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The slowly signaling G protein-biased CB_2 cannabinoid receptor agonist LY2828360 suppresses neuropathic pain with sustained efficacy and attenuates morphine tolerance and dependence

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Running title: Role of CB_2 in morphine tolerance and dependence

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Abbreviations:

AM1710, 3-(1, 1-dimethyl-heptyl)-1-hydroxy-9-methoxy-benzo(c) chromen-6-one; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CB_1 or CB_2, cannabinoid receptor 1 or 2; CHO, Chinese hamster ovary; CNS, central nervous system; CP55940, (2)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; DRG, dorsal root ganglia; Emax, maximal effect; ERK, extracellular signal-regulated kinases; HEK, human embryonic kidney; IP1, Accumulation of myo-Inositol Phosphate 1; Gi, inhibitory G proteins; GPCR, G-protein coupled receptor; KO, knockout; LY2828360 (8-(2-chlorophenyl)-2-methyl-6-(4-methylpiperazin-1-yl)-9-(tetrahydro-2H-pyran-4-yl)-9H-purine); MIA, monoiodoacetic acid; Mor, morphine; MOR, μ opioid receptor; NSAID, nonsteroidal anti-inflammatory drug; PTX, pertussis toxin; WT, wild type.

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ABSTRACT

The CB_2 cannabinoid agonist LY2828360 lacked both toxicity and efficacy in a clinical trial for osteoarthritis. Whether LY2828360 suppresses neuropathic pain has not been reported and its signaling profile is unknown. *In vitro*, LY2828360 was a slowly acting but efficacious G protein-biased CB_2 agonist, inhibiting cAMP accumulation and activating ERK1/2 signaling while failing to recruit arrestin, activate inositol phosphate signaling or internalize CB2 receptors. In wildtype (WT) mice, LY2828360 (3 mg/kg/day i.p. x 12 days) suppressed chemotherapy-induced neuropathic pain produced by paclitaxel without producing tolerance. Anti-allodynic efficacy of LY2828360 was absent in CB_2KO mice. Morphine (10 mg/kg/day i.p. x 12 days) tolerance developed in CB_2KO mice but not in WT mice with a history of LY2828360 treatment (3 mg/kg/day i.p. x 12 days). LY2828360-induced anti-allodynic efficacy was preserved in WT mice previously rendered tolerant to morphine (10 mg/kg/day i.p. x 12 days) but absent in morphine-tolerant CB_2KO mice. Coadministration of LY2828360 (0.1 mg/kg/day i.p. x 12 days) with morphine (10 mg/kg/day x 12 days) blocked morphine tolerance in WT but not CB_2KO mice. WT mice that received LY2828360 coadministered with morphine exhibited a trend (p=0.055) towards fewer naloxone-precipitated jumps compared to CB_2KO mice. In conclusion, LY2828360 is a slowly signaling, G protein-biased CB_2 agonist that attenuates chemotherapy-induced neuropathic pain without producing tolerance, and may prolong effective opioid analgesia while reducing opioid dependence. LY2828360 may be useful as a first line treatment in chemotherapy-induced neuropathic pain and may be highly efficacious in neuropathic pain states that are refractive to opioid analgesics.

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INTRODUCTION

Morphine suppresses many types of pain, but tolerance, physical dependence and unwanted side effects limit its clinical use (Trang, Sutak, & Jhamandas, 2007). Identification of therapeutic strategies for blocking opioid tolerance and dependence has, therefore, evolved as an area of intense research interest (Habibi-Asl, Vaez, Najafi, Bidaghi, & Ghanbarzadeh, 2014; Hassanipour et al., 2016; Hosseinzadeh, Parvardeh, Masoudi, Moghimi, & Mahboobifard, 2016; Mansouri, Khodayar, Tabatabaee, Ghorbanzadeh, & Naghizadeh, 2015). Adjunctive pharmacotherapies that combine mechanistically distinct analgesics represent one such approach. Opioid and cannabinoid CB_1 G-protein coupled receptors are often coexpressed in the central nervous system (CNS) (Pickel, Chan, Kash, Rodríguez, & MacKie, 2004) and can functionally interact by receptor heterodimerization or signaling cross-talk (Bushlin, Rozenfeld, & Devi, 2010). Although activation of both receptors produce analgesia, undesirable pharmacological effects limit use (Manzanares et al., 1999; Massi, Vaccani, Romorini, & Parolaro, 2001). An alternative approach aims at harnessing the therapeutic potential of cannabinoid CB_2 receptors to suppress pathological pain without producing CB_1-mediated cannabimimetic effects (for review see (Amey Dhopeshwarkar & Mackie, 2014; Guindon & Hohmann, 2008)). CB_2 receptors are primarily expressed on immune cells but may be induced in the CNS in response to injury (for review see (Mechoulam & Parker, 2011)). Activation of cannabinoid CB_2 receptors produces antinociceptive efficacy in many preclinical pain models without the unwanted side-effects associated with CNS CB_1 receptor activation. CB_2 receptors have also been implicated in facilitating morphine antinociception in normal and inflammatory conditions (Desroches, Bouchard, Gendron, & Beaulieu, 2014; Lim, Wang, & Mao, 2005; Merighi et al., 2012).

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However, whether CB_2 agonists suppress morphine tolerance or dependence in neuropathic pain models is unknown.

LY2828360 (**Fig. 1**) is a potent CB_2 receptor agonist with similar affinity for human and rat CB_2 receptors (Hollinshead et al., 2013). In a human CB_2 functional assay, approximately 87% maximal stimulation of CB_2 was observed at 20 nM concentrations whereas only 15% maximal stimulation of CB_1 was observed at 100 μM concentrations (Hollinshead et al., 2013). LY2828360 showed good CNS penetration and potent oral activity in a preclinical model of joint pain induced by intra-articular monoiodoacetic acid (MIA) (Hollinshead et al., 2013). In the MIA model, LY28282360 (0.3 mg/kg p.o.) produced a dose-related reversal of pain using incapacitance testing, demonstrating equivalent efficacy to the nonsteroidal anti-inflammatory drug (NSAID) diclofenac (Hollinshead et al., 2013). No specific risks or discomforts associated with LY2828360 were observed in patients with osteoarthritic pain who have taken LY2828360 up to a dose of 80 mg for four weeks (Pereira et al., 2013)(clinicaltrials.gov identifier: NCT01319929). Unfortunately, LY2828360 and placebo treatments did not differ in achieving the primary endpoint in patients with osteoarthritic knee pain in this phase II clinical trial. Evaluations of LY2828360 antinociceptive efficacy have not appeared in the published literature despite the fact that LY2828360-associated improvements were noted in exploratory pain models (clinicaltrials.gov identifier: NCT01319929)(Pereira et al., 2013).

The signaling profile of LY2828360 is unknown. We, therefore, performed a thorough characterization of the signaling of LY2828360 with stably expressed mouse and human CB_2 receptors by employing a range of cell based *in vitro* signaling assays: arrestin recruitment, CB_2 receptor internalization, inhibition of forskolin-stimulated cAMP (cyclase) accumulation, ERK1/2 phosphorylation, and IP1 accumulation. Moreover, to our knowledge, LY2828360 has

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never been evaluated in an animal model of neuropathic pain. Our previous studies showed that the CB_2 agonist AM1710 suppressed neuropathic pain induced by the chemotherapeutic agent paclitaxel through a CB_2-specific mechanism without producing tolerance or physical dependence (Deng et al., 2015). We, therefore, used the same paclitaxel model of peripheral neuropathy to evaluate whether LY2828360 would suppress chemotherapy-induced neuropathic pain in a CB_2-dependent manner using both CB_2KO and WT mice. We investigated whether repeated administration of LY2828360 would produce tolerance to the antinociceptive effects of the CB_2 agonist in paclitaxel-treated mice. Comparisons were made with the opioid analgesic morphine administered under identical conditions. In addition, we evaluated whether LY28282360 would produce anti-allodynic efficacy in mice that were rendered tolerant to morphine and, conversely, whether development of morphine tolerance would be attenuated in mice with a previous history of chronic LY2828360 treatment. We also evaluated whether coadministration of a low dose of LY2828360 with a maximally-efficacious dose of morphine would attenuate morphine tolerance. In all studies, pharmacological specificity was established using WT and CB_2KO mice. Finally, to assess physical dependence, we challenged mice with either vehicle or the opioid antagonist naloxone to evaluate whether LY2828360 would impact naloxone-precipitated opioid withdrawal in mice previously rendered tolerant to morphine.

METHODS AND MATERIALS

Subjects

Adult male CB_2KO mice (B6.129P2-CNR2 (tm1Dgen/J); bred at Indiana University) and WT mice (bred at Indiana University or purchased from Jackson Laboratory, Bar Harbor, ME) on a C57BL/6J background, weighing 25-33 g mice, were used in this study. Animals were single-housed several days prior to initiating pharmacological manipulations. All mice were maintained in a temperature-controlled facility ($73\pm 2\ ^0$F, 45% humidity, 12-hour light/dark

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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 (Zimmermann, 1983).

Drugs and Chemicals

Paclitaxel (Tecoland Corporation, Irvine, California) was dissolved in a cremophor-based vehicle which was made of Cremophor EL (Sigma-Aldrich, St. Louis, Missouri), ethanol (Sigma-Aldrich) and 0.9% saline (Aqualite System; Hospira, Inc, Lake Forest, Illinois) at a ratio of 1:1:18 as previously published (Deng et al., 2015). LY2828360 (8-(2-chlorophenyl)-2-methyl-6-(4-methylpiperazin-1-yl)-9-(tetrahydro-2H-pyran-4-yl)-9H-purine) was obtained from Eli Lilly and company (Indianapolis, IN) and synthesized by Eli Lilly and company as previously described (Hollinshead et al., 2013). Morphine (Sigma-Aldrich) or LY2828360 was dissolved in a vehicle containing a 2:1:1:18 ratio of dimethyl sulfoxide (Sigma-Aldrich), ALKAMULS EL-620 (Rhodia, Cranbury, New Jersey), ethanol and saline. Naloxone (Sigma-Aldrich) was dissolved in saline as indicated. Drugs were administered via intraperitoneal injection (i.p.) to mice in a volume of 10 ml/kg. CP55940 was obtained from National institute of Drug Abuse Drug Supply Service (Bethesda, MD). Pertussis toxin (Cat # BML-G100-0050) was purchased from Enzo Lifesciences (Farmingdale, NY).

Cell culture

HEK293 cells stably expressing mouse CB2 receptors (HEK mCB2) or human CB2 receptors (HEK hCB2) were generated, expanded and maintained in Dulbecco's Modified Eagle media with 10% fetal bovine serum and penicillin/streptomycin (GIBCO, Carlsbad, CA) at 37 °C

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in 5% CO_2. For ease of immuno-detection, an amino-terminal HA (hemagglutinin) epitope tag was introduced into the CB1 and CB2 receptors.

Arrestin recruitment

To determine arrestin recruitment, assays were performed using an enzyme complementation approach (A. Dhopeshwarkar & Mackie, 2016). PathHunter® CHO K1 CNR2 (Catalog No. 93-0472C2) cells were purchased from DiscoveRx (Fremont, CA). This cell line is engineered wherein an N-terminal deletion mutant of β-galactosidase (β gal) enzyme (EA or enzyme acceptor fragment) is fused with arrestin while a complimentary smaller fragment (ProLink™) is fused with C terminus domain of the mouse cannabinoid receptor. Upon receptor activation, recruitment of arrestin leads to formation of an active β galactosidase enzyme, which then acts on substrate to emit light that can be detected as luminescence. These cell lines were thawed, grown and maintained in Pathunter AssayComplete™ media (catalog # 92-0018GF2).

Quantification of cAMP levels

cAMP assays were optimized using Perkin Elmer's LANCE® ultra cAMP kit (Catalog # TRF0262, Perkin Elmer, Boston, MA) as per the manufacturer's instructions. All assays were performed at room temperature using 384-optiplates (Catalog# 6007299, Perkin Elmer). Briefly, cells were resuspended in 1X stimulation buffer (1X Hank's Balanced Salt Solution (HBSS), 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4, made fresh on the day of experiment). Cells (HEK CB2) were incubated for 1 hour at 37 °C, 5% CO_2 and humidified air and then transferred to a 384-optiplate (500 cells/µl, 10µl), followed by stimulation with drugs/compounds and forskolin (2 µM final concentration) made in 1X stimulation buffer as appropriate, for 5 minutes. For time course experiments, cells were treated with CP55940 or LY282360 (in the presence of 2 µM forskolin final concentration) for defined time points. For experiments with pertussis toxin

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(PTX), cells were treated overnight with 300 ng/ml PTX at 37°C in 5% CO_2. Cells were then lysed by addition of 10µl Eu-cAMP tracer working solution (4X, made fresh in 1X lysis buffer supplied with the kit; under subdued light conditions) and 10 µl *Ulight*™ anti-cAMP working solution (4X, made fresh in 1X lysis buffer) and further incubated for 1 hour at room temperature. Plates were then read with the TR FRET mode on an Enspire plate reader (Perkin Elmer, Boston, MA).

Detection of phosphorylated ERK1/2

HEK-mCB2 or hCB2 were seeded on poly-D-lysine coated 96-well plates (75,000 cells/well) and grown overnight at 37°C, in 5% CO_2 humidified air. The following day, media was replaced by serum free DMEM and plates were further incubated for 5 hours at 37°C in 5% CO_2 humidified air. For experiments involving PTX, cells were treated overnight with PTX (300 ng/ml) and next day serum starved for 5 hours. Following serum starvation, cells were challenged with drugs/compounds for the indicated time. After drug incubation, plates were emptied and quickly fixed with ice cold 4% PFA for 20 minutes followed by ice-cold methanol with the plate maintained at -20°C for 15 minutes. Plates were then washed with TBS/0.1% Triton X-100 for 25 minutes (5 x 5 min washes). The wash solution was then replaced by Odyssey blocking buffer and incubated further for 90 min with gentle shaking at room temperature. Blocking solution was then removed and replaced with blocking solution containing anti-phospho-ERK1/2 antibody (1:150; Cell Signaling Technology®, Danvers, MA) and was shaken overnight at 4°C. The next day, plates were washed with TBS containing 0.05% Tween-20 for 25 min (5 x 5 min washes). Secondary antibody, donkey anti-rabbit conjugated with IR800 dye (Rockland, Limerick, PA), prepared in blocking solution, was added and plates were gently shaken for 1 hour at room temperature. The plates were then again washed 5 times with

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TBS/0.05% Tween-20 solution. The plates were patted dry and scanned using LI-COR Odyssey scanner. pERK1/2 activation (expressed in %) was calculated by dividing average integrated intensities of the drug treated wells by average integrated intensities of vehicle-treated wells. All assays were performed in triplicate, unless otherwise noted.

On-cell Western for receptor internalization

HEK CB2 cells were grown to 95% confluence in DMEM + 10% FBS + 0.5% Pen/Strep. Cells were washed once with HEPES buffered saline (HBS)/bovine serum albumen (BSA) (BSA @ 0.08 mg/ml) with 200 µL per well. Drugs were applied at the indicated concentrations to cells, after which they were incubated for 90 minutes at 37°C. Cells were then fixed with 4% PFA for 20 minutes and washed 4 times (300 µl per well) with tris-buffered saline (TBS). Blocking buffer (Odyssey Blocking buffer, LI-COR, Inc. Lincoln, NE) was applied at 100 µl per well for 1h at room temperature. Anti-HA antibody, (mouse monoclonal, 1:200, Covance, Princeton, NJ) diluted in Odyssey Blocking Buffer, was then applied for one hour at room temperature. Following this, the plate was washed 5 times (300 µl per well) with TBS. Secondary antibody diluted (anti-mouse 680 antibody 1:800, LI-COR, Inc. Lincoln, NE) in blocking buffer, was then applied for one hour at room temperature. Following this, the plate was washed 5 times (300µl per well) with TBS. The plate was imaged using an Odyssey scanner (700 channel, 5.5 intensity, LI-COR, Inc. Lincoln, NE).

IP1 accumulation assay

Accumulation of myo-Inositol Phosphate 1(IP1), a downstream metabolite of IP3, was measured by using IP-One HTRF®kit (Catalog # 62, IPAPEB, Cisbio, Bedford, MA). Functional coupling of CB2 receptor to G_q G protein leads to phospholipase Cβ (PLC) activation and initiation of the inositol phosphate (IP) hydrolysis cascade. Accumulated IP3 is quickly

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dephosphorylated to IP2 and then to IP1. This assay takes advantage of the fact that accumulated IP1 is protected from further dephosphorylation by the addition of lithium chloride (LiCl) and IP1 levels can be easily quantified using an HTRF (homogeneous time-resolved fluorescence) assay. HEK mCB2 cells were detached from ~50% confluent plates using versene. Cells (10 µl, 5000 cells) were resuspended in 1X stimulation buffer (containing LiCl, supplied with the kit) and were incubated for 1 hour at 37 °C, 5% CO_2 and humidified air and then transferred to a 384-optiplate, followed by stimulation with drugs/compounds made in DMSO/ethanol as appropriate, for defined time points. Cells were then lysed with 5 µl of IP1-d2 dye (made fresh in lysis buffer, supplied with the kit) followed by addition of 5 µl Ab-Cryptate dye (made fresh in lysis buffer). Plates were incubated further for 60 minutes at room temperature and then read in HTRF mode on an Enspire plate reader. All cell- based assay experiments were performed in triplicates, unless otherwise mentioned.

General *in Vivo* Experimental Protocol

In all studies, the experimenter was blinded to the treatment condition and mice were randomly assigned to experimental conditions. Paclitaxel (4 mg/kg i.p.) was administered four times on alternate days (cumulative dose, 16 mg/kg i.p.) to induce neuropathic pain as described previously by our group (Deng et al., 2015). Control mice received an equal volume of cremophor-vehicle. Development of paclitaxel-induced allodynia was assessed on day 0, 4, 7, 11 and 14.

Effects of pharmacological manipulations were assessed at 30 min after drug administration during the maintenance phase of paclitaxel-induced neuropathy (i.e., beginning day 18-20 after initial paclitaxel injection).

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In experiment # 1, we assessed the dose response and time course of acute administration of LY2828360 on mechanical and cold allodynia in WT (C57BL/6J) mice treated with paclitaxel or its cremophor-based vehicle.

In experiment # 2 and 3, pharmacological manipulations were performed once daily for 12 consecutive days in each of the two phases of chronic treatment. Four days separated phase I and phase II chronic dosing in all studies employing two phases of chronic dosing. Experiments #2 and #3 were performed concurrently using overlapping cohorts that were tested with a single vehicle (Phase I)-vehicle (Phase II) group.

In experiment #2, we examined anti-allodynic efficacy of chronic systemic administration of LY2828360 (3 mg/kg/day i.p. x 12 days) or vehicle administered during phase I using paclitaxel-treated WT and CB_2KO mice. We then assessed the anti-allodynic efficacy of chronic systemic administration of vehicle or morphine (10 mg/kg/day i.p. x 12 days) administered during phase II in the same animals mentioned above. Responsiveness to mechanical and cold stimulation was evaluated on treatment days 1, 4, 8 and 12 during phase I and on treatment days 16, 19, 23, and 27 during phase II (i.e. phase II started on day 16).

In experiment #3, we assessed the anti-allodynic efficacy of chronic administration of LY2828360 (3 mg/kg/day i.p. x 12 days in phase II) or vehicle in paclitaxel-treated WT and CB_2KO mice that previously developed tolerance to morphine. To induce morphine tolerance, mice received repeated once daily injections of morphine (10 mg/kg/day i.p. x 12 days) in phase I treatment, while vehicle or LY2828360 (3 mg/kg/day i.p. x 12 days) was administered chronically in phase II.

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In experiment #4, we evaluated the impact of coadministration of morphine (10 mg/kg i.p. x 12 days) with a submaximal dose of LY 2828360 (0.1 mg/kg/day i.p. x 12 days) in WT and CB_2 KO mice.

In experiment #5, we evaluated whether chronic administration of LY2828360 would attenuate morphine-dependent withdrawal symptoms that were precipitated using the opioid receptor antagonist naloxone. After the last injection of morphine (on day 28 for two phase treatments, on day 13 for coadministration treatment), we challenged WT or CB_2KO mice from experiment #2, #3 and #4 with vehicle followed 30 min later by naloxone (5 mg/kg i.p.) to precipitate opioid receptor-mediated withdrawal. Mice were video-recorded for subsequent scoring of withdrawal-like behaviors for a 30 min interval following challenge with vehicle or naloxone.

Assessment of mechanical allodynia

Paw withdrawal thresholds (g) to mechanical stimulation were measured in duplicate for each paw using an electronic von Frey anesthesiometer supplied with a 90-g probe (IITC model Alemo 2390–5, Woodland Hills, CA) as described previously (Deng et al., 2012). Mice were placed on an elevated metal mesh table and were allowed to habituate under individual, inverted plastic cages to the testing platform for at least 20 minutes until exploratory behavior had ceased. After the habituation period, a force was applied to the midplantar region of the hind paw with a semi-flexible tip connected to the anesthesiometer. Mechanical stimulation was terminated when the animal withdrew its paw and the value of the applied force was recorded in grams (g). Mechanical paw withdrawal thresholds were obtained in duplicate for each paw, and are reported as the mean of duplicate determinations from each animal, averaged across animals, for each group.

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Assessment of cold allodynia

Response time (sec) 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 as previously published (Deng et al., 2012, 2015). An acetone bubble (approximately 5-6 µl) formed at the end of a blunt one C.C. syringe hub was gently applied to the plantar surface of the hind paw. Care was taken to not apply mechanical stimulation to the hindpaw with the syringe itself. The total time the animal spent attending to the acetone-stimulated paw (i.e. elevation, shaking, or licking) was recorded over one minute following acetone application. Acetone was applied three times to each paw with a 3-minute interval between applications. Values for each animal were calculated as the mean of six determinations of acetone responsiveness derived from each mouse.

Evaluation of opioid receptor-mediated withdrawal symptoms

WT (C57BL/6J) mice and CB_2KO mice that received either vehicle or morphine (10 mg/kg/day, i.p.) or a combination of morphine with LY2828360 (10 mg/kg/day i.p. morphine co-administered with 0.1 mg/kg/day i.p. LY2828360) for 12 days were challenged with vehicle followed by naloxone (5 mg/kg i.p.) to induce opioid withdrawal beginning 30 minutes following the last injection of the test drugs. Mice were videotaped and the number of jumps was scored in 5-min intervals for a total observation period of 30 min after challenge with either saline or naloxone (5 mg/kg i.p.).

Statistical analyses

Paw withdrawal thresholds (mechanical) and duration of acetone-evoked behavior (cold) were calculated for each paw and averaged. Analysis of variance (ANOVA) for repeated measures was used to determine the time course of paclitaxel-induced mechanical and cold

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allodynia. One-way ANOVA was used to identify the source of significant interactions at each time point and compare post-injection responses with baseline levels, followed by Bonferroni post hoc tests (for comparisons between groups). Appropriate comparisons were also made using Bonferroni post hoc tests or planned comparison t-tests (unpaired or paired, as appropriate). All statistical analyses were performed using IBM-SPSS Statistics version 24.0 (SPSS inc., an IBM company, Chicago, IL, USA). $p< 0.05$ was considered statistically significant. Sample size calculations and power analyses were performed using Statmate 2.0 for windows (Graphpad Prism Software, San Diego California, USA www.graphpad.com).

RESULTS

LY2828360 displays a delayed, G protein-biased signaling profile at CB_2 receptors

A range of cell-based *in vitro* signaling assays were employed to dissect the signaling of LY2828360 at CB_2 receptors.

In an arrestin recruitment assay evaluating mouse CB_2 receptors, CP55940 recruited arrestin in a concentration-dependent manner while LY2828360 failed to do so following a 90 min drug incubation (**Fig. 2A**). Recruitment of arrestin is necessary for many forms of receptor sequestration and internalization (Luttrell & Lefkowitz, 2002). In congruency, LY2828360 failed to internalize the receptor (**Fig. 2B**). Strikingly, CP55940 (1µM) induced a rapid (~5 minutes) and efficacious inhibition of forskolin-stimulated adenylyl cyclase while LY2828360 (1µM) induced an efficacious inhibition only after 30 minutes (**Fig. 2C**). CB_2 receptor inhibition of adenylyl cyclase is mediated by inhibitory Gi/o G proteins (Amey Dhopeshwarkar & Mackie, 2014). Thus, to confirm if delayed inhibition by LY2828360 was mediated by Gi/o proteins, cells were pre-treated with pertussis toxin (PTX; 300 ng/ml; overnight). After PTX treatment LY2828360 no longer inhibited cAMP accumulation at 30 minutes (**Fig. 2D**), confirming

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involvement of inhibitory G proteins. Next, full concentrations response experiments were performed at two times when maximal inhibition of forksolin-stimulated cAMP accumulation was observed. At 5 minutes, CP55940 potently and efficaciously inhibited cAMP accumulation while LY2828360 had no effect (**Fig. 2E and Table 1**). Conversely, at 30 minutes, LY2828360 was potent, efficacious and CB_2 receptor mediated (**Fig. 2F)**. CP55940 (1μM) was efficacious in stimulating ERK1/2 phosphorylation (pERK1/2) at 5, 10, 30 and 40 min. while LY2828360 (1μM) increased pERK1/2 only at later times (20, 30 and 40 min). ERK1/2 activation by LY2828360 was completely abolished by pretreatment of cells with PTX (300 ng/ml; overnight) (**Fig. 3A and 3B**), demonstrating G protein dependence. In contrast, only the early phase of CP55940 stimulation of pERK1/2 was PTX sensitive, consistent with the delayed phase of pERK1/2 activation by CP55940 being arrestin-mediated. A full concentration response experiment revealed that LY2828360 failed to increase pERK1/2 at 5 minutes but was potent and efficacious at 20 minutes and required CB_2 receptors as it was blocked by SR144528 (**Fig. 3C and 3D, Table 1**). In order to determine if the slow, biased signaling of LY2828360 was specific for mouse CB2 receptors, we next evaluated LY2828360 signaling via hCB_2 receptors. As with mCB2, LY2828360 failed to internalize hCB2 receptors (**Supplemental S1: A**), exhibited time dependent delayed inhibition of cAMP accumulation (**S1: B, D, E**) and ERK1/2 phosphorylation (**S1: F, G, I**). As with mouse CB_2, these effects were abolished by PTX (**S1: C, H**) and blocked by SR144528 (**S1: I**), confirming the involvement of Gi/o proteins and CB_2 receptors respectively. Finally, LY2828360 did not affect IP1 accumulation via mouse or human CB_2 receptors (**S2: A, B**). Potencies and efficacies of CP55940 and LY2828360 in the signaling assays described above at mouse and human CB2 receptors are summarized in Tables 1 and 2, respectively (**Table 1 and Table 2**).

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Effects of acute administration of LY2828360 in paclitaxel-treated WT mice

Paclitaxel decreased paw withdrawal thresholds ($F_{1, 10}$=249.98, p=0.0001) and increased acetone-evoked behaviors ($F_{1, 10}$=342.95, p=0.0001), consistent with our previous publications showing development of mechanical and cold allodynia following paclitaxel treatment in mice (Deng et al., 2015). Thus, mechanical (**Fig. 4A**) and cold (**Fig. 4B**) allodynia developed by day 4 (p=0.0001) following initial paclitaxel dosing and was maintained with high stability in paclitaxel-treated WT mice relative to cremophor-vehicle treatment from day 7 onward (p =0.0001).

In WT mice, acute systemic administration of LY2828360 suppressed paclitaxel-induced mechanical ($F_{1, 10}$=125.902, p=0.0001; **Fig. 4C**) and cold ($F_{1, 10}$=29.167, p=0.0001; **Fig. 4D**) allodynia in a dose-dependent manner. The high dose of LY2828360 (3 mg/kg i.p.) fully reversed paclitaxel-induced allodynia and normalized responses to pre-paclitaxel baseline levels (p=0.167 mechanical; p=0.53 cold) (**Fig. 4C, D**). However, neuropathic pain was prominent in paclitaxel-treated mice receiving doses of LY28282360 lower than 0.3 mg/kg i.p. compared to control mice that received the cremophor-vehicle in lieu of paclitaxel (p=0.001 mechanical; p=0.044 cold).

To study the duration of antinociceptive action of LY2828360, the maximally efficacious dose (3 mg/kg i.p.) was administered to paclitaxel-treated mice and responsiveness to mechanical and cold stimulation was evaluated at 0.5, 2.5, 4.5 and 24 h post injection. LY2828360 produced time-dependent suppressions of paclitaxel-evoked mechanical ($F_{1, 10}$=38.604 p=0.0001; **Fig. 4E**) and cold ($F_{1, 10}$=4.993, p<0.05 cold; **Fig. 4F**) hypersensitivities and suppression of allodynia was maintained for at least 4.5 h post-injection (p=0.001 mechanical, p=0.022 cold) relative to drug pre-injection levels (i.e. Pac). At 24 h post-injection, paclitaxel-induced mechanical allodynia

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had returned (p=1 mechanical; p=0.125 cold) to drug pre-injection levels of hypersensitivity (**Fig. 4E, F**). Residual suppression of cold allodynia was absent by 72 h post LY2828360 treatment (data not shown).

Previously chronic administration of LY2828360 blocked the development of tolerance to the antiallodynic effects of morphine in WT but not in CB_2KO mice

To study the effects of LY2828360 treatment on the development of tolerance to morphine, pharmacological manipulations were employed in two phases of treatment during the maintenance of neuropathic pain (**Fig. 5A**). In WT mice, phase I treatment with LY2828360 (3 mg/kg/day i.p. x 12 days) suppressed paclitaxel-induced mechanical ($F_{2, 15}$=183.929, p=0.0001; **Fig. 5B**) and cold ($F_{2, 15}$=64.218, p=0.0001; **Fig. 5C**) hypersensitivities relative to phase I vehicle treatments. LY2828360 markedly suppressed paclitaxel-induced mechanical and cold allodynia throughout the observation interval (p=0.0001 mechanical; p=0.016 cold; **Fig. 5B, C**). Mechanical and cold hypersensitivities were largely normalized by LY2828360 (3 mg/kg i.p. x 12 days) with responses returning to baseline (i.e. pre-paclitaxel) levels (p=0.138 mechanical; p=0.182 cold). The anti-allodynic efficacy of LY2828360 was stable throughout phase I treatment (p=0.310 mechanical, p=0.314 cold) without the development of tolerance (**Fig. 5B, C**).

On day 15, three days after completion of phase I treatment, paclitaxel-induced mechanical and cold allodynia had returned to levels comparable to those observed prior to initiation of phase I treatment (i.e. Pac; p=0.379 mechanical, p=0.62 cold; **Fig. 5B**, **C**). Mechanical and cold allodynia were maintained in these mice relative to pre-paclitaxel levels (i.e. BL; p<0.005 mechanical, p<0.006 cold). In paclitaxel-treated WT mice, chronic morphine treatment during phase II of mice previously receiving vehicle during phase I (WT/Pac: Veh (I)-

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Mor (II)) only suppressed paclitaxel-induced mechanical and cold allodynia on day 16 (p=0.0001 mechanical, p=0.0001 cold) and then failed to suppress paclitaxel-induced mechanical (p=1) and cold (p=1) allodynia on subsequent test days (i.e. day 19, 23 and 27) relative to vehicle-treated mice (WT/Pac: Veh (I)-Veh (II); **Fig. 5B, C**). Thus, morphine tolerance rapidly developed to the anti-allodynic effects of phase II morphine in paclitaxel-treated mice receiving vehicle in phase I.

By contrast, in WT mice receiving LY2828360 during phase I, phase II morphine (WT/Pac: LY (I)-Mor (II); 10 mg/kg i.p. x 12 days) sustainably suppressed paclitaxel-induced mechanical ($F_{2, 15}$=91.428, p=0.0001; **Fig. 5B**) and cold ($F_{2, 15}$=40.979, p=0.0001; **Fig. 5C**) hypersensitivities relative to mice pretreated with vehicle in phase I (WT/Pac: Veh (I)-Mor (II); p=0.0001) (**Fig. 5B** and **5C**). This suppression was present and stable throughout phase II for both mechanical (p<0.05) and cold (p<0.009) modalities when compared to drug pre-injection levels in phase II (i.e. day 15). Morphine-induced anti-allodynic efficacy was stably maintained throughout the observation interval following LY2828360 pretreatment for each stimulus modality (p= 0.222 mechanical, p=0.535 cold). Thus, a previous history of chronic treatment with LY2828360 prevented the development of morphine tolerance in paclitaxel-treated WT mice for both stimulus modalities.

In paclitaxel-treated CB_2KO mice, phase I LY2828360 (3 mg/kg/day i.p. x12 days) treatment failed to suppress mechanical (p>0.05) or cold (p>0.05) allodynia relative to vehicle treatment on any day (**Fig. 5D** and **5E**). In these same CB_2KO mice, subsequent phase II morphine treatment (CB_2KO/Pac: LY (I) - Mor (II)) only suppressed mechanical (p=0.0001) and cold (p=0.0001) allodynia on the initial day of morphine dosing (i.e. day 16), relative to vehicle treatment (CB_2KO/Pac: Veh (I)-Veh (II)). Paclitaxel-induced allodynia was fully reinstated at subsequent time points (i.e. on day 19, 23 and 27; p=1 mechanical, p=0.269 cold). The anti-

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allodynic efficacy of initial morphine administration (i.e. on day 16) was similar in WT mice and CB_2KO mice (p=0.203 mechanical; p=1 cold). Phase II morphine administration continued to suppress paclitaxel-induced allodynia (p=0.0001 mechanical; p=0.0001 cold) in WT mice previously receiving LY2828360 (WT/Pac: LY (I)-Mor (II)) but not in the CB_2KO mice at subsequent time points (i.e. day 19, 23 and 27), suggesting that pretreatment with LY2828360 did not block the development of morphine tolerance in CB_2KO mice.

Chronic LY2828360 treatment suppresses paclitaxel-induced mechanical and cold allodynia in WT mice but not in CB_2KO mice previously rendered tolerant to morphine

To evaluate whether LY2828360 has anti-allodynic efficacy in morphine-tolerant mice, we first dosed paclitaxel-treated WT and CB_2KO mice chronically with morphine during phase I (10 mg/kg/day i.p. x12 days) and continued with chronic LY2828360 administration (3 mg/kg/day i.p. x12 days, **Fig. 6A**) in phase II. In phase I, morphine administration suppressed paclitaxel-induced mechanical ($F_{1, 10}$=83.817 p=0.0001) and cold ($F_{1, 10}$=99.443, p=0.0001) allodynia relative to vehicle treatment. On day 1, morphine fully reversed paclitaxel-induced allodynia and normalized responses to pre-paclitaxel levels (i.e. BL; p=0.062 mechanical; p=1 cold), but not on subsequent test days (i.e. day 4, 8, 12; **Fig. 6B, C**). Anti-allodynic efficacy of morphine was decreased on subsequent test days relative to pre-paclitaxel levels of responsiveness (p=0.005 mechanical; p=0.0001 cold). Thus, tolerance developed to the anti-allodynic effects of morphine (i.e. on day 4, 8 and 12; **Fig. 6B, C**).

To evaluate whether LY2828360 produces anti-allodynic effects in mice previously rendered tolerant to morphine, LY2828360 (3 mg/kg/day i.p. x12 days) was administered during phase II to paclitaxel-treated mice that previously receiving morphine during phase I. Phase II LY2828360 (3 mg/kg/day i.p. x 12 days) treatment fully reversed paclitaxel-induced allodynia

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and normalized responsiveness to pre-paclitaxel baseline levels in WT mice that previously developed morphine tolerance in phase I (p=0.112 mechanical; p=0.103 cold; **Fig. 6B, C**). Thus, prior morphine tolerance does not attenuate LY2828360-induced anti-allodynic efficacy in phase II in WT mice. Anti-allodynic efficacy of LY2828360 was also stable throughout the chronic dosing period (p=1 mechanical; p=1 cold), suggesting that tolerance did not develop to phase II LY2828360 treatment in WT mice (**Fig. 6B, C**).

To further evaluate the mechanism of action underlying the anti-allodynic efficacy of LY2828360, we compared the efficacy of phase II LY2828360 treatment in CB_2KO and WT mice that were rendered tolerant to morphine during phase I. Acute morphine increased paw withdrawal thresholds and reduced cold response times in paclitaxel-treated CB_2KO mice relative to the vehicle treatment on day 1 of phase I dosing (p=0.0001 mechanical; p=0.0001 cold; **Fig. 6D** and **E**). The anti-allodynic effects of phase I morphine were attenuated on day 4 (p=0.058 mechanical; p=0.992 cold) and morphine anti-allodynic efficacy was completely absent on day 8 and day 12 of chronic dosing (p=1 mechanical; p=1 cold; **Fig. 6D** and **E**). Chronic administration of LY2828360 in phase II (3 mg/kg/day, i.p. x12 days) did not alter responsiveness to mechanical or cold stimulation in paclitaxel-treated CB_2KO mice relative to the vehicle treatment at any time point (p=0.252 mechanical; p=0.299 cold; **Fig. 6D, E**). Thus, chronic administration of LY2828360 produced anti-allodynic efficacy in paclitaxel-treated WT mice but not CB_2KO with the same prior histories of morphine treatment (p=0.0001 mechanical, p=0.0001 cold).

Chronic coadministration of low dose LY2828360 with morphine blocked morphine tolerance in WT but not in CB_2 KO mice

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In WT mice, coadministration of a submaximal dose of LY2828360 (0.1 mg/kg/day i.p. x 12 days) with morphine (10 mg/kg/day x 12 days) suppressed paclitaxel-induced mechanical ($F_{3, 20}$=111.039 p=0.0001; **Fig. 7A**) and cold ($F_{3, 20}$=56.823 p=0.0001; **Fig. 7B**) hypersensitivities relative to vehicle treatment (p=0.0001). Coadministration of the CB_2 agonist with morphine fully reversed paclitaxel-induced mechanical allodynia and normalized responses to pre-paclitaxel baseline levels throughout the observation period (p=0.078). Coadministration of the CB_2 agonist with morphine also normalized cold responsiveness on day 1 and day 4 (p=0.156) of chronic dosing to pre-paclitaxel baseline levels. By contrast, in CB_2KO mice, sustained anti-allodynic efficacy was absent in paclitaxel-treated mice receiving LY2828360 coadministered with morphine; the combination treatment only reversed paclitaxel-induced mechanical (p=0.0001) and cold (p=0.0001) allodynia relative to vehicle on day 1 (**Fig. 7A, B**). Anti-allodynic efficacy of morphine coadministered with LY2828360 was greater in WT mice relative to CB_2KO mice on subsequent days of chronic dosing (i.e. days 4, 8 and 12; p=0.0001 mechanical; p=0.0001 cold; **Fig. 7A, B**). In paclitaxel-treated WT mice, the combination of morphine with LY2828360 produced a stable and sustained anti-allodynic efficacy throughout the dosing period (p=0.344 mechanical; p=0.995 cold), demonstrating that morphine tolerance failed to develop in the coadministration condition (**Fig. 7A, 7B**).

Naloxone-precipitated withdrawal is attenuated in morphine tolerant WT but not CB_2KO mice with a prior history of LY2828360 treatment

In paclitaxel-treated WT mice, naloxone challenge produced characteristic jumping behavior that differed between groups ($F_{3, 22}$=5.657, p=0.005; **Fig. 8A**). Post hoc comparisons revealed that paclitaxel-treated WT mice that received morphine during phase II but vehicle during phase I (i.e. WT/Pac: Veh (I)-Mor (II) group) exhibited a greater number of jumps relative to

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paclitaxel-treated WT mice that received vehicle during both phases (WT/Pac: Veh (I)-Veh (II); p=0.007). The number of naloxone-precipitated jumps did not differ between groups that received phase I LY2828360 followed by phase II morphine treatment (WT/Pac: LY (I)-Mor (II)) and those that received phase I vehicle followed by phase II vehicle treatment (WT/Pac: Veh (I)-Veh (II); p=0.3). Also, the number of jumps did not differ between phase II morphine-treated mice that received either LY2828360 or vehicle during phase I (WT/Pac: VeH (I)-Mor (II) vs. WT/Pac: LY (I)-Mor (II), p=0.831). Naloxone challenge did not precipitate withdrawal in paclitaxel-treated WT mice receiving morphine in phase I (WT/Pac: Mor (I)-LY (II) vs. WT/Pac: Veh (I)-Veh (II) p=1; **Fig. 8A**).

Similarly, naloxone challenge altered the number of jumps in paclitaxel-treated CB_2KO mice ($F_{3, 21}$=5.696 p=0.005; **Fig. 8B**). In paclitaxel-treated CB_2KO mice, naloxone injection precipitated jumping in mice receiving phase I vehicle followed by phase II morphine treatment versus mice receiving vehicle during both phases of chronic dosing (CB_2KO/Pac: Veh (I)-Veh (II) vs CB_2KO/Pac: Veh (I)-Mor (II), p=0.044). The number of jumps trended higher in paclitaxel-treated CB_2KO mice receiving LY2828360 in phase I and morphine in phase II relative to CB_2KO mice that received vehicle during both phases (CB_2KO/Pac: LY (I)-Mor (II) vs. CB_2KO/Pac: Veh (I)-Veh (II) group; p=0.057). In paclitaxel-treated CB_2KO mice, the number of jumps did not differ between phase II morphine-treated mice that received either LY2828360 or vehicle during phase I (CB_2KO/Pac: LY (I)-Mor (II) vs. CB_2KO/Pac: Veh (I)-Mor (II), p=1). A trend towards a lower number of naloxone-precipitated jumps was observed in WT relative to CB_2KO mice (p=0.064; **Fig. 8C**) that received the same histories of phase I LY2828360 followed by phase II morphine treatment. Similarly, coadministration of LY2828360 with morphine also trended to produce a lower number of naloxone-precipitated

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jumps in WT compared to CB_2KO mice (p=0.055; **Fig. 8D**). The observed power of the marginally significant unpaired t-test comparing impact of LY2828360 on morphine-dependent WT and CB_2KO mice was 40%. A sample size of 20 per group would be required to detect a statistically significant impact of LY2828360 on WT and CB_2KO animals based upon the observed standard deviation, sample size and magnitude difference observed between means.

Body weight change from baseline (i.e. post vehicle) differed as a function of time post naloxone challenge ($F_{1, 48}$ = 144.18, p= 0.0001) but did not differ between groups and the interaction between time and group was not significant. A trend toward group differences in post naloxone body weight was observed at 2 h ($F_{8, 48}$=2.033, p=0.062) but not at 0.5 h ($F_{8, 48}$=1.460, p=0.197) post injection (**Fig. 8E**).

DISCUSSION

Here we show that the CB_2 agonist LY2828360 is a slowly acting but efficacious G protein-biased CB_2 agonist, inhibiting cAMP accumulation and activating ERK1/2 signaling *in vitro*. *In vivo*, chronic systemic administration of the CB_2 agonist LY2828360 suppressed chemotherapy-induced neuropathic pain without producing tolerance. The observed anti-allodynic efficacy was absent in CB_2KO mice, demonstrating mediation by CB_2 receptors. Sustained efficacy of LY2828360 was observed in mice with a history of morphine tolerance. Moreover, both chronic LY2828360 dosing completed prior to morphine dosing and coadministration of LY2828360 with morphine strongly attenuated development of tolerance of morphine. LY2828360 also trended to decrease naloxone precipitated withdrawal signs in WT but not in CB_2KO mice.

LY2828360 also displays an intriguing yet interesting signaling profile at mouse and human CB_2 receptors. Our results suggest that LY2828360 is a slowly acting CB_2 receptor agonist strongly biased towards $G_{i/o}$ G protein signaling with little effect on arrestin or G_q signaling. This

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contrasts strongly with the balanced agonist CP55940, which rapidly inhibited cAMP accumulation and increased pERK1/2. This ability of a ligand to selectively activate a subset of signaling pathways is termed as 'biased agonism or functional selectivity' (Kenakin, 2011) and has emerged as an important pharmacological concept. For example a 'biased' agonist may activate a pathway that is therapeutically more relevant and shun pathways that lead to untoward effects. More recently, 'kinetic bias' has emerged as another important pharmacological concept that emphasizes the timescale of particular pathway activation (Klein Herenbrink et al., 2016) . It remains to be determined if the marked kinetic and G protein bias of LY2828360 explains either its remarkable opioid sparing property or its failure in clinical trials for osteoarthritis pain (Pereira et al., 2013).

Tolerance limits therapeutic utility of an analgesic (Rosenblum, Marsch, Herman, & Russell, 2008). In the present study, anti-allodynic efficacy of LY2828360 was fully maintained in neuropathic mice that received once daily administration of the maximally effective dose of LY2828360 over 12 consecutive days. Anti-allodynic efficacy of LY2828360 (3 mg/kg i.p.) lasted more than 4.5 h following acute administration. Responsiveness to mechanical and cold stimulation returned to baseline after 1 and 3 days, respectively. Our data are consistent with our previous studies showing that CB2 agonist AM1710 suppresses paclitaxel-induced neuropathic pain without producing tolerance or physical dependence following either 8 days of once daily (i.p.) dosing (Deng et al., 2015) or chronic infusion over 4 weeks (Rahn et al., 2014).

A striking, novel observation of our study was that prior chronic treatment with LY2828360 for 12 days prevented subsequent development of tolerance to the anti-allodynic effect of morphine. By contrast, tolerance to morphine developed in CB_2KO mice identically treated with chronic LY2828360 in phase I followed by chronic morphine treatment in phase II. Moreover,

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in paclitaxel-treated WT mice, coadministration of morphine with a low dose of LY2828360 was fully efficacious in alleviating neuropathic pain and blocking development of morphine tolerance. These observations suggest that analgesic efficacy and, potentially, the therapeutic ratio of morphine, could be improved by adjunctive treatment that combines an opioid with a CB_2 agonist to treat neuropathic pain while simultaneously limiting development of tolerance, dependence and potentially other adverse side effects of the opioid analgesic. Our results are in line with a recent study suggesting that coadministration of a low dose of the CB_2 receptor agonist AM1241 combined with morphine reduced morphine tolerance in Walker 256 tumor-bearing rats (Zhang et al., 2016), although mediation by CB_2 receptors was not assessed. AM1241 produced a modest enhancement of opioid-mediated antinociception in the hotplate test and in a test of mechanical sensitivity in tumor-bearing rats (Zhang et al., 2016). However, tolerance developed to the anti-allodynic effects of the combination treatment assessed with mechanical but not thermal (hot plate) stimulation, suggesting that therapeutic benefit of the adjunctive treatment may be ligand and/or modality dependent. Coadministration of CB2 agonist JWH133 also exhibited opioid sparing effects in the formalin model of inflammatory pain (Yuill, Hale, Guindon, & Morgan, 2017). The mechanism underlying these therapeutically advantageous properties remains incompletely understood. In tumor-bearing mice, AM1241 upregulated μ-opioid receptor (MOR) expression in the spinal cord and dorsal root ganglia (DRG) (Zhang et al., 2016). Another study suggested CB_2 agonist upregulated MOR expression levels whereas the CB_2 antagonist inhibited the MOR expression level in Jurkat T cells (Börner, Höllt, & Kraus, 2006) and in mouse brainstem (Páldy et al., 2008). Mitogen-activated protein kinase (MAPK) activation and glial proinflammatory mediator release have also been linked to morphine tolerance (Mika, Osikowicz, Makuch, & Przewlocka, 2007;

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Raghavendra, Rutkowski, & DeLeo, 2002). CB_2 agonists could alleviate morphine tolerance by an interaction between microglial opioid and CB_2 receptors and/or by reduction of glial and MAPK activation (Badalà, Nouri-mahdavi, & Raoof, 2008; Tumati et al., 2012). CB_2 activation is correlated with increasing anti-inflammatory gene expression in the dorsal horn and reductions in mechanical and thermal hypersensitivities. Coadministration of morphine with the CB_2 agonist JWH015 synergistically inhibited preclinical inflammatory, post-operative and neuropathic-pain in a dose- and time-dependent manner (Grenald et al., 2017). The observed synergism may involve activation of CB_2 receptors on immune cells and subsequent inhibition of the inflammatory process coupled with morphine's well-characterized ability to inhibit nociceptive signaling (Grenald et al., 2017). In keratinocytes in peripheral paw tissue, AM1241 stimulated release of the endogenous opioid β-endorphin, which acted at local neuronal MORs to inhibit nociception through a naloxone-dependent mechanism (Ibrahim et al., 2005). However, naloxone-sensitivity is not a class effect of CB_2 agonists and cannot account for AM1241 antinociception (Rahn et al., 2008), but may depend upon levels of endogenous analgesic tone.

Some effects of cannabinoid receptor agonists and antagonists on morphine antinociceptive tolerance remain controversial. Coadministration of the CB_2 receptor agonist JWH-015 with morphine increased morphine analgesia and also morphine antinociceptive tolerance (Altun et al., 2015). By contrast, the CB_2 receptor antagonist JTE907 decreased morphine analgesia and attenuated morphine antinociceptive tolerance in rats using tail-flick and hot-plate tests of antinociception (Altun et al., 2015). Differences in experimental paradigms, biased signaling of the CB_2 agonist employed, or the presence/absence of a pathological pain state could account for these disparities.

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An emerging challenge for pain management is how to treat pain in the morphine-tolerant individual. Dose escalation is typically employed in early uni-modal treatment (de Leon-Casasola et al., 1993), which may enhance potential for abuse (Rosenblum et al., 2008). The combination of two or more analgesic agents with different mechanisms was proposed as an analgesic strategy (CDC, 2015). Our study has important implications for the clinical management of neuropathic pain because chronic LY2828360 treatment showed sustained anti-allodynic efficacy in neuropathic mice previously rendered tolerant to morphine. This observation is unlikely to be due to pharmacokinetic factors because morphine dosing ceased for 4 days in our study prior to introduction of phase II LY2828360 chronic treatment.

Physical dependence is another major side effect of opioid treatment, which can lead to a withdrawal syndrome when the user stops taking the drug. However, most studies of opioid dependence have used naïve animals rather than animals subjected to a neuropathic pain state (Lynch, Nicholson, Dance, Morgan, & Foley, 2010). The opioid receptor antagonist naloxone precipitates a spectrum of autonomic and somatic withdrawal signs in morphine-dependent animals (Morgan & Christie, 2011). In the present study, in paclitaxel-treated WT mice, chronic phase I pretreatment with LY2828360 produced a trend towards reducing naloxone-precipitated withdrawal jumps without reducing pain relief in the same animals where LY2828360 blocked development of morphine tolerance. This trend was absent in CB_2KO mice receiving identical treatments. In fact, our studies raise the possibility that CB_2 receptor signaling may attenuate opioid antagonist-precipitated withdrawal because CB_2KO mice trended to show higher levels of naloxone-precipitated jumping compared to WT mice when pretreated with CB_2 agonist. Moreover, coadministration of low dose LY2828360 with morphine mimicked these effects and also trended to decrease naloxone-precipitated withdrawal jumping in paclitaxel-treated WT

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mice compared with CB_2KO mice (p=0.055). Thus, LY2828360 may be efficacious in decreasing morphine withdrawal symptoms. Variability in withdrawal jumps and inadequate statistical power could account for the failure to observe more robust statistical differences in jumps between groups; the primary endpoints evaluated here were mechanical and cold responsiveness, not naloxone-induced jumping. Observations from both of these studies are, nonetheless, broadly consistent with the hypothesis that CB_2 receptor activation may attenuate signs of opioid withdrawal. Stimulation of microglial CB_2 receptors by the CB_2 agonist suppressed microglial activation (Ehrhart et al., 2005), which has been linked to morphine withdrawal behaviors. Thus, depletion of spinal lumbar microglia decreased withdrawal behaviors and attenuated the severity of withdrawal without affecting morphine antinociception (Bonin et al., 2017). The mechanism underlying these observations remains to be explored.

In summary, our observations suggest that CB_2 agonists may be useful as a first line treatment for suppressing chemotherapy-induced neuropathic pain. Our results suggest that CB_2 agonists may be useful for suppressing neuropathic pain with sustained efficacy in opioid-recalcitrant pain states without the development of tolerance or dependence.

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Authorship contributions

Participated in research design: Lin, Dhopeshwarkar, Mackie, Hohmann

Conducted experiments: Lin, Dhopeshwarkar, Huibregtse

Performed data analysis: Lin, Dhopeshwarkar

Wrote or contributed to the writing of the manuscript: Lin, Dhopeshwarkar, Mackie, Hohmann

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Footnotes

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Figure Legends

Figure 1. Chemical structure of CB_2 receptor agonist LY2828360, drawn by ChemBioDraw ultra (version 14.0).

Figure 2. LY2828360 displays a delayed signaling profile at mouse CB2 receptors. A) In CHO cells stably expressing mCB2 receptors, CP55940 recruited arrestin in a concentration-dependent manner while LY2828360 failed to do so following 90 minute drug incubation; B) In HEK cells stably transfected with mCB2, CP55940 concentration-dependently internalized the mCB2 while LY2828360 was less potent and efficacious; C) In forskolin-stimulated cAMP time course assay, CP55940 (1 μM) was efficacious and rapid in inhibiting forskolin-stimulated cAMP accumulation at 5 minutes while LY2828360 (1 μM) was efficacious only after 30 minutes; D) Following pertussis toxin (PTX) treatment, CP55940 (1 μM) modestly *increased* cAMP accumulation at 5 minutes, while LY2828360 (1 μM) failed to affect cyclase levels at all time points examined/tested; E) CP55940 was potent and efficacious in inhibiting forskolin-stimulated cAMP accumulation at 5 minutes, while LY2828360 failed to affect cAMP levels at this time point; F) However, after 30 minute incubation, LY2828360 concentration-dependently inhibited forskolin-stimulated cAMP accumulation and this inhibition was completely blocked by 1 μM SR144528(SR2). Forskolin-stimulated cAMP assays which were performed in duplicates. All other assays were performed in triplicates. All data were plotted and analyzed using GraphPad Prism 4.

Figure 3. LY282360 displays a delayed CB_2 receptor- and G protein- dependent signaling profile in activating pERK1/2. A) In HEK cells stably expressing mouse CB2 receptors,

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CP55940 (1 μM) increased phosphorylated ERK1/2 at 5 min, 10 min 30 min and 40 min time points, while LY2828360 (1 μM) had no effect at 5 min and 10 min time points but increased ERK1/2 phosphorylation at 20 min, 30 min and 40 min time points; B) Pertussis toxin (PTX) treatment abolished the 20 min phosphorylation of ERK1/2 by LY2828360 (1 μM). PTX abolished the CP55940 mediated-phosphorylation of ERK1/2 at the 5 min time point, and was retained at 40 min time point following PTX treatment; C) CP55940 concentration-dependently increased ERK1/2 phosphorylation at 5 minutes, while LY2828360 failed to affect pERK1/2 levels at this time point; D) Conversely, after 20 minutes of treatment, CP55940 decreased ERK1/2 phosphorylation while LY2828360 increased ERK1/2 phosphorylation, in a concentration dependent manner. Both effects were blocked by the CB2 receptor antagonist SR144528 (1 μM)(SR2). All pERK1/2 assays were performed in triplicates. All the experimental data were plotted and analyzed using GraphPad Prism 4.

Figure 4. Paclitaxel produced hypersensitivities to mechanical (A) and cold (B) stimulation. Non-chemotherapy control mice received cremophor-based vehicle in lieu of paclitaxel. (C, D) Dose response of LY2828360, administered systemically (i.p.), on the maintenance of (C) mechanical and (D) cold allodynia in paclitaxel-treated WT (C57BL/6J) mice. Time course of LY2828360, administered systemically (3 mg/kg i.p.), on the maintenance of (E) mechanical and (F) cold allodynia in paclitaxel-treated WT mice. BL, pre-paclitaxel baseline; Pac, baseline after paclitaxel; i.p., intraperitoneal injection; Data are expressed as mean ± SEM (n=6 per group). *$p < 0.05$ *vs*. control, one-way analysis of variance at each time point followed by Bonferroni post hoc test. # $p < 0.05$ *vs*. baseline before paclitaxel, repeated measures analysis of variance. & $p < 0.05$ *vs*. baseline after paclitaxel, repeated measures analysis of variance.

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Figure 5. Prior history of chronic LY2828360 treatment blocked the development of morphine tolerance in WT but not CB2KO mice. (A) Testing scheme used to evaluate the two phases treatment during the maintenance of neuropathic pain. Prior history of chronic LY2828360 (3 mg/kg/day i.p. x 12 days in phase I) treatment suppressed paclitaxel-induced (B) mechanical (C) cold allodynia in WT mice. Prior history of chronic LY2828360 (3 mg/kg/day i.p. x 12 days in phase I) blocked the development of tolerance to anti-allodynic effects of morphine (10 mg/kg/day x 12 days in phase II) in WT but not CB_2KO mice for both mechanical (D) and cold (E) modalities. Data are expressed as mean ± SEM (n=6 per group). *$p < 0.05$ *vs*. Veh (I)-Veh (II), one-way analysis of variance at each time point followed by Bonferroni post hoc test. # $p < 0.05$ *vs*. baseline before paclitaxel, repeated measures analysis of variance.

Figure 6. Chronic LY2828360 treatment showed sustained anti-allodynic efficacy in morphine tolerant WT mice but not CB2KO mice. (A) Testing scheme used to evaluate the two phases treatment during the maintenance of neuropathic pain. Chronic LY2828360 (3 mg/kg/day i.p. x 12 days in phase II) treatment suppressed paclitaxel-induced mechanical (B, D) and cold (C, E) allodynia in WT mice but not CB_2KO mice previously rendered tolerant to morphine (10 mg/kg/day i.p. x 12 days in phase I). Data are expressed as mean ± SEM (n=6 per group). Veh (I)-Veh (II) group is replotted from Figure 5. *$p < 0.05$ *vs.* Veh (I)-Veh (II), one-way analysis of variance at each time point followed by Bonferroni post hoc test. # $p < 0.05$ *vs.* baseline before paclitaxel, repeated measures analysis of variance.

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Figure 7. Chronic coadministration of low dose LY2828360 (0.1 mg/kg/day i.p. x 12 days) with morphine (10 mg/kg/day i.p. x 12 days) blocked development of morphine tolerance in WT but not in CB_2KO mice tested for both (A) mechanical and (B) cold allodynia. Data are expressed as mean ± SEM (n=6 per group). *$p < 0.05$ *vs.* WT-Veh, one-way analysis of variance at each time point followed by Bonferroni post hoc test. # $p < 0.05$ *vs.* baseline before paclitaxel, repeated measures analysis of variance.

Figure 8. Impact of LY2828360 treatment on naloxone-precipitated opioid withdrawal in CB_2 KO and WT mice. Naloxone (5 mg/kg i.p.) precipitates jumping in WT (A) and CB_2KO (B) mice receiving morphine (10 mg/kg/day i.p. x 12 days) during phase II of chronic dosing. (C) A trend (p=0.064) toward lower numbers of naloxone-precipitated jumps were observed in WT compared to CB_2KO mice with similar histories of LY28282360 (3 mg/kg/day x 10 days during phase I) followed by morphine (10 mg/kg/day i.p. x 12 days during phase II) treatment. (D) Naloxone-precipitated (5mg/kg i.p.) jumping trended lower in WT mice (p=0.055) receiving coadministration of LY2828360 (0.1 mg/kg/day i.p. x 12 days) with morphine (10 mg/kg/day i.p. x 12 days) compared to CB_2KO mice with the same histories of drug treatment. Naloxone did not precipitate jumping behavior in the absence of morphine. (E) Changes in body weight were greater at 2 h compared to 0.5 h following naloxone challenge. Data are expressed as mean ± SEM (n=6-8 per group) *$p < 0.05$ *vs.* Veh (I)-Veh (II), one-way analysis of variance followed by Bonferroni post hoc test or one tailed t test as appreciate.

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Table 1

Potencies and efficacies of CP55940 and LY2828360 in arrestin, internalization, cyclase and pERK1/2 assays at mouse CB2 receptors.

		CP55940				LY2828360			
	Drug incubation (mins)	EC50 (nM)	95% CI	Emax (%)	± SEM	EC50 (nM)	95% CI	Emax (%)	± SEM
Arrestin	90	2.3	0.4-12.2	125	±1.6	ND	ND	97.9*	±1.5
Internalization	90	7.4	1.1-19.3	49.1	±1.2	30.7	1.4-626.5	19.1	±2.4
Cyclase	05	6.6	1.7-12.2	52.8	±3.6	ND	ND	18.9	±5.8
	30	-	-	-	-	13.6	10.4-45.3	53.4	±1.9
pERK1/2	05	10.5	2.2-17.9	136.2	±4.1	ND	ND	4.1	±2.5
	20	1.5	0.1-3.7	20.3*	±3.4	339	128.8-345.8	43.6	±2.3

Duration of drug incubation is expressed in minutes. All assays were performed in triplicates except cAMP accumulation assays, which were performed in duplicates.EC50, 95% confidence intervals (CI), Emax (mean ± SEM) were obtained by plotting and analyzing the data using GraphPad Prism 4. ND: Not determined/cannot be determined.

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Table 2

Potencies and efficacies of CP55940 and LY2828360 in internalization, cyclase and pERK1/2 assays at human CB2 receptors.

		CP55940				LY2828360			
	Drug incubation (mins)	EC50 (nM)	95% CI	Emax (%)	± SEM	EC50 (nM)	95% CI	Emax (%)	± SEM
Internalization	90	3	0.3-15.6	33.9	±4.6	ND	ND	10.2	±7.1
Cyclase	05	12.3	2.9-18.3	59.6	±8.3	ND	ND	ND	ND
	35	-	-	-	-	16.7	4.6-59.6	42.8	±2.7
pERK1/2	05	3.77	0.4-12.7	95.7	±9.1	ND	ND	22.1*	±5.8
	30	23.3	10.1-53.9	49.4	±1.6	33.5	9.1-107.1	32.3	±1.9

Duration of drug incubation is expressed in minutes. cAMP accumulation assays were performed in duplicates. All other assays were performed in triplicates. EC50, 95% confidence intervals (CI), Emax (mean ± SEM) were obtained by plotting and analyzing the data using GraphPad Prism 4. ND: Not determined/cannot be determined.

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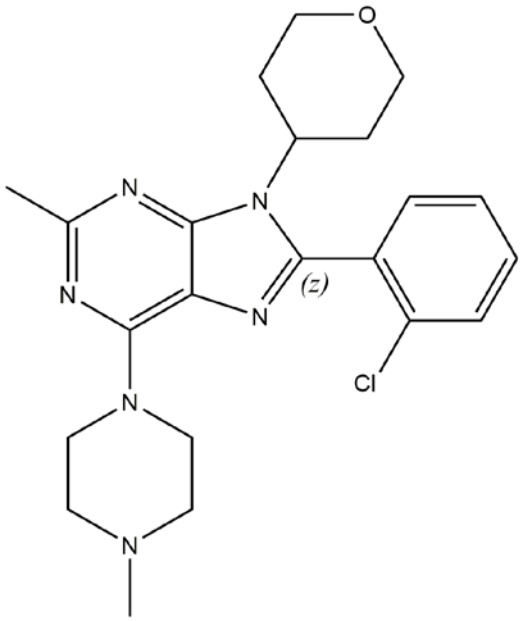
Figure 1



LY2828360

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Figure 2

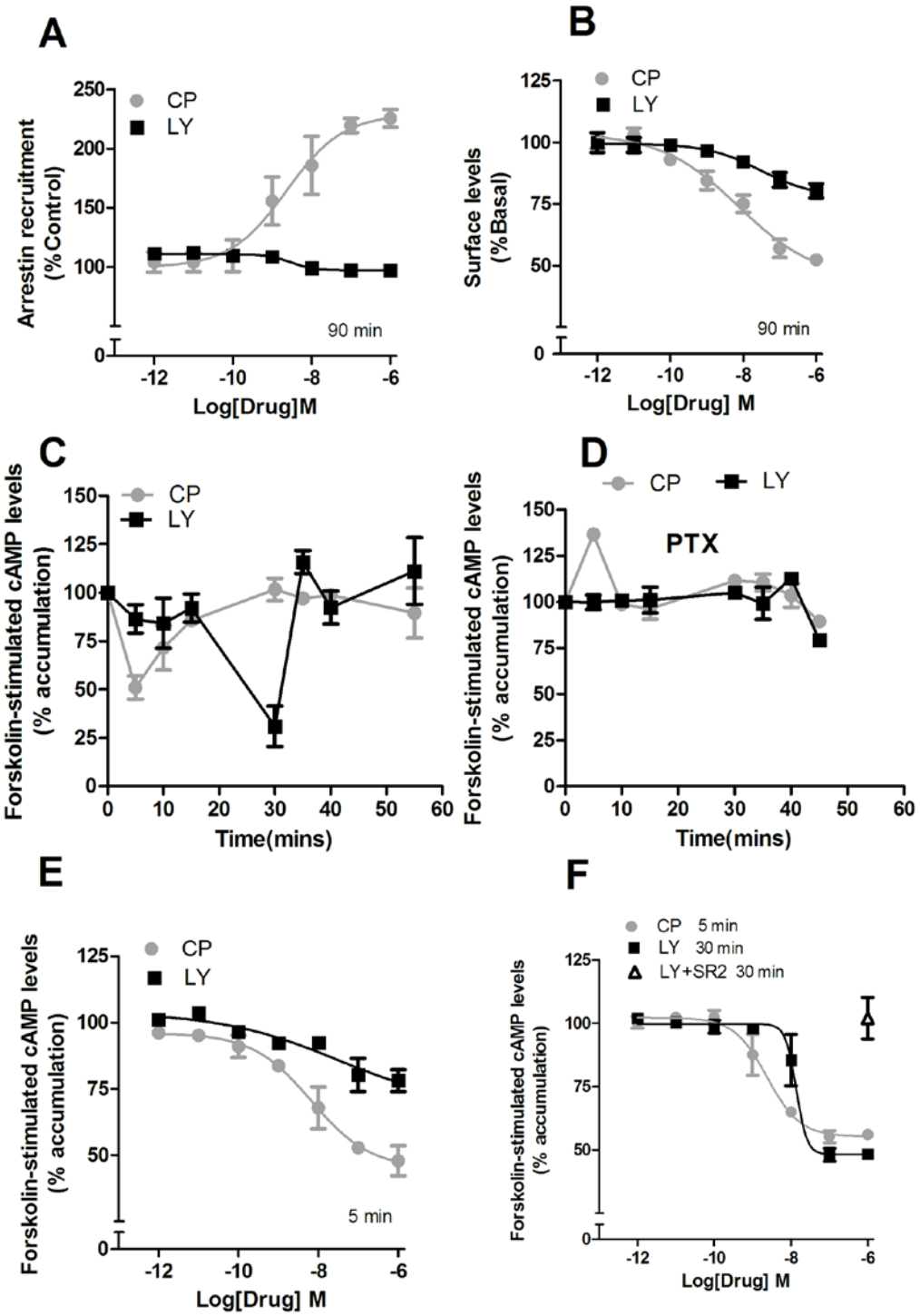

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Figure 3

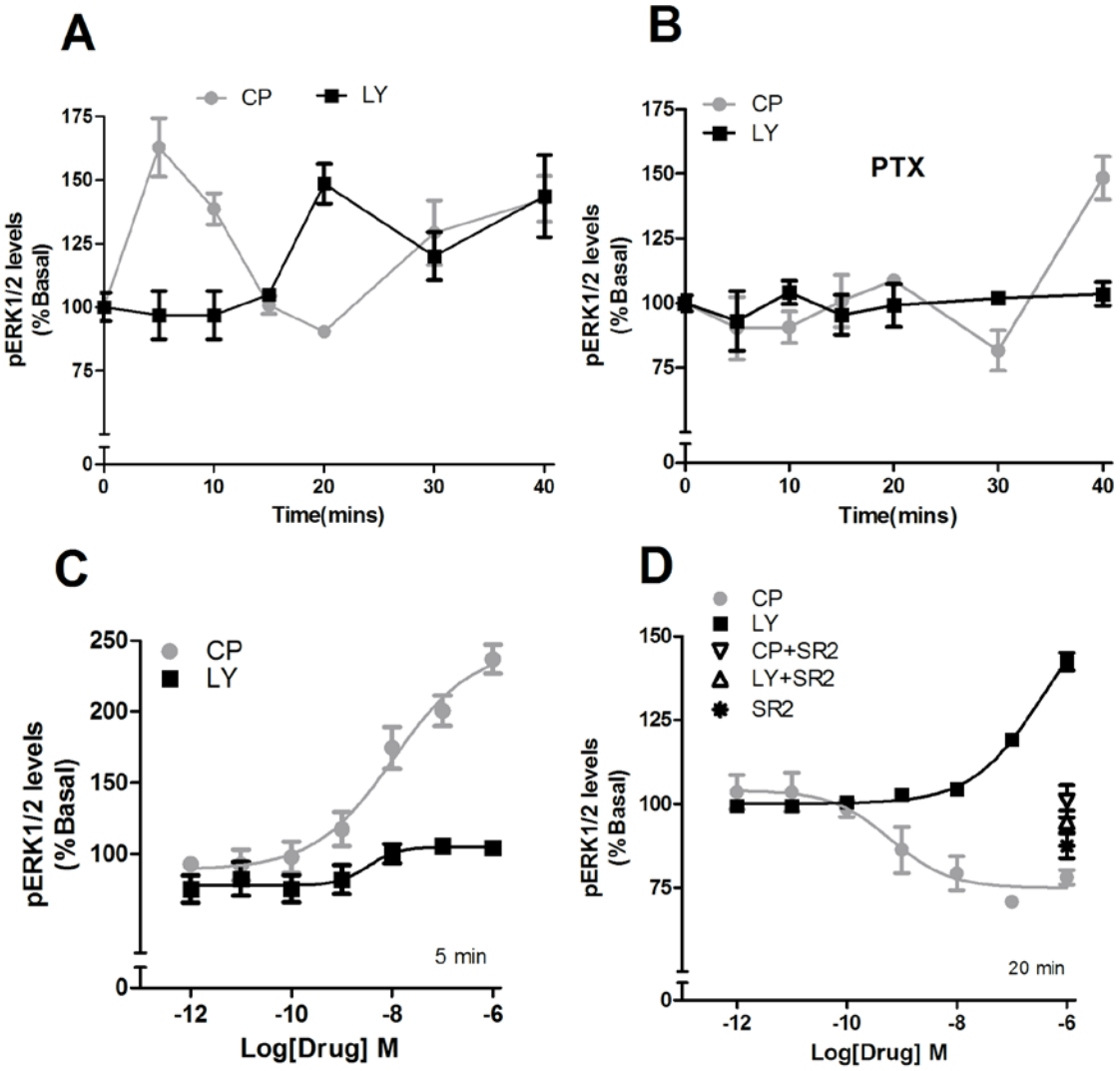

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Figure 4

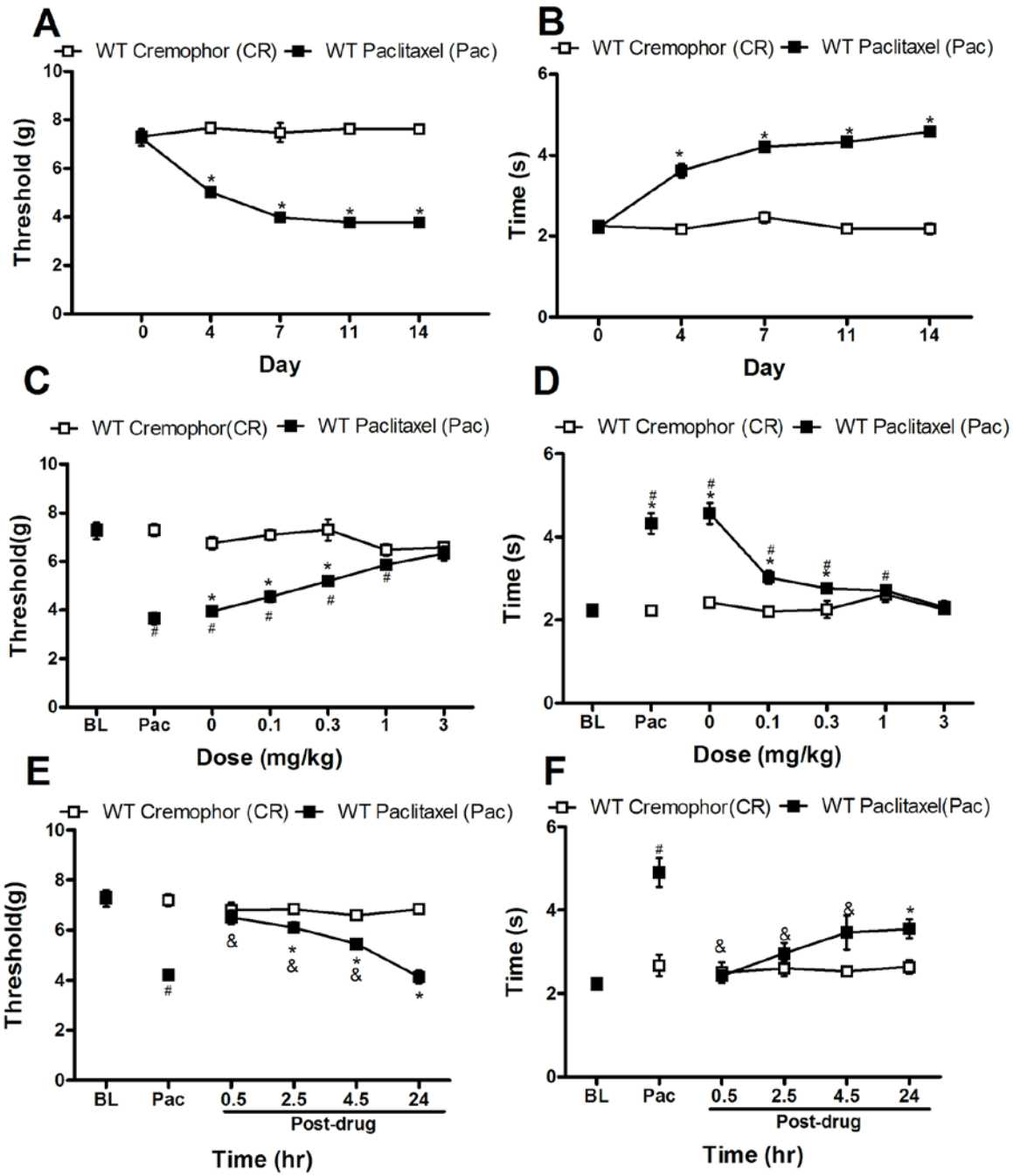

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Figure 5

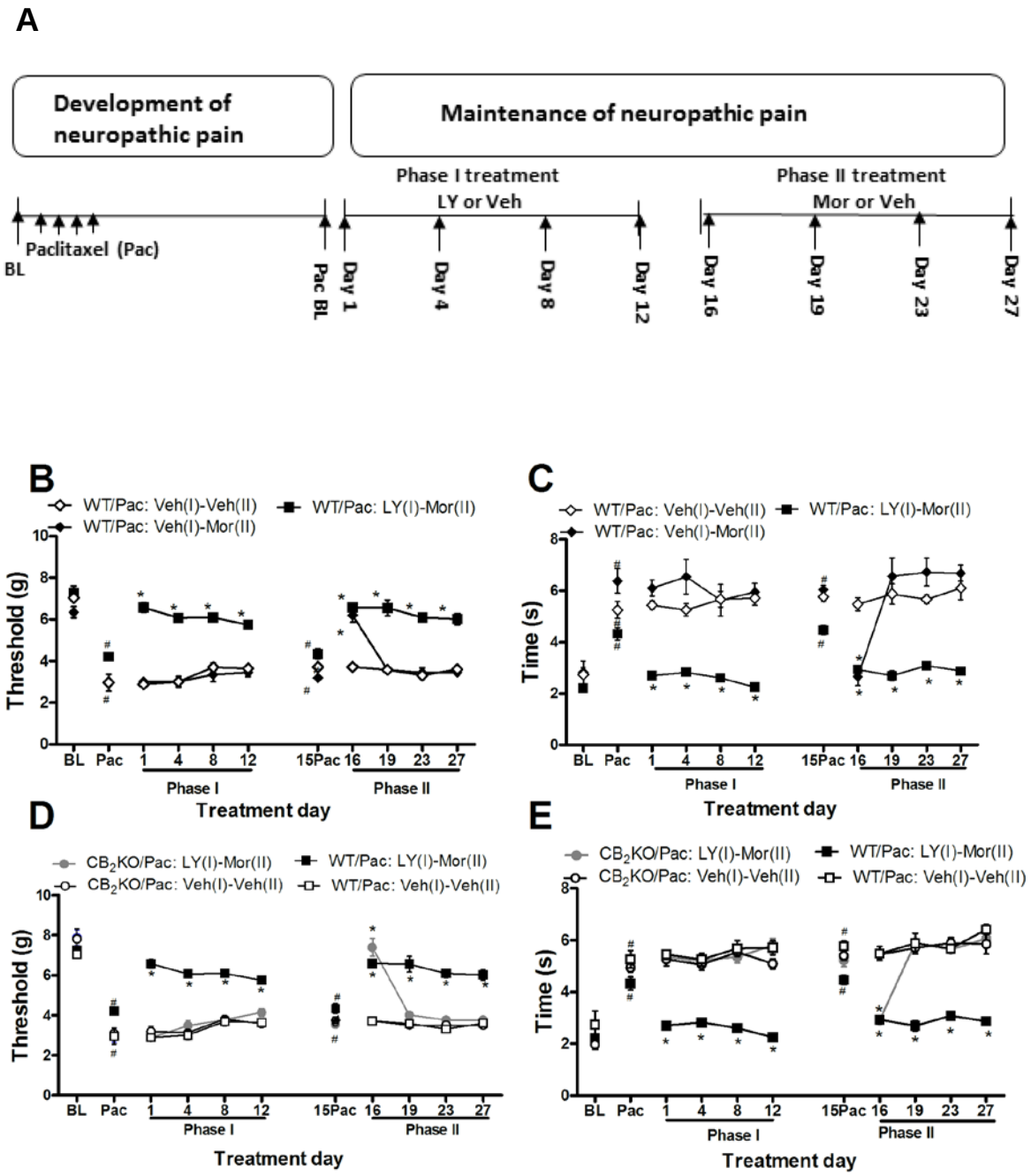

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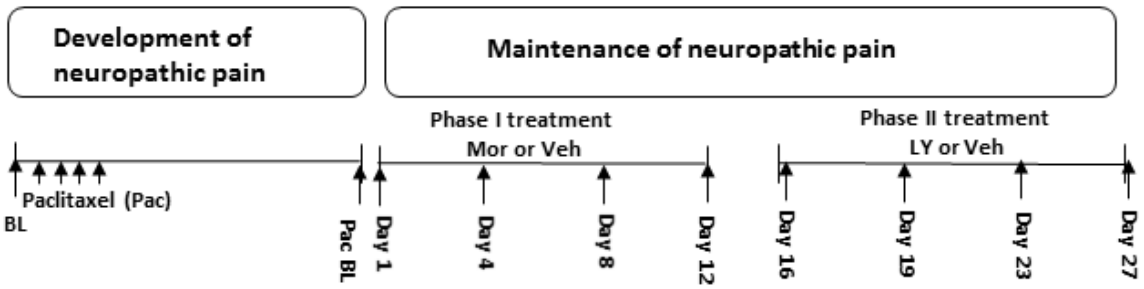
Figure 6

A



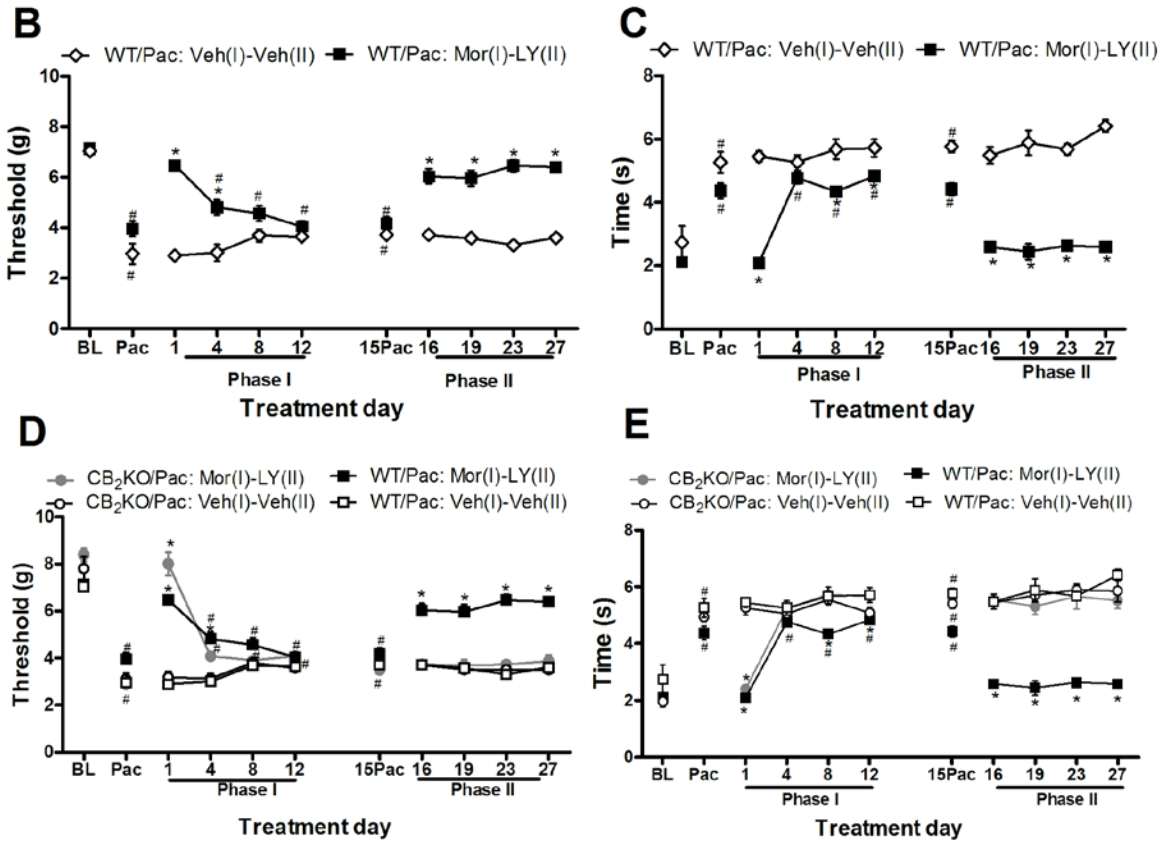


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Figure 7

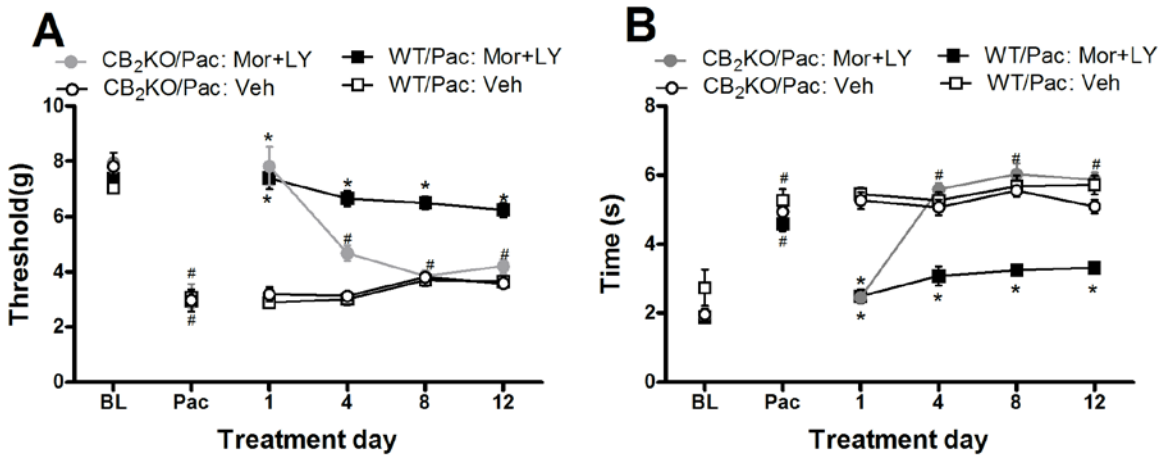

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Figure 8