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Selective Activation of Cannabinoid CB_2 Receptors Suppresses Neuropathic Nociception Induced by Treatment with the Chemotherapeutic Agent Paclitaxel in Rats

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Running Title: Cannabinoid CB_2 suppression of paclitaxel-evoked neuropathy

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Abstract

Activation of cannabinoid CB_2 receptors suppresses neuropathic pain induced by traumatic nerve injury. The present studies were conducted to evaluate the efficacy of cannabinoid CB_2 receptor activation in suppressing painful peripheral neuropathy evoked by chemotherapeutic treatment with the anti-tumor agent paclitaxel. Rats received paclitaxel (2 mg/kg i.p. per day) on four alternate days to induce mechanical hypersensitivity (mechanical allodynia). Mechanical allodynia was defined as a lowering of the threshold for paw withdrawal to stimulation of the plantar hind paw surface with an electronic von Frey stimulator. Mechanical allodynia developed in paclitaxel-treated animals relative to groups receiving the cremophor: ethanol: saline vehicle at the same times. Two structurally distinct cannabinoid CB_2 agonists — the aminoalkylindole (*R*,*S*)-AM1241 ((*R*,*S*)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl)-1*H*-indol-3-yl]-methanone) and the cannabilactone AM1714 (1,9-dihydroxy-3-(1′,1′-dimethylheptyl)-6*H*-benzo[*c*]chromene-6-one) — produced a dose-related suppression of established paclitaxel-evoked mechanical allodynia following systemic administration. Pretreatment with the CB_2 antagonist SR144528 (5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-*N*-(1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-1*H*-pyrazole-3-carboxamide), but not the CB_1 antagonist SR141716 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide), blocked the anti-allodynic effects of both (*R*,*S*)-AM1241 and AM1714. Moreover, (*R*)-AM1241, but not (*S*)-AM1241, suppressed paclitaxel-evoked mechanical allodynia relative to either vehicle treatment or pre-injection thresholds, consistent with mediation by CB_2. Administration of either the CB_1 or CB_2 antagonist alone failed to alter paclitaxel-evoked mechanical allodynia. Moreover, (*R*,*S*)-AM1241 did not alter paw withdrawal thresholds in rats that received the cremophor vehicle in lieu of paclitaxel whereas AM1714

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induced a modest antinociceptive effect. Our data suggest that cannabinoid CB_2 receptors may be important therapeutic targets for the treatment of chemotherapy-evoked neuropathy.

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Introduction

Painful peripheral neuropathy is a well documented side-effect of chemotherapeutic treatment (for review see Polomano and Bennett, 2001; Aley and Levine, 2002). The major classes of antineoplastic agents– the vinca alkaloids (e.g. vincristine), taxane (e.g. paclitaxel) and platinum-derived (e.g. cisplatin) compounds– are associated with the development of dose-limiting neuropathic pain. The chemotherapeutic agent used, dosing schedule, form of cancer, and presence of additional medical complications can impact the occurrence and severity of chemotherapy-induced neuropathy (for review see Cata et al., 2006).

Paclitaxel is commonly used for the treatment of solid tumors, ovarian and breast cancer. Paclitaxel induces antimitotic actions by impeding the cell cycle in the late phases of mitosis, stabilizing microtubule formation, and ultimately inducing apoptosis (Schiff and Horwitz, 1980). Paclitaxel preferentially impairs myelinated Aβ and Aδ fibers which carry sensory information about mechanical stimulation to the central nervous system (CNS) (Dougherty et al., 2004). Paclitaxel-evoked neuropathy is manifested as pain in the distal extremities, forming a glove and stocking pattern (Dougherty et al., 2004). Mitochondrial toxicity is also preferentially localized to long axons innervating distal extremities (Flatters and Bennett, 2006). Thus, effects of paclitaxel are evident in those areas where, due to increased distance of axonal transport and mitochondrial energy demand, disruption in sensation would first be present. Dysfunctional mitochondria could lead to low levels of energy which could potentially impair ion transporters, resulting in spontaneous neuronal firing with no concurrent receptor stimulation (i.e. paraesthesia) (Flatters and Bennett, 2006).

Peripheral neuropathy can limit dosing and duration of chemotherapeutic treatment (Holmes et al., 1991; Rowinsky et al., 1993). Pharmacotherapies for chemotherapy-induced

neuropathy are limited because the underlying cellular mechanisms remain incompletely understood. Amytriptyline, gabapentin and opioids are used to treat chemotherapy-induced neuropathy. However, none of these drugs has been shown to completely attenuate neuropathic pain (for review see Lee and Swain, 2006). The absence of approved medications available for preventing or treating this debilitating neuropathy makes the identification of alternative effective analgesics a crucial medical need.

Cannabinoids suppress neuropathic pain induced by traumatic nerve injury, toxic insults and metabolic changes (for review see Hohmann, 2002; Guindon and Hohmann, 2008). Both CB_1- (Herzberg et al., 1997; Fox et al., 2001) and CB_2 - (Ibrahim et al., 2003; Beltramo et al., 2006) specific mechanisms suppress neuropathic nociception evoked by traumatic nerve injury. CB_1 receptors are expressed primarily within the CNS (Zimmer et al., 1999). CB_2 receptors are expressed primarily, but not exclusively, outside the CNS in cells of the immune system (Munro et al., 1993). CB_2 receptors are upregulated in the CNS in neuropathic pain states (Wootherspoon et al., 2005; Beltramo et al., 2006). CB_2-selective agonists are not associated with psychoactive and motor effects typical of CB_1 receptor activation (Hanus et al., 1999; Malan et al., 2001), making the CB_2 receptor an attractive therapeutic target for the treatment of neuropathic pain.

The mixed CB_1/CB_2 agonist WIN55,212-2 suppresses neuropathic nociception induced by paclitaxel through a CB_1-specific mechanism (Pascual et al., 2005). WIN55,212-2 also suppresses vincristine-induced neuropathy through activation of both CB_1 and CB_2 receptors (Rahn et al., 2007). Activation of CB_2 receptors with (*R,S*)-AM1241 partially attenuates vincristine-induced neuropathy (Rahn et al., 2007). However, a role for CB_2 receptor activation in suppressing paclitaxel-evoked neuropathy has not been investigated. This investigation is important because distinct mechanisms may underlie development of neuropathic pain induced

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by different antineoplastic agents (for review see Cata et al., 2006). Neuropathic pain symptoms associated with each chemotherapeutic agent vary and can respond differently to pharmacological treatments (Flatters and Bennett, 2004). We used two structurally distinct CB_2-selective agonists, AM1714 and (*R*,*S*)-AM1241 (Fig. 1), to evaluate the contribution of CB_2 receptors to cannabinoid modulation of paclitaxel-induced neuropathy. AM1714 is a novel CB_2-selective agonist (K_i: CB_1 vs. CB_2: 400 nM vs. 0.8 nM) from the cannabilactone class of cannabinoids (Khanolkar et al., 2007). AM1714 has recently been shown to induce peripheral antinociception but has not previously been characterized in an animal model of pathological pain. (*R*,*S*)-AM1241 is a CB_2-selective agonist from the aminoalkylindole class of cannabinoids. (*R*,*S*)-AM1241 behaves as a protean agonist *in vitro* (Yao et al., 2006) and a CB_2 agonist *in vivo* (see Guindon and Hohmann, 2008 for review). We also compared the ability of (*R*)-AM1241 (K_i: CB_1 vs. CB_2: 139.7 nM vs. 1.4 nM), and its less active enantiomer (*S*)-AM1241 (K_i: CB_1 vs. CB_2: 2029 nM vs. 160.5 nM) (Thakur et al., 2005), to suppress paclitaxel-evoked neuropathy. Pharmacological specificity was evaluated using selective antagonist/inverse agonists for CB_1 (SR141716) and CB_2 (SR144528). Comparisons were made with the prototypical narcotic analgesic morphine.

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Methods

Subjects

One hundred and seventy-five adult male Sprague-Dawley rats (301-396g; Harlan, Indianapolis, IN) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain. Bedding containing metabolized paclitaxel was treated as biohazardous waste and disposed of according to the appropriate institutional guidelines.

Drugs and Chemicals

Paclitaxel was obtained from Tecoland (Edison, NJ). (*R,S*)-AM1241 ((*R,S*)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl)-1*H*-indol-3-yl]-methanone), (*R*)-AM1241, (*S*)-AM1241, and AM1714 (1,9-dihydroxy-3-(1′,1′-dimethylheptyl)-6*H*-benzo[*c*]chromene-6-one) were synthesized in the Makriyannis laboratory by one of the authors (by AZ and GT respectively). The (*R*)- and (*S*)-enantiomers were prepared by chiral synthesis (by AZ). SR141716 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide) and SR144528 5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-*N*-(1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-1*H*-pyrazole-3-carboxamide were provided by NIDA. Cremophor EL and morphine sulfate were obtained from Sigma Aldrich (St. Louis, MO). Dimethyl Sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Paclitaxel was dissolved as previously described (Flatters and Bennett, 2004) and administered in a volume of 1 ml/kg. Briefly, paclitaxel was dissolved in a 1:2 ratio of working stock (1:1 ratio of cremophor EL and 95% ethanol) to saline. All other drugs were dissolved in a vehicle of 100% DMSO for systemic administration and administered in a volume of 1 ml/kg bodyweight.

General Experimental Methods

Baseline withdrawal thresholds to mechanical stimulation of the hind paw were measured on day zero. Rats subsequently received four intraperitoneal (i.p.) injections of either paclitaxel (2 mg/kg/day i.p.) or cremophor: ethanol: saline vehicle (1 ml/kg/day i.p.) on alternate days, immediately following behavioral testing. The injection paradigm consisted of four once-daily injections, administered on days 0, 2, 4, and 6, as described previously (Polomano et al., 2001). Mechanical withdrawal thresholds were measured on days 0, 4, 7, 11, 14, 18, and 21. Behavioral testing was always performed just prior to paclitaxel administration (except for days 2 and 6 on which paw withdrawal thresholds were not assessed). To evaluate the possible resolution of paclitaxel-induced neuropathy, paclitaxel-treated rats were additionally evaluated weekly for the presence of mechanical allodynia for 86 days following the initial injection of paclitaxel in a pilot study. In all studies, the experimenter was blinded to the drug condition. Moreover, a single experimenter tested all animals in any given study.

Assessment of mechanical withdrawal thresholds

Mechanical withdrawal thresholds were assessed using a digital Electrovonfrey Anesthesiometer (IITC model Alemo 2290-4; Woodland Hills, CA) equipped with a rigid tip. Rats were placed underneath inverted plastic cages and positioned on an elevated mesh platform. Rats were allowed to habituate to the chamber for 10 - 15 min prior to testing. Stimulation was applied to the midplantar region of the hind paw through the floor of a mesh platform. Mechanical stimulation was terminated upon paw withdrawal; consequently, there was no upper threshold limit set for termination of a trial. On the test day (day 21), baseline mechanical withdrawal thresholds were assessed, and effects of pharmacological manipulations were subsequently evaluated. Nocifensive responses were observed in paclitaxel-treated animals at

forces (g) that failed to elicit withdrawal responses prior to chemotherapy treatment. Paclitaxel-induced decreases in mechanical paw withdrawal thresholds (assessed with the electrovonfrey anesthesiometer) were therefore defined as mechanical allodynia.

Pre-injection mechanical withdrawal thresholds were measured on day 21 prior to acute pharmacological manipulations. Paclitaxel-treated animals received systemic injections of either (*R*,*S*)-AM1241 (10 mg/kg i.p.; n = 7), AM1714 (10 mg/kg i.p.; n = 6) or DMSO (n = 7). Mechanical withdrawal thresholds were measured 30, 60, and 90 min post-injection to assess the time course of CB_2 agonist actions. Subsequent studies evaluated dose-response and pharmacological specificity by measuring paw withdrawal thresholds at the time-point of maximal cannabinoid-induced suppression of paclitaxel-evoked neuropathy (30 min post-injection).

To evaluate dose-response, separate groups of paclitaxel-treated animals received either the racemate (*R*,*S*)-AM1241 (1, 5, or 10 mg/kg i.p.; n = 6-10 per group), AM1714 (1, 5, or 10 mg/kg i.p.; n = 6 per group) or DMSO (n = 11). Separate groups of animals received the enantiomers of (*R*,*S*)-AM1241 — (*R*)-AM1241 (10 mg/kg i.p.; n = 6), or its less active enantiomer (*S*)-AM1241 (10 mg/kg i.p.; n = 6) — or the opioid agonist morphine (2 or 4 mg/kg i.p.; n = 6 per group).

To determine pharmacological specificity, separate groups of paclitaxel-treated rats received (*R*,*S*)-AM1241 (10 mg/kg i.p., n = 6), AM1714 (10 mg/kg i.p., n = 6), SR144528 (10 mg/kg i.p.) administered 20 min prior to either (*R*,*S*)-AM1241 (10 mg/kg i.p.; n = 6) or AM1714 (10 mg/kg i.p.; n = 5), SR144528 alone (10 mg/kg i.p.; n = 7) or DMSO (n = 6). In separate groups of animals, SR141716 (10 mg/kg i.p) was administered 20 minutes prior to treatment with either (*R*,*S*)-AM1241 (10 mg/kg i.p.; n = 5) or AM1714 (10 mg/kg i.p., n = 8).

Antagonist pre-treatment groups received a double volume of the DMSO vehicle. Paw withdrawal thresholds were therefore compared in animals receiving dual injections of either DMSO or saline to verify that vehicle effects could not account for the pattern of results obtained. Therefore, additional control groups received (i.p.) either saline 20 minutes prior to saline (n = 6) or DMSO 20 minutes prior to DMSO (n = 6). To evaluate possible antinociceptive effects induced by the CB_2 agonists, the maximally effective anti-allodynic dose of either AM1714 (10 mg/kg i.p.; n = 6) or (*R*,*S*)-AM1241 (10 mg/kg i.p.; n = 6) was additionally administered to cremophor-treated controls. Paw withdrawal thresholds were assessed as described above.

Statistical Analyses

Data were analyzed using analysis of variance (ANOVA) for repeated measures, one-way ANOVA or planned comparison t-tests as appropriate. The Greenhouse-Geissser correction was applied to all repeated factors. Post hoc comparisons between control groups and other experimental groups were performed using the Dunnett test. Post-hoc comparisons between different experimental groups were also performed to assess dose-response relationships and pharmacological specificity using the Tukey test. Post-drug thresholds within a given group were compared with either pre-paclitaxel (baseline) thresholds or day 21 post-paclitaxel thresholds using paired t-tests. $P < 0.05$ was considered statistically significant.

Results

General Results

Body weight did not differ between groups prior to the treatment with either paclitaxel or the cremophor: ethanol: saline vehicle. Normal weight gain was observed in groups receiving either the cremophor vehicle or paclitaxel ($F_{2, 213} = 1.3$, $P > 0.27$, Fig 2A). However, one fatality was observed in groups receiving paclitaxel.

In a pilot study conducted to evaluate the resolution of paclitaxel-evoked mechanical allodynia, paw withdrawal thresholds were lower than baseline pre-paclitaxel thresholds beginning on day 7 ($P < 0.05$ planned comparison). Paclitaxel-induced mechanical allodynia was present, relative to baseline, from days 14 – 72 following the initiation of treatment ($P < 0.05$ for all planned comparisons, data not shown). Paw withdrawal thresholds were also similar from day 14 – 72 post-paclitaxel. Therefore, day 21 post-paclitaxel was used to evaluate CB_2 agonist actions on paclitaxel-evoked mechanical allodynia in all studies reported herein. Paw withdrawal thresholds did not differ between paclitaxel-treated groups prior to cannabinoid or vehicle treatments on day 21 in any study. By contrast, thermal hyperalgesia was not observed in the present paclitaxel dosing paradigm (data not shown).

Mechanical withdrawal thresholds did not differ between either the right or the left paw for any group on any given day (days 0 – 21); therefore, withdrawal thresholds are presented as the mean of duplicate measurements, averaged across paws. Paw withdrawal thresholds were similar between groups prior to administration of paclitaxel in any given study. Paclitaxel lowered mechanical paw withdrawal thresholds (i.e. equivalently in each paw) relative to control conditions receiving the cremophor vehicle ($F_{1,115} = 10.140$, $P < 0.01$; Fig 2B). Paclitaxel

lowered paw withdrawal thresholds in all studies ($P < 0.001$ in each experiment; Fig 3 and 7A; see also Table 1).

Antagonist pretreatment conditions received dual injections of the DMSO vehicle. Paw withdrawal thresholds were therefore compared in groups receiving DMSO followed by DMSO and saline followed by saline. Post-injection paw withdrawal thresholds did not differ from day 21 pre-injection thresholds in either pretreatment group ($P > 0.54$ for both planned comparison t-tests; Table 1). Therefore, the volume of DMSO administered did not alter paclitaxel-evoked paw withdrawal thresholds in our study.

The CB_2 agonists (R,S)-AM1241 and AM1714 Suppress Paclitaxel-evoked Mechanical Allodynia

In paclitaxel-treated rats, (*R*,*S*)-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.) suppressed paclitaxel-induced mechanical allodynia relative to the vehicle condition ($F_{2,16} = 4.05$, $P < 0.05$; $P < 0.05$ for each comparison; Fig 3). Paclitaxel-induced mechanical allodynia was maximally suppressed by each agonist at 30 minutes post-injection ($F_{2,16} = 5.34$, $P < 0.05$). At this time point, both (*R*,*S*)-AM1241 and AM1714 normalized thresholds relative to pre-paclitaxel levels ($P < 0.05$ for all comparisons). (*R*,*S*)-AM1241 (10 mg/kg i.p.; n = 6) failed to induce an antinociceptive effect in animals that received cremophor: ethanol: saline vehicle in lieu of paclitaxel (Day 21 paw withdrawal threshold (Mean ± SEM) pre-injection vs. post-injection: 42.14 ± 0.36 g vs. 40.93 ± 0.78 g; $P > 0.32$; planned comparison t-test). However, AM1714 (10 mg/kg i.p. n = 6) produced a modest antinociceptive effect (Day 21 paw withdrawal threshold (Mean ± SEM) pre-injection vs. post-injection: 63.21 ± 2.98 g vs. 76.92 ± 4.22 g; $P < 0.05$; planned comparison t-test). Moreover, cremophor treatment did not alter day 21 paw withdrawal thresholds relative to day 0 baseline paw withdrawal thresholds in any group. Day 0 baseline

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paw withdrawal thresholds averaged 46.89 ± 4.23 g and 63.60 ± 4.61 g prior to initiation of cremophor treatment in groups that subsequently received (*R*,*S*)-AM1241 and AM1714, respectively on day 21. A lower baseline threshold was observed in the former compared to the latter group ($P < 0.05$, t-test). Group differences in baseline paw withdrawal thresholds may reflect individual differences combined with the sensitivity of the electrovonfrey device because each animal's threshold was highly reliable and reproducible. No differences between day 0 baseline paw withdrawal thresholds were observed for any groups tested by the same experimenter in any given study.

Effects of (R,S)-AM1241 and its Enantiomers on Paclitaxel-evoked Mechanical Allodynia

(*R*,*S*)-AM1241 increased mechanical withdrawal thresholds in a dose-related fashion relative to the vehicle condition ($F_{3,29} = 3.31$, $P < 0.05$; Fig 4A). Both the high (10 mg/kg i.p.) and middle (5 mg/kg i.p.) doses of (*R*,*S*)-AM1241 elevated paw withdrawal thresholds relative to vehicle ($P < 0.05$ for both comparisons). Effects of the low dose of (*R*,*S*)-AM1241 (1 mg/kg i.p.) did not differ from vehicle ($P > 0.12$). Both the high (10 mg/kg i.p.) and the middle (5 mg/kg i.p.) doses of (*R*,*S*)-AM1241 also elevated paw withdrawal thresholds relative to pre-injection thresholds determined 21 days following paclitaxel treatment ($F_{3,29} = 3.54$, $P < 0.05$; $P < 0.05$). Neither the low dose of (*R*,*S*)-AM1241 (1 mg/kg i.p.) nor DMSO altered paw withdrawal thresholds relative to pre-injection thresholds assessed on day 21 post-paclitaxel ($P > 0.10$). The middle and high doses of (*R*,*S*)-AM1241 normalized paw withdrawal thresholds relative to baseline (pre-paclitaxel) thresholds ($P > 0.16$), whereas DMSO failed to do so.

(*R*)-AM1241 increased paw withdrawal thresholds relative to the vehicle condition ($F_{3,25} = 4.37$, $P < 0.05$, Fig 4B) in paclitaxel-treated groups. (*S*)-AM1241 (10 mg/kg i.p.) did not significantly elevate paw withdrawal threshold relative to vehicle ($P > 0.43$). However, post hoc

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comparisons failed to reveal differential effects between (*S*)-AM1241 (10 mg/kg i.p.) and either (*R,S*)-AM1241 (10 mg/kg i.p.) or (*R*)-AM1241 (10 mg/kg i.p.) on paw withdrawal thresholds ($P > 0.24$). Both (*R*)-AM1241 (10 mg/kg i.p.) and (*R,S*)-AM1241 (10 mg/kg i.p.) significantly increased paw withdrawal thresholds relative to day 21 pre-injection thresholds ($P < 0.05$), whereas (*S*)-AM1241 failed to do so. (*R,S*)-AM1241 (10 mg/kg i.p.) and (*R*)-AM1241 (10 mg/kg i.p.) also normalized paw withdrawal thresholds relative to day 0 pre-paclitaxel thresholds ($F_{3,25} = 3.87$ $P < 0.05$; Fig 4B). By contrast, normalization of paw withdrawal thresholds was absent in groups receiving DMSO ($P < 0.001$).

The novel CB_2 agonist AM1714 suppresses paclitaxel-evoked mechanical allodynia

AM1714 suppressed paclitaxel-induced allodynia in a dose-dependent fashion ($F_{3,25} = 5.14$, $P < 0.01$, Fig 5). All three doses of AM1714 suppressed paclitaxel-evoked mechanical allodynia relative to their vehicle-treated counterparts ($P < 0.05$ for all comparisons). AM1714 (1, 5, and 10 mg/kg i.p) also normalized paclitaxel-induced mechanical allodynia relative to pre-paclitaxel baseline thresholds ($F_{3,25} = 5.63$, $P < 0.01$; $P > 0.14$ for all comparisons; Fig 5). The high dose (10 mg/kg i.p.; $P < 0.001$), but not the middle (5 mg/kg i.p.) or low dose (1 mg/kg i.p.) of AM1714 elevated paw withdrawal thresholds relative to day 21 pre-injection thresholds ($P > 0.23$ for both comparisons).

Pharmacological Specificity

Neither the CB_1-selective antagonist SR141716 (10 mg/kg i.p.) nor the CB_2-selective antagonist SR144528 (10 mg/kg i.p) altered paclitaxel-evoked mechanical allodynia relative to pre-injection thresholds ($P > 0.13$; see Table 1). The CB_2 antagonist SR144528 blocked the anti-allodynic effects of both (*R,S*)-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.; $F_{4,23} = 11.155$, $P < 0.001$; $P < 0.01$ for each comparison; Fig 6A). Paw withdrawal thresholds in agonist

groups pretreated with SR144528 did not differ from the vehicle condition ($P > 0.98$ for each comparison). Post hoc comparisons failed to reveal any differences in the anti-allodynic effects induced by either AM1714 (10 mg/kg i.p.) or (*R,S*)-AM1241 (10 mg/kg i.p.; $P > 0.98$).

SR141716 (10 mg/kg i.p.) failed to block the anti-allodynic effects produced by either (*R,S*)-AM1241 (10 mg/kg i.p.) or AM1714 (10 mg/kg i.p; $F_{4,31} = 10.788$, $P < 0.001$; Fig 6B). Paw withdrawal thresholds in paclitaxel-treated groups receiving DMSO were lower than those observed in groups receiving the CB_2 agonists in either the presence or absence of the CB_1 antagonist ($P < 0.01$ for each comparison). Paw withdrawal thresholds were similar in groups pretreated with SR141716 to those observed in groups receiving either agonist alone ($P > 0.11$ for each comparison). However, animals receiving SR141716 prior to AM1714 exhibited elevated paw withdrawal thresholds relative to baseline pre-paclitaxel thresholds ($P < 0.01$, planned comparison t-test; Fig. 7B). Post drug injection paw withdrawal thresholds were higher in all groups relative to day 21 pre-injection thresholds with the exception of vehicle ($P < 0.05$, planned comparison t-tests).

Effects of Morphine on Paclitaxel-evoked Mechanical Allodynia

The high dose of morphine (4 mg/kg i.p.) suppressed paclitaxel-induced mechanical allodynia relative to the vehicle condition ($F_{2,20} = 6.023$, $P < 0.01$; $P < 0.01$ for relevant comparison; Fig 7) and normalized paw withdrawal thresholds relative to pre-paclitaxel baseline thresholds ($P > 0.15$). The low dose of morphine (2 mg/kg i.p.) failed to alter post-paclitaxel paw withdrawal thresholds.

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Discussion

Two structurally distinct CB_2 agonists attenuated mechanical allodynia induced by treatment with the chemotherapeutic agent paclitaxel. Animals receiving paclitaxel remained in relatively good health as evidenced by the observation of normal weight gain during the course of chemotherapy treatment. However, one fatality was observed after two injections of paclitaxel. Paclitaxel-evoked mechanical hypersensitivity cannot be attributed to sensitization to repeated testing; paw withdrawal thresholds were stable in animals receiving the cremophor: ethanol: saline vehicle in lieu of paclitaxel over the same time course. Mechanical allodynia was observed in paclitaxel-treated animals tested weekly up to 3 months after the initiation of chemotherapy treatment in a pilot study. Paw withdrawal thresholds were similarly reduced relative to baseline from day 14 to 72 post-paclitaxel in this study; therefore day 21 was selected for the evaluation of drug effects on paclitaxel-evoked mechanical allodynia. Other studies have similarly reported peaks in neuropathic nociception with the present paclitaxel dosing paradigm from days 16 – 27 post initiation of paclitaxel treatment (Polomano et al., 2001; Flatters and Bennett, 2004). In all subsequent studies, mechanical allodynia developed by day 11 and continued to decrease until the final test day, day 21.

Thermal hyperalgesia was not observed in our study, consistent with previous reports employing the present paclitaxel dosing schedule (Polomano et al., 2001). A CB_1-mediated suppression of paclitaxel-induced thermal hyperalgesia has been reported using a cumulative paclitaxel dose of 4 mg/kg (Pascual et al., 2005) compared to our dose of 8 mg/kg. Differences in dosing and timing of paclitaxel injections may account for differences between these studies.

In our study, two structurally distinct cannabinoid CB_2 agonists, the aminoalklyindole (*R,S*)-AM1241 and the cannabilactone AM1714, suppressed paclitaxel-evoked mechanical

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allodynia through a CB_2-specific mechanism. All doses of AM1714 normalized paw withdrawal thresholds relative to pre-paclitaxel levels; however comparisons with day 21 pre-injection thresholds suggest that the high dose (10 mg/kg i.p.) was the most reliably effective dose. The high dose of AM1714 (10 mg/kg i.p.) produced a modest antinociceptive effect in animals treated with the cremophor vehicle in lieu of paclitaxel. By contrast, the high (10 mg/kg i.p.) and middle (5 mg/kg i.p.) but not the low (1 mg/kg i.p.) dose of (*R,S*)-AM1241 normalized paw withdrawal thresholds to pre-paclitaxel levels without inducing antinociception. Thus, AM1714 but not (*R,S*)-AM1241 produced antinociception in addition to suppression of allodynia. The mechanisms underlying these differences remain to be explored.

The suppression of paclitaxel-evoked neuropathic nociception induced by AM1241 and AM1714 is likely to be mediated by CB_2 receptors. First, multiple CB_2 agonists from different chemical classes suppressed paclitaxel-evoked neuropathic nociception. Second, (*R*)-AM1241, but not (*S*)-AM1241, suppressed paclitaxel-evoked mechanical allodynia relative to vehicle treatment and pre-injection thresholds, consistent with mediation by CB_2. Third, anti-allodynic effects of each agonist were blocked by the CB_2 antagonist SR144528. Fourth, the CB_1 antagonist SR141716 failed to block the anti-allodynic effects of either (*R,S*)-AM1241 or AM1714.

In our study, a trend toward enhanced antihyperalgesic efficacy was observed in groups pretreated with SR141716 prior to AM1714. This observation may suggest that blockade of CB_1 receptors increases endocannabinoid tone and enhances effects of the CB_2 agonist (Zhang et al., 2008). Enhancement of CB_2 agonist efficacy by CB_1 receptor blockade was apparent with AM1714, but not (*R,S*)-AM1241, suggesting possible mechanistic differences between the two agonists. More work is necessary to determine whether (*R,S*)-AM1241 and AM1714

preferentially activate different signaling pathways or whether off-target effects could contribute to these differences. (*R,S*)-AM1241, a racemic compound, may exhibit partial agonist properties that counteract this tendency. Putative changes in endocannabinoid tone may be induced by blockade of CB_1 to enhance the anti-allodynic activity of certain CB_2 agonists under conditions in which the balance between CB_1 and CB_2 receptor activation is altered. Blockade of CB_1 may also facilitate interaction of endogenous ananandamide with non-CB_1 receptors (e.g. TRPV1) to contribute to the behavioral phenotype. Nonetheless, neither the CB_1 nor the CB_2 antagonist, administered alone, increased paclitaxel-evoked mechanical allodynia. Our data extend previous work documenting that activation of CB_2 suppresses nociception and central sensitization in a variety of tissue and nerve injury models of persistent pain (Ibrahim et al., 2003; Nackley et al., 2003; Beltramo et al., 2006; Jhaveri et al., 2008).

In the present study, we compared the effects of two enantiomers of (*R,S*)-AM1241– (*R*)-AM1241 and (*S*)-AM1241 – on paclitaxel-evoked mechanical allodynia. (*R*)-AM1241 binds with 40- (Bingham et al., 2007) to 114- (Thakur et al. 2005) fold higher affinity to CB_2 receptors than (*S*)-AM1241. This observation is consistent with the ability of (*R*)-AM1241 to preferentially suppress paclitaxel-evoked mechanical hypersensitivity relative to either vehicle or day 21 pre-injection thresholds. Similar effects were not observed with administration of (*S*)-AM1241. However, both enantiomers show notable selectivity for CB_2 over CB_1. Thus, it is important to emphasize that (*S*)-AM1241 cannot be considered an inactive enantiomer of (*R*)-AM1241. This property contrasts with that of other aminoalkylinole agonists in which the enantiomer (e.g. WIN55,212-3) of the active compound (WIN55,212-2) fails to bind to cannabinoid receptors. The fact that (*S*)-AM1241 retains activity at CB_2 may account for the efficacy of (*S*)-AM1241 in models of visceral and inflammatory pain (Bingham et al., 2007) and our failure to differentiate

between effects of (*R*)-AM1241 and (*S*)-AM1241 in post hoc analyses. Our studies do not preclude the possibility that CB_2-mediated anti-allodynic effects of (*S*)-AM1241 could be detected using a higher dose of (*S*)-AM1241 or a larger sample size. It is also possible that differences in enantiomer efficacy reflect differences in agonist directed trafficking through different G proteins and signal transduction mechanisms (Shoemaker et al., 2005).

In our study, morphine (4 mg/k i.p.) suppressed paclitaxel-induced mechanical allodynia and normalized paclitaxel-evoked paw withdrawal thresholds to pre-paclitaxel levels. This same dose was previously reported to be ineffective in suppressing paclitaxel-evoked mechanical hyperalgesia (Flatters and Bennett, 2004). In this latter study, a two-fold higher dose (8 mg/kg i.p.) than that employed here (4 mg/kg i.p.) produced only a 50% reversal of paclitaxel-evoked mechanical allodynia/hyperalgesia whereas the lower dose (4 mg/kg i.p.) was ineffective. A dose of 8 mg/kg also attenuated vincristine-induced mechanical allodynia in our previous work (Rahn et al., 2007). Differences in the dependent measure (i.e. paw withdrawal frequency vs. paw withdrawal threshold in our study), method for assessing mechanical hypersensitivity (i.e. manual von Frey filaments vs. electrovonfrey device in our study) and time of testing (i.e. 1 h vs 30 min post morphine in our study) may account for these differences. Nonetheless, unwanted side-effects (i.e., sedation, nausea, altered mental status, constipation) remain associated with activation of the opioid system in humans, warranting development and validation of drug targets which lack these unwanted side-effects (Lee et al., 1995).

The mechanism by which paclitaxel induces neuropathic pain symptoms remains unknown. Paclitaxel has been reported to induce neuropathy in the absence of morphological changes in sensory or motor axons in the spinal cord (Polomano et al., 2001). This observation prompted investigations of morphological changes in the periphery. Morphological and

immunological changes in sensory nerve fibers have been reported following paclitaxel treatment (Jin et al., 2008). Abnormal calcium homeostasis may also contribute to the development of neuropathic pain symptoms associated with paclitaxel treatment (Siau and Bennett, 2006). Thus, it is noteworthy that blockade of calcium channels is effective in attenuating symptoms of peripheral neuropathy in this model, whereas an NMDA receptor antagonist was without effect (Flatters and Bennett, 2004). A reduction of mechanical hyperalgesia associated with both paclitaxel and vincristine treatment is also observed in TRPV4 knockout mice, suggesting that TRPV4 may also represent a therapeutic target for treatment of chemotherapy-evoked toxic neuropathy (Alessandri-Haber et al., 2008).

More work is necessary to identify the site of action for CB_2 agonists in suppressing paclitaxel-evoked neuropathy. Upregulation of the CB_2 receptor in the dorsal horn of the spinal cord has been reported after spinal nerve ligation injury or sciatic nerve sectioning in rats (Walczak et al., 2005; Wotherspoon et al., 2005). Moreover, CB_2 expression is upregulated in cultured DRG following prior axotomy (Wotherspoon et al., 2005). CB_2 receptors have recently been localized within the CNS, specifically on microglia which are related to macrophages (Cabral et al., 2008). Thus, it is noteworthy that paclitaxel increased the number of macrophages present in both spinal cord and the DRG (Peters et al., 2007). More work is necessary to determine whether CB_2 receptors in the CNS or DRG are upregulated by paclitaxel treatment and contribute to the observed CB_2-mediated suppression of paclitaxel-evoked neuropathy.

The recent observation of increased activation of microglia and astrocytes in paclitaxel-treated rats has led to speculation that these glial cells contribute to chemotherapy-induced neuropathic pain (Ledeboer et al., 2007). Paclitaxel increases levels of activated microglia in lamina III-VI of the spinal cord as well as astrocytes in lamina I-VI of the spinal cord (Peters et

al., 2007). Hypertrophy in both glial cell populations is observed following paclitaxel treatment (Peters et al., 2007). Moreover, pharmacologically-induced suppression of glial cells abolished and delayed the incidence of mechanical allodynia in paclitaxel-treated rats (Ledeboer et al., 2007). More work is necessary to determine whether CB_2 agonists suppress paclitaxel-evoked neuropathy by inhibiting microglial activation.

Acknowledgments. The authors thank Kenneth Maxwell for technical assistance.

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Footnotes

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Legends for Figures

Figure 1. Chemical structures of (*R,S*)-AM1241, (*R*)-AM1241, (*S*)-AM1241 and AM1714.

Figure 2A. Weight gain was observed in groups treated with either paclitaxel or cremophor: ethanol: saline vehicle. B. Time course of paclitaxel-induced mechanical allodynia, as demonstrated by a lowering of the threshold for paw withdrawal to punctuate mechanical stimulation. Data are Mean ± s.e.mean. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control condition (ANOVA). N = 6-115 per group.

Figure 3. (*R,S*)-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.) suppressed paclitaxel-induced mechanical allodynia. Both cannabinoid CB_2 agonists normalized thresholds relative to pre-paclitaxel levels at 30 minutes post injection. BL denotes baseline (day 0) paw withdrawal thresholds observed prior to paclitaxel treatment. Data are Mean ± s.e.mean. **P* < 0.05 vs. all groups (ANOVA and Dunnett's post hoc test). N = 6-7 per group.

Figure 4A. (*R,S*)-AM1241 (1, 5 and 10 mg/kg i.p.) produced a dose-related suppression of paclitaxel-evoked mechanical allodynia. In all panels, Post PTX indicates thresholds observed on day 21 post-paclitaxel. B. Both (*R,S*)-AM1241 (10 mg/kg i.p.) and its enantiomer (*R*)-AM1241 (10 mg/kg i.p.) attenuated paclitaxel-evoked mechanical allodynia. **P* < 0.05, ***P* <0.01 vs. control, ****P* <0.001 vs. baseline (ANOVA, and Dunnett post hoc test), $^{X}P < 0.05$ vs. corresponding group day 21 post-paclitaxel paw withdrawal thresholds, $^{\perp}P < 0.001$ vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). N = 6-11 per group.

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Figure 5. AM1714 (10 mg/kg) suppressed paclitaxel-induced mechanical allodynia. *P < 0.05, **P < 0.01 different from control, ***P < 0.001 vs. baseline (ANOVA, and Dunnett post hoc test) ^{X}P < 0.05 vs. corresponding group day 21 post-paclitaxel paw withdrawal thresholds, $^{\perp}P$ < 0.001 vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). N = 6-11 per group.

Figure 6A. The CB_2-selective antagonist SR144528 (SR2) blocked the suppression of paclitaxel-evoked mechanical allodynia induced by the CB_2 agonists, (*R*,*S*)-AM1241 (10 mg/kg i.p.) and AM1714 (10 mg/kg i.p.). B. The CB_1-selective antagonist SR141716 (SR1) failed to block the anti-allodynic effects of either (*R*,*S*)-AM1241 (10 mg/kg i.p.) or AM1714 (10 mg/kg i.p.) in the same model. **P <0.01 vs. all groups, ^{+}P < 0.01 vs. DMSO, AM1714 + SR2 and AM1241 + SR2 (ANOVA, and Dunnett Post Hoc Test). $^{\perp}P$ < 0.001 vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test), ^{X}P < 0.001 vs. corresponding group day 21 post-paclitaxel thresholds (t-test). N = 5-11 per group.

Figure 7. Morphine (4.0 mg/kg i.p.) blocked mechanical allodynia induced by treatment with paclitaxel. ***P <0.001 vs. baseline, *P <0.05 vs. control (ANOVA and Dunnett Post Hoc Test). $^{\perp}P$ < 0.001 vs. corresponding group baseline pre-paclitaxel paw withdrawal thresholds (t-test). N = 6-11 per group.

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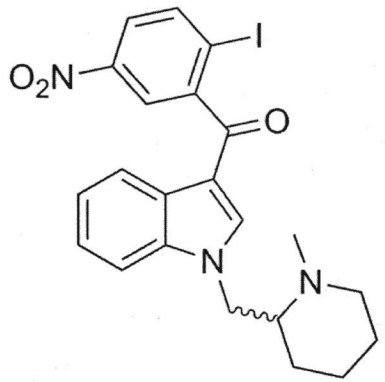
Table 1: Paw withdrawal thresholds (g) in paclitaxel-treated control conditions

	Pre-Paclitaxel	Post-Paclitaxel	
	Day 0	Day 21	Day 21
Group	Pre-injection	Pre-injection	Post-injection
Paclitaxel: DMSO-DMSO	66.915 ± 6.58	46.56 ± 4.23†	42.88 ± 3.95$^{\perp\perp}$
Paclitaxel: Saline-Saline	75.585 ± 6.32	41.20 ± 4.90†††	39.55 ± 4.40$^{\perp\perp\perp}$
Paclitaxel: DMSO	63.88 ± 2.74	32.93 ± 2.43†††	32.08 ± 5.98$^{\perp\perp\perp}$
Paclitaxel: SR141716	63.59 ± 2.20	37.34 ± 1.77†††	37.23 ± 5.05$^{\perp}$
Paclitaxel: SR144528	59.92 ± 4.10	32.64 ± 7.42††	39.78 ± 4.86$^{\perp\perp\perp}$

Data are mean ± s.e.mean; $^{\dagger\dagger\dagger}P$ <0.001, $^{\dagger\dagger}P$ <0.01, $^{\dagger}P$ <0.05 vs. baseline pre-paclitaxel paw withdrawal thresholds for corresponding group, $^{\perp\perp\perp}P$ <0.001, $^{\perp\perp}P$ <0.01, $^{\perp}P$ <0.05 vs. baseline pre-paclitaxel paw withdrawal thresholds for corresponding group (t-test).

Figure 1



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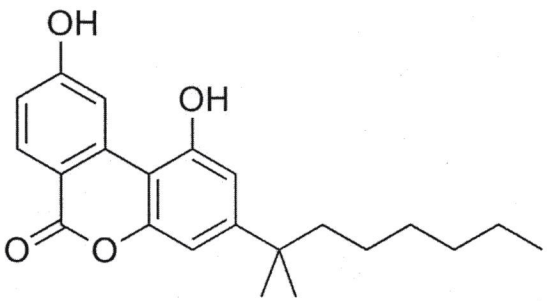
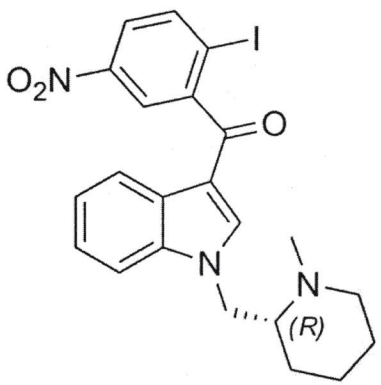


AM1714



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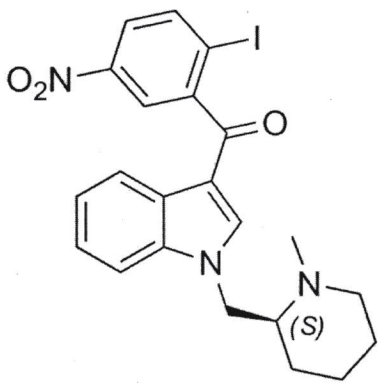
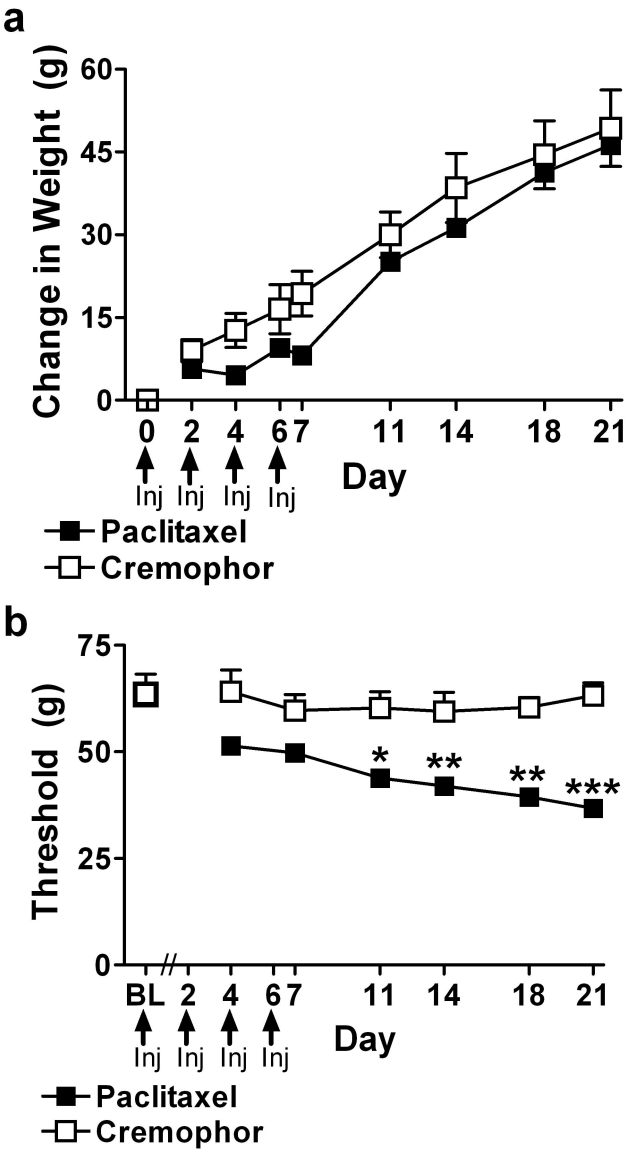


(*S*)-AM1241

Figure 2


a
Change in Weight (g)
60
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Day
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Paclitaxel
Cremophor
b
Threshold (g)
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Day
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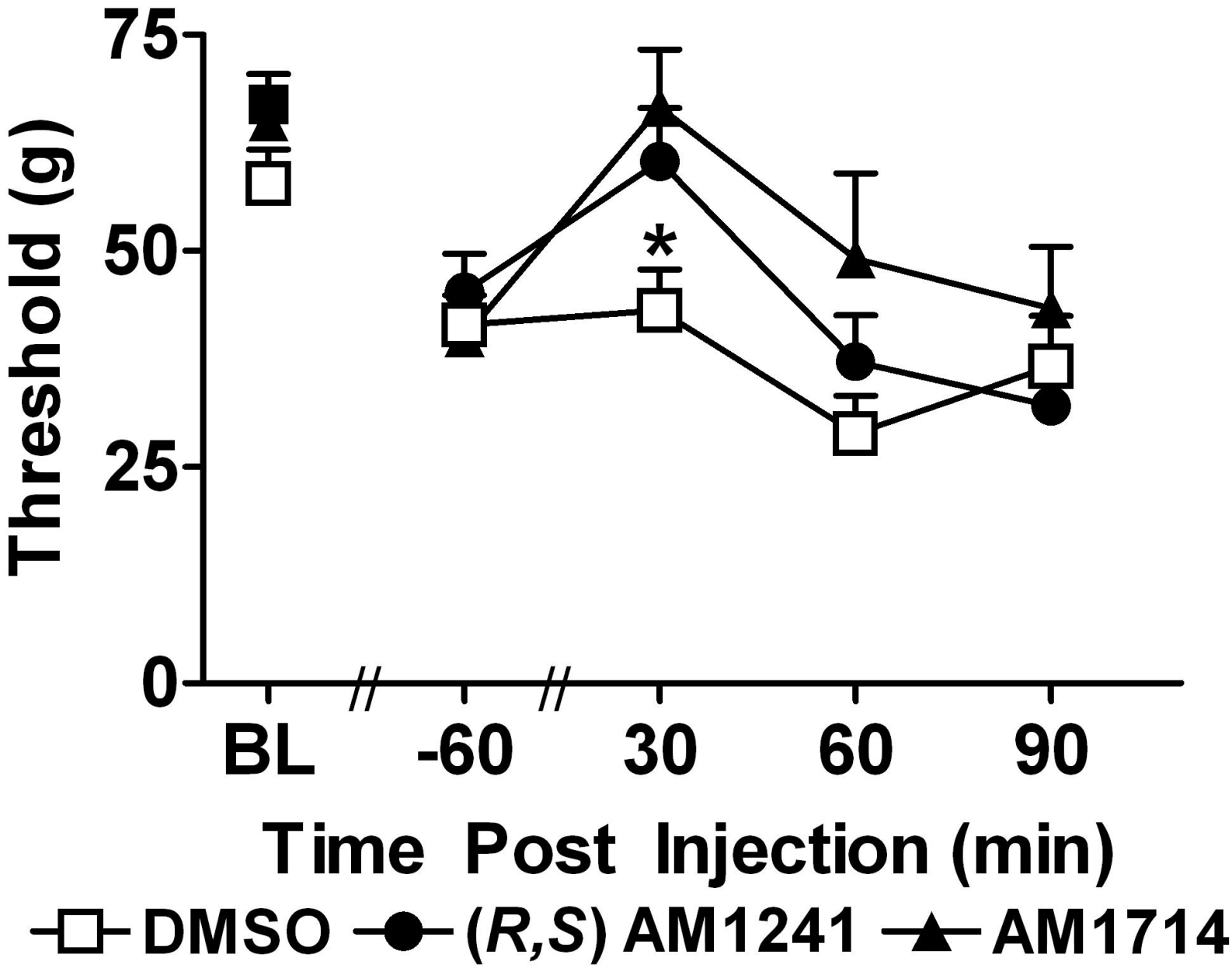
Paclitaxel
Cremophor

Figure 3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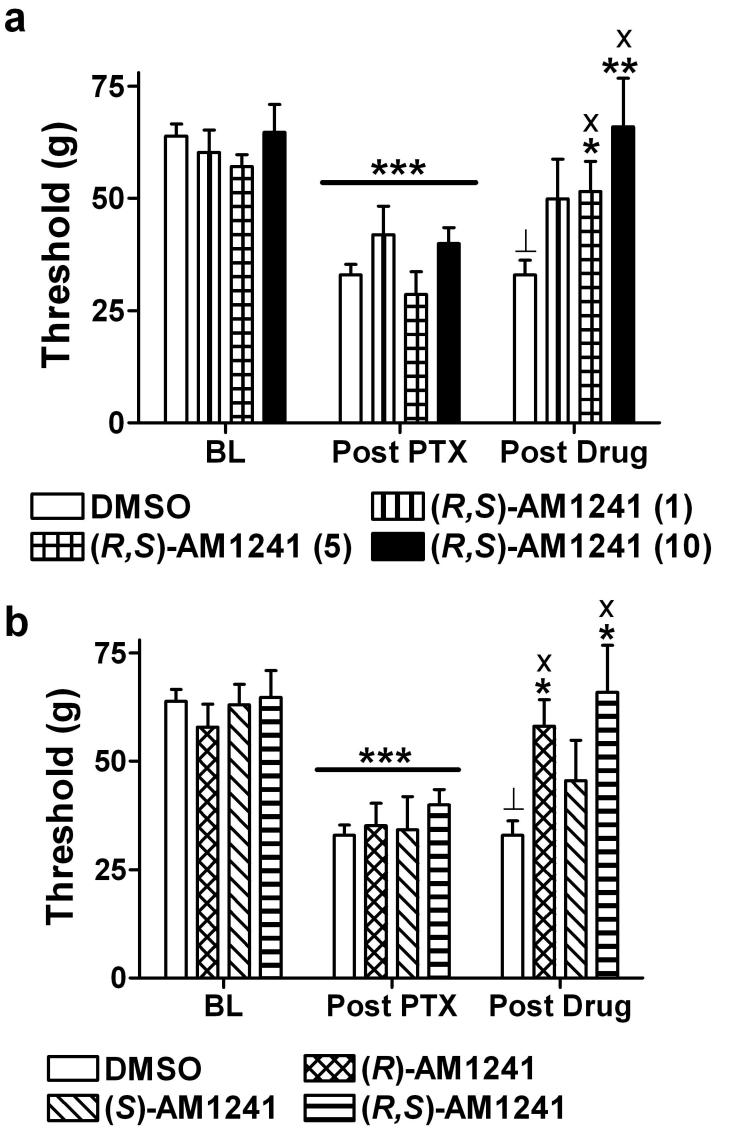
60
90
Time Post Injection (min)
DMSO
(R,S) AM1241
AM1714

Figure 4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DMSO
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(R,S)-AM1241 (10)
b
Threshold (g)
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Post PTX
Post Drug
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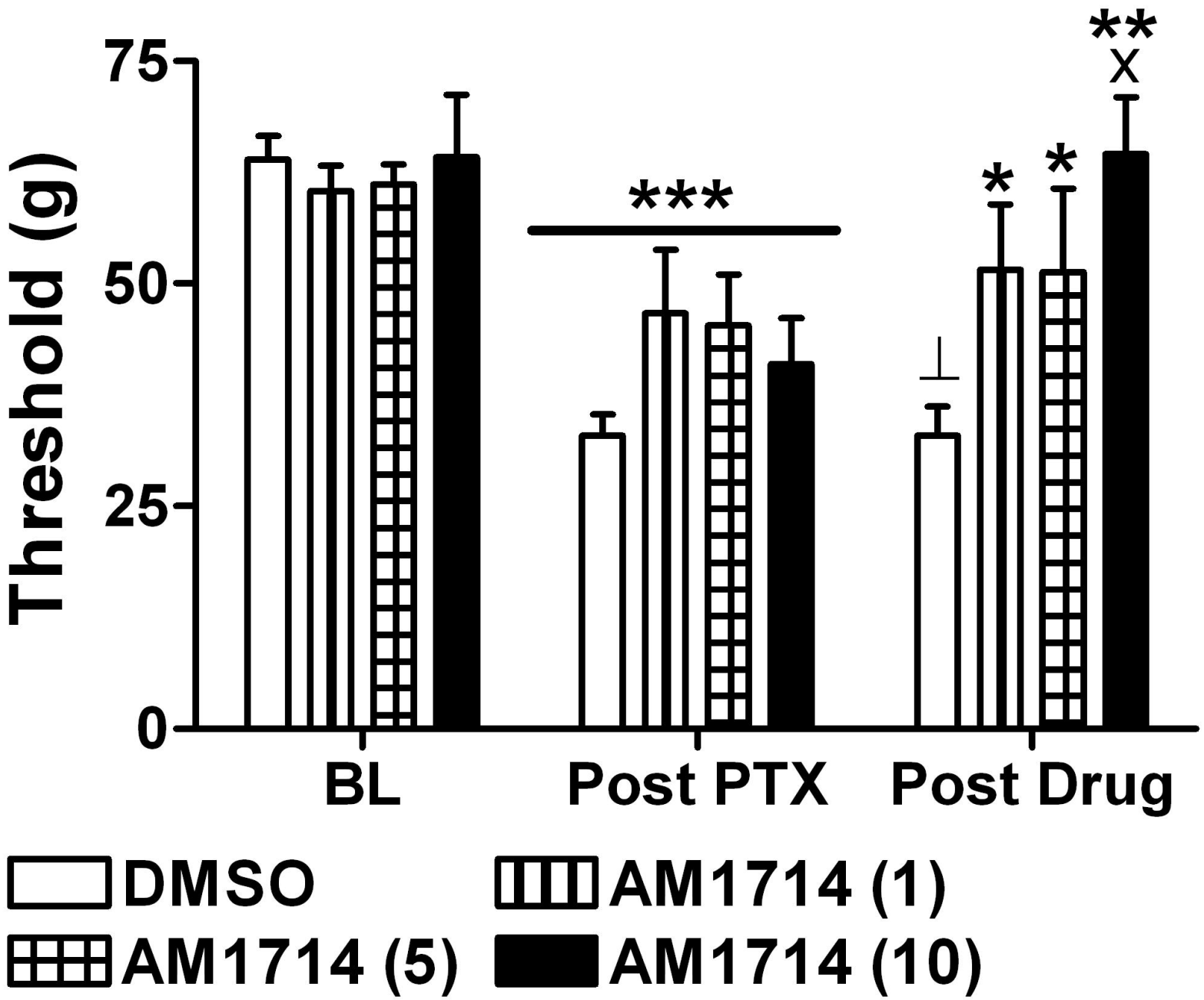
Figure 5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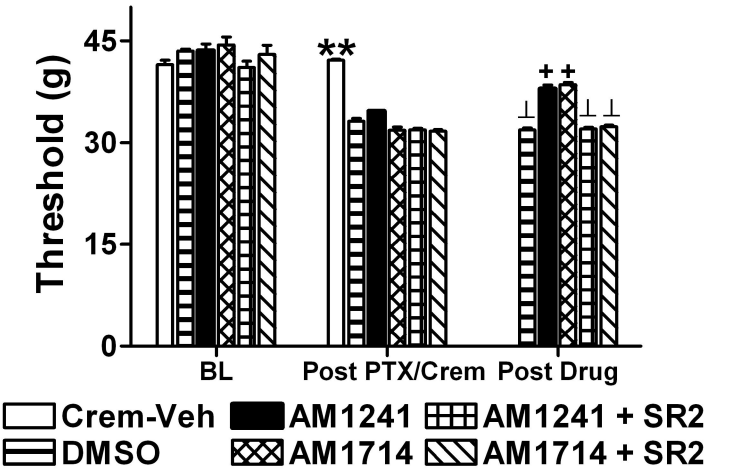
Post PTX
Post Drug
DMSO
AM1714 (1)
AM1714 (5)
AM1714 (10)

Figure 6

a


Threshold (g)
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Post PTX/Crem
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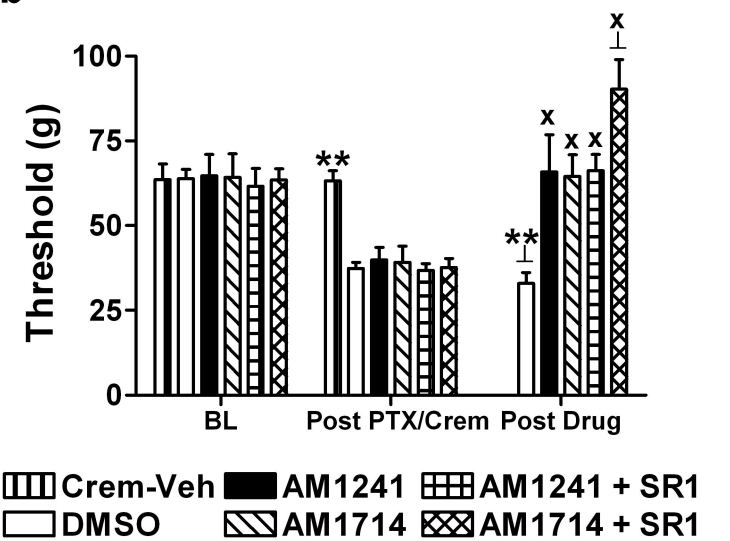
AM1241 + SR2
DMSO
AM1714
AM1714 + SR2

b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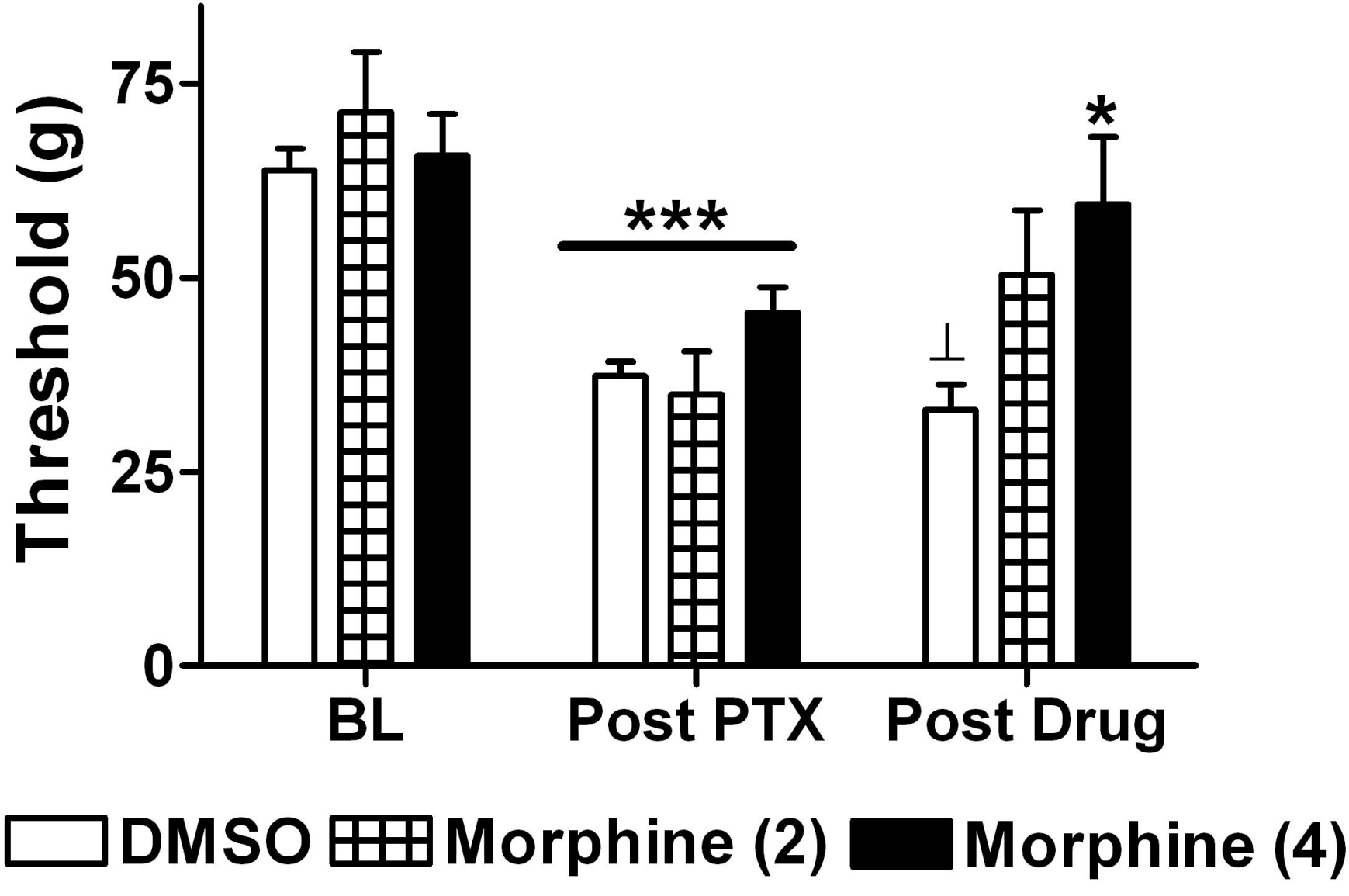
AM1241
AM1241 + SR1
DMSO
AM1714
AM1714 + SR1

Figure 7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Morphine (2)
Morphine (4)