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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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 extracellular signal-regulated kinase 1/2 signaling while failing to recruit arrestin, activate inositol phosphate signaling, or internalize CB2 receptors. In wild-type (WT) mice, LY2828360 (3 mg/kg per day i.p. × 12 days) suppressed chemotherapy-induced neuropathic pain produced by paclitaxel without producing tolerance. Antiallodynic efficacy of LY2828360 was absent in CB$_2$ knockout (KO) mice. Morphine (10 mg/kg per day i.p. × 12 days) tolerance developed in CB$_2$KO mice but not in WT mice with a history of LY2828360 treatment (3 mg/kg per day i.p. × 12 days). LY2828360-induced antiallodynic efficacy was preserved in WT mice previously rendered tolerant to morphine (10 mg/kg per day i.p. × 12 days), but it was absent in morphine-tolerant CB$_2$KO mice. Coadministration of LY2828360 (0.1 mg/kg per day i.p. × 12 days) with morphine (10 mg/kg per day × 12 days) blocked morphine tolerance in WT but not in CB$_2$KO mice. WT mice that received LY2828360 coadministered with morphine exhibited a trend ($P = 0.055$) toward fewer naloxone-precipitated jumps compared with CB$_2$KO 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 refractory to opioid analgesics.

Introduction

Morphine suppresses many types of pain, but tolerance, physical dependence, and unwanted side effects limit its clinical use (Trang et al., 2007). Identification of therapeutic strategies for blocking opioid tolerance and dependence has therefore evolved as an area of intense research interest (Habibi-Asl et al., 2014; Mansouri, et al., 2015; Hassanipour et al., 2016; Hosseinzadeh et al., 2016). 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 et al., 2004) and can

functionally interact by receptor heterodimerization or signaling cross-talk (Bushlin et al., 2010). Although activation of both receptors produces analgesia, undesirable pharmacologic effects limit their use (Manzanares et al., 1999; Massi et al., 2001). An alternative approach aims at harnessing the therapeutic potential of cannabinoid CB$_2$ receptors to suppress pathologic pain without producing CB$_1$-mediated cannabimimetic effects (for review, see Guindon and Hohmann, 2008; Dhopeshwarkar and Mackie, 2014). CB$_2$ receptors are primarily expressed on immune cells but may be induced in the CNS in response to injury (for review, see Mechoulam and Parker, 2013). 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 (Lim et al., 2005; Merighi et al., 2012; Desroches et al., 2014); however, whether CB$_2$ agonists

ABBREVIATIONS: AM1710, 3-(1, 1-dimethyl-heptyl)-1-hydroxy-9-methoxy-benzo(c) chromen-6-one; BSA, bovine serum albumin; 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; DMSO, dimethylsufoxide; ERK, extracellular signal-regulated kinases; Gi, inhibitory G proteins; HEK, human embryonic kidney; IP1, myo-inositol phosphate 1; KO, knockout; LY2828360, (8-(2-chlorophenyl)-2-methyl-6-(4-methylpiperazin-1-yl)-9-(tetrahydro-2H-pyran-4-yl)-9H-purine); pERK 1/2, phosphorylated ERK1/2; PTX, pertussis toxin; TBS, Tris-buffered saline; WT, wild type.

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 (Hollinshead et al., 2013). In the monoiodoacetic acid model, LY2828360 (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 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 4 weeks (Pereira et al., 2013) (www.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 2 clinical trial. Evaluations of LY2828360 antinociceptive efficacy have not appeared in the published literature despite 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 using a range of cell-based in vitro signaling assays: arrestin recruitment, CB_2 receptor internalization, inhibition of forskolin-stimulated cAMP (cyclase) accumulation, extracellular signal-regulated kinase (ERK1/2) phosphorylation, and myo-inositol phosphate 1 (IP1) accumulation. Moreover, to our knowledge, LY2828360 has 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 LY2828360 would produce antiallodynic efficacy in mice that were rendered tolerant to morphine and, conversely, whether development of morphine tolerance would be attenuated in mice with a 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, pharmacologic 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.

Materials and Methods

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, were used in this study. Animals were single-housed several days before initiating pharmacologic manipulations. All mice were maintained in a temperature-controlled facility (73 $\pm$ 2°F, 45% humidity, 12-hour light/dark cycle, lights on at 7 AM); food and water were provided ad libitum. All experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee of Indiana University and followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Drugs and Chemicals. Paclitaxel (Tecoland Corporation, Irvine, CA) was dissolved in a cremophor-based vehicle made of Cremophor EL (Sigma-Aldrich, St. Louis, MO), ethanol (Sigma-Aldrich), and 0.9% saline (Aqualite System; Hospira, Inc., Lake Forest, IL) 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 (Indianapolis, IN) 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 dimethylsulfoxide (DMSO) (Sigma-Aldrich), ALKAMULS EL-620 (Rhodia, Cranbury, NJ), ethanol, and saline. Naloxone (Sigma-Aldrich) was dissolved in saline as indicated. Drugs were administered via intraperitoneal injection to mice in a volume of 10 ml/kg. CP55940 [(2)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropylcyclohexanol] was obtained from the National Institute of Drug Abuse Drug Supply Service (Bethesda, MD). Pertussis toxin (PTX; cat. no. BML-G100-0050) was purchased from Enzo Lifesciences (Farmingdale, NY).
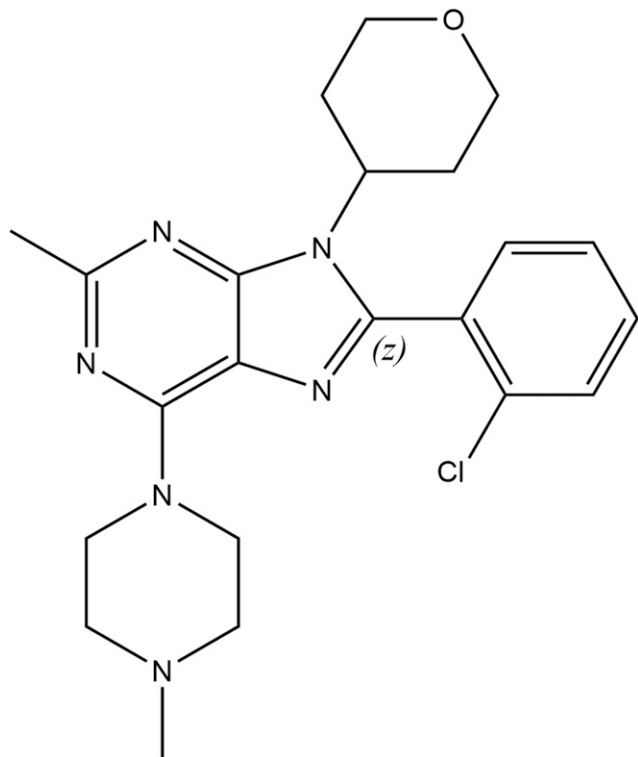

Fig. 1. Chemical structure of CB_2 receptor agonist LY2828360, drawn by ChemBioDraw Ultra (version 14.0).

Cell Culture. Human embryonic kidney (HEK) 293 cells stably expressing mouse CB2 receptors (HEK mCB2) or human CB2 receptors (HEK hCB2) were generated, expanded, and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin (GIBCO, Carlsbad, CA) at 37°C in 5% CO_2. For ease of immunodetection, an amino-terminal 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 (Dhopeshwarkar and Mackie, 2016). PathHunter Chinese hamster ovary (CHO) K1 CNR2 (cat. 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 acceptor is fused with arrestin while a complementary smaller fragment (C-terminal) is fused with C-terminal domain of the mouse CB2 cannabinoid receptor. Upon receptor activation, recruitment of arrestin leads to the 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 (cat. no. 92-0018GF2).

Quantification of cAMP Levels. cAMP assays were optimized using PerkinElmer's LANCE ultra-cAMP kit (cat. no. TRF0262; PerkinElmer, Boston, MA) per the manufacturer's instructions. All assays were performed at room temperature using 384-optiplates (cat. no. 6007299; PerkinElmer). Briefly, cells were resuspended in $1\times$ stimulation buffer ($1\times$ Hanks' balanced salt solution, 5 mM HEPES, 0.5 mM IBMX, 0.1% bovine serum albumin (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, 10 μl), followed by stimulation with drugs/compounds and forskolin (2 μM final concentration) made in $1\times$ 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 times. For experiments with PTX, cells were treated overnight with 300 ng/ml PTX at 37°C in 5% CO_2. Cells were then lysed by addition of 10 μl Eu-cAMP tracer working solution ($4\times$, made fresh in $1\times$ lysis buffer supplied with the kit, under subdued light conditions) and 10 μl *Ulight* anti-cAMP working solution ($4\times$, made fresh in $1\times$ 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 (PerkinElmer).

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 the next day serum-starved for 5 hours. After serum starvation, the cells were challenged with drugs/compounds for the indicated time. After drug incubation, plates were emptied and quickly fixed with ice-cold 4% paraformaldehyde for 20 minutes, followed by ice-cold methanol with the plate maintained at −20°C for 15 minutes. Plates were then washed with Tris-buffered saline (TBS)/ 0.1% Triton X-100 for 25 minutes (5 × 5-minute washes). The wash solution was then replaced by Odyssey blocking buffer and incubated further for 90 minutes 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 minutes (5 × 5-minute 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 five times with TBS/0.05% Tween-20 solution. The plates were patted dry and scanned using LI-COR Odyssey scanner (LI-COR, Inc., Lincoln, NE) phosphorylated ERK1/2 (pERK1/2) activation (expressed

in percentages) was calculated by dividing the average integrated intensities of the drug-treated wells by the 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% fetal bovine serum + 0.5% Pen/Strep. Cells were washed once with HEPES-buffered saline/BSA (BSA @ 0.08 mg/ml) with 200 μl/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% paraformaldehyde for 20 minutes and washed four times (300 μl per well) with TBS. Blocking buffer (Odyssey blocking buffer; LI-COR, Inc., Lincoln, NE) was applied at 100 μl per well for 1 hour at room temperature. Anti- hemagglutinin antibody (mouse monoclonal, 1: 200; Covance, Princeton, NJ) diluted in Odyssey blocking buffer was then applied for 1 hour at room temperature. After this, the plate was washed five times (300 μl/well) with TBS. Secondary antibody diluted (anti-mouse 680 antibody 1:800, LI-COR, Inc.,) in blocking buffer was then applied for 1 hour at room temperature, after which the plate was washed five times (300 μl/well) with TBS. The plate was imaged using an Odyssey scanner (700 channel, 5.5 intensity, LI-COR, Inc.,).

IP1 Accumulation Assay. Accumulation of IP1, a downstream metabolite of IP3, was measured by using IP-One HTRFkit (cat. no. 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 IP hydrolysis cascade. Accumulated IP3 is quickly 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, and IP1 levels can be easily quantified using an homogeneous time-resolved fluorescence (HTRF) assay. HEK mCB2 cells were detached from ∼50% confluent plates using versene. Cells (10 μl, 5000 cells) were resuspended in $1\times$ stimulation buffer (containing lithium chloride, 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 the 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 triplicate unless otherwise stated.

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

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 experiments 2 and 3, pharmacologic manipulations were performed once daily for 12 consecutive days in each of the two phases of chronic treatment. Four days separated phase 1 and phase 2 chronic dosing in all studies comprising two phases of chronic dosing. Experiments 2 and 3 were performed concurrently using overlapping cohorts that were tested with a single vehicle (phase 1), vehicle (phase 2) group.

In experiment 2, we examined the antiallodynic efficacy of chronic systemic administration of LY2828360 (3 mg/kg per day i.p. × 12 days) or vehicle administered during phase 1 using paclitaxel-treated WT

and CB$_2$KO mice. We then assessed the antiallodynic efficacy of chronic systemic administration of vehicle or morphine (10 mg/kg per day i.p. × 12 days) administered during phase 2 in the same animals. Responsiveness to mechanical and cold stimulation was evaluated on treatment days 1, 4, 8, and 12 during phase 1 and on treatment days 16, 19, 23, and 27 during phase 2 (i.e., phase 2 started on day 16).

In experiment 3, we assessed the antiallodynic efficacy of chronic administration of LY2828360 (3 mg/kg per day i.p. × 12 days in phase 2) or vehicle in paclitaxel-treated WT and CB$_2$KO mice that previously developed tolerance to morphine. To induce morphine tolerance, mice received repeated once daily injections of morphine (10 mg/kg per day i.p. × 12 days) in phase 1 treatment; vehicle or LY2828360 (3 mg/kg per day i.p. × 12 days) was administered chronically in phase 2.

In experiment 4, we evaluated the impact of coadministration of morphine (10 mg/kg i.p. × 12 days) with a submaximal dose of LY 2828360 (0.1 mg/kg per day i.p. × 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$_2$KO mice from experiments 2, 3, and 4 with vehicle, followed 30 minutes 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-minute interval after challenge with vehicle or naloxone.

Assessment of Mechanical Allodynia. Paw withdrawal thresholds (grams) to mechanical stimulation were measured in duplicate for each paw using an electronic von Frey anesthesiometer supplied with a 90-g probe (model Alemo 2390–5; IITC, Woodland Hills, CA) as described previously (Deng et al., 2012). Mice were placed on an elevated metal mesh table and 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 semiflexible 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. 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.

Assessment of Cold Allodynia. Response time (seconds) 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 to 6 μl) formed at the end of a blunt 1-ml syringe hub was gently applied to the plantar surface of the hind paw. Care was taken not to apply mechanical stimulation to the hind paw 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 1 minute after 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$_2$KO mice that received either vehicle or morphine (10 mg/kg per day, i.p.) or a combination of morphine with LY2828360 (10 mg/kg per day i.p. morphine coadministered with 0.1 mg/kg per 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 after the last injection of the test drugs. Mice were video-taped, and the number of jumps was scored in 5-minute intervals for a total observation period of 30 minutes 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 for repeated measures was used to determine the time course of paclitaxel-induced mechanical and cold allodynia. One-way analysis of variance was used to identify the source of significant interactions at each time point and compare postinjection responses with baseline levels, followed by Bonferroni's post hoc tests (for comparisons between groups). Appropriate comparisons were also made using Bonferroni's 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). $P < 0.05$ was considered statistically significant. Sample size calculations and power analyses were performed using Statmate 2.0 for windows (Graphpad Prism Software, San Diego CA, 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 used 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, whereas LY2828360 failed to do so after a 90-minute drug incubation (Fig. 2A). Recruitment of arrestin is necessary for many forms of receptor sequestration and internalization (Luttrell and Lefkowitz, 2002). In congruency, LY2828360 failed to internalize the receptor (Fig. 2B). Strikingly, CP55940 (1 μM) induced a rapid (~5 minutes) and efficacious inhibition of forskolin-stimulated adenylyl cyclase, and LY2828360 (1 μM) 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 (Dhopeshwarkar and Mackie, 2014). Thus, to confirm whether delayed inhibition by LY2828360 was mediated by Gi/o proteins, cells were pretreated with PTX, 300 ng/ml, overnight). After PTX treatment, LY2828360 no longer inhibited cAMP accumulation at 30 minutes (Fig. 2D), confirming involvement of inhibitory G proteins. Next, full-concentration response experiments were performed two times when maximal inhibition of forskolin-stimulated cAMP accumulation was observed. At 5 minutes, CP55940 potently and efficaciously inhibited cAMP accumulation, whereas LY2828360 had no effect (Fig. 2E; Table 1). Conversely, at 30 minutes, LY2828360 was potent, efficacious, and CB$_2$ receptor mediated (Fig. 2F). CP55940 (1 μM) was efficacious in stimulating ERK1/2 phosphorylation (pERK1/2) at 5, 10, 30, and 40 minutes, whereas LY2828360 (1 μM) increased pERK1/2 only at later times (20, 30, and 40 minutes). ERK1/2 activation by LY2828360 was completely abolished by pretreatment of cells with PTX (300 ng/ml; overnight) (Fig. 3, A and B), 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. 3, C and D; Table 1). To determine whether 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 Fig. S1A) and exhibited time- dependent delayed inhibition of cAMP accumulation (Supplemental Fig. S1, B, D, and E) and ERK1/2 phosphorylation (Supplemental Fig. S1, F, G, and I). As with mouse CB$_2$, these
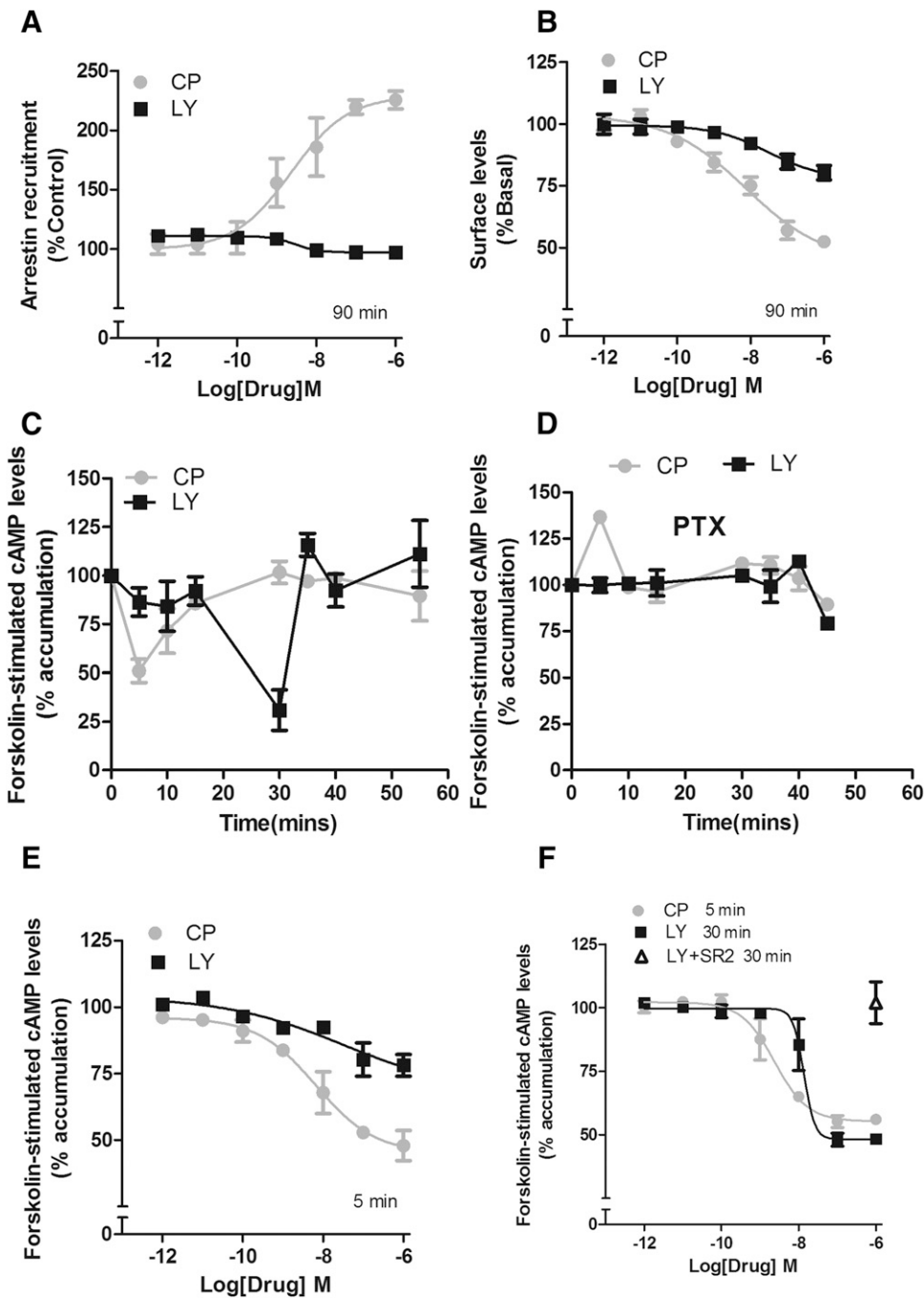
Fig. 2. LY2828360 displays a delayed signaling profile at mouse CB2 receptors. (A) In CHO cells stably expressing mCB2 receptors, CP55940 recruited arrestin in a concentration-dependent manner, whereas LY2828360 failed to do so after 90-minute drug incubation. (B) In HEK cells stably transfected with mCB2, CP55940 concentration dependently internalized the mCB2; LY2828360 was less potent and efficacious. (C) In a forskolin-stimulated cAMP time course assay, CP55940 (1 μM) was efficacious and rapid in inhibiting forskolin-stimulated cAMP accumulation at 5 minutes, whereas LY2828360 (1 μM) was efficacious only after 30 minutes. (D) After PTX treatment, CP55940 (1 μM) modestly *increased* cAMP accumulation at 5 minutes, whereas 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, whereas LY2828360 failed to affect cAMP levels at this time point. (F) After 30-minute incubation, however, LY2828360 concentration dependently inhibited forskolin-stimulated cAMP accumulation, and this inhibition was completely blocked by 1 μM SR144528(SR2). Forskolin-stimulated cAMP assays were performed in duplicate. All other assays were performed in triplicate. All data were plotted and analyzed using GraphPad Prism 4.

effects were abolished by PTX (Supplemental Fig. S1, C and H) and blocked by SR144528 (Supplemental Fig. S1I), 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 (Supplemental Fig. S2, A and B). Potencies and efficacies of CP55940 and LY2828360 in the signaling assays described at mouse and human CB2 receptors are summarized in Tables 1 and 2, respectively (Tables 1 and 2).

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 studies showing development of mechanical

and cold allodynia after paclitaxel treatment in mice (Deng et al., 2015). Thus, mechanical (Fig. 4A) and cold (Fig. 4B) allodynia developed by day 4 ($P = 0.0001$) after 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. 4, C and D);

TABLE 1
Potencies and efficacies of CP55940 and LY2828360 in arrestin, internalization, cyclase, and pERK1/2 assays at mouse CB2 receptors

Duration of drug incubation is expressed in minutes. All assays were performed in triplicates except cAMP accumulation assays, which were performed in duplicate. EC50, 95% confidence intervals (CI), and the maximal effect (Emax) (mean ± S.E.M.) were obtained by plotting and analyzing the data using GraphPad Prism 4.

		CP55940				LY2828360			
	Drug Incubation	EC50	95% CI	Emax	± S.E.M.	EC50	95% CI	Emax (%)	±S.E.M.
Arrestin	*min*	*nM*	0.4–12.2	*%*	±1.6	*nM*	ND	*%*	±1.5
	90	2.3		125		ND		97.9	
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

ND, Not determined or cannot be determined.

however, neuropathic pain was prominent in paclitaxel-treated mice receiving doses of LY2828360 lower than 0.3 mg/kg i.p. compared with 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 hours postinjection. 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 hours postinjection ($P = 0.001$ mechanical, $P = 0.022$ cold) relative to drug preinjection levels (i.e., Pac). At 24 hours postinjection,

paclitaxel-induced mechanical allodynia had returned ($P = 1$ mechanical; $P = 0.125$ cold) to drug preinjection levels of hypersensitivity (Fig. 4, E and F). Residual suppression of cold allodynia was absent by 72 hours after LY2828360 treatment (data not shown).

Previously Chronic Administration of LY2828360 Blocked the Development of Tolerance to the Anti-allodynic Effects of Morphine in WT but Not in CB₂KO Mice. To study the effects of LY2828360 treatment on the development of tolerance to morphine, pharmacologic manipulations were used in two phases of treatment during the maintenance of neuropathic pain (Fig. 5A). In WT mice, phase 1 treatment with LY2828360 (3 mg/kg per day i.p. × 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 1 vehicle treatments.
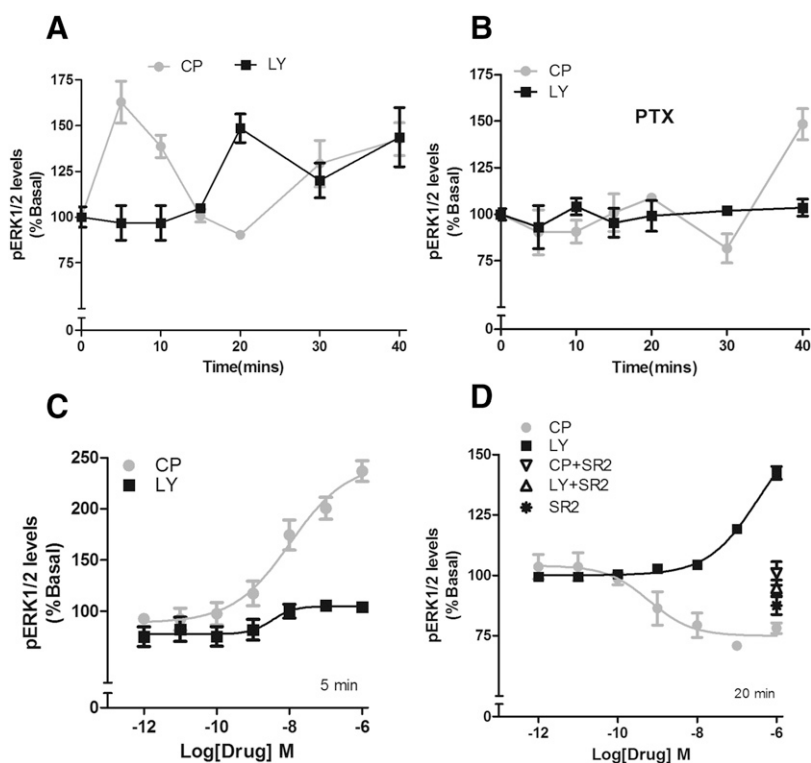

Fig. 3. LY282360 displays a delayed CB₂ receptor– and G protein–dependent signaling profile in activating pERK1/2. (A) In HEK cells stably expressing mouse CB2 receptors, CP55940 (1 μM) increased phosphorylated ERK1/2 at 5-, 10-, 30-, and 40-minute time points, whereas LY2828360 (1 μM) had no effect at 5- and 10-minute time points but increased ERK1/2 phosphorylation at 20, 30, and 40 minutes. (B) PTX treatment abolished the 20-minute phosphorylation of ERK1/2 by LY2828360 (1 μM) and abolished the CP55940-mediated phosphorylation of ERK1/2 at the 5-minute time point, but it was retained at the 40-minute time point after PTX treatment. (C) CP55940 concentration dependently increased ERK1/2 phosphorylation at 5 minutes, whereas LY2828360 failed to affect pERK1/2 levels at this time point. (D) Conversely, after 20 minutes of treatment, CP55940 decreased ERK1/2 phosphorylation, whereas 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 triplicate. All the experimental data were plotted and analyzed using GraphPad Prism 4.

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

Duration of drug incubation is expressed in minutes. cAMP accumulation assays were performed in duplicate. All other assays were performed in triplicate. EC50, 95% confidence intervals (CI), and the maximal effect (Emax, mean ± S.E.M.) were obtained by plotting and analyzing the data using GraphPad Prism 4.

	Drug Incubation	CP55940				LY2828360			
		EC50	95% CI	Emax	± S.E.M.	EC50	95% CI	Emax	±S.E.M.
Internalization	*min*	*nM*	0.3–15.6	*%*	±4.6	*nM*	ND	*%*	±7.1
	90	3		33.9		ND		10.2	
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

ND, Not determined or cannot be determined.

LY2828360 markedly suppressed paclitaxel-induced mechanical and cold allodynia throughout the observation interval ($P = 0.0001$ mechanical; $P = 0.016$ cold; Fig. 5, B and C). Mechanical and cold hypersensitivities were largely normalized by LY2828360 (3 mg/kg i.p. × 12 days) with responses returning to baseline (i.e., pre-paclitaxel) levels ($P = 0.138$ mechanical; $P = 0.182$ cold). The antiallodynic efficacy of LY2828360 was stable throughout phase 1 treatment

($P = 0.310$ mechanical, $P = 0.314$ cold) without the development of tolerance (Fig. 5, B and C).

On day 15, 3 days after the completion of phase 1 treatment, paclitaxel-induced mechanical and cold allodynia had returned to levels comparable to those observed before the initiation of phase 1 treatment (i.e., Pac; $P = 0.379$ mechanical, $P = 0.62$ cold; Fig. 5, B and C). Mechanical and cold allodynia were maintained in these mice relative to pre-paclitaxel levels
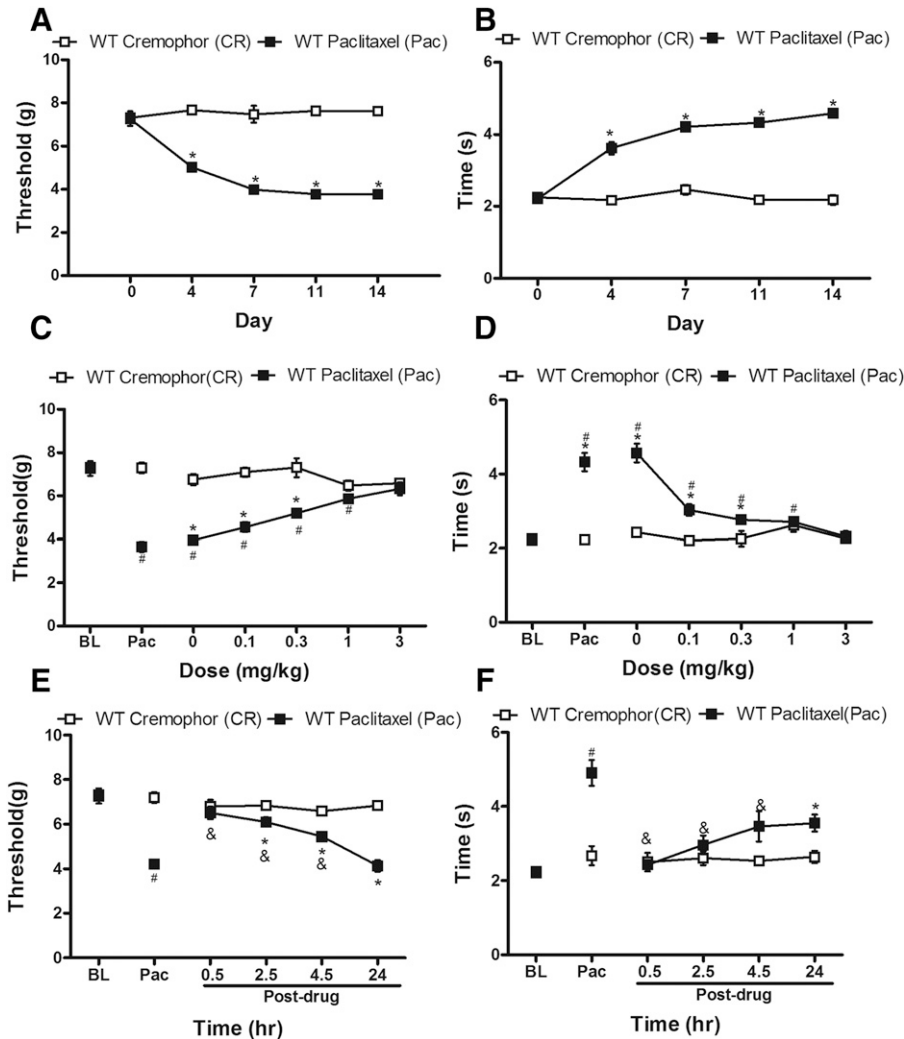

Fig. 4. Paclitaxel produced hypersensitivities to mechanical (A) and cold (B) stimulation. Non-chemotherapy control mice received cremophor-based vehicle in lieu of paclitaxel. Dose response of LY2828360, administered systemically (i.p.), on the maintenance of (C) mechanical and (D) cold allodynia in paclitaxel-treated WT (C57BL/6J) mice. The 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. Data are expressed as mean ± S.E.M. (n = 6/group). *$P <$ 0.05 vs. control, one-way analysis of variance at each time point, followed by Bonferroni's 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. BL, pre-paclitaxel baseline; Pac, baseline after paclitaxel.
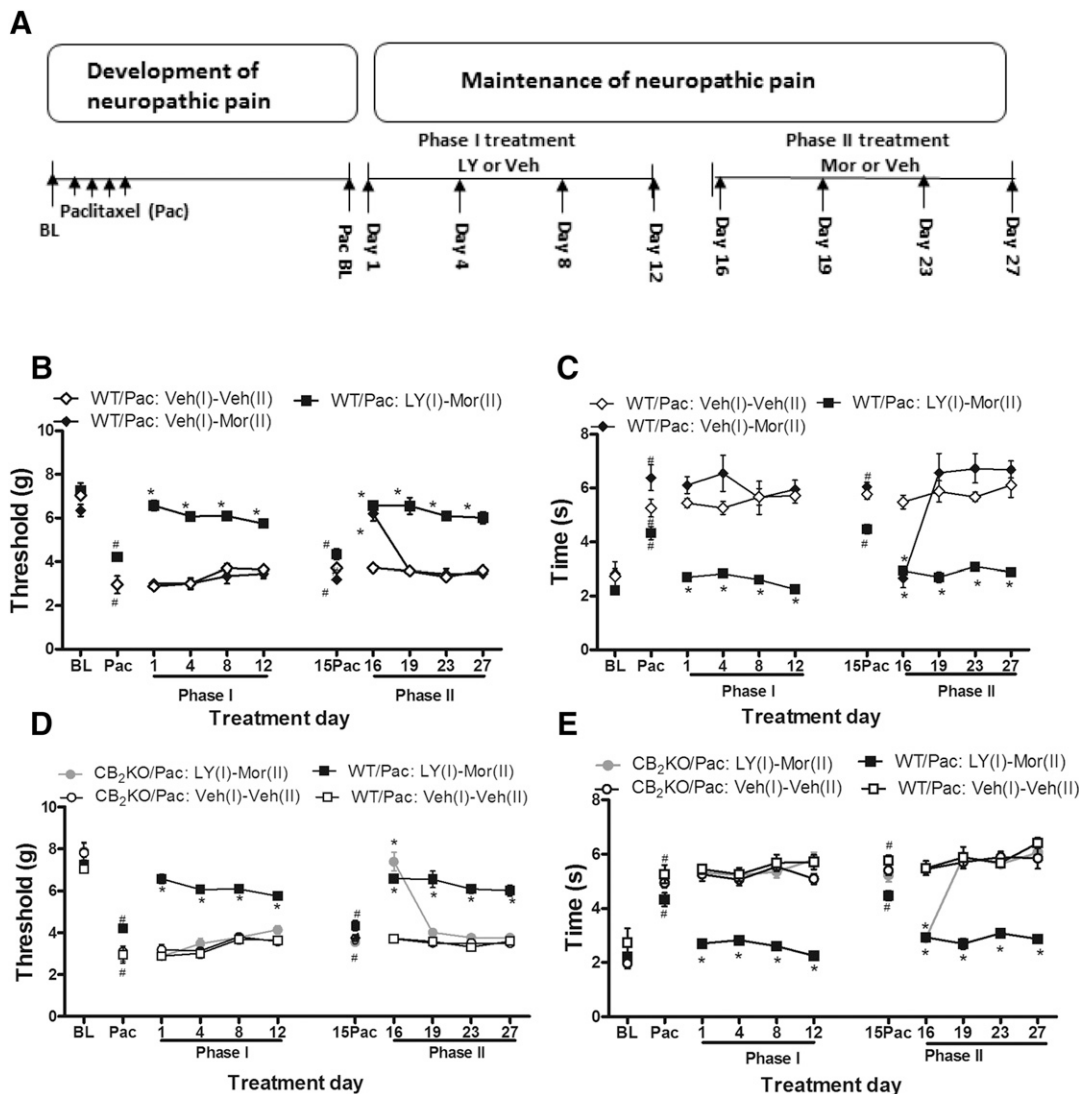
Fig. 5. History of chronic LY2828360 treatment blocked the development of morphine tolerance in WT but not in CB2KO mice. (A) The testing scheme used to evaluate the two phases of treatment during the maintenance of neuropathic pain. History of chronic LY2828360 (3 mg/kg per day i.p. × 12 days in phase 1) treatment suppressed paclitaxel-induced (B) mechanical (C) cold allodynia in WT mice. History of chronic LY2828360 (3 mg/kg per day i.p. × 12 days in phase 1) blocked the development of tolerance to the antiallodynic effects of morphine (10 mg/kg per day × 12 days in phase 2) in WT but not in CB$_2$KO mice for both mechanical (D) and cold (E) modalities. Data are expressed as mean ± S.E.M. (n = 6/group). *$P < 0.05$ versus Veh (1)-Veh (2), one-way analysis of variance at each time point, followed by Bonferroni's post hoc test. #$P < 0.05$ vs. baseline before paclitaxel, repeated measures analysis of variance.

(i.e., baseline; $P < 0.005$ mechanical, $P < 0.006$ cold). In paclitaxel-treated WT mice, chronic morphine treatment during phase 2 of mice previously receiving vehicle during phase 1 [WT/Pac: Veh (vehicle) (1)-Mor (morphine) (2)] 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., days 19, 23, and 27) relative to vehicle-treated mice [WT/Pac: Veh (1)-Veh (2); Fig. 5, B and C]. Thus, morphine tolerance rapidly developed to the antiallodynic effects of phase 2 morphine in paclitaxel-treated mice receiving vehicle in phase 1.

By contrast, in WT mice receiving LY2828360 during phase 1, phase 2 morphine [WT/Pac: LY (1)-Mor (2); 10 mg/kg i.p. × 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 1 [WT/Pac: Veh (1)-Mor (2); $P = 0.0001$] (Fig. 5, B and C). This suppression was present and stable throughout phase 2 for both mechanical ($P < 0.05$) and cold ($P < 0.009$) modalities compared with drug preinjection levels in phase 2 (i.e., day 15). Morphine-induced antiallodynic efficacy was stably maintained throughout the observation interval after 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$_2$KO mice, phase 1 LY2828360 (3 mg/kg per day i.p. × 12 days) treatment failed to suppress mechanical ($P > 0.05$) or cold ($P > 0.05$) allodynia relative to vehicle treatment on any day (Fig. 5, D and E). In these same CB$_2$KO mice, subsequent phase 2 morphine treatment [CB$_2$KO/Pac: LY (1) - Mor (2)] suppressed only 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$_2$KO/Pac: Veh (1)-Veh (2)]. Paclitaxel-induced allodynia was fully reinstated at subsequent time points (i.e., on days 19, 23, and 27; $P = 1$ mechanical, $P = 0.269$ cold). The antiallodynic efficacy of initial morphine administration (i.e., on day 16) was similar in WT mice and CB$_2$KO mice ($P = 0.203$ mechanical; $P = 1$ cold). Phase 2 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 (1)-Mor (2)] but not in the CB$_2$KO mice at subsequent time points (i.e., days 19, 23, and 27), suggesting that pretreatment with LY2828360 did not block the development of morphine tolerance in CB$_2$KO mice.

Chronic LY2828360 Treatment Suppresses Paclitaxel-Induced Mechanical and Cold Allodynia in WT Mice but Not in CB$_2$KO Mice Previously Rendered Tolerant to Morphine. To evaluate whether LY2828360 has antiallodynic efficacy in morphine-tolerant mice, we first dosed paclitaxel-treated WT and CB$_2$KO mice chronically with morphine during phase 1 (10 mg/kg per day i.p. $\times$ 12 days) and continued with chronic LY2828360 administration (3 mg/kg per day i.p. $\times$ 12 days) (Fig. 6A) in phase 2. In phase 1, 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., baseline; $P = 0.062$ mechanical; $P = 1.0$ cold) but not on subsequent test days (i.e., day 4, 8, 12; Fig. 6, B and C). Antiallodynic 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 antiallodynic effects of morphine (i.e., on day 4, 8 and 12) (Fig. 6, B and C).

To evaluate whether LY2828360 produces antiallodynic effects in mice previously rendered tolerant to morphine, LY2828360 (3 mg/kg per day i.p. $\times$ 12 days) was administered during phase 2 to paclitaxel-treated mice that previously receiving morphine during phase 1. Phase 2 LY2828360 (3 mg/kg per day i.p. $\times$ 12 days) treatment fully reversed paclitaxel-induced allodynia and normalized responsiveness to pre-paclitaxel baseline levels in WT mice that previously developed morphine tolerance in phase 1 ($P = 0.112$ mechanical; $P = 0.103$ cold; Fig. 6, B and C). Thus, prior morphine tolerance does not attenuate LY2828360-induced antiallodynic efficacy in phase 2 in WT mice. Antiallodynic efficacy of LY2828360 was also stable throughout the chronic dosing period ($P = 1.0$ mechanical; $P = 1.0$ cold), suggesting that tolerance did not develop to phase 2 LY2828360 treatment in WT mice (Fig. 6, B and C).

To further evaluate the mechanism of action underlying the antiallodynic efficacy of LY2828360, we compared the efficacy of phase 2 LY2828360 treatment in CB$_2$KO and WT mice that were rendered tolerant to morphine during phase 1. Acute morphine increased paw withdrawal thresholds and reduced cold response times in paclitaxel-treated CB$_2$KO mice relative to the vehicle treatment on day 1 of phase 1 dosing ($P = 0.0001$ mechanical; $P = 0.0001$ cold) (Fig. 6, D and E). The antiallodynic effects of phase 1 morphine were attenuated on day 4 ($P = 0.058$ mechanical; $P = 0.992$ cold) and morphine antiallodynic efficacy was completely absent on day 8 and day 12 of chronic dosing ($P = 1.0$ mechanical; $P = 1.0$ cold; Fig. 6, D and E). Chronic administration of LY2828360 in phase 2 (3 mg/kg per day, i.p. $\times$ 12 days) did not alter responsiveness to mechanical or cold stimulation in paclitaxel-treated CB$_2$KO mice relative to the vehicle treatment at any time point ($P = 0.252$ mechanical; $P = 0.299$ cold) (Fig. 6, D and E). Thus, chronic administration of LY2828360 produced antiallodynic efficacy in paclitaxel-treated WT mice but not CB$_2$KO with the same 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. In WT mice, coadministration of a submaximal dose of LY2828360 (0.1 mg/kg per day i.p. $\times$ 12 days) with morphine (10 mg/kg per day $\times$ 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 days 1 and 4 ($P = 0.156$) of chronic dosing to pre-paclitaxel baseline levels. By contrast, in CB$_2$KO mice, sustained antiallodynic efficacy was absent in paclitaxel-treated mice receiving LY2828360 coadministered with morphine; the combination treatment reversed only paclitaxel-induced mechanical ($P = 0.0001$) and cold ($P = 0.0001$) allodynia relative to vehicle on day 1 (Fig. 7, A and B). Antiallodynic efficacy of morphine coadministered with LY2828360 was greater in WT mice relative to CB$_2$KO mice on subsequent days of chronic dosing (i.e., days 4, 8, and 12; $P = 0.0001$ mechanical; $P = 0.0001$ cold) (Fig. 7, A and B). In paclitaxel-treated WT mice, the combination of morphine with LY2828360 produced a stable, sustained antiallodynic 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. 7, A and B).

Naloxone-Precipitated Withdrawal is Attenuated in Morphine Tolerant WT but Not CB$_2$KO Mice with a 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 2 but vehicle during phase 1 [i.e., WT/Pac: Veh (1)-Mor (2) group] exhibited a greater number of jumps relative to paclitaxel-treated WT mice that received vehicle during both phases [WT/Pac: Veh (1)-Veh (2); $P = 0.007$]. The number of naloxone-precipitated jumps did not differ between groups that received phase 1 LY2828360 followed by phase 2 morphine treatment [WT/Pac: LY (1)-Mor (2)] and those that received phase 1 vehicle followed by phase 2 vehicle treatment [WT/Pac: Veh (1)-Veh (2); $P = 0.3$]. Also, the number of jumps did not differ between phase 2 morphine-treated mice that received either LY2828360 or vehicle during phase 1 [WT/Pac: Veh (1)-Mor (2) vs. WT/Pac: LY (1)-Mor (2), $P = 0.831$]. Naloxone challenge did not precipitate withdrawal in paclitaxel-treated WT mice receiving morphine in phase 1 [WT/Pac: Mor (1)-LY (2) vs. WT/Pac: Veh (1)-Veh (2) $P = 1$] (Fig. 8A).
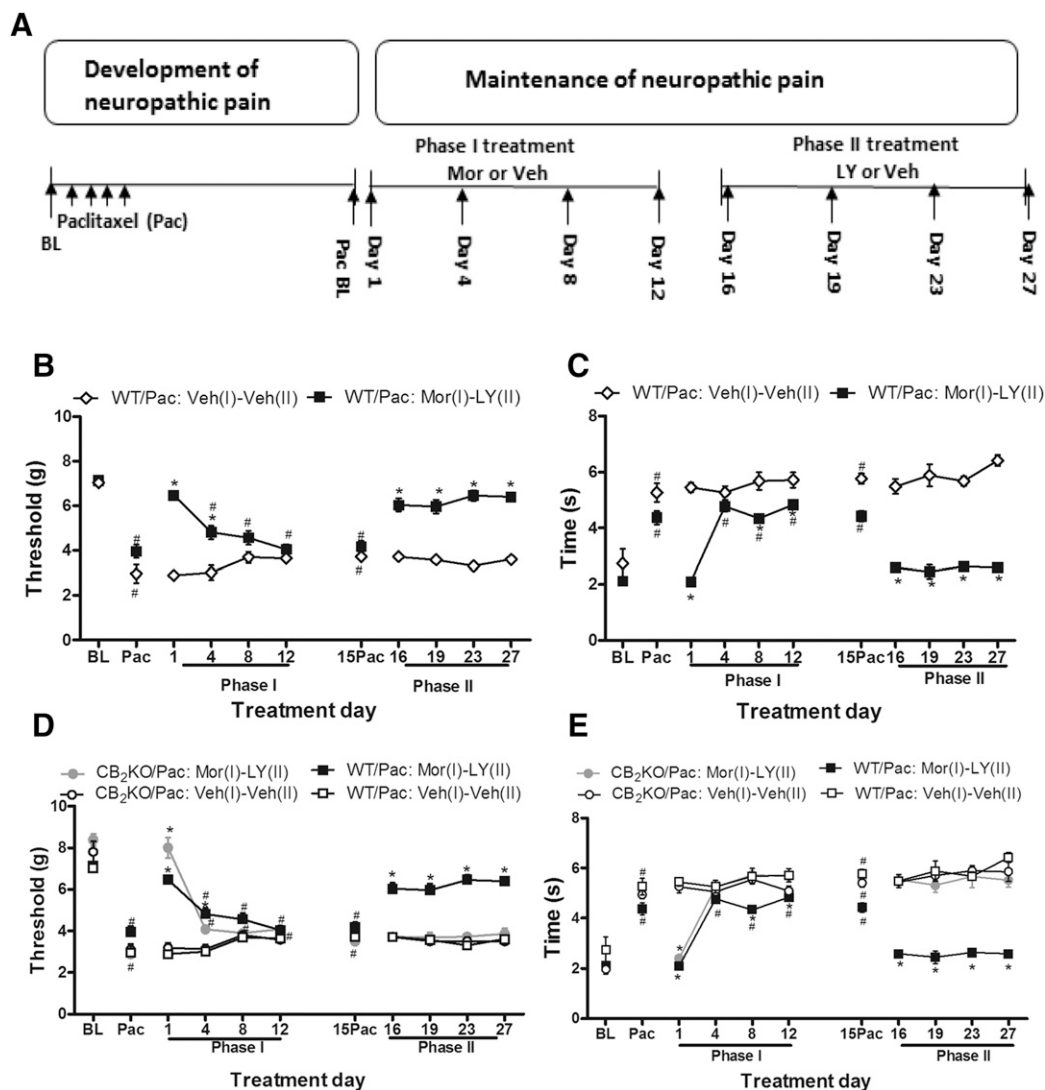
Fig. 6. Chronic LY2828360 treatment showed sustained antiallodynic efficacy in morphine-tolerant WT mice but not in CB2KO mice. (A) Testing scheme used to evaluate the two phases of treatment during the maintenance of neuropathic pain. Chronic LY2828360 (3 mg/kg per day i.p. × 12 days in phase 2) treatment suppressed paclitaxel-induced mechanical (B and D) and cold (C and E) allodynia in WT mice but not in CB_2KO mice previously rendered tolerant to morphine (10 mg/kg per day i.p. × 12 days in phase 1). Data are expressed as mean ± S.E.M. (n = 6/group). Veh (1)-Veh (2) group is replotted from Fig. 5. *$P < 0.05$ vs. Veh (1)-Veh (2), one-way analysis of variance at each time point, followed by Bonferroni's post hoc test. #$P < 0.05$ vs. baseline before paclitaxel, repeated measures analysis of variance.

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 1 vehicle followed by phase 2 morphine treatment versus mice receiving vehicle during both phases of chronic dosing [CB_2KO/Pac: Veh (1)-Veh (2) vs. CB_2KO/Pac: Veh (1)-Mor (2), $P = 0.044$]. The number of jumps trended higher in paclitaxel-treated CB_2KO mice receiving LY2828360 in phase 1 and morphine in phase 2 relative to CB_2KO mice that received vehicle during both phases [CB_2KO/Pac: LY (1)-Mor (2) vs. CB_2KO/Pac: veh (1)-Veh (2) group; $P = 0.057$]. In paclitaxel-treated CB_2KO mice, the number of jumps did not differ between phase 2 morphine-treated mice that received either LY2828360 or vehicle during phase 1 [CB_2KO/Pac: LY (1)-Mor (2) vs. CB_2KO/Pac: Veh (1)-Mor (2), $P = 1$]. A trend toward fewer naloxone-precipitated jumps was observed in WT relative to CB_2KO mice ($P = 0.064$; Fig. 8C) that received the same

histories of phase 1 LY2828360 followed by phase 2 morphine treatment. Similarly, coadministration of LY2828360 with morphine also trended to produce a lower number of naloxone-precipitated jumps in WT compared with 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/group would be required to detect a statistically significant impact of LY2828360 on WT and CB_2KO animals based on the observed S.D., sample size and magnitude difference observed between means.

Body weight change from baseline (i.e., postvehicle) differed as a function of time after 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 hours ($F_{8, 48} = 2.033$, $P = 0.062$) but not at 0.5 hour ($F_{8, 48} = 1.460$, $P = 0.197$) postinjection (Fig. 8E).
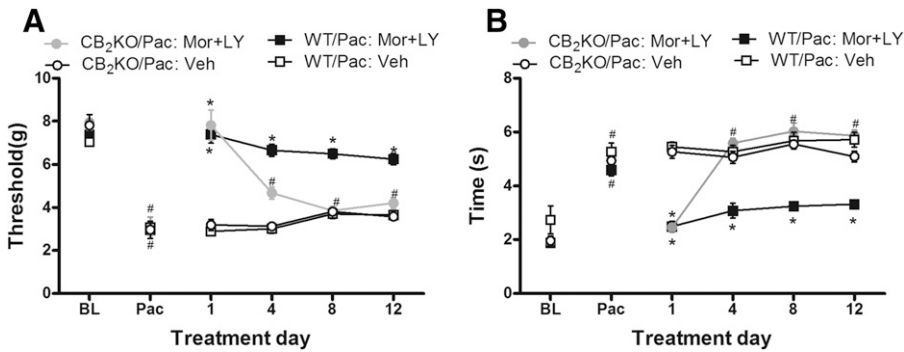
Fig. 7. Chronic coadministration of low-dose LY2828360 (0.1 mg/kg per day i.p. × 12 days) with morphine (10 mg/kg per day i.p. × 12 days) blocked development of morphine tolerance in WT but not in CB$_2$KO mice tested for both (A) mechanical and (B) cold allodynia. Data are expressed as mean ± S.E.M. (n = 6/group). *$P <$ 0.05 vs. WT-Veh, one-way analysis of variance at each time point, followed by Bonferroni's post hoc test. #$P <$ 0.05 vs. baseline before paclitaxel, repeated measures analysis of variance.

Discussion

Here we show that the CB$_2$ agonist LY2828360 is a slowly acting but efficacious G protein–biased CB$_2$ agonist that inhibits cAMP accumulation and activates 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 antiallodynic efficacy was absent in CB$_2$KO 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 before 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$_2$KO 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 toward G$_{i/o}$G protein signaling with little effect on arrestin or G$_q$ signaling, which 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 *biased agonism* or *functional selectivity* (Kenakin, 2011) and has emerged as an important pharmacologic 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 pharmacologic concept that emphasizes the time scale of the activation of a particular pathway (Klein Herenbrink et al., 2016). It remains to be determined whether 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 et al., 2008). In the present study, the antiallodynic 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. Antiallodynic efficacy of LY2828360 (3 mg/kg i.p.) lasted more than 4.5 hours after 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 after 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 antiallodynic effect of morphine. By contrast, tolerance to morphine developed in CB$_2$KO mice identically treated with chronic LY2828360 in phase 1 followed by chronic morphine treatment in phase 2. Moreover, in paclitaxel-treated WT mice, coadministration of morphine with a low dose of LY2828360 was fully efficacious in alleviating neuropathic pain and blocking the 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 the 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 the 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 antiallodynic 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 et al., 2017). The mechanism underlying these therapeutically advantageous properties remains incompletely understood. In tumor-bearing mice, AM1241 upregulated μ-opioid receptor expression in the spinal cord and dorsal root ganglia (DRG) (Zhang et al., 2016). Another study suggested CB$_2$ agonist upregulated μ-opioid receptor expression levels, whereas the CB$_2$ antagonist inhibited μ-opioid receptor expression level in Jurkat T cells (Börner et al., 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 (Raghavendra et al., 2002; Mika et al., 2007). 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 et al., 2008; Tumati et al., 2012). CB$_2$ activation is
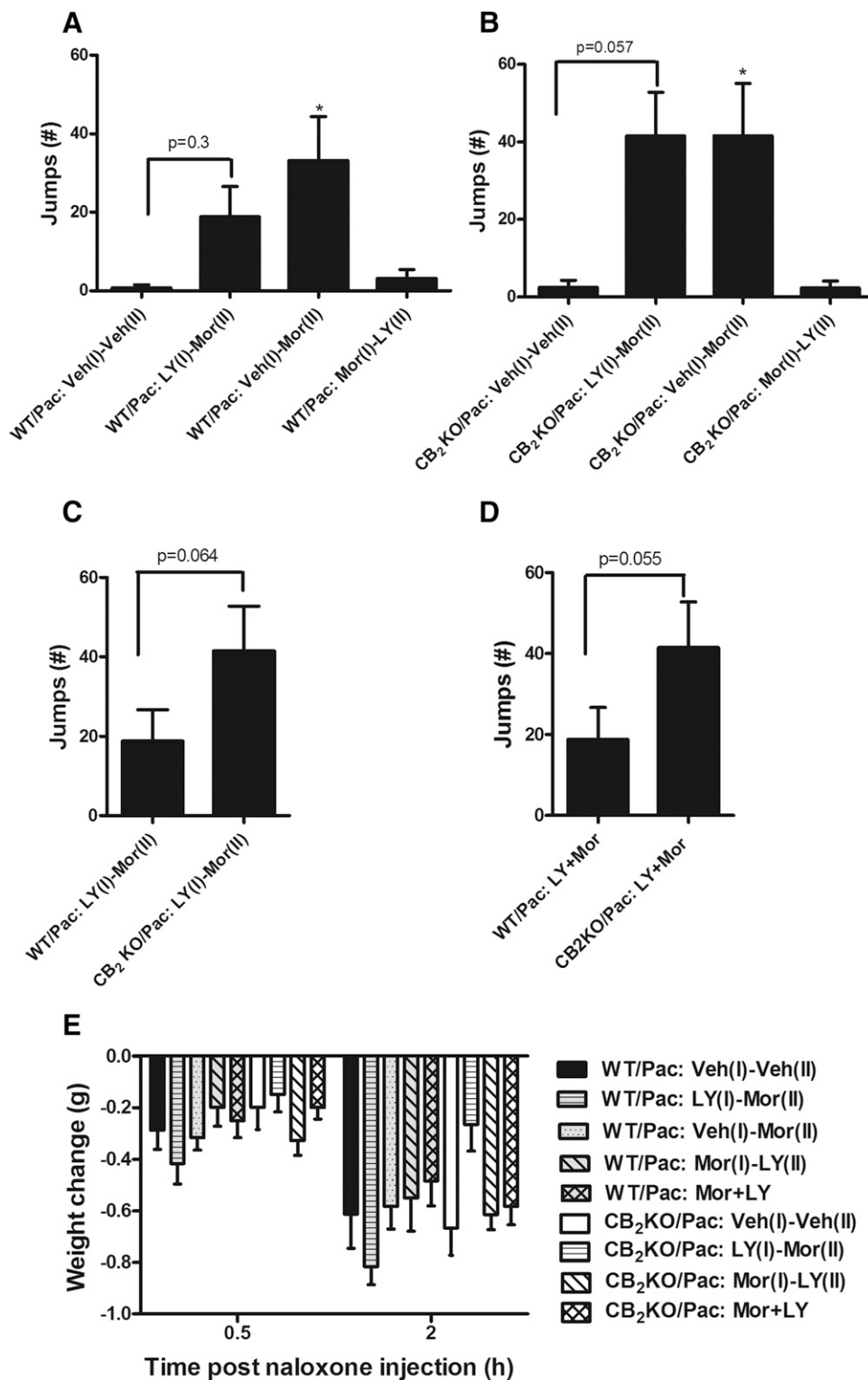
Fig. 8. Impact of LY2828360 treatment on naloxone-precipitated opioid withdrawal in CB_2 KO and WT mice. Naloxone (5 mg/kg i.p.) precipitates jumping in WT (A) and CB_2KO (B) mice receiving morphine (10 mg/kg per day i.p. $\times$ 12 days) during phase 2 of chronic dosing. (C) A trend ($P = 0.064$) toward lower numbers of naloxone-precipitated jumps was observed in WT compared with CB_2KO mice with similar histories of LY2828360 (3 mg/kg per day $\times$ 10 days during phase 1), followed by morphine (10 mg/kg per day i.p. $\times$ 12 days during phase 2) treatment. (D) Naloxone-precipitated (5 mg/kg i.p.) jumping trended lower in WT mice ($P = 0.055$) receiving coadministration of LY2828360 (0.1 mg/kg per day i.p. $\times$ 12 days) with morphine (10 mg/kg per day i.p. $\times$ 12 days) compared with 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 hours compared with 0.5 hour after naloxone challenge. Data are expressed as mean $\pm$ S.E.M. ($n = 6$–8/group) *$P < 0.05$ vs. Veh (I)-Veh (II), one-way analysis of variance followed by Bonferroni's post hoc test or one tailed t test as appreciate.

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, postoperative, 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 the 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 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 used, or the presence or absence of a pathologic pain state could account for these disparities.

An emerging challenge for pain management is how to treat pain in the morphine-tolerant individual. Dose escalation is typically used in early unimodal 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 (Raffa et al., 2010). Our study has important implications for the clinical management of neuropathic pain because chronic LY2828360 treatment showed sustained antiallodynic 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 before introduction of phase 2 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 et al., 2010). The opioid receptor antagonist naloxone precipitates a spectrum of autonomic and somatic withdrawal signs in morphine-dependent animals (Morgan and Christie, 2011). In the present study, in paclitaxel-treated WT mice, chronic phase 1 pretreatment with LY2828360 produced a trend toward 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$_2$KO mice receiving identical treatments. In fact, our studies raise the possibility that CB$_2$ receptor signaling may attenuate opioid antagonist-precipitated withdrawal because CB$_2$KO mice trended to show higher levels of naloxone-precipitated jumping compared with WT mice when pretreated with CB$_2$ agonist. Moreover, coadministration of low-dose LY2828360 with morphine mimicked these effects and trended to decrease naloxone-precipitated withdrawal jumping in paclitaxel-treated WT mice compared with CB$_2$KO 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 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 (Burma NE, 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 of 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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