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CB_1 knockout mice unveil sustained CB_2-mediated anti-allodynic effects of the mixed CB_1/CB_2 agonist CP55,940 in a mouse model of paclitaxel-induced neuropathic pain

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Running title
Distinct roles of CB_1 and CB_2 in modulating neuropathy

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Nonstandard Abbreviations
AM630, 6-iodopravadoline; ANOVA, analysis of variance; BL, baseline; cAMP, cyclic adenosine monophosphate; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CNS, central nervous system; CP55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; CR, cremophor; Δ^9-THC, Δ^9-tetrahydrocannabinol; DMSO, dimethyl sulfoxide; ERK, extracellular-signal-regulated kinase; GPCRs , G-protein-coupled receptors; i.p., intraperitoneal; KO, knock out; rimonabant, SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; NIDA, National Institute on Drug Abuse; PTX, paclitaxel; WT, wildtype.

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Abstract

Cannabinoids suppress neuropathic pain through activation of cannabinoid CB_1 and/or CB_2 receptors. However, unwanted CB_1-mediated cannabimimetic effects limit clinical use. We asked whether CP55,940, a potent cannabinoid that binds with similar affinity to CB_1 and CB_2 *in vitro*, produces functionally separable CB_1- and CB_2-mediated pharmacological effects *in vivo*. We evaluated anti-allodynic effects, possible tolerance, and cannabimimetic effects (i.e., hypothermia, catalepsy, and CB_1-dependent withdrawal signs) following systemic CP55,940 treatment in a mouse model of toxic neuropathy produced by a chemotherapeutic agent paclitaxel. The contribution of CB_1 and CB_2 receptors to *in vivo* actions of CP55,940 was evaluated using CB_1 knockout (CB_1KO), CB_2 knockout (CB_2KO), and wildtype (WT) mice. Low dose CP55,940 (0.3 mg/kg/day i.p.) suppressed paclitaxel-induced allodynia in WT and CB_2KO, but not CB_1KO mice. Low dose CP55,940 also produced hypothermia and rimonabant-precipitated withdrawal in WT, but not CB_1KO mice. In WT mice, tolerance developed to CB_1-mediated hypothermic effects of CP55,940 earlier than to anti-allodynic effects. High dose CP55,940 (10 mg/kg/day i.p.) produced catalepsy in WT mice, which precluded determination of anti-allodynic efficacy, but produced sustained CB_2-mediated suppression of paclitaxel-induced allodynia in CB_1KO mice; these anti-allodynic effects were blocked by the CB_2 antagonist AM630. High dose CP55,940 did not produce hypothermia or rimonabant-precipitated withdrawal in CB_1KO mice. Our results using the mixed CB_1/CB_2 agonist CP55,940 document that CB_1 and CB_2 receptor activations produce mechanistically distinct suppression of neuropathic pain. Our study highlights

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the therapeutic potential of targeting cannabinoid CB_2 receptors to bypass unwanted central effects associated with CB_1 receptor activation.

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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 (Guindon and Hohmann, 2009; Herzberg et al., 1997; Ibrahim et al., 2003; Kinsey et al., 2009; Pertwee, 2009; Schlosburg et al., 2009). However, unwanted psychotropic effects of cannabinoids limit their potential clinical use (Ben Amar, 2006; Dhopeshwarkar and Mackie, 2014; Pertwee, 2009). Two major subtypes of cannabinoid receptors, CB_1 and CB_2, are the key receptors responsible for the pharmacological effects of cannabinoids (Mackie, 2006). Both CB_1 and CB_2 receptors are G-protein-coupled receptors (GPCRs) whose signaling pathways include inhibition of adenylyl cyclase and activation of mitogen-activated protein kinases (Mackie, 2006). CB_1 receptors are predominantly located in the central nervous system (CNS), whereas CB_2 receptors are found primarily in the immune cells (Galiegue et al., 1995; Marsicano and Lutz, 1999; Onaivi et al., 2006; Schatz et al., 1997) and are upregulated in the CNS in response to inflammation or injury (Maresz et al., 2005; Zhang et al., 2003). Evaluation of the

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receptor mechanisms underlying therapeutic and psychotropic effects of cannabinoids, following both acute and chronic administration, may facilitate the development of safe and effective cannabinoid-based pharmacotherapies (Ben Amar, 2006; Pertwee, 2009).

CP55,940 is a potent non-selective synthetic cannabinoid that has similar affinity for both CB_1 and CB_2 receptors *in vitro* (Abood et al., 1997; Felder et al., 1995; Griffin et al., 2000). Whereas CB_2 agonists exhibit strong ligand-biased selectivity for different signal transduction pathways (Atwood et al., 2012), CP55,940 is a balanced agonist that has not been found to show functional selectivity at CB_1 or CB_2 (Atwood et al., 2012; Howlett et al., 2002). Thus, CP55,940, used in combination with CB_1 knockout (CB_1KO) and CB_2 knockout (CB_2KO) mice, represents a useful pharmacological tool for studying the functions of CB_1 and CB_2 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 (Choong et al., 2007; Hohmann et al., 1999; Lichtman and Martin, 1997; Romero et al., 2002; Sain et al., 2009; Scott et al., 2004). Pharmacological antagonism of CB_1 receptors alone (Choong et al., 2007; Lichtman and Martin, 1997; Romero et al., 2002) or of both CB_1 and CB_2 receptors (Scott et al., 2004) blocks the antinociceptive effects of CP55,940 in rats. However, a study using CB_1KO and CB_2KO mice reported that the antinociceptive effects of systemic CP55,940 (at 0.3 mg/kg i.p.), administered acutely, is mediated by CB_1, but not CB_2 receptors (Sain et al., 2009). This finding has led to the conclusion that agonist activity at CB_2 is not relevant to antinociceptive effects of mixed CB_1/CB_2 agonists, at least following systemic administration. By contrast, we hypothesized that, due to the abundance of CB_1 receptors (relative to CB_2 receptors) in the CNS, higher

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doses of mixed cannabinoids are required to activate CB_2 receptors (compared to the dose that is sufficient to activate CB_1) and that CB_2-mediated antinociceptive effects, rather than being absent, are masked by CB_1-mediated catatonia associated with mixed cannabinoid agonists. The differences between the *in vitro* and *in vivo* profiles of CP55,940 raise questions on differential roles and functions of CB_1 and CB_2 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 CB_1 and CB_2 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., CB_1KO, CB_2KO) and wildtype (WT) mice. We evaluated antinociceptive effects, possible tolerance, and typical CNS-associated side effects (i.e., hypothermia, catalepsy, and physical withdrawal) following chronic administration of CP55,940 at multiple doses. Importantly, CB_1KO mice were used to test the hypothesis that CB_2-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 CB_1 receptors are absent (i.e., in CB_1KO mice), CB_2-mediated effects can be fully characterized to ascertain the therapeutic potential of targeting CB_2 receptors.

Materials and Methods

Subjects

Adult CB_1KO and WT littermates on a CD1 background, and adult CB_2KO and WT littermates on a C57BL/6J background, weighing 26-35 g and of both sexes, were

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used in these experiments. CB_2KO (B6.129P2-CNR2(tm1Dgen/J)) and corresponding WT (C57BL/6J) mice were originally purchased from Jackson Laboratory (ME, USA). CB_1KO mice were generated as previously described (Ledent et al., 1999), whereas corresponding WT littermates were periodically outcrossed with CD1 mice (strain #022) from Charles River Laboratories (MA, USA) to maintain genetic diversity. Animals were single housed in a temperature-controlled facility (73 ± 2 °F, 45% humidity, regular 12 h light/dark cycle, lights on at 7 am), with food and water *ad libitum* provided. 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 (NJ, USA) and was dissolved in cremophor-vehicle (1:1:18 ratio of cremophor® EL/ethanol/saline). Cremophor® EL, ethanol, dimethyl sulfoxide (DMSO), and acetone were purchased from Sigma-Aldrich (MO, USA). Alkamuls EL-620 was obtained from Rhodia (NJ, USA). Saline was purchased from Aqualite System (IL, USA). (-)-CP55,940 (CP55,940) (Compton et al., 1992) was provided by the National Institute on Drug Abuse (NIDA, MD, USA) or purchased from Santa Cruz Biotechnology (TX, USA). Rimonabant (SR141716A) (Yoshioka et al., 1989) was provided by NIDA. AM630 (Ross et al., 1999) was purchased from Cayman Chemical Company (MI, USA). CP55,940, rimonabant, and AM630 were dissolved in vehicle (5:2:2:16 ratio of DMSO /alkamuls EL-620/ ethanol/ saline) and were administered intraperitoneally (i.p.) to mice in a volume of 5 ml/kg.

General experimental protocol

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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 day 0, 2, 4, 6 following 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 (Deng et al., 2015; Ward et al., 2011). The control group received an equivalent volume of cremophor-vehicle. Development of paclitaxel-induced mechanical and cold allodynia was assessed on day 0, 4, 7 and 15 following 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 post initial paclitaxel injection) and behavioral responsiveness was evaluated 30 min following injection of drug or vehicle. In Experiment #1, we evaluated anti-allodynic effects of chronic CP55,940 (0.3 mg/kg/day i.p. x 8 days) in paclitaxel-treated CB_1KO, CB_2KO, 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/day i.p. x 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/day i.p. x 9 days) in paclitaxel-treated CB_1KO and WT (CD1) mice. Rectal temperature was evaluated on treatment days 2 and 7. CB_1-mediated cannabinoid withdrawal symptoms (i.e., paw tremors, headshakes, and scratching bouts) elicited by challenge with rimonabant (10 mg/kg i.p.) in comparison to vehicle were

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examined on treatment day 9. In Experiment #3, we assessed acute effects of CP55,940 (0.3, 1, 3 and 10 mg/kg i.p.) on mechanical and cold allodynia in paclitaxel-treated CB_1KO mice. Both CB_1KO 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 anti-allodynic effects and possible side effects of chronic CP55,940 (3 mg/kg/day i.p. x 9 days) in paclitaxel-treated CB_1KO 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 anti-allodynic effects of CP55,940 (3 mg/kg/day i.p. x 8 days) in CB_1KO mice. Paclitaxel-treated CB_1KO mice that received vehicle, CP55,940 (3 mg/kg/day i.p. x 8 days) alone or co-administered with AM630 (5 mg/kg/day i.p. x 8 days) were examined.

Assessment of mechanical allodynia

Withdrawal thresholds (g) to mechanical stimulation were measured in duplicate for each paw using an electronic von Frey anesthesiometer supplied with a 90-gram range probe (IITC Life Science Inc., CA, USA) as described previously (Deng et al., 2012). Briefly, mice were individually placed in transparent plastic chambers on an elevated metal mesh table and were habituated to the testing apparatus for 30 min prior to testing. When animals ceased exploratory behaviors, a force was applied to the midplantar region

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of the hind paw by a semi-flexible tip connected to the anesthesiometer. Mechanical stimulation was terminated upon paw withdrawal.

Assessment of cold allodynia

Response time (s) 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 one 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/day i.p.) for 9 days. CB_1KO mice were treated chronically with vehicle or CP55,940 (0.3 or 3 mg/kg/day i.p.) for 9 days. On treatment day 9, at 30 min post final treatment injection, animals were first challenged with vehicle, and 30 min later challenged with the CB_1 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 min following each challenge by an experimenter blinded to experimental conditions, as previously described (Cook et al., 1998; Deng et al., 2015).

Rectal temperature

Rectal temperature (°C) was measured using a thermometer (Physitemp Instruments Inc., NJ, USA) equipped with a mouse rectal probe (Braintree Scientific Inc., MA, USA) as previously described (Deng et al., 2015).

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Ring test

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

Statistical analyses

Analysis of variance (ANOVA) 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 ANOVA was used to identify the source of significant interactions and compare post-injection responses with pre-paclitaxel baselines, followed by Bonferroni *post hoc* tests or 2-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) nor in the effects of CP55,940 ($P>0.15$ for all comparisons), and therefore results from both genders were pooled for statistical analyses. Statistical analyses were performed using IBM-SPSS Statistics version 22.0 (SPSS Inc., IL, USA). $P<0.05$ was considered significant.

Results

Paclitaxel-induced allodynia developed similarly in WT, CB_1KO, and CB_2KO mice

Prior to paclitaxel treatment, there were no genotype or gender differences in responses to mechanical (CB_1: $P=0.70$, Fig. 1A; CB_2: $P=0.22$, Fig. 1C) or cold stimulation (CB_1: $P=0.15$, Fig. 1B; CB_2: $P=0.89$, 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.

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1B) in CB_1KO 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 CB_2KO and WT littermates. Mechanical and cold allodynia were established in mice treated with paclitaxel relative to cremophor-vehicle group beginning on day 4 and were sustained throughout the testing interval in CB_1KO, CB_2KO, and WT mice (P<0.0001). Paclitaxel-induced mechanical and cold allodynia developed equivalently in CB_1KO and WT mice, as well as in CB_2KO and WT mice across all time points (P=1.000), consistent with our previously published work (Deng et al., 2015).

Chronic low dose CP55,940 suppressed paclitaxel-induced allodynia in WT but not in CB_1KO mice

In WT mice, chronic low dose treatment with CP55,940 (0.3 mg/kg/day 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 pre-injection levels ($F_{4,36}$=18.58, P<0.0001 mechanical, $F_{4,36}$=17.11, P<0.0001 cold). The anti-allodynic 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 pre-paclitaxel baseline levels ($F_{3,20}$=2.24, P=0.12 mechanical, $F_{3,20}$=0.93, P=0.44 cold, Fig. 2A-B) in WT mice.

By contrast, in CB_1KO mice, CP55,940 (0.3 mg/kg/day 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).

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Chronic low dose CP55,940 attenuated paclitaxel-induced allodynia in CB_2KO mice

Chronic low dose treatment with CP55,940 (0.3 mg/kg/day 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 pre-injection levels ($F_{12,84}$=30.20, P<0.0001 mechanical, $F_{12,84}$=20.81, P<0.0001 cold) in both CB_2KO and WT mice. CP55,940-produced anti-allodynic effects were stable from treatment day 1 to day 8 ($F_{6,42}$=0.67, P=0.67 mechanical, $F_{6,42}$=0.46, P=0.83 cold) and normalized responses to pre-paclitaxel levels (WT: $F_{3,24}$=0.15, P=0.93 mechanical, $F_{3,24}$=0.96, P=0.43 cold; CB_2KO: $F_{3,16}$=1.19, P=0.34 mechanical, $F_{3,16}$=0.41, P=0.75 cold, Fig. 2E-F). Low dose CP55,940 reversed paclitaxel-induced allodynia with similar efficacy in CB_2KO and WT mice at all time points (P=1.00 for all comparisons, Fig. 2E-F).

Tolerance developed to the anti-allodynic effects of low dose CP55,940

To further evaluate whether tolerance develops to the anti-allodynic effects of low dose CP55,940 (0.3 mg/kg/day i.p.), a subset of WT mice were tested with a 16-day once daily chronic dosing paradigm. In WT mice, CP55,940 (0.3 mg/kg/day 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/day 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. 3A-B).

Chronic low dose CP55,940 produced transient hypothermia in paclitaxel-treated WT but not CB_1KO mice

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Chronic low dose administration of CP55,940 (0.3 mg/kg/day 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 following repeated dosing. By contrast, the same dosing paradigm failed to alter body temperature in paclitaxel-treated CB_1KO mice on any day tested (P>0.07, Fig. 4B), consistent with classic CB_1-mediated hypothermia.

Chronic low dose CP55,940 produced cannabinoid withdrawal signs in paclitaxel-treated WT but not CB_1KO mice

In paclitaxel-treated WT mice, rimonabant (10 mg/kg i.p.) challenge produced prototypical CB_1-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/day 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/day i.p.) showed more rimonabant-produced scratching behaviors compared to 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 CB_1KO mice receiving either chronic CP55,940 (0.3 mg/kg/day i.p.) or chronic vehicle (Fig. 5B), suggesting that rimonabant-induced scratching in the vehicle-treated mice is mediated by CB_1 receptors.

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Acute high dose CP55,940 produced catalepsy and severe hypothermia in WT but not in CB_1KO 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 CB_1KO 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.

Acute CP55,940 dose-dependently suppressed paclitaxel-induced neuropathy in CB_1KO mice

In CB_1KO 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 CB_1KO mice (Fig. 7A-B). Medium dose CP55,940 (1 mg/kg i.p.) partially attenuated paclitaxel-induced mechanical and cold hypersensitivities in CB_1KO mice (P<0.01, Fig. 7A-B). Higher doses of CP55,940 (3 or 10 mg/kg i.p.) fully reversed paclitaxel-induced mechanical and cold allodynia in CB_1KO mice relative to vehicle (P<0.0001) and normalized responses to pre-paclitaxel levels (P>0.49, Fig. 7A-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.

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Chronic high dose CP55,940 suppressed paclitaxel-induced allodynia in CB_1KO mice

In CB_1KO mice, chronic high dose treatment with CP55,940 (3 mg/kg/day i.p.) reversed paclitaxel-induced mechanical ($F_{1,10}$=110.07, P<0.0001, 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 pre-injection levels ($F_{4,40}$=24.88, P<0.0001 mechanical, $F_{4,40}$=60.38, P<0.0001 cold). The anti-allodynic 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 pre-paclitaxel baseline levels ($F_{3,24}$=0.31, P=0.82 mechanical, $F_{3,24}$=0.97, P=0.51 cold, Fig. 8A-B).

Chronic high dose treatment with CP55,940 (3 mg/kg/day i.p.) did not alter body temperature in paclitaxel-treated CB_1KO 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 CB_1KO mice receiving chronic treatment with either CP55,940 (3 mg/kg/day i.p.) or vehicle (Fig. 8D).

Anti-allodynic effects of high dose CP55,940 in CB_1KO mice were mediated by CB_2 receptors

In paclitaxel-treated CB_1KO mice, the anti-allodynic effects of high dose CP55,940 (3 mg/kg/day 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 CB_2 antagonist AM630 (5 mg/kg/day i.p.) at all time points (P<0.0001). AM630 (5 mg/kg/day i.p.) alone

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did not alter mechanical or cold responsiveness relative to vehicle (*P*=1.00 for all comparisons) in CB_1KO mice (Fig. 9A-B).

Discussion

Psychotropic effects have represented significant hurdles for advancing cannabinoids as pharmacotherapies (Dhopeshwarkar and Mackie, 2014). Tolerance develops to CB_1-mediated locomotor effects of CP55,940 and Δ^9-tetrahydrocannabinol (Δ^9-THC) and is accompanied by down-regulation of cannabinoid receptor binding sites in the absence of nerve injury (Oviedo et al., 1993). However, 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 similar affinities for mouse CB_1 and CB_2 receptors *in vitro* (Abood et al., 1997; Griffin et al., 2000), and 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 CB_1 receptors. Interestingly, we unmasked a novel CB_2-mediated component of CP55,940-induced anti-allodynic effects through the use of CB_1KO mice. CP55,940 at a dose of 3 mg/kg i.p. (i.e., ten times higher than the dose producing CB_1-mediated pharmacological effects in WT mice) activated CB_2 receptors and produced anti-allodynic effects in CB_1KO mice.

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This desirable therapeutic profile of CB_2-mediated anti-allodynic efficacy was sustained following chronic dosing and was not accompanied by adverse cannabimimetic effects typical of CB_1 receptor activation.

In our study, low dose CP55,940 (0.3 mg/kg/day i.p.) suppressed paclitaxel-induced allodynia in WT and CB_2KO, but not in CB_1KO mice, suggesting that the anti-allodynic effects at this low dose were mediated solely by CB_1, without a contribution from CB_2 receptors. Moreover, activation of CB_1 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 CB_1-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 CB_1 receptors (Choong et al., 2007; Lichtman and Martin, 1997; Romero et al., 2002; Sain et al., 2009). Mixed cannabinoid agonists can produce CB_1–mediated antinociception through central and peripheral mechanisms (Fox et al., 2001; Lim et al., 2003), and suppress central sensitization in spinal dorsal horn neurons (Chapman, 2001) in neuropathic pain models.

CP55,940 at a ten-fold higher dose (3 mg/kg/day i.p.) produced severe catalepsy in WT but not CB_1KO mice, consistent with previous reports on CB_1-mediated catalepsy (Lichtman and Martin, 1997; Oviedo et al., 1993). Cataleptic effects of CP55,940 were absent in CB_1KO mice, we therefore used CP55,940 in conjunction with CB_1KO mice as a tool for studying CB_2-mediated signaling in isolation from CB_1 receptors (i.e., without the confounding effects of the drug on motor behavior). CP55,940 administered at the higher dose produced anti-allodynic effects in CB_1KO mice. Moreover, these anti-allodynic effects were blocked by the CB_2 antagonist AM630, suggesting that CP55,940

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at the higher dose engages CB_2 receptors to produce anti-allodynic effects. Our results are in line with a previous study showing that both CB_1 and CB_2 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 CB_2 receptors by CB_2 agonists, following acute or chronic administration, suppresses neuropathic pain (Deng et al., 2015; Hsieh et al., 2011; Landry et al., 2012; Yamamoto et al., 2008). CB_2 agonists are likely to suppress neuropathic nociception by down-regulation of pro-inflammatory cytokines and chemokines (Deng et al., 2015; Eljaschewitsch et al., 2006; Klegeris et al., 2003; Wilkerson et al., 2012) as well as inhibition of central sensitization (Elmes et al., 2004; Nackley et al., 2004).

CP55,940 binds with similar affinity to mouse CB_1 (Kd = 0.77 nM) (Abood et al., 1997) and CB_2 receptors (Kd = 0.73 nM) (Griffin et al., 2000) in *in vitro* assays. This relationship also holds for human CB_1 and CB_2 receptors (Felder et al., 1995). Interestingly, in our *in vivo* study, a ten-fold higher dose of CP55,940 was required to produce CB_2-mediated anti-allodynic efficacy relative to CB_1-mediated anti-allodynic efficacy. Thus, low dose CP55,940 preferentially engaged CB_1-mediated processes, consistent with the high expression levels of CB_1 compared to CB_2 in the CNS (Galiegue et al., 1995; Marsicano and Lutz, 1999; Onaivi et al., 2006; Schatz et al., 1997). At a higher dose, CP55,940 suppresses pain by triggering CB_2 signaling in addition to the CB_1-mediated mechanism. Indeed, in an inflammatory pain model, CB_2 receptors are involved in the peripheral antihyperalgesic actions of a mixed CB_1/CB_2 agonist WIN55,212-2, when administered locally in the paw, under conditions in which central CB_1 receptors would not be activated (Nackley et al., 2003). We postulate that the higher

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dose of systemic CP55,940 activates both CB_1 and CB_2 receptors, but the beneficial antinociceptive effects (mediated by both CB_1 and CB_2) are masked by CB_1-mediated cataleptic effects.

As expected, CP55,940 produced hypothermia in WT, but not CB_1KO mice (McGregor et al., 1996; Rawls et al., 2002; Varvel et al., 2005). By contrast, chronic CP55,940, at a dose that produced CB_2-mediated anti-allodynic efficacy, failed to decrease body temperature in CB_1KO mice, documenting that prolonged activation of CB_2 receptors does not result in hypothermia (Amenta et al., 2012; Elliott et al., 2011; Kinsey et al., 2011; Malan et al., 2001; Valenzano et al., 2005; Yao et al., 2009). Chronic CP55,940-treated WT, but not CB_1KO mice, showed profound withdrawal signs when challenged with the CB_1 antagonist rimonabant, suggesting precipitation at CB_1 receptors produces withdrawal symptoms (Aceto et al., 1996; Cook et al., 1998; Lichtman et al., 2001; Rubino et al., 1998; Tsou et al., 1995). Interestingly, although rimonabant challenge preferentially increased scratching bouts in mice treated with CP55,940 compared to vehicle, rimonabant-elicited scratching was notably absent in CB_1KO mice, demonstrating that antagonist-induced scratching [analogous to pruritis (Proietto et al., 2010)] in the absence of chronic cannabinoid dosing is mediated by CB_1 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 CB_2 receptors (present data and (Deng et al., 2015)). These studies provide strong evidence that activation of CB_2 receptors produce substantial anti-allodynic efficacy independent of CB_1 receptors.

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One of the common features of GPCRs is that prolonged exposure to their agonists lead to the development of tolerance (Martin et al., 2004; Taylor and Fleming, 2001). A striking observation of our study was that tolerance to the therapeutic effects of CP55,940 (0.3 mg/kg/day 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 CB_1-mediated pharmacological effects (e.g., analgesia, hypothermia, hypoactivity). Interestingly, using the same mouse model of paclitaxel-induced neuropathy, complete tolerance developed to both antinoiceptive 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 CB_1 receptors (Darmani et al., 2007; Wiley et al., 1995) 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 CB_1 receptors (Martin et al., 2004) and regionally specific differences in receptor density and/or efficacy (McKinney et al., 2008; Oviedo et al., 1993). For instance, CP55,940 and Δ^9-THC differ in CB_1 receptor internalization (Hsieh et al., 1999) and inhibition of adenylyl cyclase activity (Breivogel et al., 1998; Childers and Deadwyler, 1996; Fan et al., 1996; Rubino et al., 2000a; Rubino et al., 2000b; Sim et al., 1995), whereas these ligands act similarly with respect to other CB_1 signaling pathways, such as extracellular-signal-regulated kinase (ERK) activation (Daigle et al., 2008; Rubino et al., 2005). More studies that correlate behavior observations and functional signaling pathways are needed to understand tolerance associated with CB_1 receptors. In addition to CB_1-associated tolerance, the present report

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also asked whether the high dose CP55,940 treatment in CB_1KO mice would produce tolerance to CB_2-mediated anti-allodynic effects. Notably, no decrement in CB_2-mediated anti-allodynic effects was observed in CB_1KO mice treated with daily administration of the high dose CP55,940, similar to previous reports with CB_2-preferring agonists (Deng et al., 2015; Yao et al., 2009).

In conclusion, the present report demonstrated distinct roles of cannabinoid receptor subtypes in mediating the beneficial and adverse effects of CP55,940 in the animal model of paclitaxel-induced neuropathy. CP55,940 suppressed the maintenance of paclitaxel-induced neuropathic pain through both CB_1- and CB_2-dependent mechanisms. CB_2-mediated antinociceptive effects were engaged at doses approximately ten times higher than those required to produce CB_1-mediated antinociception. On the other hand, CB_1, but not CB_2 receptors were engaged in CP55,940-produced hypothermia, catalepsy, and cannabimimetic physical withdrawal. Our results further demonstrate that CB_2 receptors represent a potential therapeutic target for effectively and safely managing chemotherapy-induced neuropathic pain without unwanted effects.

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Authorship Contributions

Participated in research design: Deng, Mackie, and Hohmann.

Conducted experiments: Deng and Cornett.

Performed data analyses: Deng.

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

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Footnotes

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Figure Legends

Fig. 1. Development of paclitaxel-induced painful neuropathy. (A, C) Mechanical and (B, D) cold allodynia developed equivalently in (A, B) CB_1KO, (C, D) CB_2KO, and corresponding WT littermates following paclitaxel treatment. Non-chemotherapy controls received cremophor-vehicle in lieu of paclitaxel. Arrows show timing of paclitaxel or cremophor-vehicle injections (inj). Data are expressed as mean ± SEM (n=5-31 per group). $^{*}P<0.05$ vs. cremophor-vehicle control, repeated measures ANOVA and one-way ANOVA at each time point.

Fig. 2. Low dose CP55,940 suppressed paclitaxel-induced neuropathic pain in WT and CB_2KO, but not in CB_1KO mice. (A-D) CP55,940 (0.3 mg/kg/day i.p. x 8 days) reversed paclitaxel-induced (A, C) mechanical and (B, D) cold allodynia in (A, B) WT (CD1) littermates, but not (C, D) CB_1KO mice. (E-F) CP55,940 (0.3 mg/kg/day i.p. x 8 days) attenuated paclitaxel-induced (E) mechanical and (F) cold allodynia with similar efficacy in CB_2KO and WT (C57BL/6J) littermates. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as mean ± SEM (n=5-7 per group). $^{*}P<0.05$ vs. vehicle, 2-tailed t-test or one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P<0.05$ vs. pre-paclitaxel baseline, repeated measures ANOVA.

Fig. 3. Tolerance developed to the anti-allodynic effects of low dose CP55,940 in WT mice. CP55,940 (0.3 mg/kg/day i.p.) failed to suppress paclitaxel-induced (A) mechanical or (B) cold allodynia on day 11 of chronic dosing in WT mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as

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mean ± SEM (n=4 per group). $^{*}P<0.05$ vs. vehicle, 2-tailed t-test. $^{\#}P<0.05$ vs. pre-paclitaxel baseline, repeated measures ANOVA.

Fig. 4. Chronic low dose CP55,940 produced transient hypothermia in WT but not CB_1KO mice. (A) CP55,940 (0.3 mg/kg/day i.p. x 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/day i.p. x 8 days) did not alter body temperature in paclitaxel-treated CB_1KO mice. Data are expressed as mean ± SEM (n=4-6 per group). $^{*}P<0.05$ vs. vehicle, two-tailed t-test.

Fig. 5. Rimonabant precipitated withdrawal signs in paclitaxel-treated WT but not CB_1KO mice receiving low dose chronic CP55,940. Challenge with the CB_1 antagonist rimonabant (10 mg/kg i.p.) elicited paw tremors, head shakes, and scratching bouts in paclitaxel-treated (A) WT but not (B) CB_1KO mice receiving CP55,940 (0.3 mg/kg/day i.p. x 9 days). PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as mean ± SEM (n=4-7 per group). $^{x}P<0.05$ vs. Veh+Rim (chronic vehicle treatment and challenged by rimonabant), $^{\$}P<0.05$ vs. Veh+Veh (chronic vehicle treatment and challenged by vehicle), one-way ANOVA followed by Bonferroni *post hoc* test or two-tailed t-test.

Fig. 6. High dose CP55,940 produced catalepsy and hypothermia in paclitaxel-treated WT but not CB_1KO mice. (A, 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, D) Acute high dose treatment with CP55,940 (3 mg/kg i.p.) did not induce (B) catalepsy

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or (D) hypothermia in paclitaxel-treated CB_1KO mice. PTX, paclitaxel; Veh, vehicle. Data are expressed as mean ± SEM (n=4-7 per group). $^{*}P<0.05$ vs. vehicle, $^{+}P<0.05$ vs. CP55,940 (0.3 mg/kg i.p.), one-way ANOVA followed by Bonferroni *post hoc* test.

Fig. 7. Acute CP55,940 produced dose-dependent anti-allodynic effects in paclitaxel-treated CB_1KO 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 CB_1KO mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as mean ± SEM (n=4-7 per group). $^{*}P<0.05$ vs. vehicle, $^{+}P<0.05$ vs. CP55,940 (1 mg/kg i.p.), one-way ANOVA followed by Bonferroni *post hoc* test.

Fig. 8. In CB_1KO mice, high dose CP55,940 suppressed paclitaxel-induced allodynia without producing hypothermia or rimonabant-elicited precipitated withdrawal symptoms. (A, B) CP55,940 (3 mg/kg/day i.p. x 8 days) reversed paclitaxel-induced (A) mechanical and (B) cold allodynia in CB_1KO mice. (C, D) CP55,940 (3 mg/kg/day i.p. x 9 days) did not alter (C) body temperature or (D) produce rimonabant-elicited precipitated withdrawal symptoms in CB_1KO mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as mean ± SEM (n=4-7 per group). $^{*}P<0.05$ vs. vehicle, 2-tailed t-test. $^{\#}P<0.05$ vs. pre-paclitaxel baseline, repeated measures ANOVA.

Fig. 9. Anti-allodynic effects of high dose CP55,940 in CB_1KO mice were mediated by CB_2 receptors. In CB_1KO mice, high dose CP55,940 (3 mg/kg/day i.p. x 8 days)-

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induced suppressions of paclitaxel-evoked (A) mechanical and (B) cold allodynia were blocked by the CB_2 antagonist AM630 (5 mg/kg/day i.p. x 8 days). BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; Veh, vehicle. Data are expressed as mean ± SEM (n=4-7 per group). $^{*}P<0.05$ vs. vehicle, $^{x}P<0.05$ vs. CP55,940 (3 mg/kg i.p.), one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P<0.05$ vs. pre-paclitaxel baseline, repeated measures ANOVA.

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Figure 1

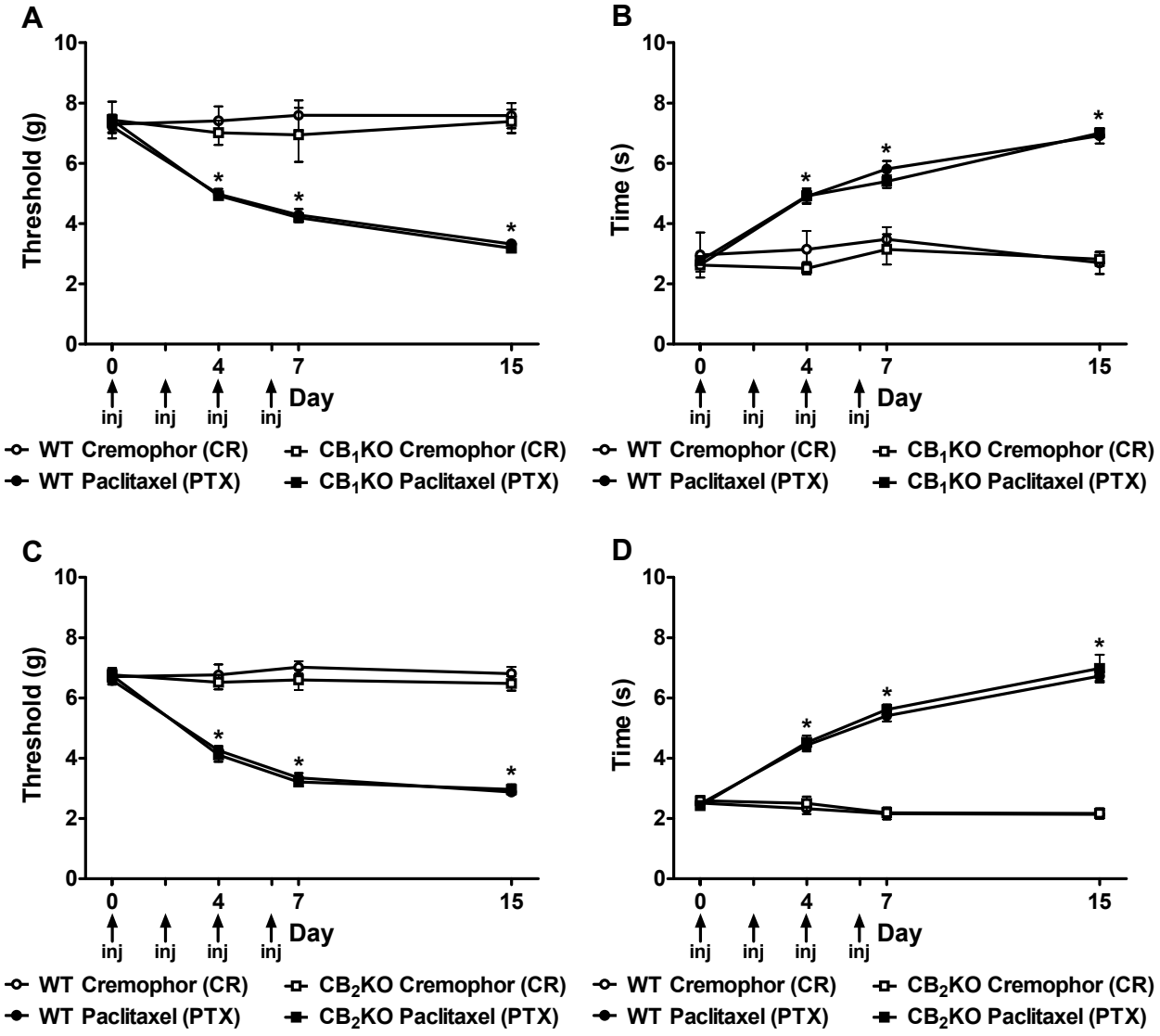

A
B
C
D
Threshold (g)
Time (s)
0
4
7
15
Day
inj
WT Cremophor (CR)
CB1KO Cremophor (CR)
WT Paclitaxel (PTX)
CB1KO Paclitaxel (PTX)
CB2KO Cremophor (CR)
CB2KO Paclitaxel (PTX)

Figure 2

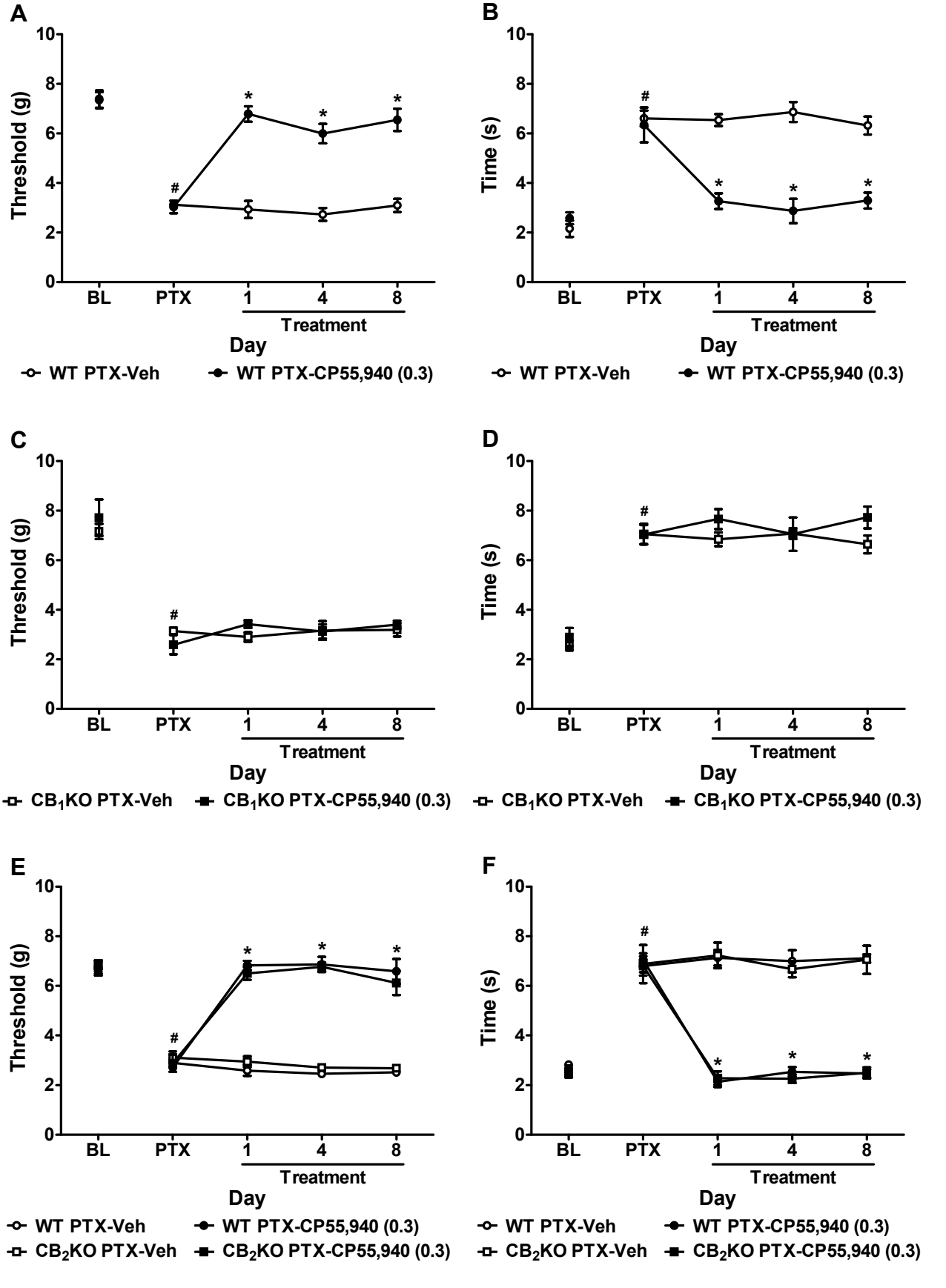

A
B
C
D
E
F
Threshold (g)
Time (s)
BL
PTX
1
4
8
Treatment
Day
WT PTX-Veh
WT PTX-CP55,940 (0.3)
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (0.3)
CB2KO PTX-Veh
CB2KO PTX-CP55,940 (0.3)

Figure 3

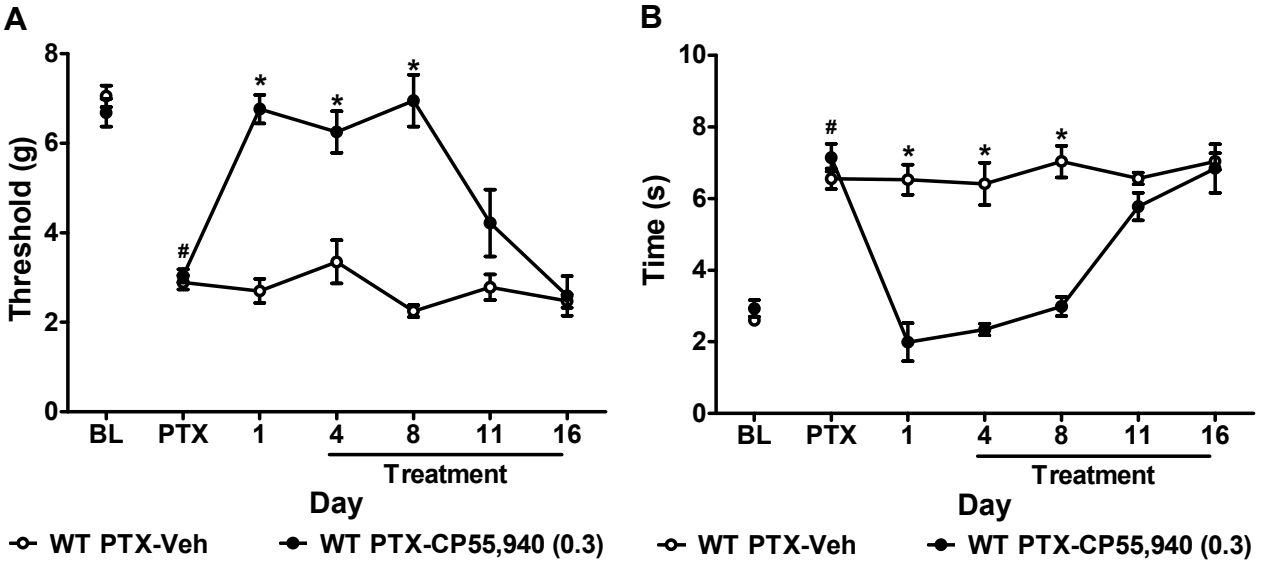

A
Threshold (g)
8
6
4
2
0
BL
PTX
1
4
8
11
16
Treatment
Day
#
*
WT PTX-Veh
WT PTX-CP55,940 (0.3)
B
Time (s)
10
8
6
4
2
0
BL
PTX
1
4
8
11
16
Treatment
Day
#
*
WT PTX-Veh
WT PTX-CP55,940 (0.3)

Figure 4

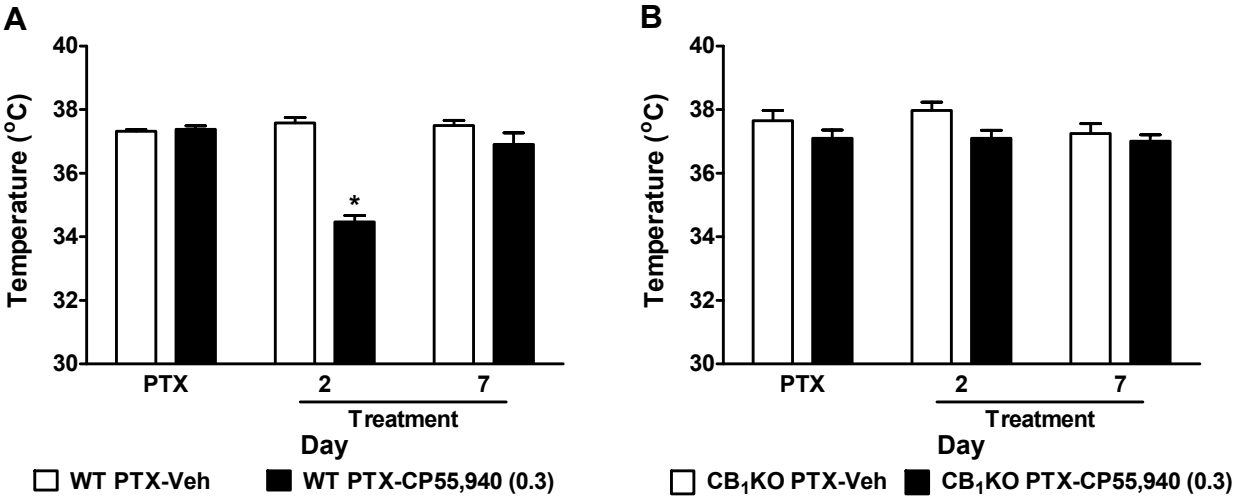

A
B
Temperature (°C)
40
38
36
34
32
30
*
PTX
2
7
Treatment
Day
WT PTX-Veh
WT PTX-CP55,940 (0.3)
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (0.3)

Figure 5

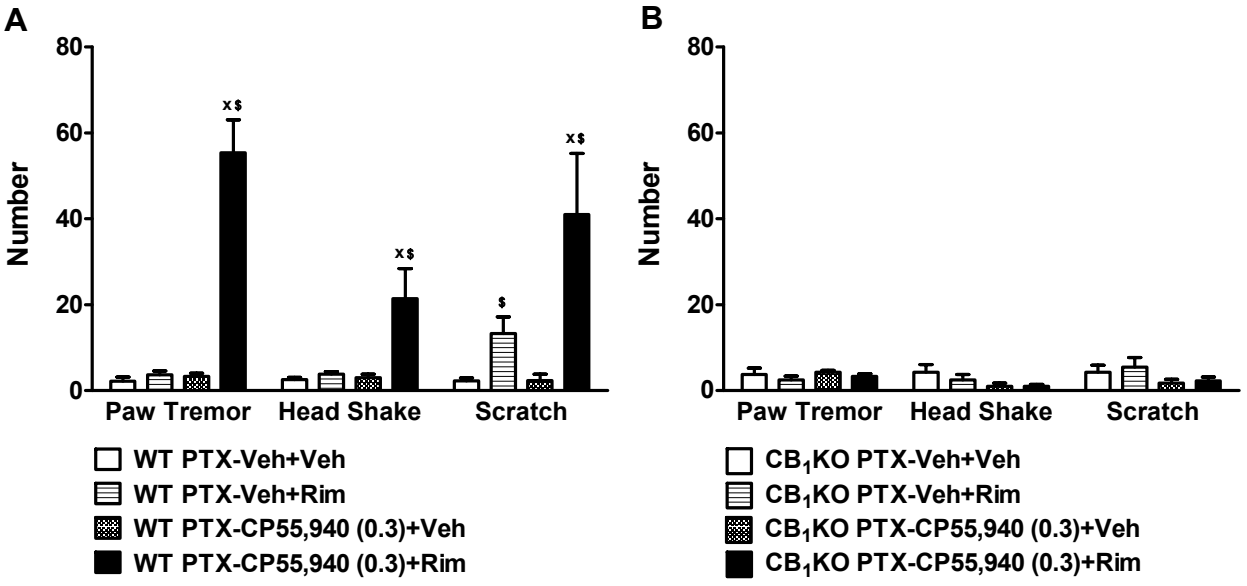

A
B
Number
80
60
40
20
0
x$
$
Paw Tremor
Head Shake
Scratch
WT PTX-Veh+Veh
WT PTX-Veh+Rim
WT PTX-CP55,940 (0.3)+Veh
WT PTX-CP55,940 (0.3)+Rim
CB1KO PTX-Veh+Veh
CB1KO PTX-Veh+Rim
CB1KO PTX-CP55,940 (0.3)+Veh
CB1KO PTX-CP55,940 (0.3)+Rim

Figure 6

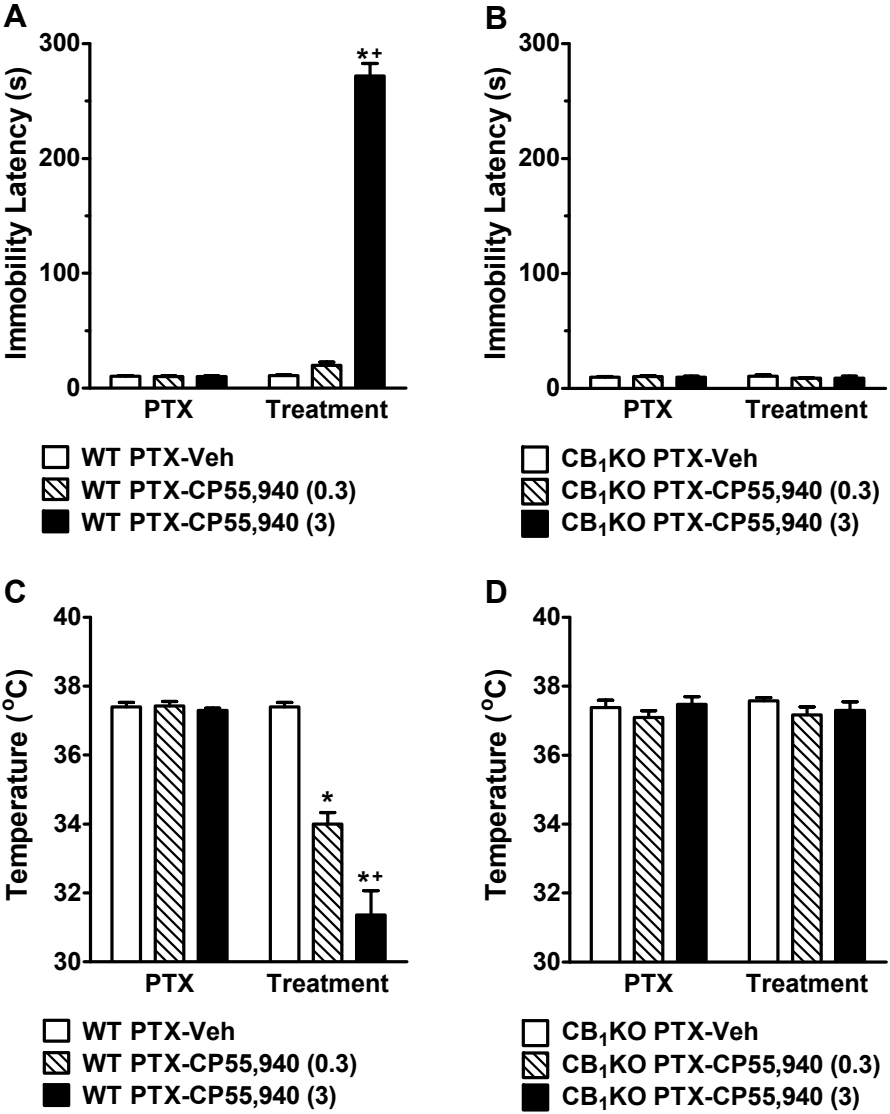

A
Immobility Latency (s)
300
200
100
0
*+
PTX
Treatment
WT PTX-Veh
WT PTX-CP55,940 (0.3)
WT PTX-CP55,940 (3)
B
Immobility Latency (s)
300
200
100
0
PTX
Treatment
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (0.3)
CB1KO PTX-CP55,940 (3)
C
Temperature (°C)
40
38
36
34
32
30
*
*+
PTX
Treatment
WT PTX-Veh
WT PTX-CP55,940 (0.3)
WT PTX-CP55,940 (3)
D
Temperature (°C)
40
38
36
34
32
30
PTX
Treatment
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (0.3)
CB1KO PTX-CP55,940 (3)

Figure 7

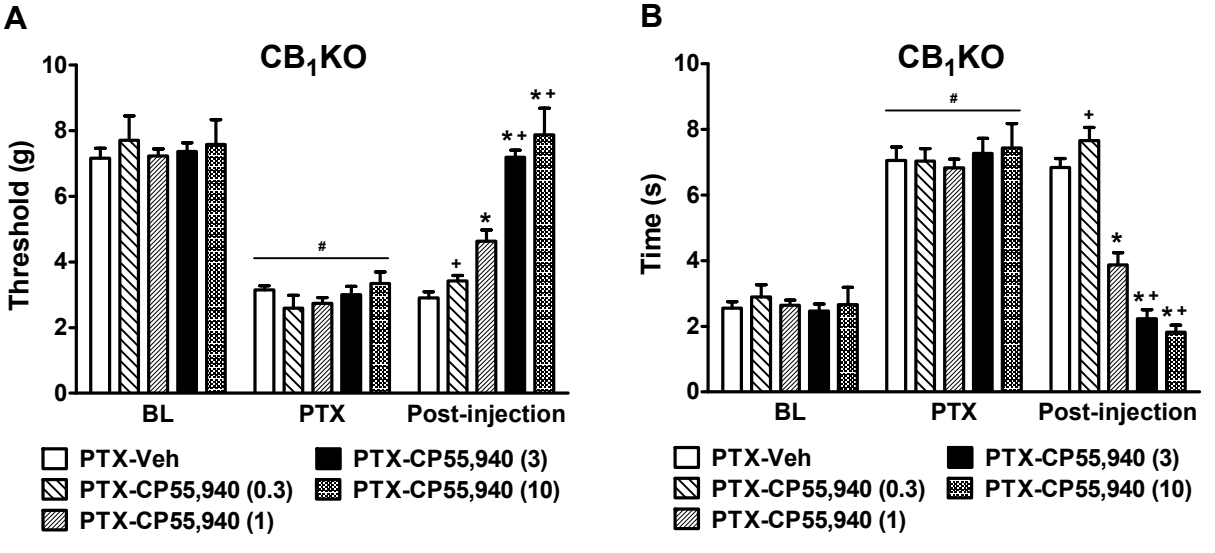

A
CB1KO
Threshold (g)
10
8
6
4
2
0
#
*
+
*+
BL
PTX
Post-injection
PTX-Veh
PTX-CP55,940 (0.3)
PTX-CP55,940 (1)
PTX-CP55,940 (3)
PTX-CP55,940 (10)
B
CB1KO
Time (s)
10
8
6
4
2
0
#
+
*
*+
BL
PTX
Post-injection
PTX-Veh
PTX-CP55,940 (0.3)
PTX-CP55,940 (1)
PTX-CP55,940 (3)
PTX-CP55,940 (10)

Figure 8

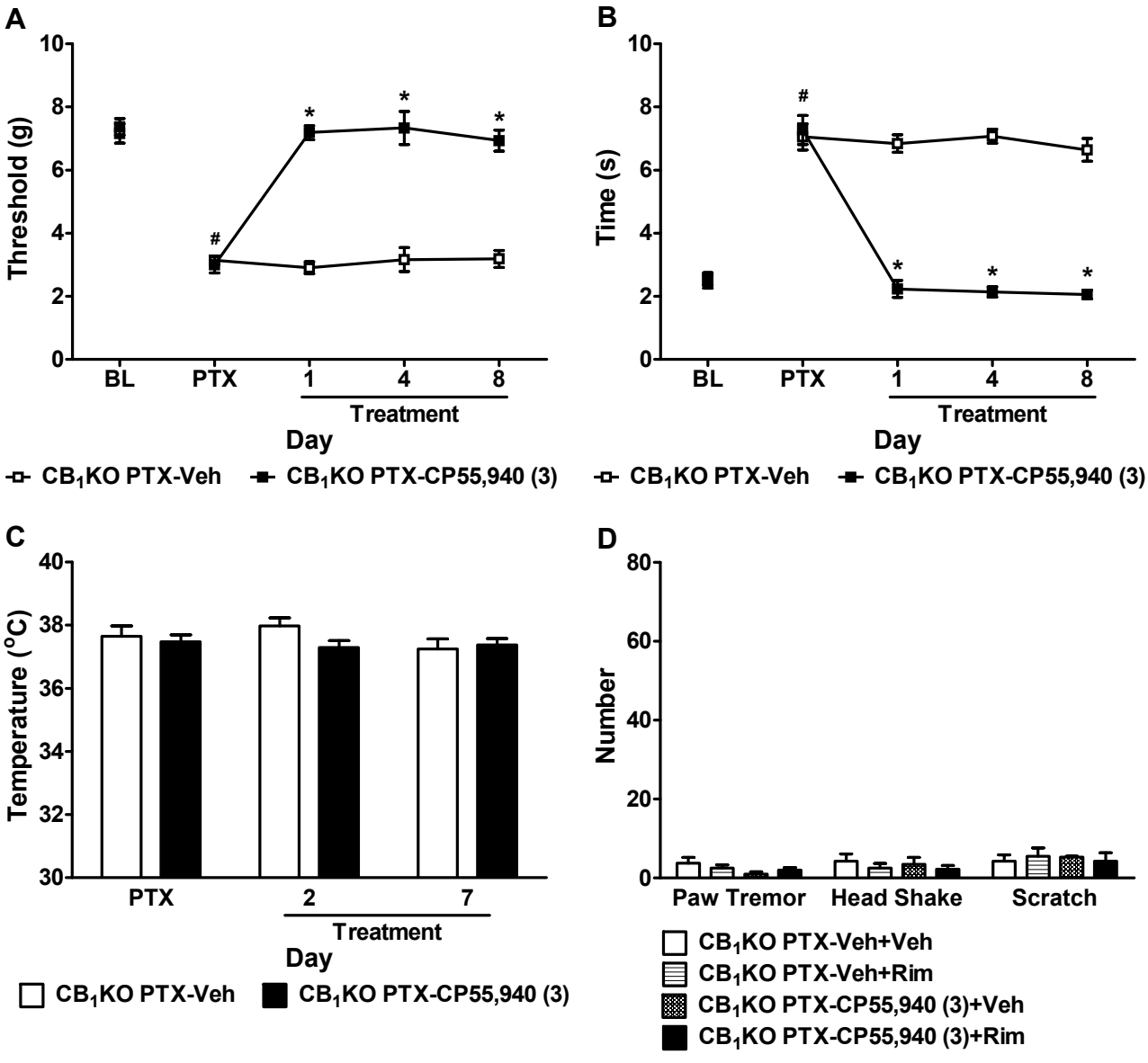
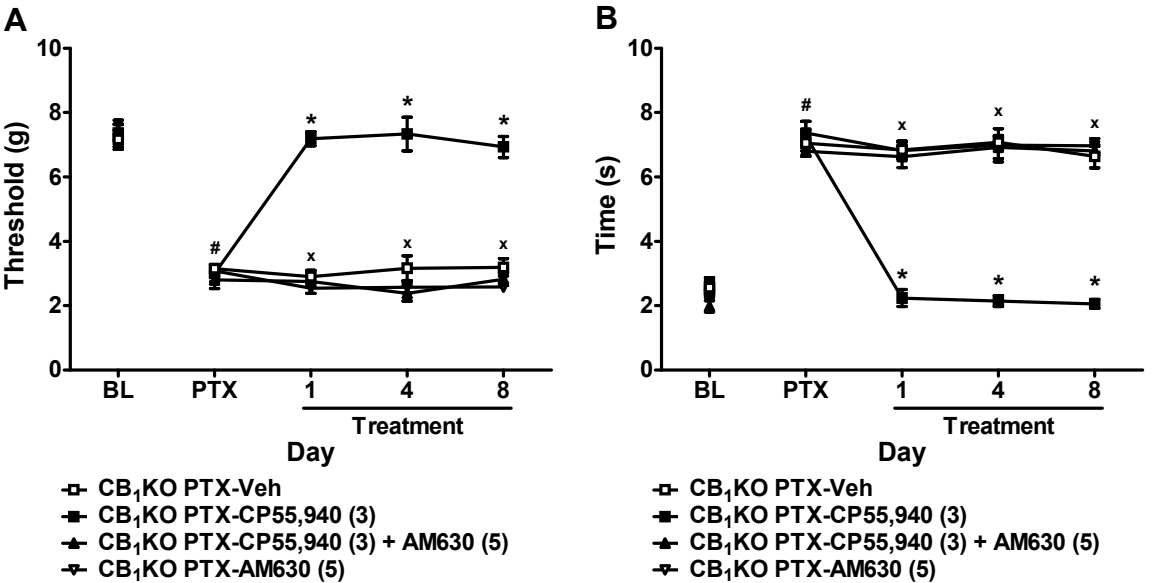

A
Threshold (g)
10
8
6
4
2
0
BL
PTX
1
4
8
Treatment
Day
#
*
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (3)
B
Time (s)
10
8
6
4
2
0
BL
PTX
1
4
8
Treatment
Day
#
*
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (3)
C
Temperature (°C)
40
38
36
34
32
30
PTX
2
7
Treatment
Day
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (3)
D
Number
80
60
40
20
0
Paw Tremor
Head Shake
Scratch
CB1KO PTX-Veh+Veh
CB1KO PTX-Veh+Rim
CB1KO PTX-CP55,940 (3)+Veh
CB1KO PTX-CP55,940 (3)+Rim

Figure 9


A
Threshold (g)
10
8
6
4
2
0
BL
PTX
1
4
8
Treatment
Day
B
Time (s)
10
8
6
4
2
0
BL
PTX
1
4
8
Treatment
Day
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (3)
CB1KO PTX-CP55,940 (3) + AM630 (5)
CB1KO PTX-AM630 (5)
CB1KO PTX-Veh
CB1KO PTX-CP55,940 (3)
CB1KO PTX-CP55,940 (3) + AM630 (5)
CB1KO PTX-AM630 (5)