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Anti-nociceptive interactions between opioids and a cannabinoid receptor 2 agonist in inflammatory pain

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Abstract

The cannabinoid 1 receptor and cannabinoid 2 receptor can both be targeted in the treatment of pain; yet, they have some important differences. Cannabinoid 1 receptor is expressed at high levels in the central nervous system, whereas cannabinoid 2 receptor is found predominantly, although not exclusively, outside the central nervous system. The objective of this study was to investigate potential interactions between cannabinoid 2 receptor and the mu-opioid receptor in pathological pain. The low level of adverse side effects and lack of tolerance for cannabinoid 2 receptor agonists are attractive pharmacotherapeutic traits. This study assessed the anti-nociceptive effects of a selective cannabinoid 2 receptor agonist (JWH-133) in pathological pain using mice subjected to inflammatory pain using the formalin test. Furthermore, we examined several ways in which JWH-133 may interact with morphine. JWH-133 produces dose-dependent anti-nociception during both the acute and inflammatory phases of the formalin test. This was observed in both male and female mice. However, a maximally efficacious dose of JWH-133 (1 mg/kg) was not associated with somatic withdrawal symptoms, motor impairment, or hypothermia. After eleven once-daily injections of 1 mg/JWH-133, no tolerance was observed in the formalin test. Cross-tolerance for the anti-nociceptive effects of JWH-133 and morphine were assessed to gain insight into physiologically relevant cannabinoid 2 receptor and mu-opioid receptor interaction. Mice made tolerant to the effects of morphine exhibited a lower JWH-133 response in both phases of the formalin test compared to vehicle-treated morphine-naïve animals. However, repeated daily JWH-133 administration did not cause cross-tolerance for morphine, suggesting opioid and cannabinoid 2 receptor cross-tolerance is unidirectional. However, preliminary data suggest co-administration of JWH-133 with morphine modestly attenuates morphine tolerance. Isobolographic analysis revealed that co-administration of JWH-133 and morphine has an additive effect on anti-nociception in the formalin test. Overall these findings show that cannabinoid 2 receptor may functionally interact with mu-opioid receptor to modulate anti-nociception in the formalin test.

Keywords

Cannabinoid 2 receptor, morphine, pain, tolerance, formalin, JWH-133, opioid, cannabinoid agonist

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Introduction

Pain is one of the most widespread and costly clinical challenges facing medicine today, afflicting an estimated 120 million Americans and costing $600 billion annually in medical expenses, loss of work productivity, and long-term insurance disability.[1,2] Long-term pain is also characterized by the high occurrence of comorbid side effects such as depression, anxiety, and suicidal ideation.[3] Opioid drugs, which exert analgesic activity through mu-opioid receptor (MOR), are the current gold standard for the treatment of acute and long-term chronic

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pain.[4] While opioid drugs have remarkable anti-nociceptive efficacy for certain types of pain, there are severe adverse consequences that can occur with prolonged use. For example, chronic use of opioids causes tolerance and a high potential risk for physical dependence and abuse.[5–7] The rate of overdose from prescription opioids has more than tripled since the early 1990s and is still on the rise.[4] More than 33,000 deaths in the United States were attributed to opioid overdose in 2015.[8] Consequently, there is a current unmet medical need to find possible alternatives or adjuvants to opioids for the treatment of chronic pain. Therefore, an interest in understanding the potential interactions of the opioid system with other pathways involved in alleviating pain is crucial.

The evidence is mounting in terms of the use of cannabinoids for the treatment of unalleviated pain. They constitute a new class of agents that can be added to the pharmaceutical toolbox for the management of chronic pain.[9,10] In human clinical trials and case studies, drugs targeting the endocannabinoid (eCB) system have shown promise for treatment of numerous pathologies, including chronic and acute pain.[11] For example, the drug Sativex (50% Δ^9-tetrahydrocannabinol (THC) and 50% cannabidiol) has been approved in Canada and several other countries for the treatment of neuropathic pain and cancer pain.[12] While cannabinoid 1 receptor (CB1R) agonists and dual cannabinoid receptor agonists (including Δ^9-THC) demonstrate potent analgesic effects in rodent models and in human use, they also have several disadvantages. Use of these drugs is associated with the development of tolerance, psychotomimetic effects, and numerous other physical side effects.[12,13] However, selective activation of cannabinoid 2 receptor (CB2R) in rats and mice does not produce these psychotropic adverse side effects,[14–16] making CB2R an attractive target for the treatment of pain and other pathologies. Indeed, CB2R agonists have been shown to alleviate acute, inflammatory, and chronic pain causing them to garner increased attention as a potential alternative to the use of opioids for treatment of pain.[15]

In recent years, mounting evidence of the importance for CB2R in pathological pain has increased interest as demonstrated by the synthesis of a variety of CB2R-selective cannabinoid agonists.[17] CB2R agonists have been shown to have efficacy in multiple models of pathological pain in preclinical rodent models including postoperative pain,[18] inflammatory pain,[15] chemotherapeutic pain,[14] and cancer-induced pain.[19] However, the mechanisms through which the anti-nociceptive and analgesic effects of CB2R agonists are mediated are not completely well characterized.

The CB2R agonists have anti-inflammatory properties, and many pain studies have suggested a mechanism of action through the regulation of inflammation (including neuroinflammation; see Benito et al.[20] and Ehrhart et al.[21]). CB2R activation attenuates the release of pro-inflammatory agents (tumor necrosis factor-α, nitric oxide) and increases release of anti-inflammatory agents (interleukin 10) from microglia and astrocytes.[21–23] The release of these pro-inflammatory factors is stimulated by activation of MOR on microglia, and this inflammatory response is thought to facilitate opioid tolerance.[24,25] As such, the use of CB2R agonists could both relieve pain and help mitigate the consequences of opioid treatment. This possibility is supported more generally by previous work both in animals and humans showing inhaled cannabis and Δ^9-THC enhance the pain relief of opioids.[26,27] However, the interaction between opioids and CB2R-selective agonists must be more extensively investigated.

Therefore, the objectives of this study were to investigate the efficacy of a CB2R agonist on inflammatory pain and to examine potential interactions between CB2R and MOR in this pathological pain model. First, we determined the anti-nociceptive efficacy of a CB2R agonist (JWH-133) in an inflammatory pain model. Second, we evaluated potential side effects and tolerance to JWH-133. Co-administration of JWH-133 and morphine was also done to assess potential functional interactions between CB2R and MOR agonists. Together, the results of this study suggest that co-administration of a CB2R agonist with an opioid could result in opioid-sparing effects in the treatment of inflammatory pain, thus minimizing adverse effects and potentially protecting against the development of opioid tolerance.

Materials and methods

Subjects

Experiments were carried out with wild-type C57BL6/J mice obtained from Jackson Laboratories (Bar Harbor, Maine). Mice used were male unless specifically noted otherwise and were tested between eight and 14 weeks of age. All mice were group-housed and were kept on a standard 12:12 h light-dark cycle with ad libitum access to standard mouse chow and water. All animal care and procedures conformed to the guidelines of the National Institutes of Health on the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the Penn State University College of Medicine.

Drugs

Morphine sulfate was obtained from the National Institute on Drug Abuse Drug Supply (Bethesda, MD). JWH-133 (CB2R agonist), SR144528 (SR2, CB2R antagonist), naloxone (MOR antagonist), and

Rimonabant (SR1, CB1R antagonist) were obtained from Cayman Chemical (Ann Arbor, MI). SP600125 (SP6), an inhibitor of c-Jun N-terminal kinases (JNK), was obtained from Sigma-Aldrich (St. Louis, MO[28]). Drugs were dissolved in isotonic 0.9% saline (90%) with Cremophor (5%) and ethanol (5%) and administered via intraperitoneal (i.p.) injection in a volume of 10 mL/kg body weight.

General experimental protocols

JWH-133 dose–response curves. To assess the anti-nociceptive efficacy of JWH-133, groups of age- and sex-matched drug-naïve mice ($n = 4$–7 per dose) were given log-scale doses of JWH-133 (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/kg) via i.p. injection. Anti-nociception was measured using the formalin test 60 min after JWH-133 injection. A similar dose–response analysis was performed for morphine (0.01–10 mg/kg, i.p.). The data from these experiments were fitted to standard sigmoidal curves with variable slope to determine the ED_{50} values and maximal doses for both drugs.

For subsequent experiments, the minimum dose of JWH-133 (1 mg/kg) required to produce a maximal anti-nociceptive response in the formalin test was used. To determine which receptor was responsible for mediating the anti-nociceptive effect of 1 mg/kg JWH-133 in the formalin test, it was co-administered with CB2R antagonist SR2 (10 mg/kg), CB1R antagonist SR1 (10 mg/kg), or MOR antagonist naloxone (10 mg/kg).

To assess tolerance, mice were injected (i.p.) once daily with either 10 mg/kg morphine or 1 mg/kg JWH-133 for up to 11 consecutive days. This duration of repeated dosing is sufficient to cause complete tolerance to the anti-nociceptive effects of morphine. The mice were assessed in the formalin model 60 min following drug administration on the final day. To assess cross-tolerance between morphine and JWH-133, some groups were given a challenge dose of the alternate drug (i.e., mice given 10 days of morphine were given a JWH-133 challenge and vice versa) on the 11th day. Some groups were co-administered 10 mg/kg morphine and 1 mg/kg JWH-133 for 10 days and then tested for anti-nociception using morphine alone on day 11 to assess the potential protective effects of JWH-133 co-administration on morphine tolerance.

Since previous published work from our laboratory has demonstrated that JNK signaling is required for morphine tolerance, we examined whether JNK signaling was required for morphine-induced cross-tolerance to JWH-133.[29,30] This experiment was done by examining the efficacy of JWH-133 (1 mg/kg) anti-nociception in the formalin model using mice that received either five days of repeated morphine alone (10 mg/kg), mice that received daily pre-treatment with SP6 (3 mg/kg) prior to morphine (10 mg/kg), and mice receiving daily vehicle injections. Pre-treatment with SP6 was given 60 min before injection of morphine.

Formalin test

The formalin test is an extensively used model of inflammatory pain.[31] This method elicits a biphasic pattern of pain behavior, with a phase of acute pain followed by a phase of inflammatory pain. The early (acute) phase is generated by the activation of C and Aδ fibers as a result of needle penetration into the hind paw. The late phase involves an inflammatory reaction due to presence of formalin in peripheral tissue,[31] the development of central sensitization, and additionally the activation of primary afferent nociceptors.[32,33] Mice were subjected to the formalin test to assess basal differences in inflammatory pain response and the anti-nociceptive effect of morphine and JWH-133 on pathological pain. Prior to testing, mice were acclimated for 20 min in a Plexiglas ($5'' \times 5'' \times 5''$) observation chamber placed on a transparent elevated platform. A mirror angled at 45° was placed below the platform to allow for constant observation of the animal's paws. Following acclimation, mice were administered 10 μL of 2.5% formalin solution into the plantar surface of a single hind paw using a 28½ gauge needle (Becton Dickinson, Franklin Lakes, NJ). Immediately after the formalin injection, mice were returned to the Plexiglas observation unit, and nociceptive behavior was continuously measured in twelve 5-min intervals for a total testing time of 60 min. During each 5-min time bin, the duration spent performing pain-response behaviors was recorded. The nociceptive behaviors were separated into three categories: (0) the injected paw has little weight placed on it; (1) the injected paw is raised off of the ground; (2) the injected paw is licked, shaken, or bitten. The amount of time spent in each category was quantified and weighted with the composite pain score-weighted scores technique ($CPS\text{-}WST_{0,1,2}$), resulting in a CPS for each 5-min interval between 0 (no pain behaviors) to 2 (maximal pain behavior[34]). The area under the curve (AUC, CPS × time (min)) was calculated using the trapezoidal rule for the acute phase (0–15 min; phase 1) and the inflammatory phase (15–60 min; phase 2). To assess the anti-nociceptive effects of drugs, mice were injected (i.p.) 60 min prior to the formalin injection.

Measurement of body temperature

Body temperature was measured using a mouse rectal thermometer probe (Physitemp, Clifton, NJ). Mouse body temperature was measured immediately prior to, and 60 min following drug administration. Hypothermia was reported as a percent change in body

temperature between pre-drug and 60 min post-drug measurements, as described by the formula:

$$(\%\Delta BT) = \frac{\left\{\begin{array}{l}(\text{pre-drug temperature}) \\ -(\text{post-drug temperature})\end{array}\right\}}{\text{pre-drug temperature}} \times 100$$

Rotarod test

Motor impairment was measured using a Med Associates ENV-577-M rotarod apparatus (St. Albans, VT). Animals were trained by undergoing six training trials per day over two consecutive days. The maximum cut-off for training trails was limited to 300 s. Mice were placed on a rotating rod (3 cm in diameter), which accelerated at a constant rate from 4 to 40 r/min over the 5-min testing period. The time spent walking on top of the rod until the mouse either fell off the rod, or slipped and held onto the rod to ride completely around was recorded. Motor impairment was determined by calculating the change in performance between the pre-test and post-test given 60 min after JWH-133, morphine, or vehicle injection.

Precipitated withdrawal

Physical dependence was induced using a series of 20 injections that were given twice daily for 10 days (5 mg/kg morphine, i.p.; 1 mg/kg JWH-133, i.p.). Following 10 days of daily drug administration, withdrawal was precipitated using an i.p. injection of vehicle, 10 mg/kg naloxone (to counter morphine), or 10 mg/kg SR2 (to counter JWH-133) 30 min after the final drug injection on the 11th day. Somatic withdrawal symptoms (paw tremors, body tremors, diarrhea, and jumps) were video recorded for 60 min after injection of naloxone, SR2, or vehicle. Withdrawal symptoms were scored in alternating 5-min time intervals (5–10, 15–20, 25–30, 35–40, 45–50, and 55–60 min, as described previously[35]).

Co-administration

When animals were given multiple i.p. injections, the second injection was administered on the opposing side of the body cavity. When testing the effects of co-administered agonists (JWH-133 and morphine), each drug was injected at the same time. When testing for agonist selectivity, antagonists were administered via i.p. injection 30 min prior to agonist treatment.

Isobolographic analysis

Isobolographic analysis was performed to determine whether the combined anti-nociceptive effects of morphine and JWH-133 were sub-additive, additive, or synergistic (super-additive). Full dose–response curves were generated in the formalin test (as described above) for JWH-133, morphine, and then a combination that was co-administered in a fixed 1:10 dose ratio (see Tallarida and Raffa,[36] Grabovsky and Tallarida,[37] and Kazantzis et al.[38] for detailed explanation and formulas). ED_{50} values for this combination were determined and compared to a theoretically calculated ED_{50} value.[39] This theoretical value was determined using the dose–response curves of JWH-133 and morphine, alone, to generate a predicted additive curve using the formula below.[36,38]

$$E(a,b) = E_B \frac{(b + b_{eq(a)})^p}{(b + b_{eq(a)})^p + C_b^p}$$

where the effect (E) of specific doses of two drugs (a, b) in combination is estimated using the dose of drug b ($b_{eq(a)}$) that gives and equivalent response to a specific dose of drug a (a), the ED_{50} of drug b (C_b), and the Hill slope of drug b (p). If the experimentally determined ED_{50} of the combination is significantly lower than the predicted value (according to a t-test), the combination is deemed synergistic. If the two ED_{50} values are equal, the combination has only an additive effect.

Data analysis

Values for anti-nociception, hypothermia, motor coordination, and precipitated withdrawal were expressed as the mean ± standard error of the mean (SEM). Data were analyzed using either one-way (Figure 3, Figure 4(c), Figure 6(d)) or two-way analysis of variance (ANOVA) (all other figures), followed by Bonferroni or Dunnett post hoc testing as appropriate. Analyses were performed using SPSS statistical software (SPSS Incorporated, Chicago, IL). $p < 0.05$ was considered significant.

Results

Anti-nociceptive effect of JWH-133 in the formalin test

The anti-nociceptive effect of JWH-133 was assessed by performing a dose–response analysis in the formalin test using male (Figure 1(a), $N = 32$) and female (Figure 1(b), $N = 28$) mice. In males, JWH-133 diminished both acute (0–15 min post-formalin, $ED_{50} = 0.23$ mg/kg; $F(7, 72) = 9.72$, $p < 0.0001$) and inflammatory pain (15–60 min post-formalin, $ED_{50} = 0.23$ mg/kg; $F(7, 216) = 8.42$, $p < 0.00001$) in a dose-dependent manner (Figure 1(a)). There was also a significant dose-dependent effect in the levels of both acute pain ($ED_{50} = 0.24$ mg/kg; $F(7, 60) = 4.62$, $p = 0.0004$) and

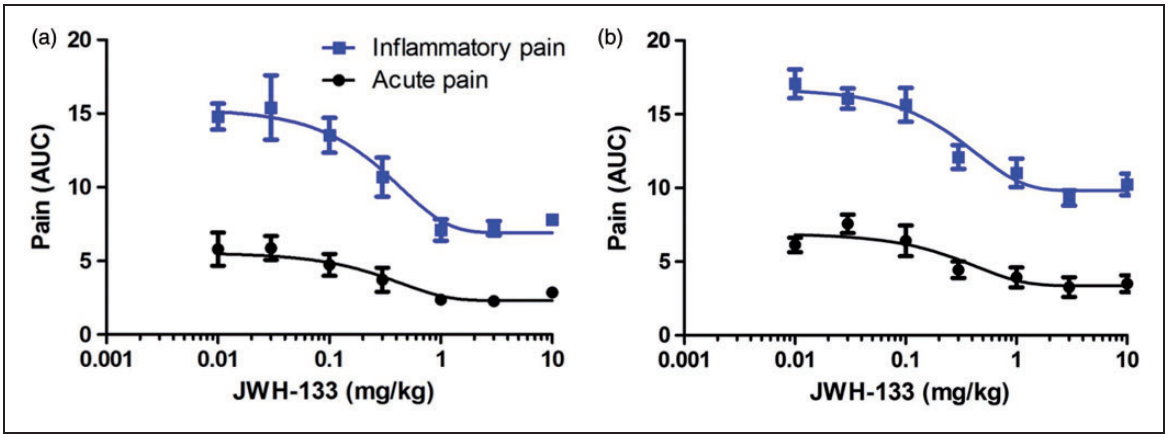

Figure 1. Anti-nociceptive efficacy of JWH-133. Wild-type male (a) and female (b) mice ($N = 4$–7 per dose) were tested using the formalin model. Testing was conducted 60 min after i.p. injection of JWH-133 (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mg/kg). The area under the curve (AUC) represents the pain behavior obtained from a composite pain score. Treatment with JWH-133 reduced both acute ($F(6, 35) = 16.02$, $p < 0.0001$) and inflammatory ($F(6, 35) = 21.53$, $p < 0.0001$) pain in a dose-dependent fashion, with a maximal effect occurring at a dose of 1 mg/kg. The calculated ED_{50} values for acute and inflammatory phases were 0.2295 mg/kg and 0.2294 mg/kg for males. For females, the ED_{50} values were 0.2431 mg/kg and 0.2030 mg/kg.

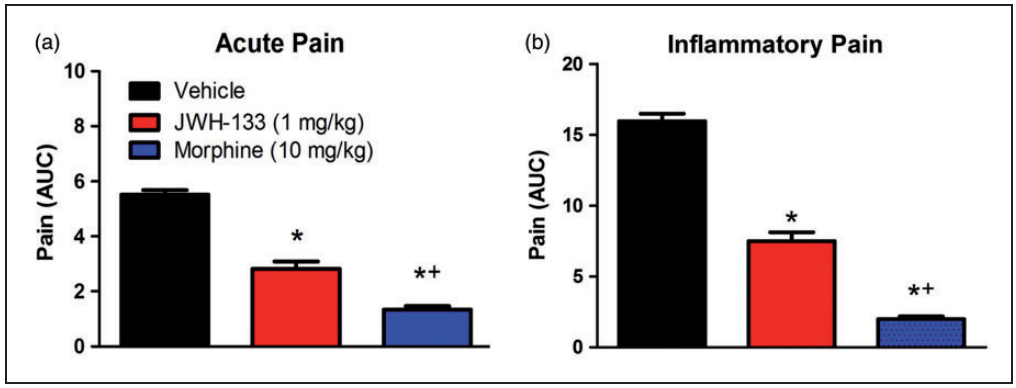

Figure 2. Comparison of morphine and JWH-133 in the formalin test. Mice ($n = 4$–6 per dose) were tested in the formalin model 60 min after i.p. administration of maximal doses of either JWH-133 (1 mg/kg) or morphine (10 mg/kg). The area under the curve (AUC) represents the pain behavior obtained from the composite pain score. Both morphine and JWH-133 reduced pain behavior relative to the vehicle group in the acute (a) and inflammatory (b) phases. Morphine produced a greater anti-nociceptive effect than JWH-133 in both phases. *$p < 0.0001$ for JWH-133 and morphine versus vehicle group (ANOVA with Bonferroni post hoc) and +$p < 0.037$ for morphine versus JWH-133 group (ANOVA with Bonferroni post hoc).
ANOVA: analysis of variance.

inflammatory pain ($ED_{50} = 0.20$ mg/kg; $F(7, 180) = 3.69$, $p = 0.0014$) in female mice (Figure 1(b)). Both male and female mice displayed maximal anti-nociceptive effects with 1 mg/kg JWH-133.

Comparison of the anti-nociceptive effect of JWH-133 and morphine in the formalin test

The anti-nociceptive effect of a maximal dose of JWH-133 (1 mg/kg) was compared to a maximal dose of morphine in groups of male mice (10 mg/kg, Figure 2, $N = 4$ per group). Analysis of the AUC of pain behavior revealed that both morphine and JWH-133 reduced pain behavior relative to the vehicle group in the acute ($F(2, 9) = 32.28$, $p < 0.0001$, Figure 2(a)) and inflammatory phases ($F(2, 9) = 132.47$, $p < 0.0001$, Figure 2(b)) of the formalin test. Moreover, morphine produced a greater anti-nociceptive effect than JWH-133 in both phases ($p < 0.037$ acute; $p < 0.0001$, inflammatory).

JWH-133 acts through the CB2 receptor

To establish that JWH-133 was selective for the CB2R, it was co-administered with antagonists for CB1R

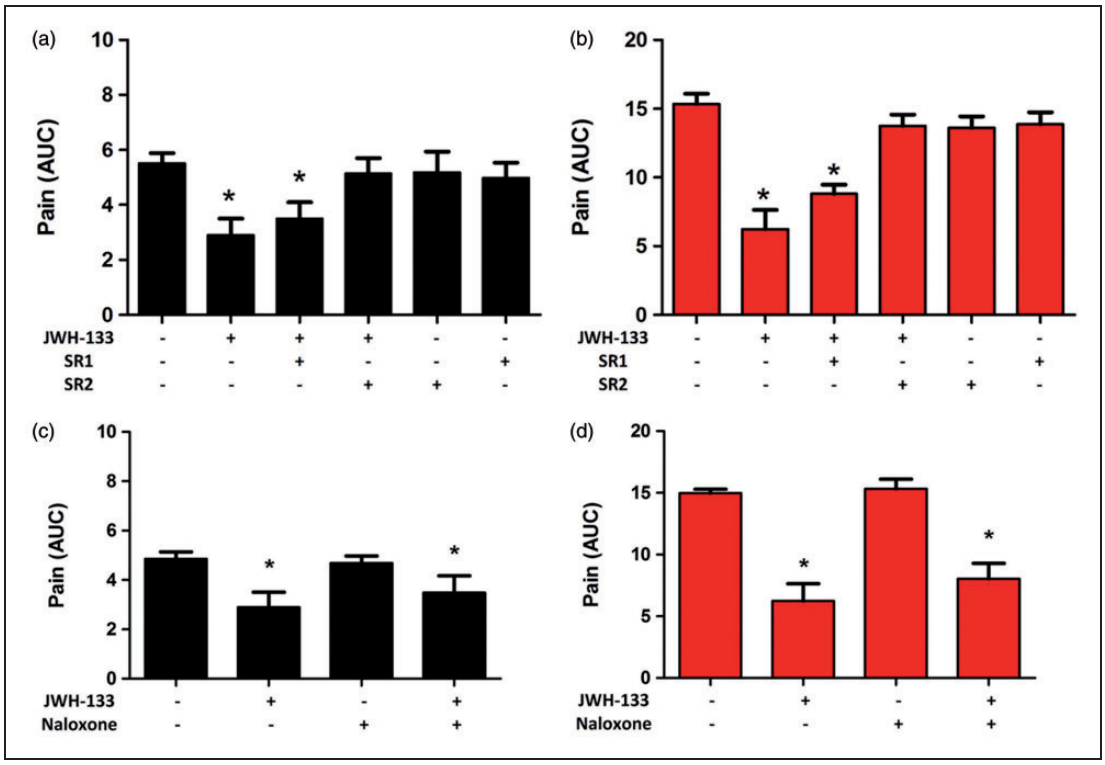

Figure 3. JWH-133 acts through CB2R. The efficacy of JWH-133 (1 mg/kg, i.p.) in the formalin test was examined in the presence of CB1R and CB2R (a and b), as well as MOR (c and d) antagonists administered 30 min prior to JWH-133 treatment ($n = 4$–5 per treatment.) The anti-nociceptive effect of JWH-133 was blocked by CB2R antagonist SR2 (10 mg/kg, i.p.) in both acute (a) and inflammatory pain (b). However, JWH-133 was not impacted by the CB1R antagonist SR1 (10 mg/kg; a and b) or by MOR antagonist naloxone (10 mg/kg; c and d). *$p < 0.046$ for JWH-133 and versus vehicle group (ANOVA with Bonferroni post hoc).
ANOVA: analysis of variance; CB1R: cannabinoid 1 receptor; CB2R: cannabinoid 2 receptor; MOR: mu-opioid receptor; AUC: area under the curve.

(SR1, 10 mg/kg) or CB2R (SR2, 10 mg/kg). The impact of these antagonists on the anti-nociceptive effects of JWH-133 was then measured using male mice in the formalin test (Figure 3, $N = 4$–5 per group). We found that JWH-133 alleviates formalin-induced pain behaviors in both the acute (Figure 3(a), $F(5, 18) = 9.21$, $p < 0.0001$) and inflammatory (Figure 3(b), $F(5, 18) = 18.69$, $p < 0.0001$) phases. The anti-nociceptive effect of JWH-133 was blocked by SR2 ($p < 0.006$, acute; $p < 0.0001$, inflammatory), but not by SR1 ($p = 1.00$, acute; $p = 0.83$, inflammatory). Indeed, the effect of JWH-133 is not mediated through CB1R since its combination with a CB1R antagonist (SR1) produced anti-nociceptive effects when compared to SR1 alone for the acute ($p < 0.012$) and inflammatory ($p < 0.03$) phases. There was no difference in pain values between JWH-133 alone or JWH-133 combined with SR1 ($p = 1.00$, acute and $p = 0.833$, inflammatory).

JWH-133 does not act through MOR

JWH-133 suppressed formalin-induced pain scores in both the acute ($F(3, 12) = 10.39$, $p < 0.001$) and inflammatory ($F(3, 12) = 58.09$, $p < 0.0001$) phases. This anti-nociceptive effect is not blocked by the MOR antagonist naloxone ($p = 0.688$, acute; $p = 0.751$, inflammatory). Formalin-induced pain behavior was similar in groups receiving JWH-133 alone or co-administered naloxone ($p = 0.69$, acute; $p = 0.75$, inflammatory (Figure 3(c) and (d)).

Evaluation of side effects

Male mice were injected daily with JWH-133 (1 mg/kg) or morphine (10 mg/kg) for seven days to examine the possibility of either acute or cumulative negative side effects such as hypothermia. JWH-133 had no impact on body temperature ($F(1, 27) = 2.78$, $p = 0.11$), and the effects of JWH-133 on body temperature were not significantly different from vehicle after one ($p = 0.530$), four ($p = 0.7359$), or seven ($p = 0.8917$) days of repeated administration according to Dunnett's post hoc testing (Figure 4(a)). By contrast, morphine does have a significant impact on hypothermia ($F(1, 27) = 9.88$, $p < 0.004$) and was significantly different from vehicle on day 1 ($p < 0.0001$) but not on days 4 ($p = 0.1334$) or 7 ($p = 0.1673$, Figure 4(a)).

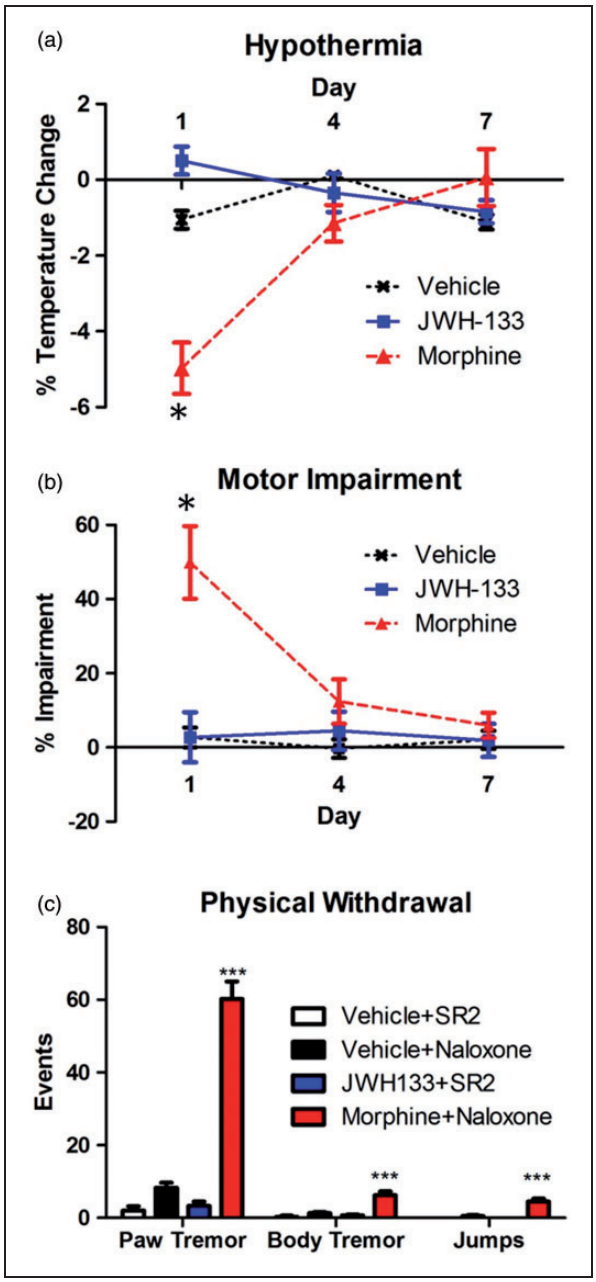

Figure 4. Lack of JWH-133-induced adverse effects. Mice ($n = 5$–7 per group) were injected with a maximal dose of JWH-133 (1 mg/kg), or morphine (10 mg/kg) for seven days to look for either acute or cumulative physical side effects. The hypothermic effects of JWH-133 were not significantly different from vehicle according to a two-way ANOVA with Dunnett's post hoc test (a). The same trends were observed when measuring the effect of JWH-133 on motor impairment using a rotarod apparatus (b). Comparatively, morphine-injected mice showed significant hypothermic effects on day 1 ($^*p = 0.001$), but not day 4 ($p = 0.1334$), or day 7 ($p = 0.1673$). Morphine also caused significant motor impairment on day 1 ($^*p = 0.001$), but not day 4 ($p = 0.2120$), or day 7 ($p = 0.8429$). Following 10 days of twice-daily drug administration, animals were administered antagonists, and frequency of physical withdrawal behaviors were recorded for 60 min (c). While SR2 administration following repeated JWH-133 did not result in withdrawal behavior, morphine-treated mice challenged with naloxone showed significant amounts of paw tremors ($^{***}p < 0.001$), body tremors ($^{***}p < 0.001$), and jumping ($^{***}p < 0.001$) relative to vehicle in a one-way ANOVA.
ANOVA: analysis of variance.

Motor impairment was measured using a rotarod apparatus because this is a common side effect for both opiates and many cannabinoids. Similarly to the hypothermia test, JWH-133 treatment did not produce motor impairment ($F(1, 27) = 0.17$, $p = 0.68$) and did not differ from vehicle on days 1 ($p = 0.999$), 4 ($p = 0.7774$), or 7 ($p = 0.9998$, Figure 4(b)). Comparatively, morphine treatment had a significant effect on motor impairment ($F(1, 27) = 21.32$, $p < 0.0001$) and was significantly different from vehicle on day 1 ($p = 0.0001$) but not on days 4 ($p = 0.2120$) or 7 ($p = 0.8429$, Figure 4(b)).

Precipitated somatic withdrawal

Following 10 days of twice-daily injections of either JWH-133 or morphine, antagonists were given to determine whether somatic withdrawal symptoms could be precipitated. Precipitation of physical withdrawal from JWH-133 using the CB2R antagonist SR2 did not result in any detectable somatic withdrawal events following repeated JWH-133. Similarly, SR2 did not elicit somatic withdrawal symptoms in mice treated with daily saline (Figure 4(c), $N = 4$ per group). However, treatment of morphine-tolerant mice with naloxone elicited an increase in paw tremors ($p < 0.001$), body tremors ($p < 0.001$) and jumping behavior ($p < 0.001$) compared to vehicle-treated mice.

Lack of observed tolerance to 11 once-daily injections of JWH-133

In order to investigate the development of tolerance, male mice ($N = 3$–5 per group) were assessed using the formalin model following one, six, or 11 days of JWH-133 (1 mg/kg; Figure 5(a)) or morphine (10 mg/kg; Figure 5(b)) treatment. Analysis of the AUC of pain behavior revealed that JWH-133 reduced pain behavior relative to the vehicle group in the acute ($F(3, 15) = 15.01$, $p < 0.0001$) and inflammatory phases ($F(3, 15) = 41.21$, $p < 0.0001$) of the formalin test after one, six, or 11 days of repeated administration ($p < 0.0002$, acute and $p < 0.0001$, inflammatory). The magnitude of JWH-133's anti-nociceptive efficacy did not diminish following one, six, or 11 days of continuous administration ($p = 1.000$). In the acute phase, morphine reduced pain behavior in comparison to the vehicle

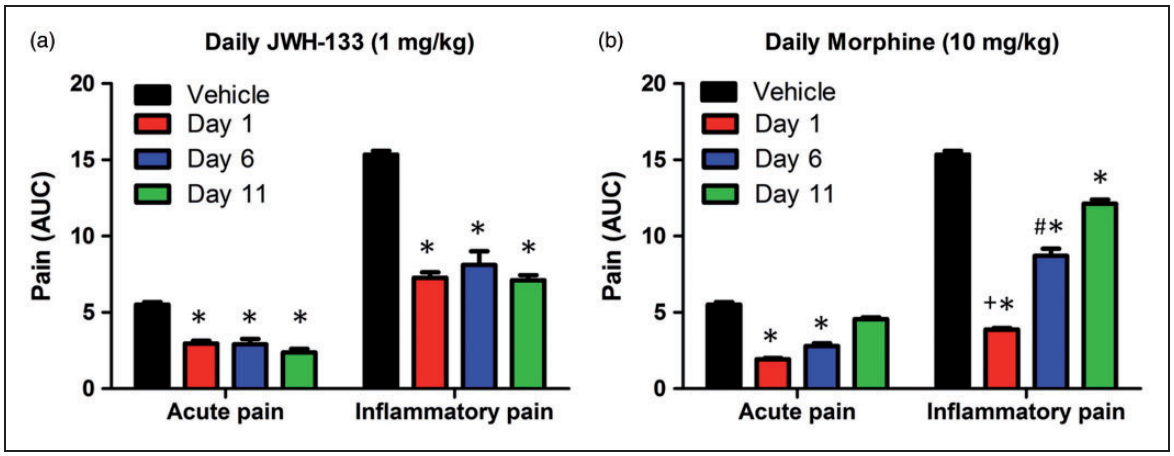

Figure 5. Lack of observed tolerance to JWH-133. Mice ($n = 3$–5 per group) were measured in the formalin test following 1, 6, or 11 days of JWH-133 (1 mg/kg; a) or morphine (10 mg/kg; b) administration. JWH-133 (a) and morphine (b) reduce pain behavior relative to vehicle group. This anti-nociceptive effect of JWH-133 is similar on all days examined. For morphine, a greater anti-nociceptive effect is observed at day 1 relative to day 6 and from day 6 relative to day 11. Data are expressed as mean ± SEM. $*p < 0.0001$ for JWH-133 or morphine versus vehicle group (ANOVA with Bonferroni post hoc); $+p < 0.001$ for morphine day 1 versus morphine day 6 group (ANOVA with Bonferroni post hoc); $\#p < 0.039$ for morphine day 6 versus morphine day 11 group (ANOVA with Bonferroni post hoc).
ANOVA: analysis of variance; AUC: area under the curve; SEM: standard error of the mean.

group ($F(3, 15) = 47.05$, $p < 0.0001$) after one and six days of repeated administration ($p < 0.0001$) but not after 11 days ($p = 0.149$). In the inflammatory phase, morphine reduced pain behavior relative to the vehicle group ($F(3, 15) = 55.03$, $p < 0.0001$) after one, six, or 11 days of repeated administration ($p < 0.031$). However, this anti-nociceptive effect of morphine was different in a time-dependent manner since greater anti-nociceptive effect is observed at day 1 relative to day 6 ($p < 0.001$) and from day 6 relative to day 11 ($p < 0.039$).

JWH-133 and morphine cross-tolerance

Male mice ($N = 5$–8 per group) were injected for 10 days with either vehicle or JWH-133 (1 mg/kg) and then given a challenge dose of morphine (10 mg/kg) on day 11 to assess cross-tolerance in the formalin test (Figure 6(a)). The repeated (10 days) administration of vehicle or JWH-133 and a challenge dose (on day 11) of morphine ($F(2, 13) = 325.04$, $p < 0.0001$) suppressed CPS relative to control group in a time-dependent manner ($F(22, 143) = 11.46$, $p < 0.0001$, Figure 6(a)). This suppression was observed from 5 to 15 min (acute phase; $F(2, 13) = 22.24$ (5), 5.86 (10), and 4.57 (15); $p < 0.0001$ for all time points) and from 20 to 50 min (inflammatory phase 2; $F(2, 13) = 25.92$ (20), 37.56 (25), 71.57 (30), 71.67 (35), 57.10 (40), 12.30 (45), and 4.37 (50); $p < 0.035$ for all time points) post-formalin injection compared to the control group.

Analysis of the AUC of pain behavior revealed that both the repeated vehicle group and the repeated JWH-133 group showed anti-nociceptive efficacy of morphine challenge relative to the vehicle-only control group in both phase 1 ($F(2, 13) = 63.58$, $p < 0.0001$; Figure 6(b)) and phase 2 ($F(2, 13) = 356.04$, $p < 0.0001$; Figure 6(c)) of the formalin test.

Male mice were also injected for 10 days with either vehicle or morphine and then given a challenge dose of the JWH-133 drug on day 11 to assess cross-tolerance in the opposing direction. Only the repeated (10 days) administration of vehicle and a challenge dose (on day 11) of JWH-133 ($F(2, 14) = 17.61$, $p < 0.0001$) suppressed CPS relative to control group in a time-dependent manner ($F(22, 154) = 6.30$, $p < 0.0001$, Figure 6(d)). This suppression was observed at 5 (acute phase; $F(2, 14) = 5.65$; $p < 0.001$) min and from 30 to 50 min (inflammatory phase 2; $F(2, 14) = 5.65$ (30), 24.97 (35), 24.93 (40), and 13.84 (45); $p < 0.016$ for all time points) post-formalin injection compared to the control or repeated morphine + JWH-133 challenge groups. There was no difference in formalin-induced pain values between the control and repeated morphine + JWH-133 challenge groups from 5 to 60 min ($p > 0.064$ for all time points) except at 20 ($p < 0.001$) and 45 ($p < 0.004$) min.

In these mice, analysis of the AUC of pain behavior revealed that only the repeated vehicle with JWH-133 challenge group produced anti-nociception relative to the control or repeated morphine + JWH-133 challenge groups in both phase 1 ($F(2, 14) = 22.39$, $p < 0.0001$; Figure 6(e)) and phase 2 ($F(2, 14) = 6.57$, $p < 0.01$; Figure 6(f)) of the formalin test. Similar formalin-induced pain values are observed in the control and repeated morphine + JWH-133 challenge groups in both phases ($p = 1.00$ acute and inflammatory).

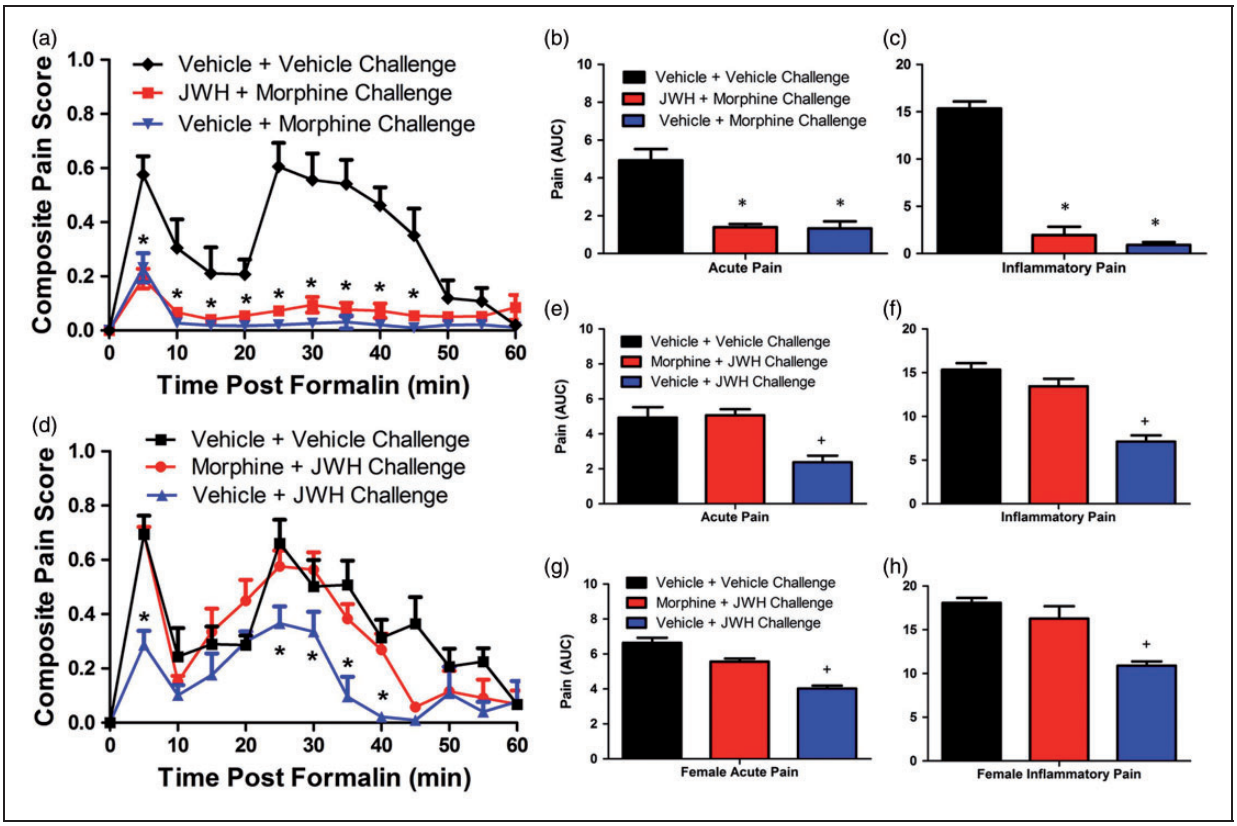

Figure 6. Cross-tolerance between JWH-133 and morphine. The formalin model of inflammatory pain was utilized to examine cross-tolerance between morphine and JWH-133. The composite pain score (a) and AUC for acute (b) and inflammatory pain (c) is shown for male mice that received a challenge dose of morphine (10 mg/kg, i.p.) after 10 days of repeated JWH-133 (1 mg/kg, i.p.) administration. A second group received a challenge dose of JWH-133 following repeated morphine and is displayed as a composite pain score (d) and AUC for acute (e) and inflammatory pain (f). A group of female mice also received a challenge dose of JWH-133 following repeated morphine and are displayed as AUC for acute (g) and inflammatory pain (h). Data are expressed as mean ± SEM ($n = 5$–8 per group). *$p < 0.0001$ versus control group (ANOVA with Bonferroni post hoc); +$p < 0.01$ versus control or repeated morphine + JWH-133 challenge groups (ANOVA with Bonferroni post hoc).
ANOVA: analysis of variance; AUC: area under the curve; SEM: standard error of the mean.

In groups of female mice ($N = 4$–7), analysis of the AUC of pain behavior revealed that only the repeated vehicle with JWH-133 challenge group produced anti-nociception relative to the control or repeated morphine + JWH-133 challenge groups in both phase 1 ($F(2, 8) = 13.72$, $p < 0.003$; Figure 6(g)) and phase 2 ($F(2, 8) = 9.71$, $p < 0.01$; Figure 6(h)) of the formalin test. Similar formalin-induced pain values were observed in the control and repeated morphine + JWH-133 challenge groups in both phases ($p = 0.164$ acute; 1.00 inflammatory).

JWH-133 co-administration modestly attenuates morphine tolerance

Male mice ($N = 4$ per group) were co-administered 10 mg/kg morphine and 1 mg/kg JWH-133, given morphine alone, or given vehicle for up to 10 days. All groups were then tested for morphine-induced anti-nociception using a challenge dose morphine alone on (days 2, 6, 11) to assess potential protective effects of JWH-133 on morphine tolerance.

On day 2, analysis of the AUC of pain behavior revealed that vehicle + morphine challenge, morphine + morphine challenge, or combination (morphine + JWH-133)+morphine challenge groups showed lower pain values relative to the control group for both phases ($F(3, 12) = 60.26$ acute and 360.55 inflammatory, $p < 0.0001$, Figure 7). In the inflammatory phase, the vehicle + morphine challenge group shows lower pain values relative to morphine + morphine challenge group ($p < 0.004$).

On day 6, the AUC of pain behavior also revealed that vehicle + morphine challenge, morphine + morphine challenge, or combination (morphine + JWH-133) + morphine challenge groups showed lower pain values

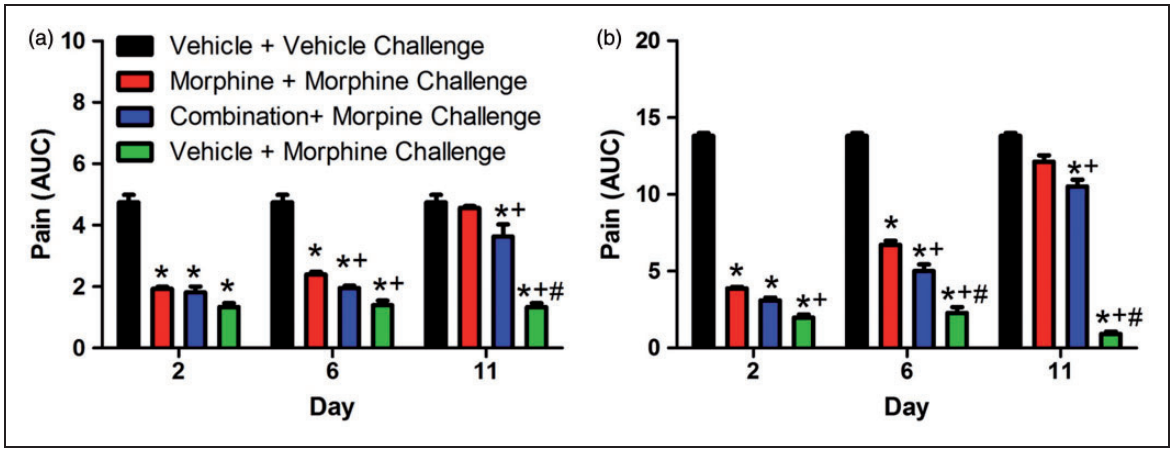

Figure 7. JWH-133 co-administration modestly protects against morphine tolerance. The efficacy of morphine (10 mg/kg) in the formalin model was compared between mice receiving daily morphine alone (10 mg/kg) and mice receiving daily morphine (10 mg/kg) and daily JWH-133 (1 mg/kg). Co-administration of JWH-133 with morphine resulted in significantly greater acute phase 1 (a) and phase 2 (b) anti-nociception on day 6 and day 11. Data are expressed as mean ± SEM ($n = 4$ per group). *$p < 0.004$ versus vehicle group (ANOVA with Bonferroni post hoc); +$p < 0.047$ versus morphine + morphine challenge group (ANOVA with Bonferroni post hoc); #$p < 0.003$ versus combination + morphine challenge group (ANOVA with Bonferroni post hoc).
ANOVA: analysis of variance; AUC: area under the curve; SEM: standard error of the mean.

relative to the control group for both phases ($F(3, 12) = 117.69$ acute and 165.98 inflammatory, $p < 0.0001$). In the acute and inflammatory phases, the vehicle + morphine challenge or the combination+morphine challenge groups showed lower pain values relative to morphine + morphine challenge group ($p < 0.002$, acute and $p < 0.047$, inflammatory). These results show the protective effect of JWH-133 co-administration with repeated administration of morphine on both phases of the formalin test. In the inflammatory phase only, the vehicle + morphine challenge group showed the lowest pain values ($p < 0.004$).

On day 11, the AUC of pain behavior revealed that vehicle + morphine challenge and combination (morphine + JWH-133)+morphine challenge groups showed lower pain values relative to the control group for both phases ($F(3, 13) = 74.44$ acute and 179.36 inflammatory, $p < 0.0001$). The control group showed similar values to the morphine + morphine challenge group for both phases ($p = 1.00$, acute and $p = 0.05$, inflammatory). In the acute and inflammatory phases, the vehicle + morphine challenge or the combination + morphine challenge groups show lower pain values relative to morphine + morphine challenge group ($p < 0.019$, acute and $p < 0.037$, inflammatory). In both phases, the vehicle + morphine challenge group shows the lowest pain values ($p < 0.0001$ acute and inflammatory phases).

JNK signaling is partially responsible for morphine-induced cross-tolerance to JWH-133

The efficacy of a challenge dose of JWH-133 (1 mg/kg) in the formalin model was compared between groups of male mice that received either five days of morphine alone (10 mg/kg), five days of the JNK inhibitor SP600125 (3 mg/kg) and morphine (10 mg/kg), or five days of vehicle (Figure 8, $N = 4$ per group). Pre-treatment with SP600125 prior to morphine caused a significant increase in JWH-133 efficacy ($F(2, 9) = 91.86$ acute, and $F(2, 9) = 55.37$ inflammatory, $p < 0.0001$ for both phases). Therefore, repeated co-administration of SP600125 (3 mg/kg) with morphine appears to reduce observed cross-tolerance to JWH-133. In both phases of the formalin test, the vehicle and JWH-133 challenge group shows the lowest pain values ($p < 0.0001$ acute and inflammatory phases).

Isobolographic analysis

To examine the potential synergy between JWH-133 and morphine, isobolographic analysis was used to compare the theoretical and experimental dose–response curves for a 1:10 fixed ratio dose combination of JWH-133 and morphine in the formalin test (Figure 9(a) and (b)). In this analysis, there is a substantial difference in the maximal efficacies of JWH-133 and morphine. Therefore, a non-linear isobolographic analysis[36] was used. In phase 1 of the formalin test, the theoretical ED_{50} of 1:10 JWH-133/morphine combination (0.7765 mg/kg) was found to overlap with the experimentally determined value (0.7236 mg/kg, Figure 9(c)). This indicates that the 1:10 fixed ratio combination is likely to be additive but not synergistic. In phase 2 of the formalin test, the experimentally determined ED_{50} (0.6211 mg/kg) appears to be lower than the predicted ED_{50} (0.9258 mg/kg, Figure 9(d)). However, the 95%

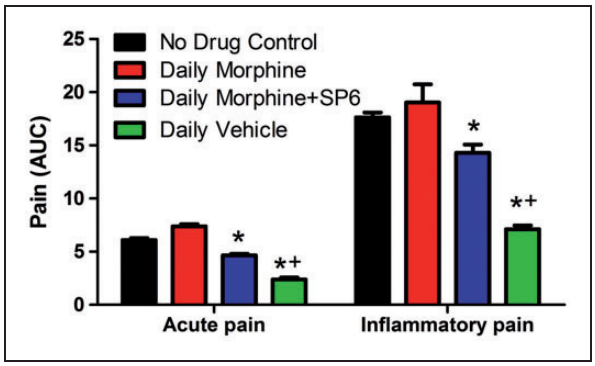

Figure 8. JNK signaling is partially responsible for morphine-induced cross-tolerance to JWH-133. The efficacy of a challenge dose of JWH-133 (1 mg/kg) in the formalin model was compared between mice that received five days of daily morphine alone (10 mg/kg), mice receiving daily morphine (10 mg/kg) and SP6 (3 mg/kg), and mice receiving daily vehicle. Co-administration of SP6 with morphine resulted in a significant increase of JWH-133 efficacy both in acute and inflammatory pain. Data are expressed as mean ± SEM ($n = 3$–5 per group). *$p < 0.0001$ versus chronic morphine group (ANOVA with Bonferroni post hoc); +$p < 0.001$ versus chronic morphine + SP6 group (ANOVA with Bonferroni post hoc).
JNK: c-Jun N-terminal kinase; ANOVA: analysis of variance; AUC: area under the curve; SEM: standard error of the mean.

confidence intervals of the two values are overlapping. Thus, our results demonstrate that the combined anti-nociceptive effects of JWH-133 and morphine are additive but likely not synergistic.

Discussion

The primary goal of this study was to test the hypothesis that CB2R functionally interacts with the opioid system to modulate inflammatory pain. First, we examined the effects of CB2R activation alone and found that JWH-133 causes dose-dependent anti-nociception in both the acute and inflammatory phases of the formalin test (Figure 1). Using the CB2R-seletive antagonist SR2, we demonstrated that the anti-nociceptive effects of JWH-133 occur through activation of CB2R (Figure 3). The efficacy of JWH-133 is consistent with previous work demonstrating that CB2R agonists can produce robust anti-nociception in other inflammatory and neuropathic pain models.[40–42] For example, systemic administration of the CB2R agonist GW405833 to mice and rats was able to reverse hyperalgesia in a model of inflammatory pain involving complete Freund's adjuvant (CFA).[43] In addition, two other CB2R agonists A-836339 and AM-1241 were anti-nociceptive in a rat CFA model when administered spinally (intrathecally) or via direct intraplantar injection to the inflamed paw.[42] Previous studies report that AM-1710 was able to completely reverse paclitaxel-induced neuropathic pain in mice.[14] Interestingly, continuous administration of AM-1710 before and after paclitaxel treatment can prevent the development of neuropathic pain for several weeks.[44]

Additionally, we sought to examine potential sex differences in JWH-133 anti-nociception between males and females using the formalin test (Figure 1(a) and (b)). Females displayed higher levels of pain behavior at every JWH-133 dose. However, this difference appears to be due to higher basal levels of pain in female mice rather than reduced sensitivity to the anti-nociceptive effects of JWH-133. The finding that females generally exhibit more pain behaviors is consistent with animal and human studies of pain.[45–49] Our study did not suggest that female mice were more sensitive to JWH-133, as the calculated ED_{50} values and maximally efficacious doses did not differ between male and female mice. However, there is evidence for sex differences in the response to cannabinoids. Previous studies have shown that female rats are more sensitive to Δ^9-THC and develop tolerance to it more quickly.[50] It is possible that this discrepancy is due to the fact that many previously reported sex differences in cannabinoid response involved compounds that also activate CB1R. It is also possible that previously reported sex differences in rats are partially species-dependent.

We also sought to assess potential negative side effects of JWH-133. Our results closely aligned with previous studies that failed to demonstrate adverse effects of other CB2R agonists (AM-1241, AM-1710, and HU-308) in rodents.[14,16,51] We did not observe negative side effects typically associated with agonists for the CB1R and/or opioid receptors which includes hypothermia, motor incoordination, or antagonist-induced somatic withdrawal symptoms (tremors, jumping, diarrhea) for JWH-133 (Figure 4). However, it is important to recognize that this list of potential adverse effects is not exhaustive, and more research studies are needed to assess the possibility of other adverse effects that might also be sex-specific. In particular, the short duration of repeated JWH-133 administration and limited number of observed somatic withdrawal symptoms here are not sufficient to fully assess the possibility that subtle somatic symptoms might occur. Furthermore, since we only examined possible somatic symptoms, it is possible that precipitated JWH-133 withdrawal could cause affective and behavioral withdrawal symptoms such as anxiety, depression, and anhedonia.

Regardless of the pain model used, multiple studies observe a lack of tolerance to repeated administration of CB2R agonists. For example, daily systemic administration of AM-1710 attenuated chemotherapy-evoked neuropathic pain for up to eight days with no evidence of tolerance.[11] Similarly, the CB2R agonist, JWH015 (intrathecal, once daily), reversed

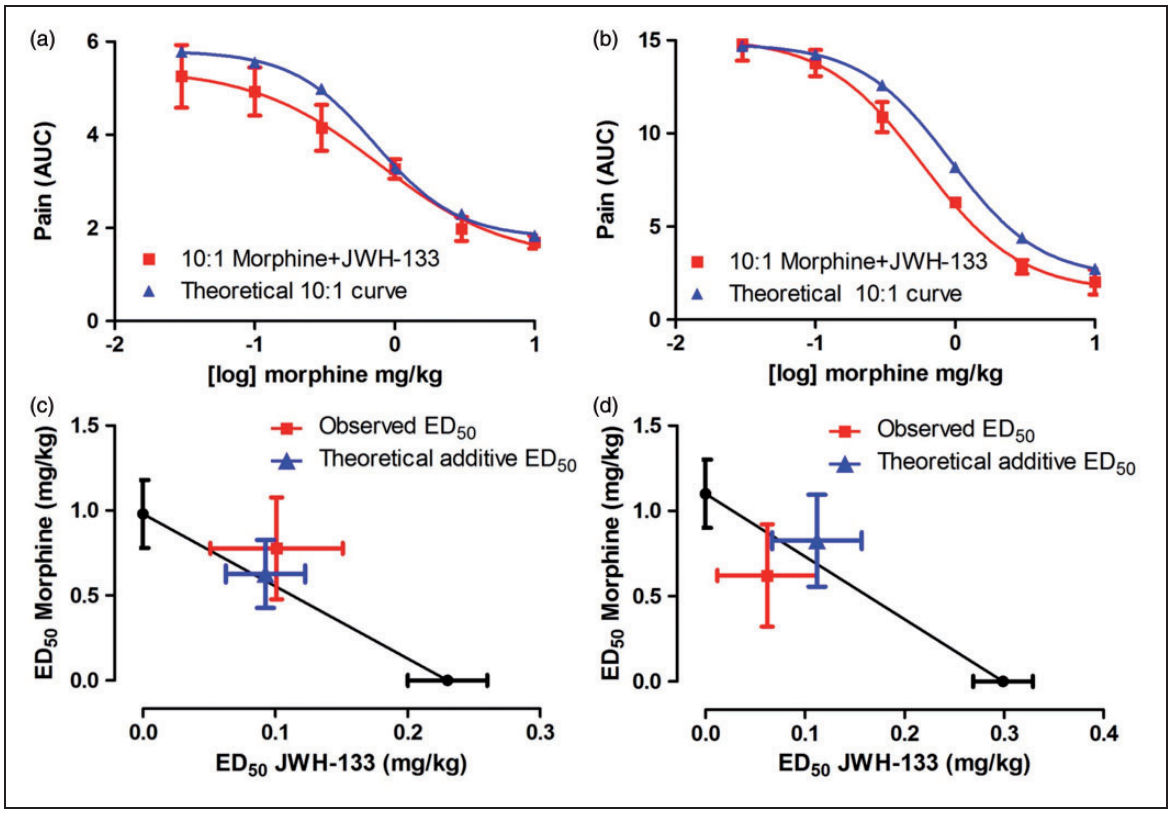

Figure 9. The anti-nociceptive effects of JWH-133 and morphine are additive in the formalin test. A dose–response curve was generated in the formalin test for co-administration of JWH-133 and morphine in a 1:10 fixed dose ratio ($n = 3$–6 per dose). Non-linear isobolographic analysis was used to compare the experimentally determined ED_{50} values with theoretical ED_{50} values if the combination were exactly additive. In phase 1 (c), the theoretical ED_{50} (0.7765 ± 0.18 mg/kg) was found to overlap with the experimentally determined value (0.7236 ± 0.11 mg/kg), suggesting additivity. In phase 2 (d) of the formalin test, the experimentally determined ED_{50} (0.6211 ± 0.063 mg/kg) appears to be lower than the predicted ED_{50} (0.9258 ± 0.12 mg/kg). However, the 95% confidence intervals of the two values are overlapping.
AUC: area under the curve.

surgery-induced allodynia for up to five days without tolerance.[52] In this study, we report a lack of tolerance to the anti-nociceptive effects of JWH-133 after 11 consecutive days of administration (Figure 5). However, it is possible that tolerance to JWH-133 may occur with longer periods of chronic dosing and/or may occur for the affective effects of JWH-133 on pain (anxiety, depression, and anhedonia). Previous studies evaluating the absence of tolerance for CB2 agonists failed to evaluate the affective components of pain.

To fully understand why CB2R agonists do not cause obvious tolerance, it is important to increase our understanding of the mechanisms through which they produce anti-nociception and possible effects on the affective component of pain and/or sex differences. First, the anti-nociceptive effect of JWH-133 might be caused by the ability of CB2R activation to suppress inflammation at the site of injury.[20,21] For example, paclitaxel-induced neuropathic pain in rats and mice can be prevented if the CB2R agonist, MDA7, is co-administered during treatment with paclitaxel.[53] Rats receiving MDA7 did not display the expected increase of markers for microglial and astrocyte activation in the spinal cord that are associated with inflammatory response during paclitaxel treatment. One possible explanation for this finding is that CB2R activation blocked the normal inflammatory response to paclitaxel, thus preventing inflammation, nerve damage, and pain.

Beyond mediating inflammation, a second proposed mechanism for this crosstalk between cannabinoid and the opioid systems is agonist-stimulated release of endogenous ligands. We have directly examined the possibility that JWH-133-mediated anti-nociception might be mediated through endogenous opioid release. Our results indicate that anti-nociceptive efficacy of JWH-133 was not prevented by antagonism of MOR with naloxone (Figure 3). This is consistent with previous studies using CB2R agonist GW405833 to reduce CFA-induced inflammatory allodynia in the presence

of naltrexone, another MOR antagonist.[43] These findings suggest that certain CB2R agonists (including JWH-133) can attenuate inflammatory pain through a MOR-independent mechanism. Differences in the ability of CB2R agonists to stimulate endogenous opioid release might be due to signaling bias. For example, JWH-133 appears to be strongly biased toward G protein-dependent mechanisms and biased against signaling through β-arrestin recruitment.[54–56] However, there are other mechanisms of action that could explain these results including morphine-stimulated eCB release and/or morphine-stimulated downregulation of CB2R.

Despite the lack of tolerance to the anti-nociceptive effects of JWH-133, we observe morphine-induced cross-tolerance for JWH-133 following repeated daily injections of morphine (Figure 6). However, this effect is unidirectional since repeated daily injections of JWH-133 do not cause cross-tolerance to a challenge dose of morphine. Previous work has demonstrated that tolerance to the anti-nociceptive and anti-allodynic effects of morphine require JNK signaling.[29,57,58] Therefore, we examined the possibility that morphine-induced cross-tolerance to JWH-133 was also mediated through a JNK signaling mechanism. We found that this cross-tolerance to JWH-133 is only partially attenuated by SP600125, a broad-spectrum inhibitor of all three JNK isoforms (Figure 8). However, there are other mechanisms of action that could explain these results including morphine-stimulated eCB release and/or morphine-stimulated downregulation of CB2R. Thus, the specific mechanism of action for opioid-induced cross-tolerance to JWH-133 is not understood and requires additional study.

Interestingly, our results suggest that JWH-133 co-administration may be protective against the development of morphine tolerance (Figure 7). This finding is in agreement with a previous study of cancer pain that showed a delay in morphine tolerance when morphine was co-administered with a sub-analgesic dose of AM-1241.[59] While intriguing, the magnitude of the JWH-133 effect on morphine tolerance is quite modest in our study.

Co-administration of CB2R agonists with opioids may also result in increased anti-nociceptive efficacy due to drug synergism. One potential benefit of a synergistic interaction between JWH-133 is that it could allow effective anti-nociception with considerably lower morphine doses. Such an opioid-sparing effect for JWH-133 could help minimize the adverse effects of opioids. Human studies have shown increased pain relief in patients when inhaled cannabis use is combined with opioids.[60] Therefore, we performed isobolographic analysis of morphine and JWH-133 in combination to test for potential synergy (Figure 9). We were able to demonstrate an additive effect of this combination but were not able to demonstrate a greater-than-additive (synergistic) effect. However, only one fixed ratio (10:1) drug combination was used. Nonetheless, even an additive effect of CB2R agonists could be beneficial due to the lack of adverse effects.[61]

Conclusion

Our study demonstrates unidirectional morphine-induced cross-tolerance to JWH-133. This finding raises the possibility that CB2R may functionally interact with the opioid system to modulate inflammatory pain. Interestingly, we also find that co-administration of JWH-133 with morphine produces an additive anti-nociceptive effect in the formalin test while also potentially protecting against morphine tolerance. Taken together, these findings highlight the potential therapeutic applications for different CB2R ligands in pathological pain states without tolerance or adverse effects associated with currently available treatments. In particular, further investigation into the use of CB2R agonist as adjuvant treatments to opioid therapy should be investigated.

Author Contributions

MBY and DEH conducted the behavioral tests and pharmacological studies. MBY, DJM, and JG designed the project. DJM and JG oversaw the project and assisted in data analysis. MBY, DEH, DJM, and JG interpreted the research and wrote the paper. All authors read and approved the final manuscript. JG and DJM contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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