steroidal hormones, including estrogen [60–62]. The inflammatory response, as measured by plasma extravasation, was lower in proestrus, the phase of the estrous cycle when estrogen levels peak, compared to other stages [59]. These findings indicate that gonadal hormones may modify the pathophysiological characteristic of pain, which could also influence the pharmacological actions of CBD.

In our study, we observed that the peak antinociceptive effect of CBD in females during late diestrus phase was achieved at a 100-fold lower dose than in males. Sex differences in CBD sensitivity have been observed by Britch and Craft [33]. Plasma concentrations of CBD and its metabolites were not significantly different in males and females, at least after acute inhalation of vaporized CBD [63]. However, CBD has a complex pharmacological profile. It has limited direct effects on cannabinoid receptors actions at a wide range of ionotropic and G-protein coupled receptors have been reported [64–66]. Notably, CBD interacts with $5HT_{1A}$ receptors and transient receptor potential vanilloid (TRPV) channels [64,67,68]. Studies conducted in male rodents utilizing neuropathic pain models have reported the involvement of TRPV subtype 1 channels in the antinociceptive effect of CBD on mechanical allodynia [35,36,44,69], whilst others have reported $5HT_{1A}$ receptor-mediated antiallodynic effects [37,48,69,70].

Comparable data for female rats is currently unavailable. However, in mice, it has been suggested that males may utilize serotonergic systems more efficiently to attenuate nociceptive behavior, whereas female mice rely more on alternative mechanisms such as GABA [71]. Differential engagement of 5-HT and GABAergic control systems in males and females may contribute to sex differences in the mechanism of action of CBD regarding pain.

In our study, females in late diestrus were sensitive to CBD at a 10-fold lower dose than in proestrus or males. CBD has been shown to act as a positive allosteric modulator at $GABA_A$ receptors [72,73]. GABAergic transmission displays considerable plasticity during the estrous cycle, particularly in the late diestrus phase, when the rapid decline in progesterone secretion leads to the upregulation of extrasynaptic $GABA_A$ receptors [74]. Further studies are needed to understand how these changes impact pain control circuitry and the antinociceptive actions of CBD.

In the present study, we used the von Frey test as the sole indicator of post-operative pain. Although commonly used to quantify mechanical allodynia, it has some limitations since it focuses only on evoked withdrawals and does not consider other important aspects of the post-operative pain experience. To more fully understand post-operative pain in females, future studies should consider incorporating other assessments such as conditioned place preference, as previously described in male rats [18]. Another paradigm to explore is the utilization of the mechanical conflict system, a relatively new operant procedure, to assess mechanical sensitivity and recovery by quantifying the motivation to avoid neuropathic pain [75]. Evaluating emotional distress, such as anxiety and depressive behaviors associated with pain in animal models, could also provide further insight.

The mechanisms underlying the antinociceptive action of CBD, the factors that give rise to sex differences in sensitivity, and why CBD appears to be more potent in counteracting chronic rather than acute pain are still unclear. Nevertheless, understanding these factors is crucial for optimizing the therapeutic potential of CBD. The multimodal actions of CBD on mechanical allodynia, inflammation, and anxiety confer enormous potential to combat the adverse effects of chronic pain. Importantly, our findings suggest that CBD has the potential to alleviate persistent pain in both females and males. In females, sensitivity to CBD changes significantly during the estrous cycle. If this finding translates to humans, personalized dosing regimens may need to be developed to treat pain in women. To optimize future clinical use, it is essential that further work be carried out in both sexes.

Author contributions

KG and WAP conceptualized and guided the project design. ALFA conducted behavioral experiments. MCC, WAP, MLB, JAC, and TAL contributed with their academic and scientific expertise, physical infrastructure, supply support, and revising the manuscript. K Genaro and TA Lovick wrote the manuscript.

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CRediT authorship contribution statement

Genaro Karina: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Lovick Thelma Anderson:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Crippa José Alexandre:** Funding acquisition, Resources, Writing – review & editing. **Carvalho Milene Cristina:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Arantes Ana Luisa Ferreira:** Investigation, Methodology. **Prado Wiliam Alves:** Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. **Brandão Marcus Lira:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

Conflict of Interest

JA Crippa is a member of the International Advisory Board of the Australian Centre for Cannabinoid Clinical and Research Excellence (ACRE) – National Health and Medical Research Council (NHMRC). JA Crippa has received travel support to attend scientific meetings and personal consultation fees from BSPG-Pharm. JA Crippa is a coinventor of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023," Def. US number Reg. 62193296; July 29, 2015; INPI on August 19, 2015 (BR1120150164927; Mechoulam R, Zuardi AW, Kapczinski F, Hallak JEC, Guimarães FS, Crippa JA, Breuer A). Universidade de São Paulo (USP) has licensed this patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1) and has an agreement with Prati-Donaduzzi to "develop a pharmaceutical product containing synthetic CBD and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders." JAC is a coinventor of the patent "Cannabinoid-containing oral pharmaceutical composition, method for preparing and using same," INPI on September 16th, 2016 (BR 112018005423-2). The other authors declare that they have no conflicts of interest. JA Crippa is a consultant and/or has received speaker fees and/or sits on the advisory board and/or receives research funding from Janssen-Cilag, Torrent Pharm, Prati-Donaduzzi, PurMed Global, and BSPG Pharm over the past 3 years.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2023.114793.

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