

# Evaluating the Antinociceptive Efficacy of Cannabidiol Alone or in Combination with Morphine Using the Formalin Test in Male and Female Mice

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## Abstract

**Introduction:** Phytocannabinoids have emerged as a potential alternative treatment option for individuals experiencing persistent pain. However, evidence-based research regarding their clinical utility in both males and females remains incomplete. In addition, it is unknown whether combining readily available cannabinoids with opioids has a synergistic or subadditive effect on pain modulation. To begin to fill this knowledge gap, we investigated the antinociceptive effects of the phytocannabinoid, CBD, either alone or in combination with opioids in male and female C57BL/6J mice.

**Results:** Using the formalin test, our results show that CBD (10 mg/kg, i.p.) treatment evoked antinociception in phase I, but not in phase II, of the formalin test in male mice. However, in female mice, CBD showed no significant antinociceptive effect. In addition, a direct sex comparison showed that CBD evoked a significant increase in nociceptive behaviors in female versus male mice during phase I of the formalin test. Furthermore, we show that CBD (10 mg/kg, i.p.) in combination with low-dose morphine (1 mg/kg, i.p.) was ineffective at eliciting a synergistic antinociceptive response in both male and female mice. Lastly, consistent with previous literature, we showed that females treated with a relatively higher dose of morphine (10 mg/kg, i.p.) displayed a significant increase in the variability of nociceptive behaviors compared to morphine-treated male mice.

**Conclusion:** Overall, our results suggest that CBD treatment may have beneficial antinociceptive effects during the acute phase of persistent pain, but these effects are more beneficial to males than females. We provide further pre-clinical support that treatments geared toward reducing nociceptive behaviors differentially affect males and females.

**Key words:** cannabinoids; formalin assay; CBD; male and female mice; pain

## Introduction

Currently, opioids are considered the gold standard for pain treatment.<sup>1</sup> However, the side effects, including sedation, respiratory depression, tolerance, and abuse liability, reduce the effectiveness of opioids.<sup>1,2</sup> In addition, it has been demonstrated in pre-clinical studies that opioids are more effective in males than in females, with females requiring higher doses of opioids for a comparable therapeutic effect.<sup>3–8</sup> This has the potential to lead to an increased risk of negative side effects. Due

to the ongoing opioid epidemic, there is a need to identify opioid alternatives for pain management that take into account sex-specific outcomes.

There is mixed evidence that activation of the endocannabinoid system is effective in reducing pain, which is based on clinical studies testing the effectiveness of *Cannabis sativa* and its main active ingredient,  $\Delta$ 9-THC.<sup>9–13</sup> Unfortunately, the use of  $\Delta$ 9-THC comes with cognitive risks, organ-specific toxicity, and high abuse potential making it an ineffective substitute for

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opioids.<sup>14</sup> Despite this, *C. sativa* contains numerous other cannabinoids that are potentially effective in treating pain due to their binding affinity to receptors within the endocannabinoid system as well as the binding affinity to nociceptive receptors.<sup>15–17</sup>

In pre-clinical models, it has been shown that CBD reduces pain alone or in combination with other cannabinoids.<sup>18–20</sup> In addition, in clinical and pre-clinical research, it has been shown that CBD has low abuse potential.<sup>21,22</sup> This has led to a growing market of pure CBD available for over-the-counter use, but evidence-based research regarding the clinical utility of pure CBD remains incomplete. In addition, it is unknown whether combining readily available cannabinoids with opioids will have synergistic effects on pain modulation, thus enabling CBD to act as a potential opioid limiting therapeutic in both males and females.

To fill this knowledge gap and assess the effects of CBD on pain, we used the formalin test. The formalin test is a widely used model of persistent pain that evokes two distinct phases of nociceptive behavior, with an initial acute phase (phase I) followed by an inflammatory phase (phase II).<sup>23</sup> Phase I occurs 0–15 min after formalin injection into the rodent paw with pain behaviors associated predominantly with chemical stimulation of C fiber nociceptors,<sup>24</sup> which are involved in slow, poorly localized pain perception.<sup>25</sup> Phase II occurs 15–60 min postformalin injection and is associated with peripheral inflammatory processes produced by inflammatory mediators released following tissue injury.<sup>23</sup>

Here, using the formalin test, we assessed the antinociceptive potential of pure CBD both alone and in combination with morphine in male and female mice.

## Methods

### Animals

All experiments were performed in accordance with procedures approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Mice used in this study included male and female C57BL/6 wild-type mice age matched (10–12 weeks; Jackson Laboratory, Bar Harbor, ME). All mice were group housed on a 12-h light/dark cycle with *ad libitum* food and water.

### Drugs

(–)-morphine sulfate pentahydrate was provided by the National Institute on Drug Abuse Drug Supply Program. CBD was purchased from Cayman Chemical (Ann Arbor, MI; Cat. # 90080). CBD-d3 (HPLC stan-

dard) was purchased from Sigma Aldrich (St. Louis, MO). Formalin solution was prepared from 37% formaldehyde stock solution (Cat. # F79; Thermo Fisher Scientific, Waltham, MA).

### Formalin testing

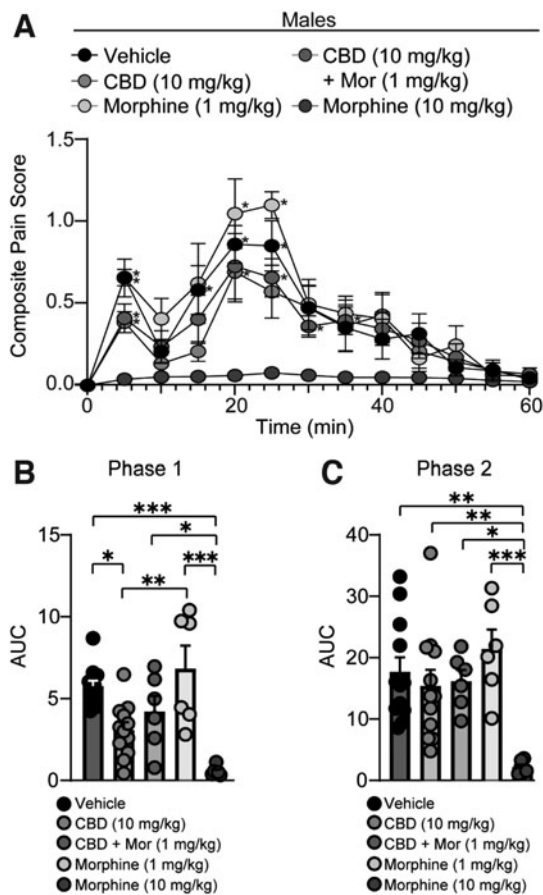
Mice received injections of vehicle (DMSO, Tween 80, saline [1:1:18], i.p.), CBD (10 mg/kg, i.p.), morphine (1 mg/kg, i.p.) (low-dose morphine), or morphine (10 mg/kg, i.p.) (high-dose morphine) 1 h before tests by an experimenter blinded to treatment. High-dose morphine (10 mg/kg) was used as a positive control due to its antinociceptive effects in both male and female mice (Figs. 1 and 2), while low-dose morphine (1 mg/kg) was used due to the lack of antinociceptive effects observed in the formalin assay in both male and female mice (Figs. 1 and 2). Mice were randomly assigned to groups.

Mice were acclimated to a plexiglass observation chamber (in cm: 13×13×13) on a transparent table for 20 min. After the 20-min acclimation period, 2.5% formalin solution (10 μL) was injected to the plantar surface of the right hind paw. Mice were immediately placed back into the plexiglass observation chamber. Mouse behavior was recorded for 60 min using a GoPro camera (San Mateo, CA), which was placed underneath the observation chamber.

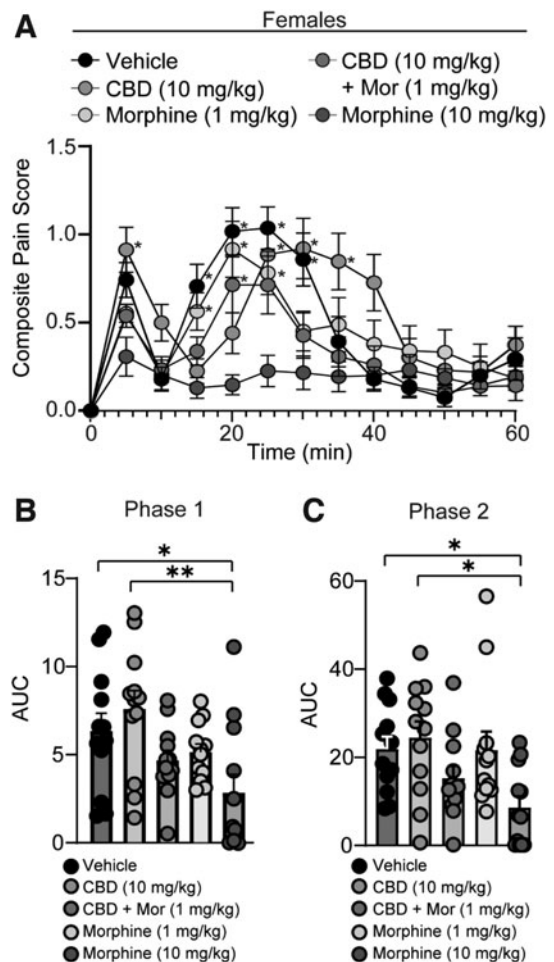
Four behavioral categories were observed by a trained observer (blinded to condition), including (1) no behavior, (2) little or no weight placed on the injected paw, (3) the injected paw is raised, or (4) licking, shaking, biting, or rapid lifting of the injected paw. These behaviors were assessed during twelve 5-min (i.e., 300 sec) bins.

The time that the animal spent displaying each behavior was recorded and a weighted composite score was calculated based on the following formula for each 5-min bin, as previously published<sup>26–28</sup>:  $((a + b) + (2 \times c))/d$ , where *a* refers to the time the animal spent placing little or no weight on the injected paw, *b* refers to the time the animal spent raising the injected paw, *c* refers to the time the animal spent licking, shaking, biting, or rapid lifting of the injected paw, and *d* refers to the total time (i.e., 300 sec). The weighted composite score was selected based on the severity of pain associated with *c*.

Based on this formula, the maximum composite score would equal 2, signifying a maximum pain state. The area under the curve was calculated for the acute phase (phase I; 0–15 min) and the inflammatory phase (phase II; 15–60 min) using GraphPad Prism (9.1.2).



**FIG. 1.** CBD reduces nociceptive behaviors in male mice, but in combination with low-dose morphine, CBD does not evoke synergistic antinociceptive effects. **(A)** Summary of the time course corresponding to the composite pain score from 0 to 60 min after formalin injection ( $F_{(48,444)} = 1.424$ ,  $p = 0.0374$ ; two-way repeated-measures ANOVA with Tukey's post-test) (Vehicle:  $n = 12$ ; CBD:  $n = 12$ ; CBD + Mor:  $n = 6$ ; Mor [1 mg/kg]:  $n = 6$ ; Mor [10 mg/kg]:  $n = 6$ ). Asterisks denote significant difference from high-dose morphine (10 mg/kg). **(B)** Summary graph showing the AUC during phase I of the formalin assay ( $F_{(4,37)} = 11.78$ ,  $p < 0.0001$ ; one-way ANOVA with Tukey's post-test). **(C)** Summary graph showing the AUC during phase II of the formalin assay ( $F_{(4,37)} = 5.999$ ,  $p = 0.0008$ ; one-way ANOVA with Tukey's post-test). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . ANOVA, analysis of variance; AUC, area under the curve.



**FIG. 2.** CBD alone or in combination with low-dose morphine does not reduce pain during phase I or phase II of the formalin test in female mice. **(A)** Summary of the time course corresponding to the composite pain score from 0 to 60 min after formalin injection ( $F_{(48,660)} = 4.181$ ,  $p < 0.0001$ ; two-way repeated-measures ANOVA with Tukey's post-test) (Vehicle:  $n = 12$ ; CBD:  $n = 12$ ; CBD + Mor:  $n = 12$ ; Mor [1 mg/kg]:  $n = 12$ ; Mor [10 mg/kg]:  $n = 12$ ). Asterisks denote significant difference from high-dose morphine (10 mg/kg). **(B)** Summary graph showing the AUC during phase I of the formalin assay ( $F_{(4,55)} = 4.236$ ,  $p = 0.0046$ ; one-way ANOVA with Tukey's post-test). **(C)** Summary graph showing the AUC during phase II of the formalin assay ( $F_{(4,55)} = 3.820$ ,  $p = 0.0082$ ; one-way ANOVA with Tukey's post-test). \* $p < 0.05$ , \*\* $p < 0.01$ .

### Analysis of plasma drug levels

Two hours following CBD (10 mg/kg, i.p.) treatment, mice were anesthetized with isoflurane and tail blood was collected in a microtube coated with EDTA-K2 (Cat. # 041-TOM-14C; Milian Dutscher Group). Whole blood was spun at 4°C–2000 RPM for 10 min at which point serum (supernatant) was pipetted off and placed in sterile tubes stored at –80°C until analysis.

Plasma concentrations of CBD were determined using mass spectrometry. Standard curves were constructed by plotting the ratio of the analyte peak area to internal standard peak area versus analyte concentration. The standard working solution (4  $\mu$ L) and internal standard (4  $\mu$ L) were spiked into control plasma (10  $\mu$ L), and after vortexing, acetonitrile/H<sub>2</sub>O/formic acid (90/10/0.1) (22  $\mu$ L) was added to extract the analytes from plasma. Proteins were precipitated by vortexing with subsequent centrifugation at 8765 g for 10 min at 4°C. The supernatant was taken and loaded to the high-performance liquid chromatography/mass spectrometry/mass spectrometry (HPLC/MS/MS) system, with final concentrations of 0.025 ng/mL to 1000 ng/mL for CBD.

Treated plasma was processed the same way as standards: after spiking internal standards (4  $\mu$ L) into plasma (10  $\mu$ L), samples were vortexed and acetonitrile/H<sub>2</sub>O/formic acid (90/10/0.1) (26  $\mu$ L) was added for extraction. The calculated concentrations from the standard curves were multiplied by 4 to reflect the *in vivo* levels of CBD in plasma.

CBD in plasma was analyzed using a Sciex QTRAP 6500+ mass spectrometer coupled with a Sciex EXion HPLC separation system. A 1.7  $\mu$ m Acquity UPLC BEH C18 analytical column (2.1  $\times$  100 mm; Waters, Ireland) was used to separate CBD with other isomers as well as impurities. The gradient elution was conducted using a flow rate of 0.4 mL/min with the following conditions: initial at 70% mobile phase B (acetonitrile) and 30% mobile phase A (0.1% formic acid in water), followed by a linear gradient to 90% mobile phase B in 1 min, and kept at 90% mobile phase B for three additional minutes to flush the column before back to initial conditions to equilibrate the column.

The Sciex QTrap 6500+ mass spectrometer was equipped with an electrospray ionization probe operated in positive active mode. The decluster potential was 70 V for CBD; the entrance potential was 10 V, the collision energy was 33 V, and the collision cell exit potential was 12 V for CBD while the curtain gas

was 35 L/h, and the collision gas (CAD) was medium. The ion spray voltage was 5500 V, the temperature was 550°C, gas 1 was 15 L/h, and gas 2 was 15 L/h.

The multiple reaction monitoring mode was used to analyze and quantify CBD as well as CBD-d3, with the transitions of *m/z* 315 > 193 for CBD and 318 > 196 for CBD-d3. All peaks were integrated and quantified by Sciex OS 1.5 software.

### Statistical analysis

All results are shown as mean  $\pm$  SEM. Each experimental group consisted of at least six mice. No data points were excluded. Statistical significance was assessed in GraphPad Prism software (9.1.2) using a one-way analysis of variance (ANOVA), two-way ANOVA, or a two-way repeated-measures ANOVA with Tukey's correction for multiple comparisons to identify differences as specified. *F*-values for two-way ANOVA statistical comparisons represent interactions between variables unless otherwise stated. Two-tail tests were performed for all studies.

### Results

Male and female mice respond differently to CBD administration during phase I of the formalin test

We investigated how male and female mice responded to CBD treatment (10 mg/kg, i.p.) during phase I and phase II of the formalin test. The CBD dose of 10 mg/kg was selected based on our observation that systemic injections of CBD (10 mg/kg, i.p.) in female mice produced blood plasma concentrations that were within the range observed in humans after smoking CBD-containing cigarettes (152.2  $\pm$  15.5 ng/mL, 483.99  $\pm$  49.28 nM; Table 1).<sup>29</sup> In addition, the CBD blood plasma concentrations observed were within the range shown to reduce pain and decrease depressive-like behaviors in rats and male mice, respectively (Table 1).<sup>30,31</sup>

Furthermore, it has been previously shown that this dose of CBD is effective at reducing mechanical allodynia in female neuropathic mice,<sup>32,33</sup> and is below the plasma concentrations known to produce adverse effects in mammals.<sup>34,35</sup> Using this dose, we found that CBD was effective at reducing nociceptive behaviors in

**Table 1. Plasma Concentrations of CBD in Females at 2 h After i.p. Injection**

Plasma level		
Drug	Dose (mg/kg)	Steady state (ng/mL)
CBD ( <i>n</i> =8)	10	152.2 $\pm$ 15.5

Mean  $\pm$  SEM.

male mice during phase I (vehicle- vs. CBD-treated male mice:  $p=0.0105$ , Tukey's post-test), but not phase II (vehicle- vs. CBD-treated male mice:  $p=0.9429$ , Tukey's post-test), of the formalin test (Fig. 1A–C).

In contrast, we found that CBD was ineffective at preventing nociceptive behaviors in female mice during both phase I (vehicle- vs. CBD-treated female mice:  $p=0.8473$ , Tukey's post-test) and phase II (vehicle- vs. CBD-treated female mice:  $p=0.9802$ , Tukey's post-test) of the formalin test (Fig. 2A–C). These data demonstrate that males and females respond differently to CBD treatment.

Based on this finding, we next performed analyses to directly compare sex differences between males and females treated with CBD using the data shown in Figures 1 and 2. First, we found that there were no significant sex differences in nociceptive behaviors between vehicle control groups during phase I ( $p=0.9553$ , Tukey's post-test) (Fig. 3A, B) or II of the formalin test ( $p=0.7849$ , Tukey's post-test) (Fig. 3A, C), suggesting that male and female mice express similar nociceptive responses to formalin injection.

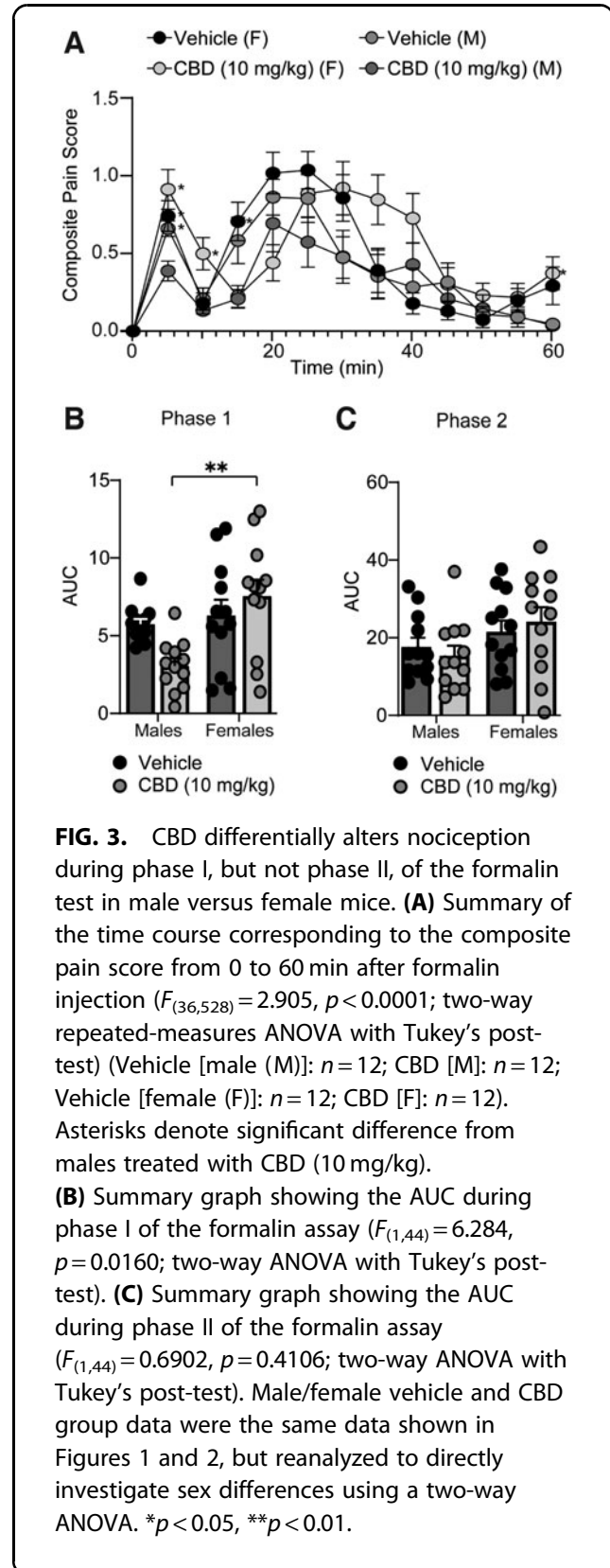
We next performed a pairwise comparison to statistically compare the effects of CBD treatment between male and female mice. We observed that CBD-treated female mice displayed a significant increase in nociceptive behaviors compared to CBD-treated male mice during phase I of the formalin test ( $p=0.0011$ , Tukey's post-test) (Fig. 3B). In contrast, during phase II of the formalin test, no sex differences were observed between CBD-treated mice ( $p=0.1651$ , Tukey's post-test) (Fig. 3C).

CBD in combination with low-dose morphine has no effect on formalin-induced nociception in male or female mice

To investigate whether a synergistic antinociceptive effect exists when coadministering CBD (10 mg/kg) with low-dose morphine (1 mg/kg), we measured nociceptive behaviors in male and female mice using the formalin test.

In male mice, we found that CBD (10 mg/kg, i.p.) in combination with low-dose morphine (1 mg/kg, i.p.), which does not evoke antinociception in the formalin assay (Figs. 1 and 2), did not further reduce formalin-induced nociception (Fig. 1A–C). In contrast, morphine, at the higher dose of 10 mg/kg, abolished formalin-induced nociception during both phase I and phase II of the formalin test (Fig. 1A–C).

In female mice, we found that CBD in combination with low-dose morphine (1 mg/kg, i.p.) did not reduce nociceptive behaviors during either phase I or phase II



of the formalin test (Fig. 2A–C). However, we did observe that high-dose morphine (10 mg/kg, i.p.) significantly reduced formalin-induced nociception during phase I and phase II of the formalin test in female mice (Fig. 2A–C).

Lastly, we found that, in comparison with females, males displayed significantly less variability in their antinociceptive response to high-dose morphine (10 mg/kg) treatment during both phases of the formalin test (Phase I:  $F_{(11,5)} = 159.4$ ,  $p < 0.0001$ ; Phase II:  $F_{(11,5)} = 70.01$ ,  $p = 0.0002$ ;  $F$  test to compare variances).

## Discussion

In this study, first, we found that CBD was effective at reducing nociceptive behaviors in male mice, but only during phase I of the formalin test. Second, we observed that CBD treatment did not reduce nociceptive behaviors in female mice during phase I or II of the formalin test. Third, we found that CBD-treated females showed a significant increase in nociceptive behaviors compared to CBD-treated male mice, but only during phase I of the formalin test. Fourth, we found that CBD (10 mg/kg) in combination with low-dose morphine (1 mg/kg) was ineffective at producing synergistic antinociception responses in both male and female mice.

Lastly, we showed that female mice responded to the high-dose morphine (10 mg/kg, i.p.) treatment with a significantly greater variability than male mice during both phases of the formalin test.

### CBD effects on nociception in the formalin test

Here, we show that CBD, in male mice, is effective at attenuating acute pain (i.e., phase I) in the formalin test. Our results partly align with another study that demonstrated that CBD (5 and 50 mg/kg, i.p.) did not reduce nociceptive behaviors in the formalin test in male rats.<sup>36</sup> The reason that we observed a significant antinociceptive effect in CBD-treated male mice during phase I of the formalin assay may be due to the species used (rat vs. mouse) or the dose of CBD, as evidence suggests that the dose/effect curve of CBD in relation to behavioral effects follows a U shape rather than a sigmoidal curve.<sup>37</sup>

An important feature of the formalin test is that it includes two distinct nociceptive phases. The first phase, which represents acute pain, begins immediately after injection, likely through formalin-induced C fiber activation.<sup>23,24</sup> The second phase of the formalin test is mediated by inflammatory factors, including substance P, bradykinin, histamine, serotonin, and prostaglandins,<sup>38</sup>

which activate, in part, transient receptor potential vanilloid (TRPV) and transient receptor potential subfamily A (TRPA) channels directly or indirectly through the activation of downstream signaling pathways.<sup>25,39</sup>

Importantly, the representative acute and inflammatory pain states of the formalin test do not actually represent disease-specific acute or inflammatory pain. Clinically, acute pain is mediated by nociceptor activation following exposure to a mechanical, thermal, or chemical stimulus, while inflammatory pain is mediated by the release of endogenous signaling molecules (i.e., inflammatory soup) that sensitize primary afferent nerve fibers.<sup>25</sup>

Although the formalin test encompasses many similar underlying mechanisms of acute and inflammatory pain as those manifested clinically (e.g., activation of nociceptive C fibers during the acute phase and peripheral inflammatory processes during phase II<sup>23</sup>), the results may not translate to the human condition due to the engagement of highly plastic molecules and circuits that are unique to each individual's response to a given pain condition. Even in animal models of disease, antinociceptive responses will vary to treatments that target the endocannabinoid system.<sup>40</sup>

For example, a systematic review and meta-analysis of modulators of the endocannabinoid system found variability in treatment outcomes based on the pain model implemented with the largest attenuation of pain-associated behavior reported in models of burn injury and the smallest significant attenuation reported in models of inflammation.<sup>40</sup> Furthermore, it was found that CBD significantly attenuated nocifensive behaviors in neuropathic pain models with mixed results in inflammatory pain models.<sup>40</sup> These findings suggest that treatment options targeting the endocannabinoid system may be more effective for some forms of pain rather than others.

Another interesting aspect to our findings was that attenuation of the first phase of the formalin test in males was not sufficient to prevent the transition to the second phase of persistent pain. Given that persistent pain is a consequence of the initial acute response to a nociceptive stimulus, it would be expected that attenuating the nociceptive response to phase I of the formalin test would mitigate the nociceptive response to phase II.

It has been previously shown that local anesthesia at the site of the formalin injection or spinal cord anesthesia given during the first phase is sufficient to prevent or reduce the second nociceptive phase of the formalin

test.<sup>41–43</sup> Future studies will need to address the mechanisms mediating CBD-induced reduction in phase I of the formalin test to help understand the lack of effect as acute pain transitions to persistent pain.

Despite our findings that show a partial effect of CBD treatment on persistent pain, other clinical and pre-clinical studies have found CBD to be a promising antinociceptive agent for the reduction of inflammatory and neuropathic pain.<sup>17,19,30,44–48</sup> Therefore, it is plausible that the formalin test initiates a cascade of nociceptive-related signals that require the simultaneous activation and/or inhibition of multiple receptor targets. Because of this, CBD in combination with other drugs may be required to reduce persistent tonic pain.

#### CBD as an opioid sparing therapeutic

In addition to investigating the effects of CBD alone, we investigated whether CBD in combination with low-dose morphine had synergistic effects on reversing formalin-induced nociceptive behaviors. Evidence supports the opioid-sparing potential of CBD, but these observations are not consistent across multiple pre-clinical pain models.<sup>49,50</sup>

Our results show that CBD in combination with low-dose morphine produces neither synergistic nor subadditive effects on formalin-induced nociception. This observed lack of effect is unlikely due to metabolic drug/drug interactions as CBD is metabolized by cytochrome P450 enzymes, while morphine is metabolized exclusively by glucuronidation via UGT2B7.<sup>51,52</sup> In addition, CBD has a long half-life.<sup>31,53</sup> Therefore, we expect that the physiological effects of CBD were active throughout the entirety of our experiments, which is supported by our observed plasma CBD concentrations (Table 1).

#### Sex differences following CBD treatment in the formalin test

We identified a significant difference between male and female mice treated with CBD during phase I, but not phase II, of the formalin assay, suggesting that CBD (10 mg/kg) differentially alters the pain response in male versus female mice during formalin-induced acute pain.

Importantly, we did not perform a dose response to find an optimal dose of CBD for both males and females undergoing the formalin test. Instead, our intention was to investigate a dose of CBD that is known to produce (1) blood plasma concentrations that are similar to those observed in humans 2 h after CBD administration<sup>54,55</sup> and (2) antinociceptive behaviors in

arthritic male rodents and neuropathic female rodents.<sup>30,32,33</sup> Therefore, these investigations highlight that one dose may not be optimal for all individuals or all pain states as many clinical and pre-clinical studies have shown that females respond differently compared to males following pain treatment.<sup>56–61</sup>

In line with this, we observed a significant sex difference between male and female responses to high-dose morphine (10 mg/kg), with females displaying greater variability compared to males. This is consistent with other studies showing that females require a higher dose of morphine to reach analgesic effects that are equivalent to males.<sup>3–7,62</sup>

We cannot definitively conclude from our investigation that CBD is ineffective for females in a model of persistent pain as sex-specific differences may be attributed to the dosing schedule, pain model used, dose administered, and/or localization of the injection site. For example, in models of neuropathic pain, it has been shown that CBD (10 mg/kg, i.p.) was effective at reducing paclitaxel-induced cold and mechanical allodynia in female mice.<sup>32</sup>

However, CBD was not administered acutely, as in our study, but rather CBD was repeatedly administered once a day for 14 consecutive days before paclitaxel injections.<sup>32</sup> Yet, in a similar dosing schedule through which naive rodents received repeated CBD treatment, it was observed that CBD (10 mg/kg, i.p.) administered twice a day for four days was only effective at increasing tail withdrawal latencies in males, with no effect observed in females.<sup>63</sup> These results suggest that the antinociceptive effects of CBD (10 mg/kg, i.p.) are dependent upon the dosing schedule or pain state.

Interestingly, in contrast to the sex differences observed in our study, others have found comparable outcomes between males and females following CBD treatment. For example, following acute CBD treatment (10 or 30 mg/kg, i.p.), in naive rodents, a previous report found no change in response latency to paw pressure or tail withdrawal tests in both male and female rodents.<sup>64</sup> In another study implementing a model of Parkinson's-induced myofascial pain, the authors discovered that localized injection of CBD into the masseter muscle (the site of formalin injection) evoked antinociceptive behaviors in both phases of the formalin test as well as increased mechanical allodynia in both male and female rodents.<sup>65</sup>

Taking these previous findings into account, our observations that an acute systemic injection of CBD (10 mg/kg) was more effective at reducing nociception

in phase I of the formalin test in males rather than in females may not hold true when varying the dosing schedule, dose used, or placement of injection (i.e., localized to the site of injury versus systemic).

In addition to only using one dose of CBD in our assessments, another limitation to our study is that we did not monitor the estrus phase of the female mice, which may explain the variability observed following treatment with either CBD or morphine. It is known that the estrus phase is accompanied by shifts in hormones, which regulate receptor expression/function,<sup>66–72</sup> drug metabolism,<sup>73–75</sup> and neuroimmune function,<sup>76–78</sup> all factors that contribute to a pain response.<sup>79–81</sup> Therefore, future studies addressing the mechanisms of CBD action and how the estrus cycle may influence CBD's effects are required.

Despite these sex differences in response to pain treatment, we observed that both males and females displayed comparable nocifensive behaviors to formalin injection, as demonstrated by a lack of significant difference between vehicle-treated male and female mice. Our results are consistent with studies in mice, showing that male nociceptive responses to formalin injection during phase I and II of the formalin test were no different than female mice across all stages of the estrus cycle.<sup>82</sup>

## Conclusion

In conclusion, we interpret our results to indicate that CBD (10 mg/kg) in combination with low-dose morphine (1 mg/kg) is ineffective at reducing nociception in the formalin test and that CBD may have sex-specific effects on nociceptive severity. Importantly, our results, in combination with other published findings, suggest that CBD has the potential to be an effective antinociceptive therapeutic for some, but not all, forms of pain.

In addition, caution should be implemented in self-medicating with CBD, as CBD when combined with morphine has shown to evoke subadditive effects in distinct forms of pain.<sup>49</sup> This subadditive behavioral phenotype is supported by a pharmacological study, which showed that CBD decreased *in vitro* binding of morphine to opioid receptors, which would reduce morphine-induced opioid receptor activation and likely diminish morphine-induced analgesia.<sup>83,84</sup> Moreover, given its metabolism by CYP3A4 and CYP2C19, self-medication may interfere with the metabolism of many prescription drugs.<sup>85</sup>

Given the inconsistent effects of CBD on reducing pain across multiple pain models, it is clear that future work, in

addition to the important contributions already made,<sup>86</sup> is required to understand the mechanisms by which CBD evokes antinociceptive responses in both males and females. This will help clinicians effectively implement CBD-based modalities for pain management.

## Authors' Contributions

D.E.S., W.M.R.-K., K.E.V., and N.M.G. designed the experiments. D.E.S., D.P.M., and D.S. performed the experiments. D.E.S., D.P.M., W.M.R.-K., D.S., K.E.V., and N.M.G. performed the analyses. D.E.S., W.M.R.-K., K.E.V., D.S., and N.M.G. wrote the article.

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## Author Disclosure Statement

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#### Abbreviations Used

$\Delta 9$ -THC = delta-9-tetrahydrocannabinol  
ANOVA = analysis of variance  
AUC = area under the curve  
CBD = cannabidiol  
HPLC/MS/MS = high-performance liquid chromatography/mass spectrometry/mass spectrometry  
TRPV = transient receptor potential vanilloid channel