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Chronic cannabinoid CB_2 activation reverses paclitaxel neuropathy without tolerance or CB_1-dependent withdrawal

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Abstract

Background—Mixed cannabinoid CB_1/CB_2 agonists such as Δ^9-tetrahydrocannabinol (Δ^9-THC) can produce tolerance, physical withdrawal, and unwanted CB_1-mediated central nervous system side effects. Whether repeated systemic administration of a CB_2-preferring agonist engages CB_1 receptors or produces CB_1-mediated side effects is unknown.

Methods—We evaluated anti-allodynic efficacy, possible tolerance, and cannabimimetic side effects of repeated dosing with a CB_2-preferring agonist AM1710 in a model of chemotherapy-induced neuropathy produced by paclitaxel using CB_1KO, CB_2KO, and WT mice. Comparisons were made with the prototypic classical cannabinoid Δ^9-THC. We also explored the site and possible mechanism of action of AM1710.

Results—Paclitaxel-induced mechanical and cold allodynia developed equivalently in CB_1KO, CB_2KO, and WT mice. Both AM1710 and Δ^9-THC suppressed established paclitaxel-induced allodynia in WT mice. Unlike Δ^9-THC, chronic AM1710 did not engage CB_1 activity or produce antinociceptive tolerance, CB_1-mediated cannabinoid withdrawal, hypothermia, or motor dysfunction. Anti-allodynic efficacy of systemic AM1710 was absent in CB_2KO mice or WT mice receiving the CB_2 antagonist AM630, administered either systemically or intrathecally. Intrathecal AM1710 also attenuated paclitaxel-induced allodynia in WT but not CB_2KO mice, implicating a possible role for spinal CB_2 receptors in AM1710 anti-allodynic efficacy. Finally, both acute and

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chronic treatment with AM1710 decreased mRNA levels of tumor necrosis factor alpha and monocyte chemoattractant protein-1 in lumbar spinal cord of paclitaxel-treated WT mice.

Conclusions—Our results highlight the potential of prolonged use of CB_2 agonists for managing chemotherapy-induced allodynia with a favorable therapeutic ratio marked by sustained efficacy and absence of tolerance, physical withdrawal, or CB_1-mediated side effects.

Keywords

Cannabinoid CB_2; chemotherapy-induced neuropathic pain; knockout mouse; tolerance; precipitated withdrawal; side effect

Introduction

Cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive component of cannabis, are used clinically to treat neuropathic pain and chemotherapy-induced nausea and vomiting (1, 2). However, unwanted psychotropic side effects limit widespread therapeutic use (1). These side effects (e.g. psychoactivity, dizziness, physical dependence) are centrally mediated by cannabinoid CB_1 receptors (3, 4). A preferable strategy that avoids safety and efficacy concerns while preserving antinociceptive property is to target cannabinoid CB_2 receptors.

CB_2 receptors are found predominantly in immune cells and tissues and also occur at low levels, relative to CB_1, in the central nervous system (CNS) (5, 6). In preclinical studies, CB_2-preferring agonists promote neuroprotection (7–9) and produce antinociception (10–19). However, CB_2-preferring agonists often have significant affinity at CB_1 receptors. Given the high abundance of CB_1 in the CNS, even low-level CB_1-occupancy by CB_2-preferring agonists could eliminate the benefits of receptor selectivity and/or produce adverse side effects following chronic treatment (2). Whether it is possible to obtain therapeutic benefits from repeated systemic administration of CB_2-preferring agonists without engaging CB_1 receptors or producing unwanted CB_1-mediated side effects remains poorly understood.

Dose-limiting peripheral neuropathy can develop in cancer patients receiving chemotherapeutic agents (paclitaxel, cisplatin, vincristine, etc.) (20). Side effects and limited efficacy of clinically available medications make this neuropathy difficult to manage (21). Thus, there is a significant need to identify novel analgesics for treating chemotherapy-evoked neuropathic pain. CB_2-preferring agonists exhibit antinociceptive properties in animal models of chemotherapy-induced neuropathy (22–27). However, the site of action and mechanism by which CB_2 receptors modulate chemotherapy-induced neuropathy are not yet clear. Several proinflammatory cytokines (e.g. tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin 6 (IL-6)) and downstream chemokines (e.g. monocyte chemoattractant protein-1 (MCP-1)) are implicated in mechanisms of neuropathic pain (28–35) and CB_2-mediated actions (36). The potential contributions of such cytokines and chemokines in the antinociceptive action of CB_2 agonist on chemotherapy-induced neuropathy remain unknown.

Here, we characterized antinociceptive efficacy of the CB_2-preferring agonist AM1710 in a model of paclitaxel-induced neuropathy using CB_2 knockout (CB_2KO), CB_1 knockout (CB_1KO), and wildtype (WT) mice. We evaluated whether repeated administration of AM1710 would produce antinociceptive tolerance or CB_1-mediated side effects (i.e. physical withdrawal, motor ataxia, and hypothermia). In addition, we investigated the site of action and the impact of AM1710 on mRNA levels of pro-inflammatory cytokines and chemokine in lumbar spinal cords of paclitaxel-treated mice.

Methods and Materials

Subjects

Adult CB_2KO (B6.129P2-CNR2(tm1Dgen/J), Jackson, ME, USA) and WT littermates (Jackson) on C57BL/6J background, and CB_1KO (generated as previously described (4)) and WT littermates (Charles River, MA, USA) on CD1 background, weighing 25–33g and of both sexes, were used in these experiments. Mice were periodically backcrossed to maintain genetic integrity. Animals were single-housed in a temperature-controlled facility (73±2 °F, 45% humidity, 12h light/dark cycle, lights on at 7am), with food and water *ad libitum* provided. All experimental procedures were approved by Bloomington Institutional Animal Care and Use Committee of Indiana University and followed guidelines of the International Association for the Study of Pain (37).

Drugs and chemicals

Paclitaxel (Tecoland, NJ, USA) was dissolved in cremophor-vehicle (1:1:18 ratio of cremophor® EL (Sigma-Aldrich, MO, USA)/ethanol (Sigma-Aldrich)/saline (Aqualite System, IL, USA)). AM1710 (Makriyannis lab), AM630 (Cayman, MI, USA) and rimonabant (SR141716A, National Institute on Drug Abuse (NIDA), MD, USA) were dissolved in vehicle (5:2:2:16 ratio of dimethyl sulfoxide (DMSO, Sigma-Aldrich)/ alkamuls® EL-620 (Rhodia, NJ, USA)/ethanol/saline). Δ^9-THC (NIDA) was dissolved in vehicle (1:1:18 ratio of ethanol/cremophor/saline). Drugs were administered intraperitoneally (i.p.) to mice in a volume of 5 ml/kg. AM1710 and AM630 were also dissolved in vehicle (1:1:1:17 ratio of DMSO/alkamuls/ethanol/saline) and administered intrathecally (i.t.) to animals in a volume of 5 μl (38).

General experimental protocol

All experiments were conducted double-blinded with mice randomly assigned to experimental conditions. Prior to paclitaxel treatment, no genotype or gender differences were detected in any dependent measure ($P>0.26$ for all comparison). Paclitaxel (4 mg/kg i.p.) was administered four times on alternate days (cumulative dose: 16 mg/kg i.p.) to induce neuropathy (39). Controls received an equal volume of cremophor-vehicle. Development of paclitaxel-induced allodynia was assessed every two days.

Effects of pharmacological manipulations were assessed at 30 min post drug administration during the maintenance phase of paclitaxel-induced neuropathy (day 15 post initial paclitaxel injection). In Experiment #1, we assessed the dose responses of acute AM1710 on mechanical and cold allodynia in paclitaxel-treated WT (C57BL/6J) animals. In Experiment

#2, we examined anti-allodynic efficacy and possible side effects of chronic AM1710 (5 mg/kg/day i.p. × 9 days) in paclitaxel-treated CB_2KO, CB_1KO, and respective WT littermates. Effects of chronic Δ^9-THC (5 or 10 mg/kg/day i.p. × 9 days) in paclitaxel-treated WT (C57BL/6J) animals were also evaluated. Responsiveness to mechanical and cold stimulation was evaluated on treatment days 1, 4 and 8. Motor performance and rectal temperature were measured on treatment days 2 and 7. We also assessed whether chronic AM1710 would activate CB_1 receptors sufficiently to produce CB_1-dependent withdrawal symptoms following treatment with a CB_1 antagonist. Thus, after the last injection of AM1710 (treatment day 9), we challenged CB_2KO and WT mice with the CB_1 antagonist rimonabant (10 mg/kg i.p.) to precipitate CB_1-mediated withdrawal. We also challenged CB_1KO and WT mice receiving chronic AM1710 with the CB_2 antagonist AM630 (5 mg/kg i.p.) to determine if this treatment elicits behavioral signs reminiscent of CB_1 or opioid receptor-mediated withdrawal. In Experiment #3, we examined pharmacological specificity of AM1710 in paclitaxel-treated WT or CB_1KO mice that received vehicle, AM1710 (5 mg/kg/day i.p. × 8 days) alone or co-administered with AM630 (5 mg/kg/day i.p. × 8 days). In Experiment #4, we investigated the site of action of AM1710. We evaluated whether antagonism of spinal CB_2 receptors by AM630 (5μg i.t.) would block the anti-allodynic effects of systemic AM1710 (5 mg/kg i.p.) in paclitaxel-treated WT mice. We also examined effects of intrathecal AM1710 (5μg i.t.) on paclitaxel-evoked allodynia in CB_2KO and WT mice. In Experiment #5, we explored the impact of paclitaxel and AM1710 on spinal mRNA levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6), chemokine (MCP-1), and markers of the endocannabinoid system (CB_1, CB_2, fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MGL)) in WT (C57BL/6J) mice.

Assessment of mechanical allodynia

Withdrawal thresholds (g) to mechanical stimulation were measured in duplicate for each paw using electronic von Frey anesthesiometer supplied with 90-gram probe (IITC, CA, USA) (25). See Supplementary Material.

Assessment of cold allodynia

Response time (s) spent attending to (i.e. elevating, licking, biting, or shaking) the paw stimulated with acetone (Sigma-Aldrich) was measured in triplicate for each paw to assess cold allodynia (39). See Supplementary Material.

Evaluation of cannabinoid CB_1 withdrawal symptoms

WT (C57BL/6J) mice receiving vehicle or Δ^9-THC (5 or 10 mg/kg/day i.p. × 9 days) were challenged with vehicle or rimonabant (10 mg/kg i.p.). CB_2KO and WT littermates receiving vehicle or AM1710 (5 mg/kg/day i.p. × 9 days) were challenged with rimonabant (10 mg/kg i.p.). CB_1KO and WT mice receiving vehicle or AM1710 (5 mg/kg/day i.p. × 9 days) were challenged with AM630 (5 mg/kg i.p.). Challenge compounds were given 45 min post final injection. Mice were videoed and the number of paw tremors, headshakes, and scratching bouts were scored over 30 min following challenge (40).

Rotarod test

Motor performance was assessed using an accelerating rotarod (IITC) (4–40 rpm with cut-off time of 300 s) (41). See Supplementary Material.

Rectal temperature

Rectal temperature (°C) was measured using a thermometer (Physitemp, NJ, USA) with mouse rectal probe (Braintree, MA, USA).

RNA extraction and qRT-PCR

Total RNAs were extracted from lumbar spinal cords (42). One-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using PowerSYBR green PCR kit (Applied Biosystems, CA, USA) to quantify mRNA levels (43). The quantified mRNA levels were expressed as fold induction relative to control. Primer sequences (Table S1) see Supplementary Material.

Statistical analyses

The dose-response curves and ED_{50} values for AM1710 were determined using GraphPad Prism (CA, USA). Analysis of variance (ANOVA) for repeated measures was used to determine time course of paclitaxel-induced allodynia and drug effects. One-way ANOVA was used to identify the source of significant interactions at each timepoint and compare post-injection responses with baselines, followed by Bonferroni *post hoc* tests or two-tailed t-tests, as appropriate. Impact of paclitaxel or AM1710 on mRNA levels was analyzed using two-tailed t-tests or one-way ANOVA, respectively. Statistical analyses were performed using IBM-SPSS Statistics V21.0 (IL, USA). $P<0.05$ was considered significant.

Results

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

In both CB_2KO and WT mice, paclitaxel decreased mechanical thresholds ($F_{3,20}=519.03$, $P<0.0001$, Figure 1A) and increased response time to cold stimulation ($F_{3,20}=553.78$, $P<0.0001$, Figure 1B). Similarly, paclitaxel induced mechanical ($F_{3,20}=426.66$, $P<0.0001$, Figure 1C) and cold ($F_{3,20}=707.28$, $P<0.0001$, Figure 1D) allodynia in CB_1KO and WT littermates. Mechanical and cold allodynia were present in paclitaxel-treated CB_2KO, CB_1KO, and WT mice relative to cremophor-vehicle since day 4 ($P<0.0001$). Responsiveness to paclitaxel did not differ between CB_2KO and WT mice, or between CB_1KO and WT mice ($P=1.000$).

Effects of Δ^9-THC in paclitaxel-treated WT mice

In WT mice, Δ^9-THC (5 or 10 mg/kg/day i.p.) suppressed paclitaxel-evoked mechanical ($F_{2,14}=26.57$, $P<0.0001$) and cold allodynia ($F_{2,14}=13.58$, $P<0.002$) relative to vehicle in a dose-and time-dependent manner ($F_{8,56}=27.97$, $P<0.0001$ mechanical, $F_{8,56}=24.44$, $P<0.0001$ cold, Figure 2A–B). The high dose of Δ^9-THC (10 mg/kg/day i.p.) produced greater antinociceptive effects than the low dose (5 mg/kg/day i.p.) ($P<0.01$ mechanical, $P<0.03$ cold) and normalized responses to pre-paclitaxel levels ($P=0.13$ mechanical, $P=0.07$

cold) on treatment day 1. Tolerance developed more rapidly to the high dose of Δ^9-THC. The high (P=1.00 day 4 and 8) and low (P<0.0001 day 4, P=1.00 day 8) doses of Δ^9-THC failed to produce antinociception relative to vehicle after 4 or 8 days of injections, respectively. Both doses of Δ^9-THC decreased motor performance and produced hypothermia in paclitaxel-treated WT mice relative to vehicle on day 2 (P<0.04), but not day 7 (P>0.11), of chronic dosing (Figure 2C–D). Thus, over 8 days of Δ^9-THC (5 or 10 mg/kg/day i.p.) administration, tolerance developed to antinociceptive efficacy, motor ataxia, and hypothermia in paclitaxel-treated animals.

In paclitaxel-treated WT mice, chronic Δ^9-IHC (5 or 10 mg/kg/day i.p.) produced cannabinoid withdrawal signs following rimonabant (10 mg/kg i.p.) challenge, characterized by paw tremors ($F_{5,26}$=65.60, P<0.0001) and headshakes ($F_{5,26}$=38.13, P<0.0001) relative to vehicle (P<0.0001, Figure 2E). Rimonabant, but not vehicle, produced scratching behaviors ($F_{5,26}$=10.34, P<0.0001) in animals receiving chronic vehicle or Δ^9-THC (Figure 2E).

Effects of acute AM1710 in paclitaxel-treated WT mice

In WT mice, acute systemic AM1710 dose-dependently suppressed paclitaxel-induced mechanical (ED_{50}: 1.14±0.07 mg/kg i.p.) and cold (ED_{50}: 1.49±0.06 mg/kg i.p.) allodynia (Figure S1). AM1710 (5 mg/kg i.p.) produced maximal anti-allodynic efficacy and was used for chronic dosing.

Chronic AM1710 suppressed paclitaxel-induced allodynia in WT but not CB_2KO mice

In WT mice, chronic AM1710 (5 mg/kg/day i.p.) suppressed paclitaxel-induced mechanical ($F_{1,13}$=98.97, P<0.0001) and cold ($F_{1,13}$=249.03, P<0.0001) hypersensitivities relative to vehicle (P<0.0001) and pre-injection levels ($F_{4,52}$=67.12, P<0.0001 mechanical, $F_{4,52}$=62.04, P<0.0001 cold, Figure 3A–B). AM1710 anti-allodynic efficacy was stable throughout the chronic dosing paradigm (P=0.75 mechanical, P=1.00 cold). AM1710 fully reversed paclitaxel-induced allodynia and normalized responses to pre-paclitaxel baselines (P=0.86 mechanical, P=0.46 cold, Figure 3A–B).

By contrast, in CB_2KO mice, AM1710 (5 mg/kg/day i.p.) failed to suppress paclitaxel-induced mechanical (P=0.22) or cold (P=0.79) allodynia relative to vehicle (P>0.20) on any day (P=1.00 mechanical, P=0.59 cold, Figure 3C–D). AM1710 did not alter responsiveness to mechanical (P=0.94) or cold (P=0.66) stimulation in CB_2KO or WT littermates treated with cremophor-vehicle at any timepoint (P=0.84 mechanical, P=0.89 cold, Figure 3E–F).

Anti-allodynic effects of AM1710 were independent of CB_1 signaling

In both CB_1KO and WT littermates, AM1710 (5 mg/kg/day i.p.) reversed paclitaxel-induced mechanical ($F_{3,17}$=112.37, P<0.0001) and cold ($F_{3,17}$=29.24, P<0.0001) allodynia relative to vehicle (P<0.0001) and pre-injection levels ($F_{12,68}$=17.04, P<0.0001 mechanical, $F_{12,68}$=21.97, P<0.0001 cold, Figure 4A–B). AM1710-induced anti-allodynic effects were stable throughout the treatment paradigm (P=0.97 mechanical, P=0.12 cold). AM1710 fully reversed paclitaxel-induced mechanical (P>0.88) and cold (P>0.052) allodynia and normalized responses to pre-paclitaxel baselines in both CB_1KO and WT littermates. Anti-

allodynic efficacy of AM1710 did not differ between CB_1KO and WT littermates at any timepoint (P>0.99, Figure 4A–B). AM1710 did not alter mechanical (P=0.72) or cold (P=0.11) responsiveness in CB_1KO or WT littermates treated with cremophor-vehicle on any day (P=0.88 mechanical, P=0.53 cold, Figure 4C–D).

Anti-allodynic effects of AM1710 were mediated by CB_2 receptors

In paclitaxel-treated WT (C57BL/6J) mice, AM1710 (5 mg/kg/day i.p.)-produced suppressions of mechanical ($F_{3,19}$=65.57, P<0.0001) and cold ($F_{3,19}$=95.35, P<0.0001) allodynia were blocked by the CB_2 antagonist AM630 (5 mg/kg/day i.p.) at all timepoints (P<0.0001, Figure 5A–B). Identical results were obtained in WT (CD1) mice (data not shown).

In paclitaxel-treated CB_1KO mice, the anti-allodynic effects of AM1710 (5 mg/kg/day i.p.) on mechanical ($F_{3,16}$=111.06, P<0.0001) and cold ($F_{3,16}$=37.02, P<0.0001) hypersensitivities were blocked by AM630 (5 mg/kg/day i.p.) at all timepoints (P<0.0001, Figure 5C–D). AM630 alone did not alter mechanical or cold responsiveness relative to vehicle in WT or CB_1KO mice (P=1.00, Figure 5A–D).

Chronic AM1710 did not produce motor dysfunction or hypothermia

Paclitaxel did not alter motor performance or body temperature in CB_2KO, CB_1KO or corresponding WT littermates relative to cremophor-vehicle (P>0.11, Figure S2). Moreover, AM1710 (5 mg/kg/day i.p.) did not produce motor dysfunction or hypothermia in either paclitaxel- or cremophor-treated groups in CB_2KO, CB_1KO, or WT littermates on treatment day 2 or 7 (P>0.95, Figure S2).

CB_1 antagonism did not elicit classic cannabinoid withdrawal signs in mice receiving chronic AM1710

We asked whether the CB_1 antagonist rimonabant would elicit cannabinoid CB_1-dependent withdrawal symptoms in mice receiving chronic AM1710. In paclitaxel-treated WT mice that received chronic Δ^9-THC (10 mg/kg/day i.p.), rimonabant (10 mg/kg i.p.) challenge produced paw tremors ($F_{4,20}$=272.81, P<0.0001) and headshakes ($F_{4,20}$=32.10, P<0.0001, Figure 6A). Rimonabant challenge did not elicit paw tremors or headshakes in CB_2KO or WT mice receiving chronic AM1710 (5 mg/kg/day i.p.) relative to vehicle (P=1.00, Figure 6A, S3A). Neither Δ^9-THC nor AM1710 treatment altered rimonabant-induced scratching (P=0.22) compared to vehicle (Figure 6A).

We next asked whether the CB_2 antagonist AM630 could precipitate paw tremors, headshakes and/or scratching behaviors in mice receiving chronic AM1710. AM630 (5 mg/kg i.p.) challenge did not elicit paw tremors (P=0.29), headshakes (P=0.88), or scratching (P=0.96) relative to vehicle in CB_1KO or WT mice receiving chronic AM1710 (5 mg/kg/day i.p.) (Figure 6B, S3B). In addition, no autonomic signs (e.g. diarrhea, eyelid ptosis) or writhing behaviors were observed following AM630 challenge.

Spinal CB_2 receptors were necessary for the anti-allodynic effect of systemic AM1710

In WT mice, anti-allodynic effects of AM1710 (5 mg/kg i.p.) on paclitaxel-induced mechanical ($F_{3,20}$=16.51, P<0.0001) and cold ($F_{3,20}$=30.93, P<0.0001) allodynia were blocked by intrathecal AM630 (5 μg i.t.) (P<0.0001, Figure 7). Intrathecal AM630 alone did not alter paclitaxel-evoked mechanical (P=1.00) or cold (P>0.72) allodynia relative to vehicle (Figure 7).

Intrathecal AM1710 suppressed paclitaxel-induced neuropathy in WT but not CB_2KO mice

We asked whether activation of spinal CB_2 receptors was sufficient to suppress paclitaxel-induced allodynia. In WT mice, intrathecal AM1710 (5 μg i.t.) suppressed paclitaxel-induced mechanical ($F_{1,10}$=42.42, P<0.0001) and cold ($F_{1,10}$=78.99, P<0.0001) allodynia compared to vehicle (P<0.0001); intrathecal AM1710 fully reversed paclitaxel-evoked allodynia and normalized responses to pre-paclitaxel levels (P=0.89 mechanical, P=0.87 cold, Figure 8A–B). By contrast, in CB_2KO mice, AM1710 (5 μg i.t.) failed to attenuate paclitaxel-induced mechanical (P=0.85) or cold (P=0.46) allodynia relative to vehicle (Figure 8C–D).

Impact on spinal mRNA levels of markers of the endocannabinoid system, cytokines, and chemokine

In WT mice, paclitaxel increased MCP-1 (P<0.004), but not IL-1β (P=0.52), IL-6 (P=1.00), TNFα (P=0.83), CB_1(P=0.34), CB_2 (P=0.26), FAAH(P=0.28), or MGL (P=0.18) mRNA levels in spinal cords relative to cremophor-vehicle (Figure 9A, S4) during the maintenance phase of paclitaxel-induced neuropathy. In paclitaxel-treated WT mice, both acute and chronic (8 days) AM1710 (5 mg/kg/day i.p.) decreased TNFα ($F_{2,9}$=19.52, P<0.002) and MCP-1 ($F_{2,9}$=15.00, P<0.002), but not IL-1β (P=0.38) or IL-6 (P=0.68) spinal mRNA levels (Figure 9B).

Discussion

Drug development for neuropathic pain management has proved a challenge due in part to limited efficacy and troubling side-effect profiles. Indeed, these challenges also apply to potential therapeutic use of cannabinoids (44). Here, we showed that repeated systemic administration of the CB_2-preferring agonist AM1710 suppressed chemotherapy-induced allodynia without tolerance or significant CB_1 involvement (i.e. the absence of CB_1 antagonist-precipitated withdrawal symptoms, motor ataxia, and hypothermia). We also confirmed a CB_2-mediated mechanism of antinociceptive action for AM1710 both pharmacologically and through use of knockout mice. Moreover, we identified a spinal site of action of AM1710 and explored AM1710-mediated regulation of pro-inflammatory cytokines and chemokine mRNA levels following paclitaxel treatment.

CB_2 receptors are implicated in pain mechanisms following sciatic nerve injury (45) and joint pain (46). In our study, neither the development nor the maintenance of paclitaxel-induced allodynia differed between CB_2KO and WT mice. CB_2 receptors are highly inducible and are expressed in spinal microglia upon inflammation (47) or neuropathic pain (48–52). However, we did not detect changes in CB_2 or FAAH mRNA levels in lumbar

spinal cords of WT animals following paclitaxel treatment. By contrast, cisplatin alters endocannabinoid tone (43, 53), highlighting distinct mechanisms underlying neuropathies produced by these two chemotherapeutic agents (20). More work is needed to understand the role of the endocannabinoid system in induction and maintenance of chemotherapy-induced neuropathy.

In our study, both acute and chronic systemic treatment with the CB_2-preferring agonist AM1710 attenuated paclitaxel-induced allodynia in WT mice. Notably, deletion of CB_2 receptors or pharmacological blockade with the CB_2 antagonist AM630 prevented the anti-allodynic effects of AM1710. Thus, AM1710 suppressed chemotherapy-induced allodynia via CB_2 receptor activation, consistent with previous observations on anti-allodynic efficacies of other CB_2 agonists (14, 24, 26, 54) in neuropathic or inflammatory pain models. Taken together, these studies suggest therapeutic potential of CB_2 agonists in managing a wide spectrum of pain states.

Most CB_2 agonists identified to date exhibit low affinity for CB_1 (2). Indeed, it has been speculated that antinociceptive therapeutic efficacy of CB_2 agonists is mediated by CB_1 receptors (2, 55). In our study, AM1710 fully reversed paclitaxel-induced allodynia with similar efficacy in both CB_1KO and WT mice following either acute or chronic administration, consistent with a previous study showing that CB_2 agonist AM1241 retained antinociceptive efficacy in CB_1KO mice subjected to spinal nerve ligation (56). We also showed that antinociceptive effects of chronic AM1710 were blocked by a CB_2 antagonist in CB_1KO mice, further demonstrating that CB_2, but not CB_1, receptors mediate the anti-allodynic effects of the CB_2 agonist AM1710 on paclitaxel-induced neuropathy.

Tolerance may limit an analgesic's therapeutic use (57–59). It occurs following prolonged exposure of CB_1 receptors to cannabinoids in preclinical (60–63) and clinical (44) studies. Here, we showed that chronic dosing over 4 to 8 days with Δ^9-THC was sufficient to produce tolerance to both anti-allodynic efficacy and CB_1-mediated side effects in the paclitaxel-induced neuropathy model. However, no decrement in anti-allodynic efficacy was observed in animals received daily administration of the maximally effective dose of AM1710 over 8 days. Our data are in line with previous works showing that intrathecal JWH015 (17) or systemic A-836339 (64) does not produce antinociception tolerance following traumatic nerve injury.

In binding assays, the CB_2-preferring agonist AM1710 exhibits 54-fold selectivity for CB_2 over CB_1 receptors (65). This limited selectivity raises the possibility that a low level of CB_1 occupancy by this compound could potentially activate CB_1 receptors and translate into unwanted CB_1-mediated side effects following chronic administration, negatively impacting its therapeutic ratio and hindering its clinical acceptance. We evaluated this possibility in two ways. The first was that in our study, chronic AM1710 did not result in motor deficits or hypothermia, hallmarks of CB_1 agonists, consistent with previous observations with other CB_2 agonists (11, 54, 64, 66–69). The second was to detect signs of CB_1-mediated withdrawal. Physical dependence, quantified by signs of withdrawal following antagonist administration, has been reported after chronic cannabinoid (3, 58) and opioid (70–72) use. For example, challenge with the CB_1 antagonist rimonabant elicits profound withdrawal

symptoms in animals treated chronically with CB_1 agonists (40, 72–74). However, no study has examined whether prolonged treatment with a CB_2-preferring agonist results in a state where cannabinoid withdrawal signs through residual CB_1 activity can be elicited. In theory, this would be a very sensitive way to detect low levels of sustained CB_1 receptor activation. Here, we showed that unlike with chronic Δ^9-THC, mice treated with chronic AM1710 did not exhibit signs of rimonabant-precipitated withdrawal. Importantly, we assessed withdrawal signs in a neuropathic pain model to mimic a common clinical scenario. Coupled with the observation that CB_2 agonists show little intrinsic reward (75, 76), this class of compounds may lack drug abuse liability. These findings collectively support the clinical potential of prolonged use of CB_2 agonists.

Whether withdrawal symptoms could be elicited by precipitation at CB_2 receptors in animals receiving chronic CB_2 agonists is an important question that has never been studied. Here, we evaluated behaviors (i.e. paw tremors, headshakes, scratching) that are signs common to withdrawal precipitated by CB_1 or opioid receptor antagonists (40, 70–72). These behaviors were absent in AM1710-treated WT or CB_1KO mice following CB_2 antagonist AM630 challenge (CB_1KO mice were used to avoid potential residual CB_1-mediated component of AM1710). Interestingly, scratching was produced independent of withdrawal by rimonabant, but not AM630, consistent with pruritis as a common response to CB_1 antagonists (77). More work is necessary to further investigate possible withdrawal signs at CB_2 receptors.

Here, we reported the first evaluation of the site of action of a CB_2 agonist in the chemotherapy-induced neuropathy model. We showed that anti-allodynic effects of systemic AM1710 were blocked by intrathecal administration of a CB_2 antagonist. Moreover, a systemically inactive dose of AM1710 (5 μg/animal, equivalent to 0.16–0.2 mg/kg), administered intrathecally, produced robust antinociception in WT but not CB_2KO mice. Thus, activation of spinal CB_2 receptors by AM1710 is sufficient to reverse paclitaxel-induced allodynia. Peripheral (11, 12), spinal (14–17), or both peripheral and spinal (13, 18) sites of action are implicated in CB_2 agonist efficacy in various preclinical pain models. The differences in site of action could be attributed to different functional properties of the CB_2 agonists or distinct mechanisms produced by the specific pain state. Interestingly, in line with our results in chemotherapy-induced neuropathy, spinal site of CB_2 agonist action has been implicated in models of traumatic nerve injury (13–17). Therefore, CB_2 agonists may possess a shared mechanism of action in suppressing neuropathic pain through activation of spinal CB_2 receptors.

To further explore the mechanism of CB_2-mediated antinociception, we studied the impact of AM1710 on expression of cytokines and a chemokine in paclitaxel-induced neuropathy. Pro-inflammatory cytokines (e.g. IL-1β (28), IL-6 (29), TNFα (30–34)), and the chemokine MCP-1 (35) are implicated in mechanisms of neuropathic pain produced by traumatic nerve injury. Inflammatory processes are also generated by chemotherapy treatments (78–81). We did not detect alterations of spinal mRNA levels of IL-1β, IL-6 or TNFα during the maintenance phase of paclitaxel-induced allodynia. Transient upregulations of TNFα (81) or IL-6 (29) have been observed during the development of neuropathy induced by vincristine or nerve injury. It is possible that earlier timepoints during the development of paclitaxel-

induced neuropathy would be sensitive to transient alterations in cytokine production. Nonetheless, AM1710 robustly decreased spinal mRNA levels of TNFα in paclitaxel-treated WT mice. Our results, along with published report on CB_2 agonist JWH015-induced TNFα downregulation *in vitro* (36), suggest possible TNFα involvement in CB_2 activity. We also observed that spinal MCP-1 mRNA levels were elevated by paclitaxel and were decreased by AM1710. Thus, suppression of MCP-1 may contribute to the mechanism of CB_2-mediated anti-allodynic efficacy in chemotherapy-induced neuropathy (79, 80). In inflammatory and neuropathic pain, TNFα upregulates MCP-1 (82) and modulates central sensitization (83–85) and c-fiber responses (86, 87). CB_2 agonists suppress central sensitization (88–91). More studies are necessary to identify the source of spinal TNFα and MCP-1, their regulation, and their potential roles in CB_2-mediated suppression of central sensitization and chemotherapy-induced neuropathy.

In conclusion, chronic systemic treatment with the CB_2 agonist AM1710 suppressed chemotherapy-induced allodynia without producing tolerance, CB_1-mediated cannabinoid withdrawal or CNS side effects associated with CB_1 activation. The observed anti-allodynic efficacy required activation of spinal CB_2 receptors and was independent of CB_1 signaling. Furthermore, the pro-inflammatory cytokine TNFα and chemokine MCP-1 are likely involved in CB_2-mediated anti-allodynic efficacy. Together, our results support the therapeutic potential of prolonged use of CB_2 agonists for managing toxic neuropathic pain without apparent adverse effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2-AG	2-arachidonoyl glycerol
AEA	anandamide
ANOVA	analysis of variance
BL	baseline
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CNS	central nervous system

CR	cremophor
DMSO	dimethyl sulfoxide
Δ^9-THC	Δ^9-tetrahydrocannabinol
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
FAAH	fatty acid amide hydrolase
i.p	intraperitoneal
i.t	intrathecal
IL-1β	interleukin-1 beta
IL-6	interleukin 6
KO	knock out
MCP-1/CCL2	monocyte chemoattractant protein-1
MGL	monoacylglycerol lipase
NIDA	National Institute on Drug Abuse
PTX	paclitaxel
qRT-PCR	quantitative reverse transcription polymerase chain reaction
TNFα	tumor necrosis factor alpha
WT	wildtype

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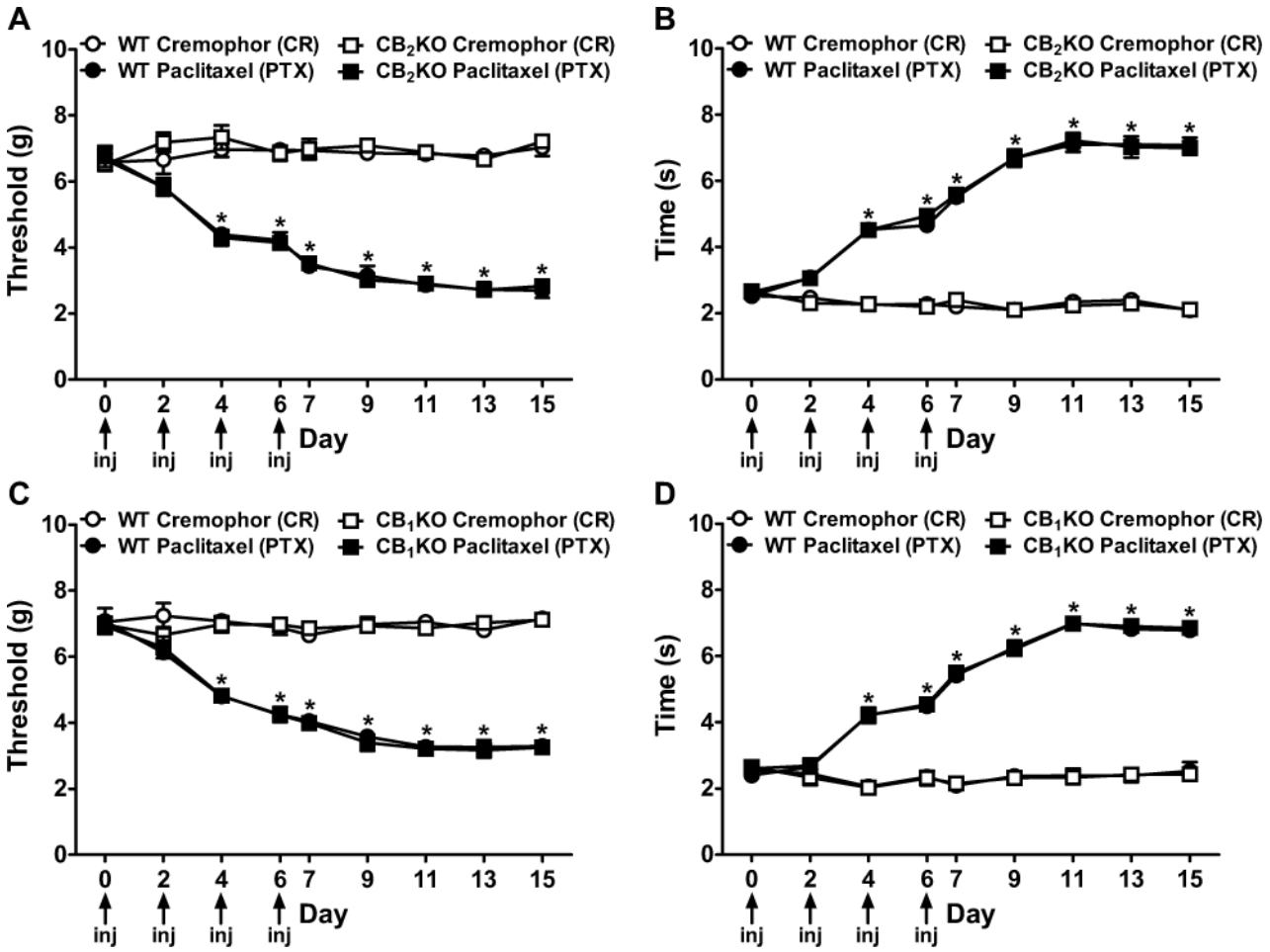

Figure 1. Paclitaxel produced hypersensitivities to mechanical and cold stimulation

(A, C) Mechanical and (B, D) cold allodynia developed equivalently in (A, B) CB_2KO, (C, D) CB_1KO, and corresponding WT littermates following paclitaxel treatment. Non-chemotherapy controls received cremophor-vehicle in lieu of paclitaxel. Arrows show timing of paclitaxel or cremophor injections (inj). Data are expressed as mean ± SEM (n=6 per group). *P<0.05 vs. control, repeated measures ANOVA and one-way ANOVA at each timepoint.

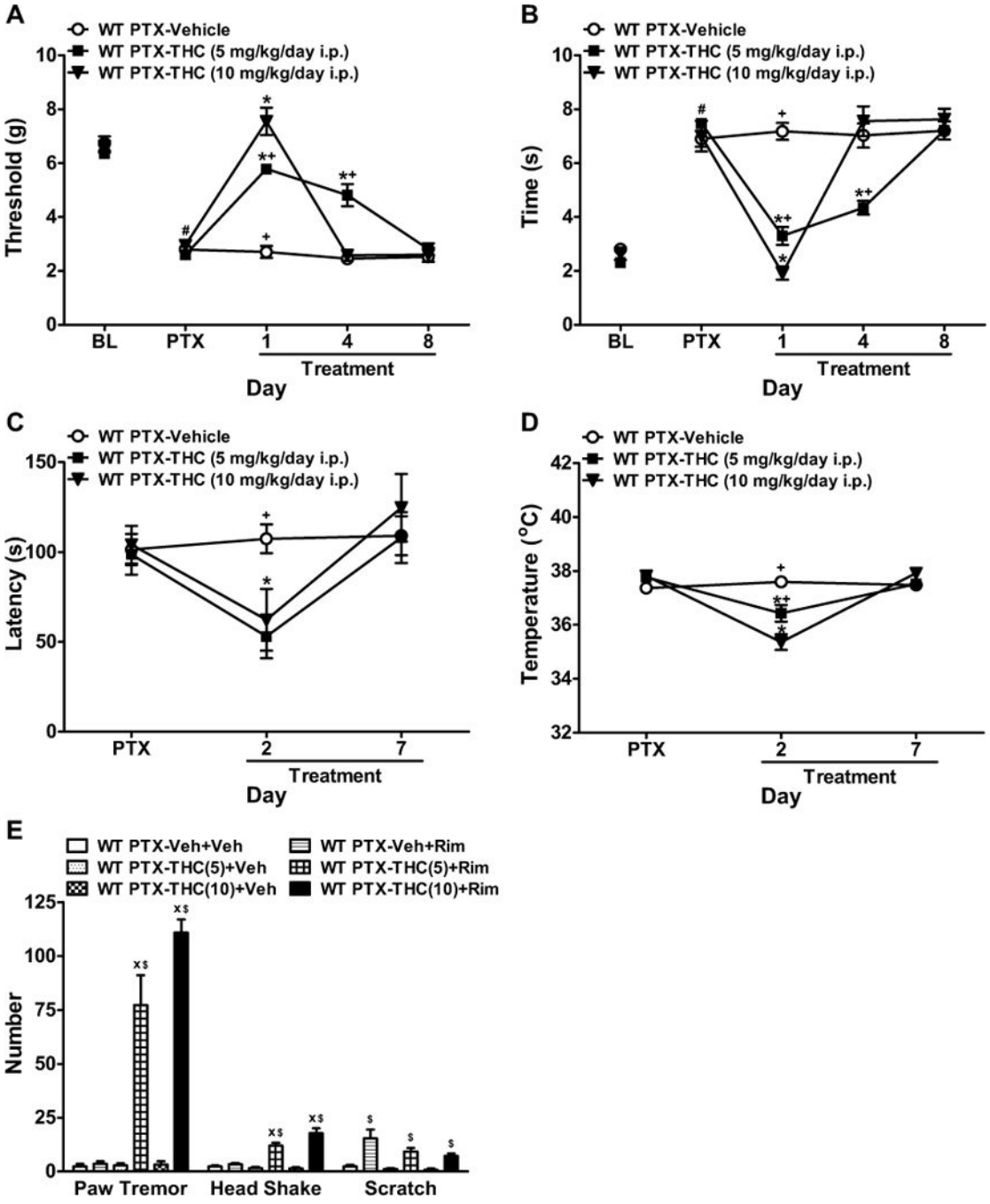

Figure 2. Effects of Δ^9-THC in paclitaxel-treated WT mice

(A, B) Δ^9-THC (5 or 10 mg/kg/day i.p.) attenuated paclitaxel-induced (A) mechanical and (B) cold allodynia in WT (C57BL/6J) mice in a dose- and time-dependent manner. (C, D) Δ^9-THC (5 or 10 mg/kg/day i.p.) decreased (C) motor performance and (D) body temperature in paclitaxel-treated WT mice relative to vehicle on treatment day 2, but not day 7. (E) Δ^9-THC (5 or 10 mg/kg/day i.p.) produced withdrawal symptoms when challenged with the CB_1 antagonist rimonabant. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline. Data are expressed as mean ± SEM (n=5–6 per group). *P<0.05 vs.

vehicle, $^{+}P<0.05$ vs. Δ^9-THC (10 mg/kg/day i.p.), $^{x}P<0.05$ vs. Veh+Rim (chronic vehicle and challenge by rimonabant), $^{\$}P<0.05$ vs. Veh+Veh (chronic vehicle and challenge by vehicle), one-way ANOVA followed by Bonferroni *post hoc* test or two-tailed t-test. $^{\#}P<0.05$ vs. BL, repeated measures ANOVA.

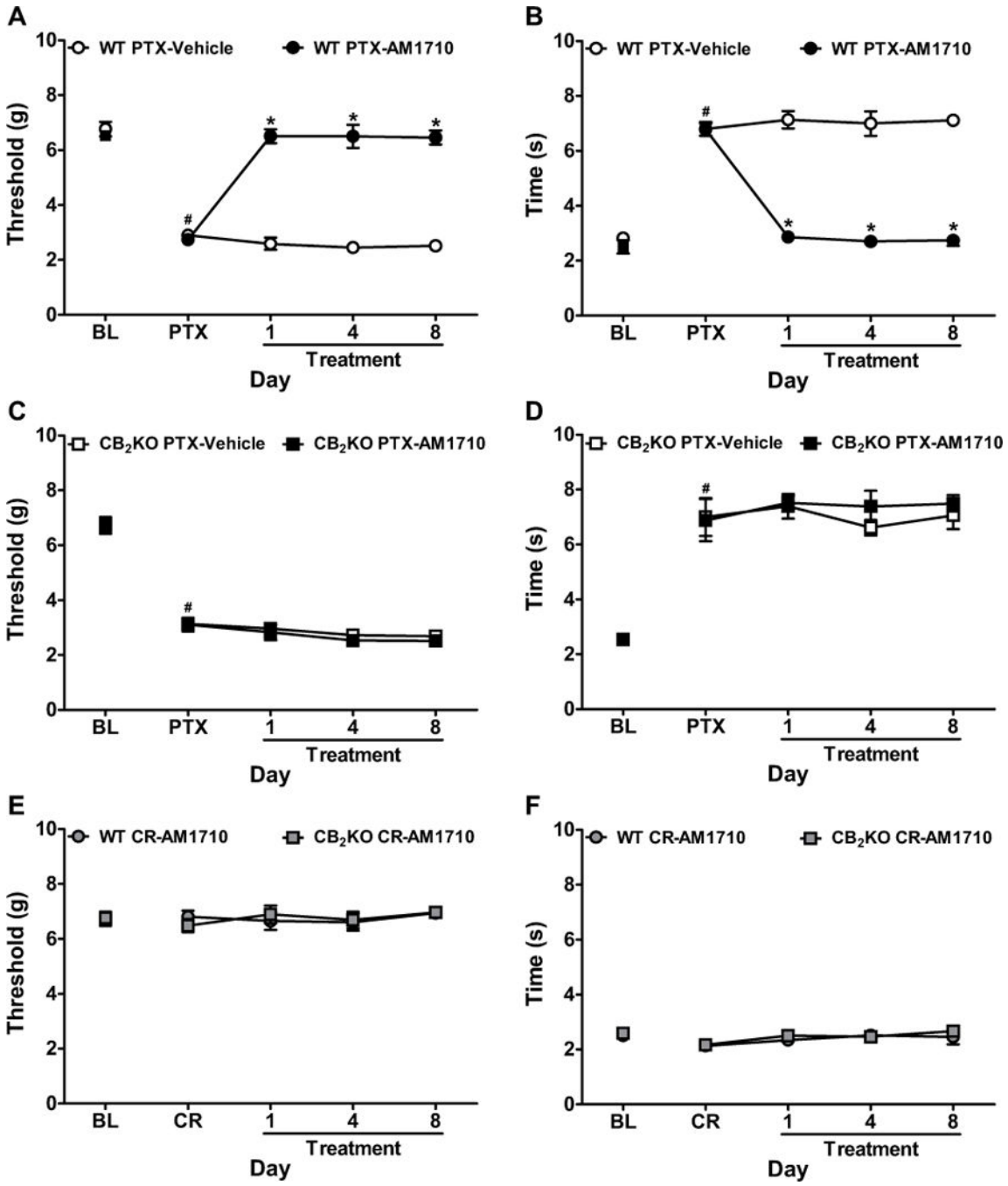

Figure 3. Chronic systemic administration of AM1710 suppressed paclitaxel-induced neuropathy in WT but not CB_2KO mice

(A, B) AM1710 (5 mg/kg/day i.p. × 8 days) reversed paclitaxel-induced (A) mechanical and (B) cold allodynia in WT littermates. (C, D) AM1710 (5 mg/kg/day i.p. × 8 days) did not suppress paclitaxel-induced (C) mechanical or (D) cold allodynia in CB_2KO mice. (E, F) AM1710 (5 mg/kg/day i.p. × 8 days) did not alter (E) mechanical or (F) cold responsiveness in cremophor-treated CB_2KO or WT mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; CR, post-cremophor baseline. Data are expressed as mean ± SEM (n=4–8 per

group). *P<0.05 vs. vehicle, one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P$<0.05 vs. pre-paclitaxel baseline, repeated measures ANOVA.

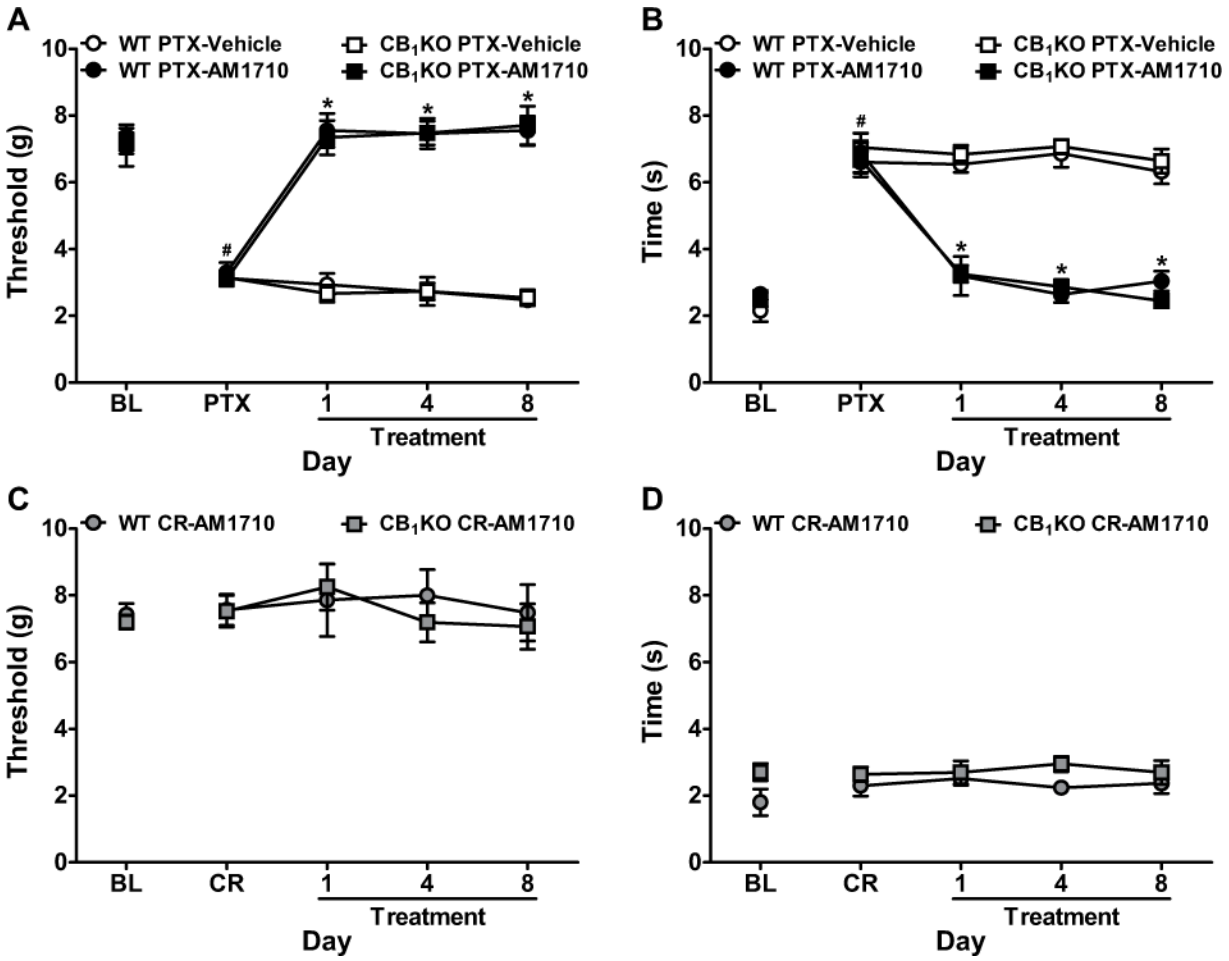

Figure 4. Chronic systemic administration of AM1710 reversed paclitaxel-induced neuropathic pain with similar efficacy in CB_1KO and WT mice

(A, B) AM1710 (5 mg/kg/day i.p. × 8 days) reversed paclitaxel-induced (A) mechanical and (B) cold allodynia in both CB_1KO and WT littermates. (C, D) AM1710 (5 mg/kg/day i.p. × 8 days) did not alter (C) mechanical or (D) cold responsiveness in cremophor-treated CB_1KO or WT mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline; CR, post-cremophor baseline. Data are expressed as mean ± SEM (n=4–8 per group). *P<0.05 vs. vehicle, one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P$<0.05 vs. pre-paclitaxel baseline, repeated measures ANOVA.

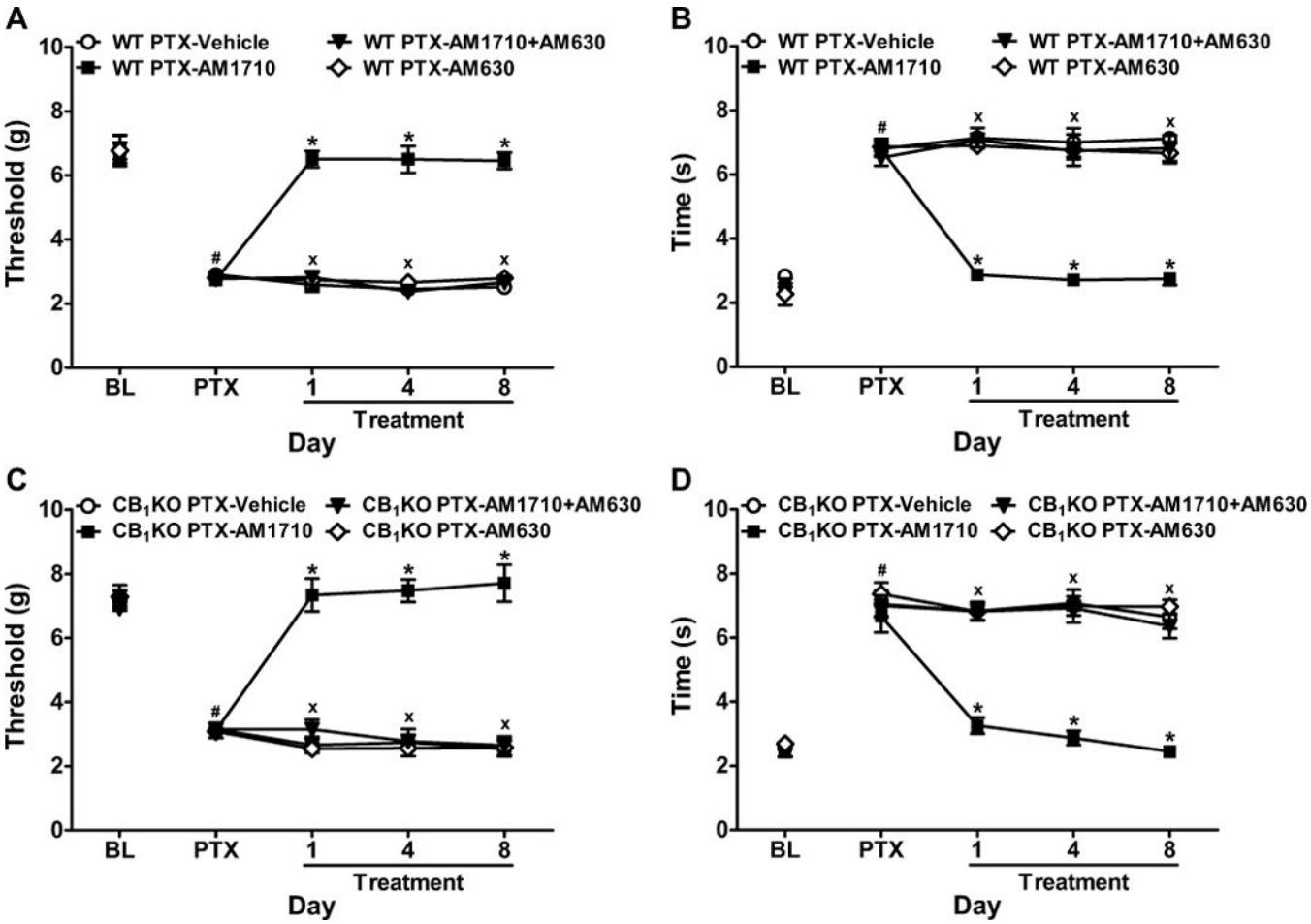

Figure 5. Anti-allodynic effects of chronic systemic AM1710 were mediated by CB_2 receptors

AM1710 (5 mg/kg/day i.p. × 8 days)-induced suppressions of paclitaxel-evoked (A, C) mechanical and (B, D) cold allodynia were blocked by the CB_2 antagonist AM630 (5 mg/kg/day i.p. × 8 days) in both (A, B) WT (C57BL/6J) and (C, D) CB_1KO mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline. Data are expressed as mean ± SEM (n=4–9 per group). **P*<0.05 vs. vehicle, ^{x}P <0.05 vs. AM1710 (5 mg/kg i.p.), one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P$<0.05 vs. pre-paclitaxel baseline, repeated measures ANOVA.

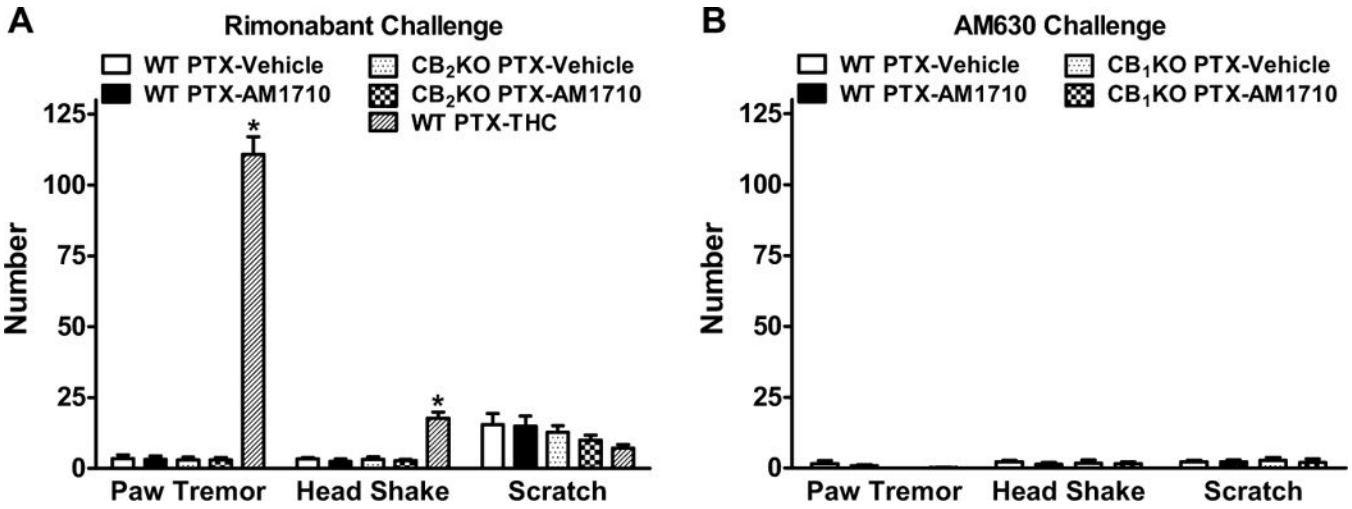

Figure 6. Chronic systemic AM1710 treatment did not produce cannabinoid CB_1-dependent withdrawal signs

(A) AM1710 (5 mg/kg/day i.p. × 9 days) did not produce CB_1-dependent withdrawal signs (i.e. paw tremors, headshakes) when precipitated with the CB_1 antagonist rimonabant (10 mg/kg i.p.) in CB_2KO or WT littermates. (B) Challenge with the CB_2 antagonist AM630 (5 mg/kg i.p.) did not produce paw tremors, headshakes, or scratching behaviors in CB_1KO or WT littermates treated chronically with AM1710 (5 mg/kg/day i.p. × 9 days). Data are expressed as mean ± SEM (n=4–5 per group). *P<0.05 vs. vehicle, one-way ANOVA followed by Bonferroni *post hoc* test.

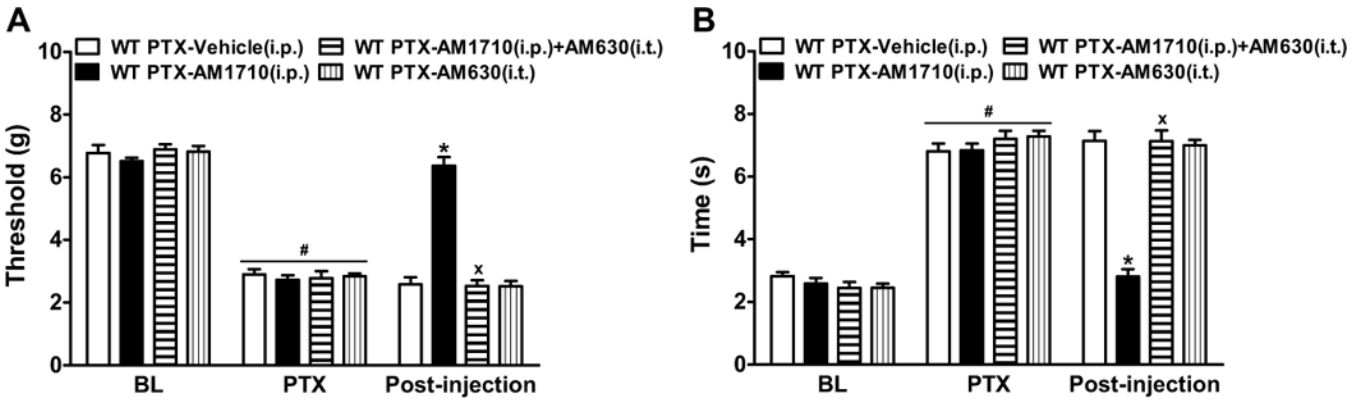

Figure 7. Antagonism of spinal CB_2 receptors blocked anti-allodynic effects of systemic AM1710 in WT mice

Intrathecal administration of the CB_2 antagonist AM630 (5 μg i.t.) blocked AM1710 (5 mg/kg i.p.)-induced suppressions of (A) mechanical and (B) cold allodynia in paclitaxel-treated WT (C57BL/6J) mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline. Data are expressed as mean ± SEM (n=6 per group). **P*<0.05 vs. vehicle, ^{x}P<0.05 vs. AM1710 (5 mg/kg i.p.), one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P$<0.05 vs. pre-paclitaxel baseline, repeated measures ANOVA.

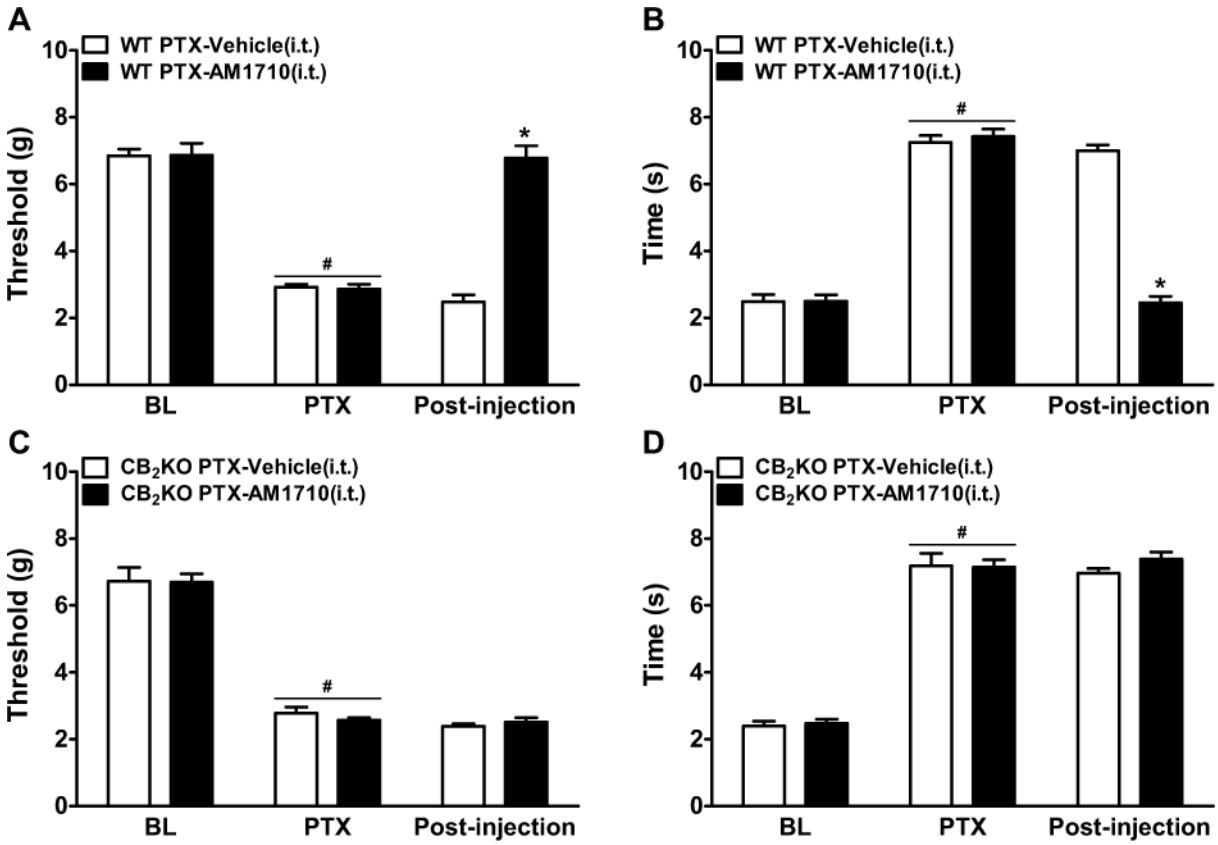

Figure 8. Activation of spinal CB_2 receptors suppressed paclitaxel-induced allodynia in WT but not CB_2KO mice

Intrathecal administration of AM1710 (5 μg i.t.) suppressed paclitaxel-induced (A, C) mechanical and (B, D) cold allodynia in (A, B) WT, but not (C, D) CB_2KO mice. BL, pre-paclitaxel baseline; PTX, post-paclitaxel baseline. Data are expressed as mean ± SEM (n=6 per group). **P*<0.05 vs. vehicle, one-way ANOVA followed by Bonferroni *post hoc* test. $^{\#}P$<0.05 vs. pre-paclitaxel baseline (BL), repeated measures ANOVA.

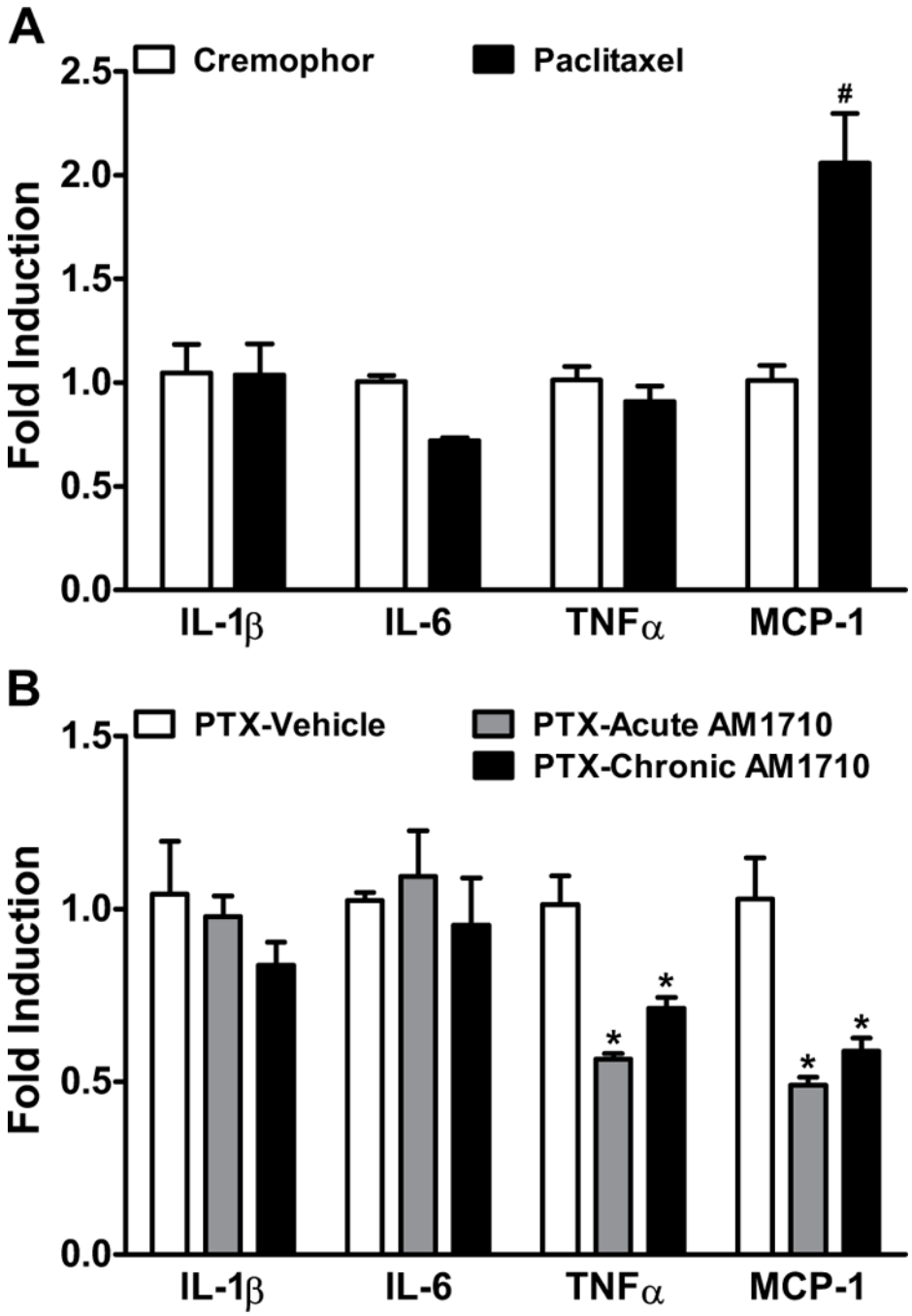

Figure 9. Impact of paclitaxel and AM1710 on cytokine and chemokine mRNA levels in lumbar spinal cord

(A) Paclitaxel increased the spinal mRNA levels of MCP-1, but not IL-1β, IL-6, or TNFα relative to cremophor in WT mice (day 15 post initial paclitaxel dosing). (B) Both acute (once daily injections of vehicle × 7 days followed by a terminal injection of AM1710 (5 mg/kg i.p.) on the 8th day, grey bar) and chronic (5 mg/kg/day i.p. × 8 days, black bar) administrations of AM1710 decreased the spinal mRNA levels of TNFα and MCP-1, but not IL-1β or IL-6 relative to vehicle (once daily × 8 days, white bar) in paclitaxel-treated WT animals. IL-1β, interleukin-1 beta; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha;

MCP-1, monocyte chemoattractant protein-1. Data are expressed as mean ± SEM (n=4 per group). $^{\#}P<0.05$ vs. cremophor vehicle in lieu of paclitaxel, one-tailed t-test. $^{*}P<0.05$ vs. vehicle in lieu of AM1710, one-way ANOVA followed by Bonferroni *post hoc* test.