CB₁ Knockout Mice Unveil Sustained CB₂-Mediated Antiallodynic Effects of the Mixed CB₁/CB₂ Agonist CP55,940 in a Mouse Model of Paclitaxel-Induced Neuropathic Pain

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ABSTRACT

Cannabinoids suppress neuropathic pain through activation of cannabinoid CB₁ and/or CB₂ receptors; however, unwanted CB₁-mediated cannabimimetic effects limit clinical use. We asked whether CP55,940 [(–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexanol], a potent cannabinoid that binds with similar affinity to CB₁ and CB₂ in vitro, produces functionally separable CB₁- and CB₂-mediated pharmacological effects in vivo. We evaluated antiallodynic effects, possible tolerance, and cannabimimetic effects (e.g., hypothermia, catalepsy, CB₁-dependent withdrawal signs) after systemic CP55,940 treatment in a mouse model of toxic neuropathy produced by a chemotherapeutic agent, paclitaxel. The contribution of CB₁ and CB₂ receptors to in vivo actions of CP55,940 was evaluated using CB₁ knockout (KO), CB₂KO, and wild-type (WT) mice. Low-dose CP55,940 (0.3 mg/kg daily, i.p.) suppressed paclitaxel-induced allodynia in WT and CB₂KO mice, but not CB₁KO mice. Low-dose

CP55,940 also produced hypothermia and rimonabant-precipitated withdrawal in WT, but not CB₁KO, mice. In WT mice, tolerance developed to CB₁-mediated hypothermic effects of CP55,940 earlier than to antiallodynic effects. High-dose CP55,940 (10 mg/kg daily, i.p.) produced catalepsy in WT mice, which precluded determination of antiallodynic efficacy but produced sustained CB₂-mediated suppression of paclitaxel-induced allodynia in CB₁KO mice; these antiallodynic effects were blocked by the CB₂ antagonist 6-iodopravadoline (AM630). High-dose CP55,940 did not produce hypothermia or rimonabant-precipitated withdrawal in CB₁KO mice. Our results using the mixed CB₁/CB₂ agonist CP55,940 document that CB₁ and CB₂ receptor activations produce mechanistically distinct suppression of neuropathic pain. Our study highlights the therapeutic potential of targeting cannabinoid CB₂ receptors to bypass unwanted central effects associated with CB₁ receptor activation.

Introduction

Peripheral neuropathic pain induced by chemotherapy detrimentally impacts the lives of cancer patients and is one of the major side effects responsible for discontinuation of anticancer treatment (Windebank and Grisold, 2008). To date, the mechanisms underlying chemotherapy-induced neuropathic pain remain poorly understood (Windebank and Grisold, 2008). Additional challenges for the management of chemotherapy-induced neuropathy include limited efficacy and significant side effects of existing medications (Pachman et al., 2011). Thus, identification of therapeutic strategies that are both safe and effective for managing chemotherapy-evoked neuropathic pain remains an unmet clinical need.

Cannabinoids produce antinociceptive effects in preclinical models of neuropathic pain (Herzberg et al., 1997; Ibrahim et al., 2003; Guindon and Hohmann, 2009; Kinsey et al., 2009; Pertwee, 2009; Schlosburg et al., 2009); however, unwanted

psychotropic effects of cannabinoids limit their potential clinical use (Ben Amar, 2006; Pertwee, 2009; Dhopeswarkar and Mackie, 2014). Two major subtypes of cannabinoid receptors, CB₁ and CB₂, are the key receptors responsible for the pharmacological effects of cannabinoids (Mackie, 2006). Both CB₁ and CB₂ receptors are G-protein-coupled receptors whose signaling pathways include inhibition of adenylyl cyclase and activation of mitogen-activated protein kinases (Mackie, 2006). CB₁ receptors are predominantly located in the central nervous system (CNS), whereas CB₂ receptors are found primarily in immune cells (Galieue et al., 1995; Schatz et al., 1997; Marsicano and Lutz, 1999; Onaivi et al., 2006) and are upregulated in the CNS in response to inflammation or injury (Zhang et al., 2003; Maresz et al., 2005). Evaluation of the receptor mechanisms underlying therapeutic and psychotropic effects of cannabinoids, after both acute and chronic administration, may facilitate the development of safe and effective cannabinoid-based pharmacotherapies (Ben Amar, 2006; Pertwee, 2009).

CP55,940 [(–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexanol] is a potent nonselective

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ABBREVIATIONS: AM630, 6-iodopravadoline; CNS, central nervous system; CP55,940, (–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexanol; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; KO, knockout; WIN55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; WT, wild-type.

synthetic cannabinoid that has equal affinity for both CB₁ and CB₂ receptors *in vitro* (Felder et al., 1995; Abood et al., 1997; Griffin et al., 2000). Whereas CB₂ agonists exhibit strong ligand-biased selectivity for different signal transduction pathways (Atwood et al., 2012), CP55,940 is a balanced agonist that has not shown functional selectivity at CB₁ or CB₂ (Howlett et al., 2002; Atwood et al., 2012). Thus, CP55,940, used in combination with CB₁ knockout (KO) and CB₂KO mice, represents a useful pharmacological tool for studying the functions of CB₁ and CB₂ receptors *in vivo*. CP55,940 has been reported to possess antinociceptive efficacy in various preclinical pain models, including acute pain, inflammatory pain, and neuropathic pain induced by traumatic nerve injury (Lichtman and Martin, 1997; Hohmann et al., 1999; Romero et al., 2002; Scott et al., 2004; Choong et al., 2007; Sain et al., 2009). Pharmacological antagonism of CB₁ receptors alone (Lichtman and Martin, 1997; Romero et al., 2002; Choong et al., 2007) or of both CB₁ and CB₂ receptors (Scott et al., 2004) blocks the antinociceptive effects of CP55,940 in rats; however, a study using CB₁KO and CB₂KO mice reported that the antinociceptive effects of systemic CP55,940 (at 0.3 mg/kg, *i.p.*), administered acutely, is mediated by CB₁ but not by CB₂ receptors (Sain et al., 2009). This finding led to the conclusion that agonist activity at CB₂ is not relevant to antinociceptive effects of mixed CB₁/CB₂ agonists, at least following systemic administration. By contrast, we hypothesized that, due to the abundance of CB₁ receptors (relative to CB₂ receptors) in the CNS, higher doses of mixed cannabinoids are required to activate CB₂ receptors (compared with the dose that is sufficient to activate CB₁) and that CB₂-mediated antinociceptive effects, rather than being absent, are masked by CB₁-mediated catatonia associated with mixed cannabinoid agonists. The differences between the *in vitro* and *in vivo* profiles of CP55,940 raise questions about the differential roles and functions of CB₁ and CB₂ receptors *in vivo*, particularly in persistent pain states in which chronic dosing is required for clinical use.

In the present study, we investigated the contribution of cannabinoid CB₁ and CB₂ receptor subtypes to the *in vivo* actions of the mixed cannabinoid CP55,940 in a mouse model of chemotherapy-induced neuropathy using transgenic (*i.e.*, CB₁KO, CB₂KO) and wild-type (WT) mice. We evaluated antinociceptive effects, possible tolerance, and typical CNS-associated side effects (*e.g.*, hypothermia, catalepsy, and physical withdrawal) associated with chronic administration of CP55,940 at multiple doses. CB₁KO mice were used to test the hypothesis that CB₂-mediated signaling can be engaged by mixed cannabinoids *in vivo* to produce sustained anti-allodynic efficacy without producing side effects. Thus, under conditions in which confounding effects of CP55,940 at CB₁ receptors are absent (*e.g.*, in CB₁KO mice), CB₂-mediated effects can be fully characterized to ascertain the therapeutic potential of targeting CB₂ receptors.

Materials and Methods

Subjects. Adult CB₁KO and WT littermates on a CD1 background and adult CB₂KO and WT littermates on a C57BL/6J background weighing 26–35 g and of both sexes, were used in these experiments. CB₂KO (B6.129P2-CNR2(tm1Dgen/J)) and corresponding WT (C57BL/6J) mice were originally purchased from Jackson Laboratory (Bar Harbor, ME). CB₁KO mice were generated as previously described (Ledent et al.,

1999), whereas corresponding WT littermates were periodically outcrossed with CD1 mice (strain number 022) from Charles River Laboratories (Wilmington, MA) to maintain genetic diversity. Animals were single housed in a temperature-controlled facility (73 ± 2°F, 45% humidity, regular 12-hour light/dark cycle, lights on at 7 AM), with food and water *ad libitum*. All experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee of Indiana University and followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Drugs and Chemicals. Paclitaxel was purchased from Tecoland Corporation (Irvine, CA) and was dissolved in cremophor-vehicle (1:1:18 ratio of cremophor/ethanol/saline). cremophor EL, ethanol, dimethyl sulfoxide, and acetone were purchased from Sigma-Aldrich (St. Louis, MO). Alkamuls EL-620 was obtained from Rhodia (Cranbury, NJ). Saline was purchased from Aqualite System (Hospira, Lake Forest, IL). (–)-CP55,940 (CP55,940) (Compton et al., 1992) was provided by the National Institute on Drug Abuse or purchased from Santa Cruz Biotechnology (Dallas, TX). Rimonabant (Yoshioka et al., 1989) was provided by the National Institute on Drug Abuse. AM630 (6-iodopravadoline) (Ross et al., 1999) was purchased from Cayman Chemical Company (Ann Arbor, MI). CP55,940, rimonabant, and AM630 were dissolved in vehicle (5:2:2:16 ratio of dimethylsulfoxide/alkamuls EL-620/ethanol/saline) and were administered intraperitoneally to mice in a volume of 5 ml/kg.

General Experimental Protocol. Animals were randomly assigned to experimental groups and tested by an experimenter blinded to experimental conditions. Paclitaxel (4 mg/kg, *i.p.*) was administered four times on days 0, 2, 4, and 6 after initiation of paclitaxel dosing in a volume of 6.67 ml/kg (cumulative dose: 16 mg/kg, *i.p.*) to induce neuropathy, as previously described (Ward et al., 2011; Deng et al., 2015). The control group received an equivalent volume of CR-vehicle. Development of paclitaxel-induced mechanical and cold allodynia was assessed on days 0, 4, 7, and 15 after the initiation of paclitaxel dosing.

Effects of pharmacological manipulations were evaluated over 9 consecutive days of repeated once-daily injections. Chronic dosing was initiated during the maintenance phase of paclitaxel-induced allodynia (*i.e.*, from day 16 to day 24 after initial paclitaxel injection), and behavioral responsiveness was evaluated 30 minutes after injection of drug or vehicle. In experiment 1, we evaluated the antiallodynic effects of chronic CP55,940 (0.3 mg/kg daily, *i.p.* × 8 days) in paclitaxel-treated CB₁KO, CB₂KO, and respective WT littermates. Responsiveness to mechanical and cold stimulation was evaluated on treatment days 1, 4, and 8. To examine the time course of the development of antinociceptive tolerance, a subset of WT animals were treated with CP55,940 (0.3 mg/kg daily, *i.p.* × 16 days) and assessed for mechanical and cold responsiveness on treatment days 1, 4, 8, 11, and 16.

In experiment 2, we investigated possible side effects of CP55,940 (0.3 mg/kg daily, *i.p.* × 9 days) in paclitaxel-treated CB₁KO and WT (CD1) mice. Rectal temperature was evaluated on treatment days 2 and 7. CB₁-mediated cannabinoid withdrawal symptoms (*i.e.*, paw tremors, headshakes, and scratching bouts) elicited by challenge with rimonabant (10 mg/kg, *i.p.*) in comparison with vehicle were examined on treatment day 9.

In experiment 3, we assessed the acute effects of CP55,940 (0.3, 1, 3, and 10 mg/kg, *i.p.*) on mechanical and cold allodynia in paclitaxel-treated CB₁KO mice. Both CB₁KO and WT mice receiving CP55,940 (0.3 and 3 mg/kg, *i.p.*) were evaluated for catalepsy in the ring test. CP55,940 (3 mg/kg, *i.p.*) produced motor impairment and sedation in WT littermates that precluded the assessment of responsiveness to mechanical and cold stimulation.

In experiment 4, we assessed the antiallodynic effects and possible side effects of chronic CP55,940 (3 mg/kg daily, *i.p.* × 9 days) in paclitaxel-treated CB₁KO mice. Responsiveness to mechanical and cold stimulation was evaluated on treatment days 1, 4, and 8. Rectal temperature was evaluated on treatment days 2 and 7. Withdrawal symptoms evoked by rimonabant (10 mg/kg, *i.p.*) challenge were examined on treatment day 9.

In experiment 5, we examined the receptor mechanism underlying the antiallodynic effects of CP55,940 (3 mg/kg daily, i.p. $\times$ 8 days) in CB₁KO mice. Paclitaxel-treated CB₁KO mice that received vehicle, CP55,940 (3 mg/kg daily, i.p. $\times$ 8 days) alone or coadministered with AM630 (5 mg/kg daily, i.p. $\times$ 8 days) were examined.

Assessment of Mechanical Allodynia. Withdrawal thresholds (in grams) to mechanical stimulation were measured in duplicate for each paw using an electronic von Frey anesthesiometer supplied with a 90-g range probe (IITC Life Science Inc., Woodland Hills, CA) as described previously (Deng et al., 2012, 2015). Briefly, mice were individually placed in transparent plastic chambers on an elevated metal mesh table and habituated to the testing apparatus for 30 minutes before testing. When animals ceased exploratory behaviors, a force was applied to the midplantar region of the hind paw by a semiflexible tip connected to the anesthesiometer. Mechanical stimulation was terminated on paw withdrawal.

Assessment of Cold Allodynia. Response time (in seconds) to cold stimulation was assessed in triplicate for each paw by the acetone method as described previously (Deng et al., 2015). Briefly, mice were individually placed underneath transparent plastic chambers on an elevated metal mesh table. After habituation, an acetone bubble that formed at the end of a blunt 1-ml syringe was gently presented onto the plantar surface of the hind paw. Time that the animal spent attending to (i.e., elevating, licking, biting, or shaking) the acetone-stimulated paw was measured over a 60-second observation period.

Evaluation of Cannabinoid Withdrawal Symptoms. WT mice were treated chronically with vehicle or CP55,940 (0.3 mg/kg per day, i.p.) for 9 days. CB₁KO mice were treated chronically with vehicle or CP55,940 (0.3 or 3 mg/kg daily, i.p.) for 9 days. On treatment day 9, 30 minutes after the final treatment injection, animals were first challenged with vehicle and 30 minutes later challenged with the CB₁ antagonist rimonabant (10 mg/kg, i.p.). Mice were video-recorded, and the numbers of paw tremors, headshakes, and scratching bouts were scored over 30 minutes after each challenge by an experimenter blinded to experimental conditions, as previously described (Cook et al., 1998; Deng et al., 2015).

Rectal Temperature. Rectal temperature ($^{\circ}$ C) was measured using a thermometer (Physitemp Instruments Inc., Clifton, NJ) equipped with a mouse rectal probe (Braintree Scientific Inc., Braintree, MA) as previously described (Deng et al., 2015).

Ring Test. Catalepsy was assessed using the ring test as previously described (Pertwee, 1972). Immobility latency (in seconds) that the animal spent motionless on the ring during a 5-minute observation period was recorded.

Statistical Analyses. Analysis of variance for repeated measures was used to determine the time course of allodynia and chronic drug effects. The sphericity-assumed correction was applied to all repeated factors; degrees of freedom for significant interactions are reported as uncorrected values. One-way analysis of variance was used to identify the source of significant interactions and compare postinjection responses with prepaclitaxel baselines, followed by Bonferroni post hoc tests or two-tailed *t* tests, as appropriate. No gender differences were detected in chemotherapy-induced responses to mechanical or cold stimulation ($P > 0.41$ for all comparisons) or in the effects of CP55,940 ($P > 0.15$ for all comparisons); therefore, results from both genders were pooled for statistical analyses. Statistical analyses were performed using IBM-SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered significant.

Results

Paclitaxel-Induced Allodynia Developed Similarly in WT, CB₁KO, and CB₂KO Mice. Before paclitaxel treatment, there were no genotype or gender differences in responses to mechanical (CB₁: $P = 0.70$, Fig. 1A; CB₂: $P = 0.22$; Fig. 1C) or cold stimulation (CB₁: $P = 0.15$, Fig. 1B; CB₂: $P = 0.89$; Fig. 1D).

Paclitaxel decreased thresholds to mechanical stimulation ($F_{3,62} = 72.33$, $P < 0.0001$; Fig. 1A) and increased response

time to cold stimulation ($F_{3,62} = 19.04$, $P < 0.0001$; Fig. 1B) in CB₁KO and WT littermates. Similarly, paclitaxel induced hypersensitivity to mechanical ($F_{3,33} = 140.24$, $P < 0.0001$; Fig. 1C) and cold ($F_{3,88} = 71.71$, $P < 0.0001$; Fig. 1D) stimulation in both CB₂KO and WT littermates. Mechanical and cold allodynia were established in mice treated with paclitaxel relative to CR-vehicle group beginning on day 4 and were sustained throughout the testing interval in CB₁KO, CB₂KO, and WT mice ($P < 0.0001$). Paclitaxel-induced mechanical and cold allodynia developed equivalently in CB₁KO and WT mice, as well as in CB₂KO and WT mice across all time points ($P = 1.00$), consistent with our previously published work (Deng et al., 2015).

Chronic Low-Dose CP55,940 Suppressed Paclitaxel-Induced Allodynia in WT but Not in CB₁KO Mice. In WT mice, chronic low-dose treatment with CP55,940 (0.3 mg/kg daily, i.p.) reversed paclitaxel-induced mechanical ($F_{1,9} = 91.73$, $P < 0.0001$; Fig. 2A) and cold ($F_{1,9} = 26.84$, $P < 0.002$; Fig. 2B) allodynia relative to vehicle ($P < 0.0001$ for all comparisons) and preinjection levels ($F_{4,36} = 18.58$, $P < 0.0001$ mechanical; $F_{4,36} = 17.11$, $P < 0.0001$ cold). The antiallodynic effects of low-dose CP55,940 were stable from treatment day 1 to day 8 ($F_{2,18} = 0.42$, $P = 0.66$ mechanical; $F_{2,18} = 1.50$, $P = 0.25$ cold). Low-dose CP55,940 fully suppressed paclitaxel-induced allodynia and restored responses to prepaclitaxel baseline levels ($F_{3,2} = 2.24$, $P = 0.12$ mechanical; $F_{3,20} = 0.93$, $P = 0.44$ cold; Fig. 2, A and B) in WT mice.

By contrast, in CB₁KO mice, CP55,940 (0.3 mg/kg daily, i.p.) failed to attenuate paclitaxel-evoked mechanical ($F_{1,8} = 0.31$, $P = 0.60$; Fig. 2C) or cold hypersensitivities ($F_{1,8} = 1.79$, $P = 0.22$; Fig. 2D) relative to vehicle ($P > 0.07$ for all comparisons) on any day ($F_{4,32} = 0.93$, $P = 0.46$ mechanical; $F_{4,32} = 0.98$, $P = 0.43$ cold).

Chronic Low-Dose CP55,940 Attenuated Paclitaxel-Induced Allodynia in CB₂KO Mice. Chronic low-dose treatment with CP55,940 (0.3 mg/kg daily, i.p.) suppressed paclitaxel-induced mechanical ($F_{3,21} = 88.71$, $P < 0.0001$; Fig. 2E) and cold ($F_{3,21} = 62.59$, $P < 0.0001$; Fig. 2F) allodynia relative to vehicle ($P < 0.0001$ for all comparisons) and preinjection levels ($F_{12,84} = 30.20$, $P < 0.0001$ mechanical; $F_{12,84} = 20.81$, $P < 0.0001$ cold) in both CB₂KO and WT mice. CP55,940-induced antiallodynic effects were stable from treatment day 1 to 8 ($F_{6,42} = 0.67$, $P = 0.67$ mechanical; $F_{6,42} = 0.46$, $P = 0.83$ cold) and normalized responses to prepaclitaxel levels (WT: $F_{3,24} = 0.15$, $P = 0.93$ mechanical, $F_{3,24} = 0.96$, $P = 0.43$ cold; CB₂KO: $F_{3,16} = 1.19$, $P = 0.34$ mechanical, $F_{3,16} = 0.41$, $P = 0.75$ cold; Fig. 2, E and F). Low-dose CP55,940 reversed paclitaxel-induced allodynia with similar efficacy in CB₂KO and WT mice at all time points ($P = 1.00$ for all comparisons; Fig. 2, E and F).

Tolerance Developed to the Antiallodynic Effects of Low-Dose CP55,940. To further evaluate whether tolerance develops to the antiallodynic effects of low-dose CP55,940 (0.3 mg/kg daily, i.p.), a subset of WT mice were tested in a 16-day once-daily chronic dosing paradigm. In WT mice, CP55,940 (0.3 mg/kg daily, i.p.) suppressed paclitaxel-evoked mechanical ($F_{1,6} = 78.01$, $P < 0.0001$; Fig. 3A) and cold ($F_{1,6} = 28.58$, $P < 0.01$; Fig. 3B) allodynia relative to vehicle in a time-dependent manner ($F_{6,36} = 14.53$, $P < 0.0001$ mechanical; $F_{6,36} = 25.08$, $P < 0.0001$ cold). CP55,940 (0.3 mg/kg daily, i.p.) no longer suppressed paclitaxel-induced allodynia relative to vehicle after 11 days of chronic injection ($P > 0.10$ for all comparisons) in WT mice (Fig. 3, A and B).

Fig. 1. Development of paclitaxel-induced painful neuropathy. (A and C) Mechanical and (B and D) cold allodynia developed equivalently in (A and B) CB₁KO, (C and D) CB₂KO, and corresponding WT littermates after paclitaxel treatment. Nonchemotherapy controls received CR-vehicle in lieu of paclitaxel. Arrows show timing of paclitaxel or CR-vehicle injections (inj). Data are expressed as mean $\pm$ S.E.M. ($n = 5-31$ per group). * $P < 0.05$ versus CR-vehicle control, repeated measures analysis of variance (ANOVA), and one-way ANOVA at each time point.

Chronic Low-Dose CP55,940 Produced Transient Hypothermia in Paclitaxel-Treated WT but Not CB₁KO Mice. Chronic low-dose administration of CP55,940 (0.3 mg/kg daily, i.p.) decreased body temperature relative to vehicle on treatment day 2 ($P < 0.0001$), but not day 7 ($P = 0.20$), in paclitaxel-treated WT mice (Fig. 4A). Thus, tolerance developed to the hypothermic effect of low-dose CP55,940 after repeated dosing. By contrast, the same dosing paradigm failed to alter body temperature in paclitaxel-treated CB₁KO mice on any day tested ($P > 0.07$; Fig. 4B), consistent with classic CB₁-mediated hypothermia.

Chronic Low-Dose CP55,940 Produced Cannabinoid Withdrawal Signs in Paclitaxel-Treated WT but Not CB₁KO Mice. In paclitaxel-treated WT mice, rimonabant (10 mg/kg, i.p.) challenge produced prototypical CB₁-mediated withdrawal signs, such as paw tremors ($F_{3,20} = 57.79$, $P < 0.0001$) and headshakes ($F_{3,20} = 8.59$, $P < 0.002$) in mice treated chronically with low-dose CP55,940 (0.3 mg/kg daily, i.p.) relative to vehicle challenge ($P < 0.01$) and relative to the vehicle group ($P < 0.01$; Fig. 5A). Challenge with rimonabant, but not vehicle, elicited scratching behaviors in paclitaxel-treated WT animals that received chronic administration of

vehicle or CP55,940 ($F_{3,20} = 7.88$, $P < 0.002$); WT mice treated with chronic CP55,940 (0.3 mg/kg daily, i.p.) showed more rimonabant-produced scratching behaviors compared with the vehicle group ($P < 0.05$; Fig. 5A).

By contrast, rimonabant challenge did not elicit paw tremors ($F_{3,12} = 0.62$, $P = 0.62$), headshakes ($F_{3,12} = 1.80$, $P = 0.20$), or scratching bouts ($F_{3,12} = 1.34$, $P = 0.31$) relative to vehicle challenge in CB₁KO mice receiving either chronic CP55,940 (0.3 mg/kg daily, i.p.) or chronic vehicle (Fig. 5B), suggesting that rimonabant-induced scratching in the vehicle-treated mice is mediated by CB₁ receptors.

Acute High-Dose CP55,940 Produced Catalepsy and Severe Hypothermia in WT but Not in CB₁KO Mice. In paclitaxel-treated WT mice, a higher dose (3 mg/kg, i.p.) of CP55,940, administered acutely, induced catalepsy ($F_{2,14} = 391.89$, $P < 0.0001$; Fig. 6A) and hypothermia ($F_{2,14} = 35.59$, $P < 0.0001$; Fig. 6C) relative to either vehicle ($P < 0.0001$) or low-dose CP55,940 (0.3 mg/kg, i.p.) treatment ($P < 0.02$). By contrast, in paclitaxel-treated CB₁KO mice, neither doses of CP55,940 (0.3 or 3 mg/kg, i.p.) altered immobility latency ($F_{2,12} = 0.75$, $P = 0.50$; Fig. 6B) or body temperature ($F_{2,12} = 1.19$, $P = 0.34$; Fig. 6D) relative to vehicle treatment.

Fig. 2. Low-dose CP55,940 suppressed paclitaxel-induced neuropathic pain in WT and CB₂KO but not in CB₁KO mice. (A–D) CP55,940 (0.3 mg/kg daily, i.p. × 8 days) reversed paclitaxel-induced (A and C) mechanical and (B and D) cold allodynia in (A and B) WT (CD1) littermates but not in (C and D) CB₁KO mice. (E–F) CP55,940 (0.3 mg/kg daily, i.p. × 8 days) attenuated paclitaxel-induced (E) mechanical and (F) cold allodynia with similar efficacy in CB₂KO and WT (C57BL/6J) littermates. BL, prepaclitaxel baseline; PTX, postpaclitaxel baseline; Veh, vehicle. Data are expressed as mean ± S.E.M. ($n = 5–7$ per group). * $P < 0.05$ versus vehicle, two-tailed t test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. # $P < 0.05$ versus prepaclitaxel baseline, repeated measures ANOVA.

Acute CP55,940 Dose-Dependently Suppressed Paclitaxel-Induced Neuropathy in CB₁KO Mice. In CB₁KO mice, acute systemic treatment with CP55,940 suppressed paclitaxel-induced mechanical ($F_{4,21} = 11.19$, $P <$

0.0001; Fig. 7A) and cold ($F_{4,21} = 12.61$, $P < 0.0001$; Fig. 7B) allodynia in a dose-dependent manner. Low-dose CP55,940 (0.3 mg/kg, i.p.) failed to attenuate paclitaxel-evoked allodynia relative to vehicle ($P > 0.84$ for both modalities) in CB₁KO mice

Fig. 3. Tolerance developed to the anti-allodynic effects of low-dose CP55,940 in WT mice. CP55,940 (0.3 mg/kg per day, i.p.) failed to suppress paclitaxel-induced (A) mechanical or (B) cold allodynia on day 11 of chronic dosing in WT mice. BL, prepaclitaxel baseline; PTX, postpaclitaxel baseline; Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4$ per group). * $P < 0.05$ versus vehicle, two-tailed t test. # $P < 0.05$ versus prepaclitaxel baseline, repeated measures analysis of variance.

(Fig. 7, A and B). Medium-dose CP55,940 (1 mg/kg, i.p.) partially attenuated paclitaxel-induced mechanical and cold hypersensitivities in CB₁KO mice ($P < 0.01$; Fig. 7, A and B). Higher doses of CP55,940 (3 or 10 mg/kg, i.p.) fully reversed paclitaxel-induced mechanical and cold allodynia in CB₁KO mice relative to vehicle ($P < 0.0001$) and normalized responses to prepaclitaxel levels ($P > 0.49$; Fig. 7, A and B). WT mice treated with CP55,940 (3 mg/kg, i.p.) exhibited severe catalepsy and thus were not used for the assessments of mechanical and cold allodynia.

Chronic High-Dose CP55,940 Suppressed Paclitaxel-Induced Allodynia in CB₁KO Mice. In CB₁KO mice, chronic high-dose treatment with CP55,940 (3 mg/kg daily, i.p.) reversed paclitaxel-induced mechanical ($F_{1,10} = 110.07$, $P < 0.000$; Fig. 8A) and cold ($F_{1,10} = 98.41$, $P < 0.0001$; Fig. 8B) hypersensitivities relative to vehicle ($P < 0.0001$ for all comparisons) and preinjection levels ($F_{4,40} = 24.88$, $P < 0.0001$ mechanical; $F_{4,40} = 60.38$, $P < 0.0001$ cold). The antiallodynic effects of high-dose CP55,940 were stable throughout the chronic dosing regimen ($F_{2,20} = 0.41$, $P = 0.67$ mechanical; $F_{2,20} = 0.74$, $P = 0.49$ cold). High-dose CP55,940 fully suppressed paclitaxel-induced allodynia and restored responses to prepaclitaxel baseline levels ($F_{3,24} = 0.31$, $P = 0.82$ mechanical; $F_{3,24} = 0.97$, $P = 0.51$ cold; Fig. 8, A and B).

Chronic high-dose treatment with CP55,940 (3 mg/kg daily, i.p.) did not alter body temperature in paclitaxel-treated CB₁KO mice on any day tested ($F_{1,6} = 0.79$, $P = 0.41$; Fig. 8C). Moreover, rimonabant challenge did not induce paw tremors ($F_{3,12} = 1.37$, $P = 0.30$), headshakes ($F_{3,12} = 0.40$, $P = 0.75$), or scratching bouts ($F_{3,12} = 0.14$, $P = 0.93$) relative to vehicle

challenge in CB₁KO mice receiving chronic treatment with either CP55,940 (3 mg/kg daily, i.p.) or vehicle (Fig. 8D).

Antiallodynic Effects of High-Dose CP55,940 in CB₁KO Mice Were Mediated by CB₂ Receptors. In paclitaxel-treated CB₁KO mice, the antiallodynic effects of high-dose CP55,940 (3 mg/kg daily, i.p.) on mechanical ($F_{3,17} = 88.50$, $P < 0.0001$; Fig. 9A) and cold ($F_{3,17} = 59.44$, $P < 0.0001$; Fig. 9B) hypersensitivities were blocked by the CB₂ antagonist AM630 (5 mg/kg daily, i.p.) at all time points ($P < 0.0001$). AM630 (5 mg/kg daily, i.p.) alone did not alter mechanical or cold responsiveness relative to vehicle ($P = 1.00$ for all comparisons) in CB₁KO mice (Fig. 9, A and B).

Discussion

Psychotropic effects have represented significant hurdles for advancing cannabinoids as pharmacotherapies (Dhopeswarkar and Mackie, 2014). Tolerance develops to CB₁-mediated locomotor effects of CP55,940 and Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and is accompanied by downregulation of cannabinoid receptor binding sites in the absence of nerve injury (Oviedo et al., 1993). If tolerance develops differentially to psychotropic effects and therapeutic effects of cannabinoids, the clinical potential of these agents would be enhanced. A better understanding of the receptor mechanisms underlying the therapeutic and side effect profiles of cannabinoids observed with chronic dosing may improve current pharmacotherapies and validate novel targets (Ben Amar, 2006; Pertwee, 2009). In the present study, we used CP55,940, a potent synthetic cannabinoid with equal affinities for mouse CB₁ and CB₂ receptors in vitro (Abood et al., 1997; Griffin et al., 2000), and

Fig. 4. Chronic low-dose CP55,940 produced transient hypothermia in WT but not CB₁KO mice. (A) CP55,940 (0.3 mg/kg daily, i.p. $\times$ 8 days) decreased body temperature in paclitaxel-treated WT mice on treatment day 2 but not day 7. (B) CP55,940 (0.3 mg/kg daily, i.p. $\times$ 8 days) did not alter body temperature in paclitaxel-treated CB₁KO mice. Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4-6$ per group). * $P < 0.05$ versus vehicle, two-tailed t test.

Fig. 5. Rimonabant precipitated withdrawal signs in paclitaxel-treated WT but not CB₁KO mice receiving chronic low-dose CP55,940. Challenge with the CB₁ antagonist rimonabant (10 mg/kg, i.p.) elicited paw tremors, headshakes, and scratching bouts in paclitaxel-treated (A) WT but not (B) CB₁KO mice receiving CP55,940 (0.3 mg/kg daily, i.p. $\times$ 9 days). PTX, postpaclitaxel baseline; Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4-7$ per group). ^{*} $P < 0.05$ versus Veh + Rim (chronic vehicle treatment and challenged by rimonabant); ^s $P < 0.05$ versus Veh + Veh (chronic vehicle treatment and challenged by vehicle), one-way analysis of variance followed by Bonferroni post hoc test or two-tailed t test.

knockout mice to study the pharmacological effects associated with activation of distinct cannabinoid receptor subtypes in a model of paclitaxel-induced neuropathic pain. CP55,940 at a dose of 0.3 mg/kg i.p. suppressed paclitaxel-induced allodynia and produced undesirable side effects (i.e., hypothermia and physical withdrawal) through activation of CB₁ receptors. Interestingly, we unmasked a novel CB₂-mediated component of CP55,940-induced antiallodynic effects through the use of

CB₁KO mice. CP55,940 at a dose of 3 mg/kg i.p. (i.e., 10 times higher than the dose producing CB₁-mediated pharmacological effects in WT mice) activated CB₂ receptors and produced antiallodynic effects in CB₁KO mice. This desirable therapeutic profile of CB₂-mediated antiallodynic efficacy was sustained after chronic dosing and was not accompanied by adverse cannabinimimetic effects typical of CB₁ receptor activation.

Fig. 6. High-dose CP55,940 produced catalepsy and hypothermia in paclitaxel-treated WT but not CB₁KO mice. (A and C) Acute high-dose treatment with CP55,940 (3 mg/kg, i.p.) induced (A) catalepsy and (C) hypothermia in paclitaxel-treated WT mice. (B and D) Acute high-dose treatment with CP55,940 (3 mg/kg, i.p.) did not induce (B) catalepsy or (D) hypothermia in paclitaxel-treated CB₁KO mice. PTX, paclitaxel; Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4-7$ per group). ^{*} $P < 0.05$ versus vehicle; ^{**} $P < 0.05$ versus CP55,940 (0.3 mg/kg, i.p.), one-way analysis of variance followed by Bonferroni post hoc test.

Fig. 7. Acute CP55,940 produced dose-dependent anti-allodynic effects in paclitaxel-treated CB₁KO mice. Dose response of CP55,940 (0.3, 1, 3, and 10 mg/kg, i.p.) on paclitaxel-evoked (A) mechanical and (B) cold allodynia in CB₁KO mice. BL, prepaclitaxel baseline; Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4-7$ per group). * $P < 0.05$ versus vehicle; * $P < 0.05$ versus CP55,940 (1 mg/kg, i.p.), one-way analysis of variance followed by Bonferroni post hoc test.

In our study, low-dose CP55,940 (0.3 mg/kg per day, i.p.) suppressed paclitaxel-induced allodynia in WT and CB₂KO, but not in CB₁KO mice, suggesting that the antiallodynic effects at this low dose were mediated solely by CB₁, without a contribution from CB₂ receptors. Moreover, activation of CB₁ receptors was sufficient to fully reverse paclitaxel-induced neuropathic pain behaviors. Our results confirmed a previous study showing that CP55,940 at this dose produces

CB₁-mediated antinociception in various pain models (Sain et al., 2009). These results are in agreement with previous reports suggesting that CP55,940 produces antinociceptive effects via CB₁ receptors (Lichtman and Martin, 1997; Romero et al., 2002; Choong et al., 2007; Sain et al., 2009). Mixed cannabinoid agonists can produce CB₁-mediated antinociception through central and peripheral mechanisms (Fox et al., 2001; Lim et al., 2003) and suppress central

Fig. 8. In CB₁KO mice, high-dose CP55,940 suppressed paclitaxel-induced allodynia without producing hypothermia or rimonabant-elicited precipitated withdrawal symptoms. (A and B) CP55,940 (3 mg/kg day, i.p. $\times$ 8 days) reversed paclitaxel-induced (A) mechanical and (B) cold allodynia in CB₁KO mice. (C and D) CP55,940 (3 mg/kg day, i.p. $\times$ 9 days) did not alter (C) body temperature or (D) produce rimonabant-elicited precipitated withdrawal symptoms in CB₁KO mice. BL, pre paclitaxel baseline; PTX, postpaclitaxel baseline; Veh, vehicle. Data are expressed as mean $\pm$ S.E.M. ($n = 4-7$ per group). * $P < 0.05$ versus vehicle, two-tailed t test. * $P < 0.05$ versus prepaclitaxel baseline, repeated-measures analysis of variance.

Fig. 9. Antiallodynic effects of high-dose CP55,940 in CB₁KO mice were mediated by CB₂ receptors. In CB₁KO mice, high-dose CP55,940 (3 mg/kg daily, i.p. × 8 days)-induced suppressions of paclitaxel-evoked (A) mechanical and (B) cold allodynia were blocked by the CB₂ antagonist AM630 (5 mg/kg daily, i.p. × 8 days). BL, prepaclitaxel baseline; PTX, postpaclitaxel baseline; Veh, vehicle. Data are expressed as mean ± S.E.M. ($n = 4-7$ per group). * $P < 0.05$ versus vehicle; * $P < 0.05$ versus CP55,940 (3 mg/kg, i.p.), one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. # $P < 0.05$ versus prepaclitaxel baseline, repeated measures ANOVA.

sensitization in spinal dorsal horn neurons (Chapman, 2001) in neuropathic pain models.

CP55,940 at a 10-fold higher dose (3 mg/kg daily, i.p.) produced severe catalepsy in WT but not in CB₁KO mice, consistent with previous reports on CB₁-mediated catalepsy (Oviedo et al., 1993; Lichtman and Martin, 1997). Cataleptic effects of CP55,940 were absent in CB₁KO mice; we therefore used CP55,940 in conjunction with CB₁KO mice as a tool for studying CB₂-mediated signaling in isolation from CB₁ receptors (i.e., without the confounding effects of the drug on motor behavior). CP55,940 administered at the higher dose produced antiallodynic effects in CB₁KO mice. Moreover, these antiallodynic effects were blocked by the CB₂ antagonist AM630, suggesting that CP55,940 at the higher dose engages CB₂ receptors to produce antiallodynic effects. Our results are in line with a previous study showing that both CB₁ and CB₂ receptors are involved in the actions of CP55,940 on acute pain and spinal nerve ligation-induced neuropathic pain (Scott et al., 2004). Activation of spinal and/or peripheral CB₂ receptors by CB₂ agonists, after acute or chronic administration, suppresses neuropathic pain (Yamamoto et al., 2008; Hsieh et al., 2011; Landry et al., 2012; Deng et al., 2015). CB₂ agonists are likely to suppress neuropathic nociception by downregulation of proinflammatory cytokines and chemokines (Klegeris et al., 2003; Eljaschewitsch et al., 2006; Wilkerson et al., 2012; Deng et al., 2015) as well as inhibition of central sensitization (Elmes et al., 2004; Nackley et al., 2004).

CP55,940 binds with similar affinity to mouse CB₁ ($K_d = 0.77$ nM) (Abood et al., 1997) and CB₂ receptors ($K_d = 0.73$ nM) (Griffin et al., 2000) in *in vitro* assays. This relationship also holds for human CB₁ and CB₂ receptors (Felder et al., 1995). Interestingly, in our *in vivo* study, a 10-fold higher dose of CP55,940 was required to produce CB₂-mediated antiallodynic efficacy relative to CB₁-mediated antiallodynic efficacy. Thus, low-dose CP55,940 preferentially engaged CB₁-mediated processes, consistent with the high expression levels of CB₁ compared with CB₂ in the CNS (Galiegue et al., 1995; Schatz et al., 1997; Marsicano and Lutz, 1999; Onaivi et al., 2006). At a higher dose, CP55,940 suppresses pain by triggering CB₂ signaling in addition to the CB₁-mediated mechanism. Indeed, in an inflammatory pain model, CB₂ receptors are involved in the peripheral antihyperalgesic actions of a mixed CB₁/CB₂ agonist WIN55,212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-

naphthalenyl-methanone], when administered locally in the paw, under conditions in which central CB₁ receptors would not be activated (Nackley et al., 2003). We postulate that the higher dose of systemic CP55,940 activates both CB₁ and CB₂ receptors, but the beneficial antinociceptive effects (mediated by both CB₁ and CB₂) are masked by CB₁-mediated cataleptic effects.

As expected, CP55,940 produced hypothermia in WT, but not CB₁KO mice (McGregor et al., 1996; Rawls et al., 2002; Varvel et al., 2005). By contrast, chronic CP55,940, at a dose that produced CB₂-mediated antiallodynic efficacy, failed to decrease body temperature in CB₁KO mice, documenting that prolonged activation of CB₂ receptors does not result in hypothermia (Malan et al., 2001; Valenzano et al., 2005; Yao et al., 2009; Elliott et al., 2011; Kinsey et al., 2011; Amenta et al., 2012). Chronic CP55,940-treated WT, but not CB₁KO mice, showed profound withdrawal signs when challenged with the CB₁ antagonist rimonabant, suggesting precipitation at CB₁ receptors produces withdrawal symptoms (Tsou et al., 1995; Aceto et al., 1996; Cook et al., 1998; Rubino et al., 1998; Lichtman et al., 2001). Interestingly, although rimonabant challenge preferentially increased scratching bouts in mice treated with CP55,940 compared with vehicle, rimonabant-elicited scratching was notably absent in CB₁KO mice, demonstrating that antagonist-induced scratching [analogous to pruritis (Proietto et al., 2010)] in the absence of chronic cannabinoid dosing is mediated by CB₁ receptors, rather than an off-target effect of rimonabant. Our studies are the first to evaluate possible signs of physical dependence in animal pain models associated with repeated systemic activation of CB₂ receptors [present data and (Deng et al., 2015)]. These studies provide strong evidence that activation of CB₂ receptors produce substantial antiallodynic efficacy independent of CB₁ receptors.

One of the common features of G-protein-coupled receptors is that prolonged exposure to their agonists lead to the development of tolerance (Taylor and Fleming, 2001; Martin et al., 2004). A striking observation of our study was that tolerance to the therapeutic effects of CP55,940 (0.3 mg/kg daily, i.p.) occurred later than tolerance to its psychotropic effects. Our results, along with published reports (Bass and Martin, 2000; McKinney et al., 2008; Nguyen et al., 2012), suggested that the time course of tolerance may vary between different CB₁-mediated pharmacological effects (e.g., analgesia,

hypothermia, hypoactivity). Interestingly, using the same mouse model of paclitaxel-induced neuropathy, complete tolerance developed to both antinociceptive and hypothermic effects of the prototypical cannabinoid Δ^9 -THC over 8 days (Deng et al., 2015). CP55,940 and Δ^9 -THC differ in potency at CB₁ receptors (Wiley et al., 1995; Darmani et al., 2007) and tolerance development (De Vry et al., 2004). Ligand-dependent differences in tolerance development for different pharmacological effects may be attributed to the different signaling pathways recruited by CB₁ receptors (Martin et al., 2004) and regionally specific differences in receptor density and/or efficacy (Oviedo et al., 1993; McKinney et al., 2008). For instance, CP55,940 and Δ^9 -THC differ in CB₁ receptor internalization (Hsieh et al., 1999) and inhibition of adenylyl cyclase activity (Sim et al., 1995; Childers and Deadwyler, 1996; Fan et al., 1996; Breivogel et al., 1998; Rubino et al., 2000a,b), whereas these ligands act similarly with respect to other CB₁ signaling pathways, such as extracellular-signal-regulated kinase activation (Rubino et al., 2005; Daigle et al., 2008). More studies that correlate behavior observations and functional signaling changes are needed to understand tolerance associated with CB₁ receptors. In addition to CB₁-associated tolerance, the present report also asked whether the high-dose CP55,940 treatment in CB₁KO mice would produce tolerance to CB₂-mediated antiallodynic effects. Notably, no decrement in CB₂-mediated antiallodynic effects was observed in CB₁KO mice treated with daily administration of the high-dose CP55,940, similar to previous reports with CB₂-preferring agonists (Yao et al., 2009; Deng et al., 2015).

In conclusion, the present report demonstrated distinct roles of cannabinoid receptor subtypes in mediating the beneficial and adverse effects of CP55,940 in an animal model of paclitaxel-induced neuropathy. CP55,940 suppressed the maintenance of paclitaxel-induced neuropathic pain through both CB₁- and CB₂-dependent mechanisms. CB₂-mediated antinociceptive effects were engaged at doses approximately 10 times higher than those required to produce CB₁-mediated antinociception. On the other hand, CB₁ but not CB₂ receptors were engaged in CP55,940-produced hypothermia, catalepsy, and cannabimimetic physical withdrawal. Our results further demonstrate that CB₂ receptors represent a potential therapeutic target for effectively and safely managing chemotherapy-induced neuropathic pain without unwanted effects.

Authorship Contributions

Participated in research design: Deng, Mackie, Hohmann.

Conducted experiments: Deng, Cornett.

Performed data analyses: Deng.

Wrote or contributed to the writing of the manuscript: Deng, Mackie, Hohmann.

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