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# Monoacylglycerol Lipase Inhibitors Reverse Paclitaxel-Induced Nociceptive Behavior and Proinflammatory Markers in a Mouse Model of Chemotherapy-Induced Neuropathy[S]

Zachary A. Curry, Jenny L. Wilkerson, Deniz Bagdas, S. Lauren Kyte, Nipa Patel, Giulia Donvito, Mohammed A. Mustafa, Justin L. Poklis, Micah J. Niphakis, Ku-Lung Hsu, Benjamin F. Cravatt, David A. Gewirtz, M. Imad Damaj, and Aron H. Lichtman

*Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia (Z.A.C., J.L.W., D.B., S.L. K., N.P., G.D., M.A.M., J.L.P., D.A.G., M.I.D., A.H.L.); The Skaggs Institute for Chemical Biology and Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California (M.J.N., B.F.C.); and Department of Chemistry, University of Virginia, Charlottesville, Virginia (K.-L.H.)*

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## ABSTRACT

Although paclitaxel effectively treats various cancers, its debilitating peripheral neuropathic pain side effects often persist long after treatment has ended. Therefore, a compelling need exists for the identification of novel pharmacologic strategies to mitigate this condition. As inhibitors of monoacylglycerol lipase (MAGL), the primary hydrolytic enzyme of the endogenous cannabinoid, 2-arachidonoylglycerol, produces antinociceptive effects in numerous rodent models of pain, we investigated whether inhibitors of this enzyme (i.e., JZL184 and MJN110) would reverse paclitaxel-induced mechanical allodynia in mice. These drugs dose dependently reversed allodynia with respective $ED_{50}$ values (95% confidence limit) of 8.4 (5.2–13.6) and 1.8 (1.0–3.3) mg/kg. Complementary genetic and pharmacologic approaches revealed that the antiallodynic effects of each drug require both cannabinoid receptors, $CB_1$ and $CB_2$. MJN110 reduced paclitaxel-mediated increased expression of monocyte chemoattractant protein-1 (MCP-1, CCL2) and phospho-p38 MAPK in dorsal root ganglia as well as MCP-1 in spinal dorsal horn. Whereas the antinociceptive effects of high dose JZL184 (40 mg/kg) underwent tolerance following 6 days of repeated dosing, repeated administration of a threshold dose (i.e., 4 mg/kg) completely reversed paclitaxel-induced allodynia. In addition, we found that the administration of MJN110 to control mice lacked intrinsic rewarding effects in the conditioned place preference (CPP) paradigm. However, it produced a CPP in paclitaxel-treated animals, suggesting a reduced paclitaxel-induced aversive state. Importantly, JZL184 did not alter the antiproliferative and apoptotic effects of paclitaxel in A549 and H460 non-small cell lung cancer cells. Taken together, these data indicate that MAGL inhibitors reverse paclitaxel-induced neuropathic pain without interfering with chemotherapeutic efficacy.

## Introduction

Paclitaxel is a widely prescribed chemotherapeutic for the treatment of breast, lung, and other cancers, but causes a

variety of serious side effects, including peripheral neuropathy, leukopenia, joint or muscle pain, vomiting, and alopecia (Ghersi et al., 2015). Chemotherapy-induced peripheral neuropathy (CIPN) causes severe sensory disturbances that range from mild tingling to spontaneous painful burning paresthesia affecting the longest sensory nerves to the hands and feet (Dougherty et al., 2004) and can persist long after treatment cessation (Tanabe et al., 2013) in up to 68% of chemotherapy cancer patients (Seretny et al., 2014). As traditional analgesics generally lack efficacy in treating this condition (Kim et al., 2015), a pressing need exists for novel analgesic strategies. However, the endogenous cannabinoid system contains several potential targets of promise to treat CIPN. Here, we employ a mouse paclitaxel model of CIPN to explore whether inhibition of monoacylglycerol lipase (MAGL) (Dinh et al., 2002), the primary hydrolytic enzyme of the endogenous cannabinoid 2-arachidonoylglycerol (2-AG) (Mechoulam et al.,

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**ABBREVIATIONS:** ABHD6, alpha beta hydrolase domain-containing protein 6; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variance; CIPN, chemotherapy-induced peripheral neuropathy; CPP, conditioned place preference; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; MAGL, monoacylglycerol lipase; MCP-1, monocyte chemoattractant protein-1; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; $PGD_2$, prostaglandin D2.

1995; Sugiura et al., 1995; Blankman et al., 2007), will reduce nociceptive behavior and associated inflammatory responses.

MAGL inhibitors increase 2-AG, decrease arachidonic acid levels in the central nervous system (Long et al., 2009), and produce antiallodynic effects in rodent neuropathic pain models, including chronic constriction injury of the sciatic nerve (Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016b; Crowe et al., 2017) and cisplatin neuropathy (Guindon et al., 2013). Importantly, the $CB_2$ receptor agonist AM1710 (Deng et al., 2015b) and the MAGL inhibitor JZL184 (Slivicki et al., 2017) reverse paclitaxel-induced allodynia in mice. In the present study, we build upon this work using the MAGL inhibitors JZL184 (Long et al., 2009) and MJN110 (Niphakis et al., 2013) in the paclitaxel mouse model of CIPN. Although MJN110 is more potent than JZL184 (Ignatowska-Jankowska et al., 2015), it also inhibits $\alpha/\beta$-hydrolase domain 6 (ABHD6) (Niphakis et al., 2013), which metabolizes a much smaller percentage of 2-AG than MAGL (Blankman et al., 2007). To control for this off-target effect, we tested the selective ABHD6 inhibitor [4-(4′-methoxy[1,1′-biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone (KT195) (Hsu et al., 2012; Wilkerson et al., 2016a). We used complementary genetic and pharmacologic approaches to assess the contribution of $CB_1$ and $CB_2$ receptors to observed pharmacological effects of the inhibitors. Because CIPN persists in patients long after chemotherapy treatment, we explored whether the antinociceptive effects of JZL184 would undergo tolerance or be maintained after repeated administration. We also explore whether repeated administration of JZL184 increases endocannabinoid levels and decreases arachidonic acid levels, along with prostaglandin D2 ($PGD_2$) (Nomura et al., 2011b), in the lumbar spinal cord of paclitaxel-treated mice.

In addition to neurotoxicity (Bobylev et al., 2015), paclitaxel causes inflammation within the dorsal root ganglia (DRG) and activates toll-like receptor 4 (Li et al., 2014) with subsequent p38 MAPK expression and activation through phosphorylation, increasing phosphorylated p38 MAPK (phospho-p38) (Li et al., 2015). Paclitaxel also increases the chemokine monocyte chemoattractant protein-1 (MCP-1)/(C-C motif) ligand 2 (CCL2) expression in the DRG (Zhang et al., 2013; Makker et al., 2017) and the spinal cord (Pevida et al., 2013; Zhang et al., 2013), causing sensory neuron sensitization through transient receptor potential channel activation (Jung et al., 2008) and increased intracellular calcium (Zhang et al., 2013). Inhibition of MCP-1 signaling reverses and prevents paclitaxel-induced allodynia (Zhang et al., 2013). In contrast, inhibition of phospho-p38 does not reverse but prevents (Li et al., 2015) paclitaxel-induced allodynia. Although a $CB_2$ receptor agonist inhibits spinal MCP-1 expression associated with paclitaxel (Deng et al., 2015b), MAGL inhibitors remain unexplored. Accordingly, we tested whether MAGL inhibition would ameliorate paclitaxel-induced expression of MCP-1 and phospho-p38 in the DRG and spinal dorsal horn.

Along with mechanical allodynia, we assessed whether MJN110 produces a conditioned place preference (CPP) in paclitaxel-treated mice. The CPP paradigm allows for disassociation of a drug's analgesic profile and its intrinsic rewarding effects, as shown for cisplatin neuropathy (Park et al., 2013; Krukowski et al., 2017) and osteoarthritis (Havelin et al., 2016). However, CPP has not been used in paclitaxel-induced CIPN to examine MAGL inhibitors. Here, we test whether MJN110 produces a CPP in paclitaxel-treated mice, indicating relief from an aversive state not observed in control mice.

Finally, as paclitaxel-induced CIPN develops during chemotherapy treatment, it is important to test whether candidate antinociceptive agents, such as MAGL inhibitors, interfere with paclitaxel's antitumor effects. Thus we examined whether JZL184 affects the antiproliferative and apoptotic activity of paclitaxel in the human cell lines of non-small cell lung cancer (NSCLC) (Johnson et al., 2006) A549 (Marostica et al., 2015) and H460 (Huisman et al., 2002) cells.

## Materials and Methods

**Animals.** Adult male C57BL/6J mice with starting weights of $24.8 \pm 0.16$ g (Jackson Laboratories, Bar Harbor, ME) were used in all studies except in experiments employing $CB_1$ $(-/-)$ and $CB_2$ $(-/-)$ mice. Male and female $CB_1$ $(-/-)$ (Zimmer et al., 1999) and $CB_2$ $(-/-)$ (Jackson Laboratories) and matched wild-type control mice were received from the Virginia Commonwealth University (VCU) Massey Cancer Center Transgenic/Knockout Mouse Shared Resource. Both lines of transgenic mice have been backcrossed with C57BL/6J mice. Based on genotyping analysis, $CB_1$ $(-/-)$ mice were 90% similar to C57BL/6J, 6% similar to C57BL/6N, and 4% similar to 129/Sv mice. $CB_2$ $(-/-)$ mice were 71% similar to C57BL/6J and 27% similar to C57BL/6N and 2% similar to 129/Sv mice. Animals were housed four per cage, with separation as needed when fighting, and maintained in the AAALAC-approved vivarium at Virginia Commonwealth University. Animals were provided with water and Teklad LM-485 Mouse Diet (7012; Envigo/Teklad Diets, Madison, WI) chow ad libitum with a 12-hour light/dark cycle beginning at 0600. Animal studies were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the VCU Institutional Animal Care and Use Committee. Before the studies were started employing the transgenic mice, the VCU vivarium was undergoing renovation. Thus, these mice were moved between vivarium rooms and exposed to construction noise. For all other studies, mice were housed undisturbed in the vivarium. Following behavioral testing, animals were euthanized using $CO_2$ asphyxiation and cervical dislocation unless tissue collection was performed. Tissue collection was performed after either rapid decapitation or isoflurane anesthesia.

**Assessment of Mechanical Allodynia.** Mice received a minimum of 4 days prior to experimentation to acclimate to the vivarium. Prior to behavioral assessment, mice were acclimated to the von Frey mesh elevated platform for a minimum of 40 min/day for a minimum of 3 days. During acclimation and testing, each mouse was placed in a ventilated Plexiglas cylinder, approximately 3 inches in diameter, minimizing locomotor activity. To assess paw withdrawal thresholds, von Frey filaments (North Coast Medical, Gilroy, CA) were applied to the hind paw for 3 seconds on each of three trials until a positive response was noted as a paw withdrawal to the stimulus. Testing began at the 0.6 g filament and moved sequentially to higher values (1, 1.4, 2, 4 g) until five of six withdrawals were recorded. If a response occurred at 0.6 g, the 0.4, 0.16, and 0.07 g filaments were sequentially applied until five of six positive responses were obtained. A sixth test was not conducted if the first five tests were positive. A maximum of 4 g was used to prevent sensitization. Results for each animal are reported as the average of both hind paws.

**Drugs and Dosing.** Paclitaxel (Taxol; Tocris Bioscience, Bristol, UK) was dissolved in a vehicle solution containing a 1:1:18 ratio of ethanol, emulphor-620 (Rhodia, Cranbury, NJ), and saline (0.9% NaCl). A cycle of paclitaxel consisted of a total of four intraperitoneal injections of paclitaxel (8 mg/kg per injection) in which injections were given every other day (Toma et al., 2017). Control (no paclitaxel) mice were given four injections of vehicle. The injection volumes were 0.01 ml/g of body mass. The Cravatt Laboratory at Scripps Research

Institute, La Jolla, CA, synthesized MJN110 and KT195, and the Drug Supply Program at the National Institute on Drug Abuse (Bethesda, MD) provided JZL184, rimonabant, and SR1445528. Doses of JZL184 (Long et al., 2009; Ghosh et al., 2013; Ignatowska-Jankowska et al., 2015), MJN110 (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016b), and KT-195 (Hsu et al., 2012; Wilkerson et al., 2016a) were selected based on the results of previous in vivo and in vitro studies using these agents.

Following basal paw withdrawal assessment, paclitaxel or corresponding vehicle was administered as described above. Paclitaxel-treated and vehicle-pretreated control mice were separately housed to prevent cross-contamination. After confirmation of paw withdrawal thresholds, drugs were administered intraperitoneally and mice were placed on the mesh for a minimum of 30 minutes prior to drug testing. Testing was conducted in a blinded manner with respect to acute drug treatment, and a 72-hour washout period was imposed between drug treatments as previously described (Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016a). All experiments were completed within 8 weeks of the last paclitaxel/vehicle injection with a minimum 5-day washout period before switching drugs. The same cohorts of $CB_1 (-/-)$ and $CB_2 (-/-)$ and $(+/+)$ mice were used to test MJN110 and JZL184 within 5 weeks following paclitaxel cessation. A 1-week washout period was used before switching drugs in these animals. For time course studies, testing began 0.5 hours after drug administration and was finished 24 hour posttreatment. Unless otherwise indicated, the antinociceptive effects of MJN110 were tested 3 hours postadministration, while those of JZL184 was tested 2 hours posttreatment. Rimonabant or SR144528 was administered 30 minutes prior to JZL184 or MJN110 (Wilkerson et al., 2016b). Allodynia was confirmed prior to each test and persisted at 8 weeks postpaclitaxel when testing was completed.

In the repeated JZL184 dosing experiment, C57BL/6J mice received a cycle of paclitaxel or vehicle and 10–12 days later received repeated intraperitoneal injections of JZL184 or vehicle as previously described (Kinsey et al., 2013). Mice in the repeated JZL184 conditions received a daily intraperitoneal injection of JZL184 (4 or 40 mg/kg) for 6 days. For the acute conditions, each mouse was given a daily intraperitoneal injection of vehicle for 5 days and administered vehicle or JZL184 (4 or 40 mg/kg) on day 6. All mice were tested for mechanical allodynia 2 hours following the final injection on day 6.

**Immunohistochemistry.** Immunohistochemistry was conducted as previously described (Wilkerson et al., 2012, 2016a) with slight modifications. Briefly, 9 days after the cycle of paclitaxel or vehicle, mice were given an intraperitoneal injection of MJN110 (5 mg/kg) or vehicle, and 3 hours later the mice were deeply anesthetized with isoflurane and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 (Thermo Fisher Scientific, Waltham, MA). Whole spinal columns were collected and fixed overnight in 4% paraformaldehyde at 4°C. Spinal columns then underwent EDTA decalcification (8% in PBS) for approximately 60 days and were sliced in 7-$\mu$m-thick sections. DRG sections corresponding to the L5–L6 spinal cord were selectively examined. Tissue processing, paraffin embedding, and slide preparation was provided by the Virginia Commonwealth University Cancer Mouse Models Core.

Samples were deparaffinized in Hemo De (Electron Microscopy Sciences, Hatfield, PA) and rehydrated in 100% and 70% (v/v) ethanol (Pharmco-AAPER, Brookfield, CT) followed by PBS (Abcam, Cambridge, MA). Antigen retrieval was conducted in a pressure cooker (10 PSI) for 10 minutes in citrate buffer (2.1 g/100 ml deionized water, pH 6) followed by 10 minutes of cooling and 5 minutes in room temperature PBS. Slides were blocked for 5 to 6 hours in 4.6% normal donkey serum/PBS solution. Primary antibodies were applied in 0.5% bovine serum albumin/PBS solution with 1% sodium azide and incubated overnight at 4°C. The following primary antibodies were used: rat anti-MCP-1 (1:50, ab8101; Abcam) (Zoja et al., 1997; Park et al., 2011) and rabbit anti-phospho-p38 MAPK (1:800, 4511S; Cell Signaling, Danvers, MA) (Wilkerson et al., 2012; Shi et al., 2017). On

the following day, slides were incubated in secondary antibodies at room temperature. For MCP-1, rhodamine red donkey anti-rat (1:2000, 712-295-153; Jackson ImmunoResearch Laboratories, West Grove, PA) was used for 2 hours. For phospho-p38 MAPK, samples were incubated in biotinylated donkey anti-rabbit (1:1000 for DRG,1: 1300 for dorsal horn; 711-065-152; Jackson ImmunoResearch Laboratories) for 1 hour, followed by processing with the Ultra-Sensitive ABC Peroxidase Standard Staining Kit (32050; Thermo Fisher Scientific) and TSA Plus Cyanine 3 and Fluorescein System (PerkinElmer, Waltham, MA). Slides were then incubated in PBS, followed by a dip in deionized water and placed under a cover slip using Vectashield Antifade Mounting Medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA). Imaging was done at 40× with a Zeiss AxioObserver A1 inverted microscope equipped with an Axiocam MRc5 color CCD camera and ZEN 2012 software (Carl Zeiss AG, Thornwood, NY) at the Virginia Commonwealth University Microscopy Facility. Images were converted to grayscale and underwent rethresholding to reduce background fluorescence using Zeiss ZEN lite software (Carl Zeiss AG) in a manner that was consistent across all treatment groups for each study. Spinal cord images were rotated or inverted as necessary for consistent orientation.

Densitometry analysis was conducted as previously described (Wilkerson et al., 2012, 2016a; Bagdas et al., 2016) according to the methods of Samudio-Ruiz et al. (2009), Zhang et al. (2012). Densitometry analysis was conducted using Image J by selecting the anatomic location of the DRG or spinal dorsal horn in each image in an unbiased manner. For phospho-p38 MAPK analysis, four stained nuclei were selected at random from each image. Results are reported as the average of four separate sections per animal minus the average of four sections from a control slide lacking primary antibody. A sample size of four animals for each treatment condition was used.

For confocal microscopy and colocalization analysis, images were acquired at 63× using a Zeiss AxioObserver inverted LSM710 META confocal microscope and ZEN 2012. The entire z-stack of a region was collected, and final images were generated from a single image along the z-plane.

**Lipid Quantification.** Quantification of endogenous cannabinoids was conducted as previously described (Ramesh et al., 2011; Ignatowska-Jankowska et al., 2015). Briefly, mice in the repeated administration study underwent mechanical allodynia testing and were euthanized by decapitation approximately 3–6s hour after drug administration, a time point known to produce reliable elevations in 2-AG and decreased arachidonic acid (Long et al., 2009). Spinal cord tissues were rapidly collected by hydraulic extrusion flushing the spinal canal with saline. The spinal cord was dissected to isolate the lumbar enlargement region (L4–L6), which was immediately frozen and stored at −80°C until further processing.

On the day of lipid extraction, the pre-weighed mouse samples were homogenized with 1.4 ml chloroform:methanol (containing 0.0348 g PMSF/ml), as previously described (Kinsey et al., 2013). Six-point calibration curves ranged from 0.078 to 10 pmol for AEA, 0.125 to 16 nmol for 2-AG, arachidonic acid and 1.75 to 140 pmol PGD$_2$, a negative control, and blank control were prepared. Internal standards (50 $\mu$l of each of 1 pmol AEA-d$_8$, 1 nmol 2-AG-d$_8$, 1 nmol arachidonic acid-d$_8$ and 14 pmol PGD$_2$-d$_4$) were added to each calibrator, control and sample, except the blank control. Each calibrator, control, and sample was then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and centrifuged (10 minutes at 4000 $g$ and 4°C). The aqueous phase plus debris were collected and extracted again twice with 0.8 ml chloroform, the organic phases were pooled and organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform, mixed with 1 ml cold acetone, and centrifuged (10 minutes at 4000$g$ and 4°C) to precipitate proteins. The upper layer was collected and evaporated to dryness and reconstituted with 0.1 ml methanol and placed in auto-sample vials for analysis.

The ultraperformance liquid chromatography-tandem mass spectrometer analysis of AEA, 2-AG, arachidonic acid and PGD$_2$ was performed on a Sciex 6500 QTRAP system with an IonDrive Turbo
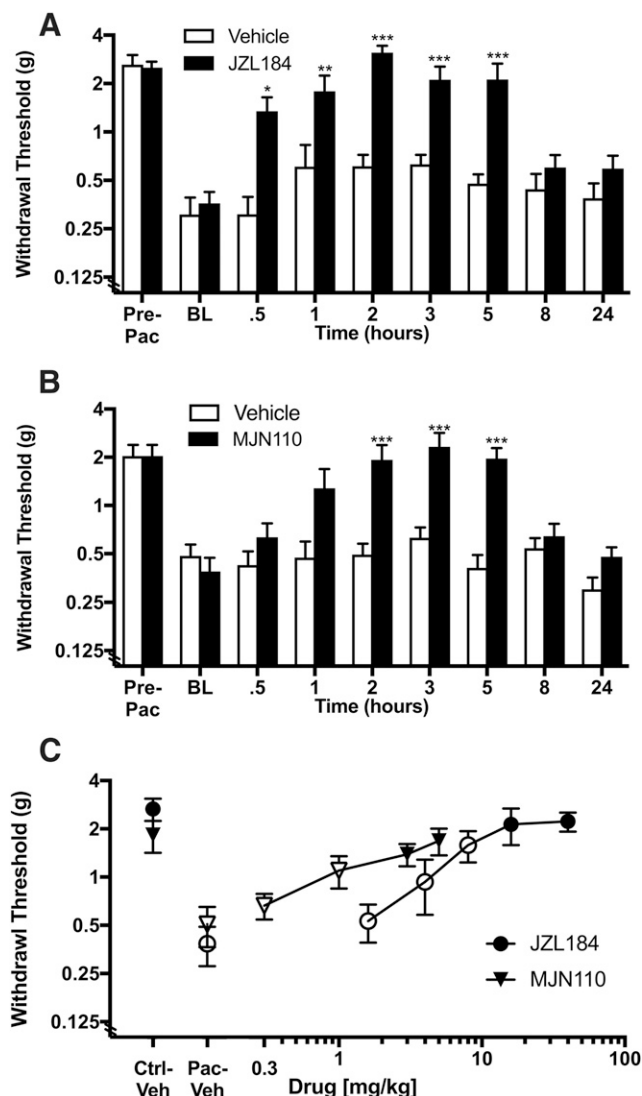
**Fig. 1.** MAGL inhibitors significantly reverse mechanical allodynia in paclitaxel-treated mice. (A) JZL184 (40 mg/kg) reverses paclitaxel-induced allodynia with maximal antiallodynic effectU+0073 occurring from 0.5 to 5 hours postadministration compared with baseline (BL). (B) MJN110 reverses mechanical allodynia with maximal effects occurring 2–5 hours postinjection. Pre-Pac = baseline prior to paclitaxel (Pac) treatment. (C) JZL184 and MJN110 dose-dependently reverse mechanical allodynia in separate cohorts of paclitaxel-treated mice. Maximum reversal of allodynia was comparable to vehicle control mice treated with vehicle (Ctrl-Veh) for both drugs, as they did not significantly differ. Data are reported as mean ± S.E.M., $n$ = 7 or 8 mice/group. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001 vs. vehicle-treated mice at the respective time point (A and B). Filled symbols indicate a significant effect ($P$ < 0.05) of drug vs. vehicle-treated mice that received a cycle of paclitaxel (C).



**Fig. 2.** Antiallodynic effects of MAGL inhibitors require $CB_1$ receptor activation. (A) A cycle of paclitaxel (Pac) leads to the development of mechanical allodynia in $CB_1$ (+/+) and (−/−) mice. JZL184 and MJN110 significantly reverse mechanical allodynia in $CB_1$ (+/+) mice, but not in $CB_1$ (−/−) mice. The $CB_1$ receptor antagonist rimonabant significantly blocks the antiallodynic effects of JZL184 (B) and MJN110 (C). Control (Ctrl) mice and vehicle (Veh) treatment groups are shown for comparison. Data are reported as mean ± S.E.M., $n$ = 7 or 8 mice/group. *$P$ < 0.05 vs. $CB_1$ (+/+) mice (A). ***$P$ < 0.001 vs. vehicle pre-treatment (B and C).

V source for TurbolonSpray (ON, Canada) attached to a Shimadzu UPLC system (Kyoto, Japan) controlled by Analyst software (ON, Canada). Chromatographic separation of AEA, 2-AG, and arachidonic acid was performed on a Discovery HS C18 column 15 cm × 2.1 mm, 3 $\mu$m (Supelco, Bellefonte, PA) kept at 25°C and an injection volume of 10 $\mu$l. The mobile phase consisted of A: acetonitrile and B: water with 1 g/l ammonium acetate and 0.1% formic acid. The following gradient was used: 0.0–2.4 minutes at 40% A, 2.5–6.0 minutes at 40% A, hold for 2.1 minutes at 40% A, then 8.1–9 minutes 100% A, hold at 100% A for 3.1 minutes, and return to 40% A at 12.1 minutes with a flow rate was 1.0 ml/min. The source temperature was set at 600°C and had a curtain gas at a flow rate of 30 ml/min. The ionspray voltage was
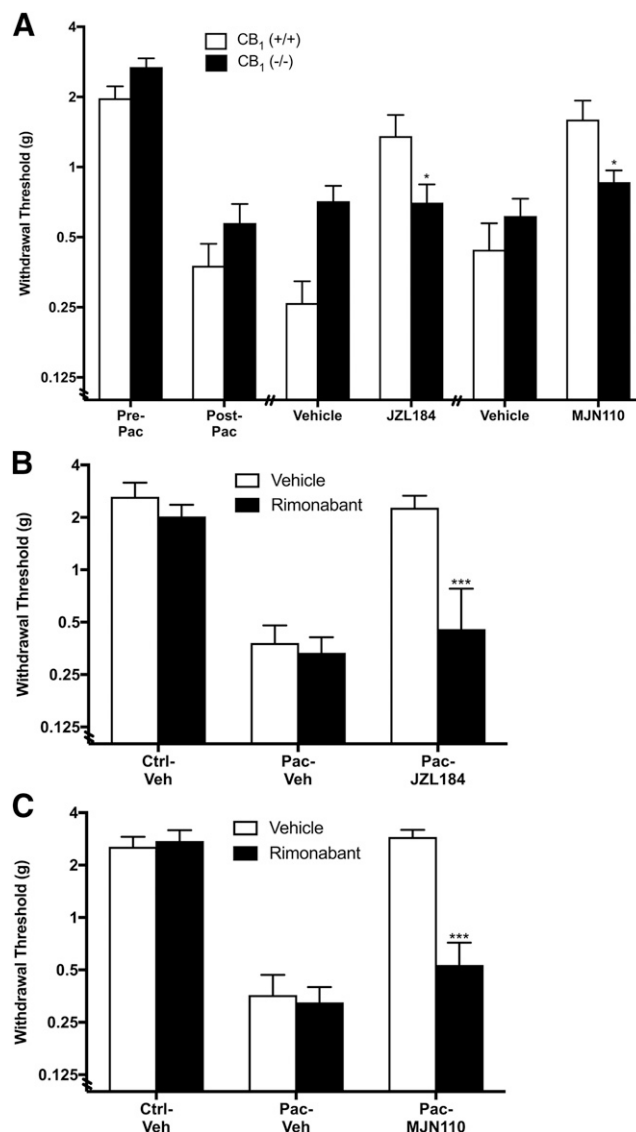
5000 V with ion source gases 1 and 2 flow rates of 60 and 50 ml/min, respectively. The mass spectrometer was run in positive ionization mode for AEA and 2-AG and in negative ionization mode for arachidonic acid, and the acquisition mode used was multiple reaction monitoring. The following transition ions ($m/z$) were monitored with their corresponding collection energies (eV) in parentheses: AEA: 348 > 62 (13) and 348 > 91 (60); AEA-$d_8$: 356 > 63 (13); 2-AG: 379 > 287 (26) and 379 > 296 (28); 2-AG-$d_8$: 384 > 287 (26); arachidonic acid: 303 > 259 (−25) and 303 > 59 (−60); arachidonic acid-$d_8$: 311 > 267 (−25); $PGD_2$: 351 > 271 (−23) and 351 > 315 (−15); $PGD_2$-$d_4$: 355 > 275 (−23). The total run time for the analytical method was 14 minute. Calibration curves were analyzed with each analytical batch for each analyte. A linear regression of the ratio of the peak area counts of analyte and the corresponding deuterated internal standards versus concentration was used to construct the calibration curves.
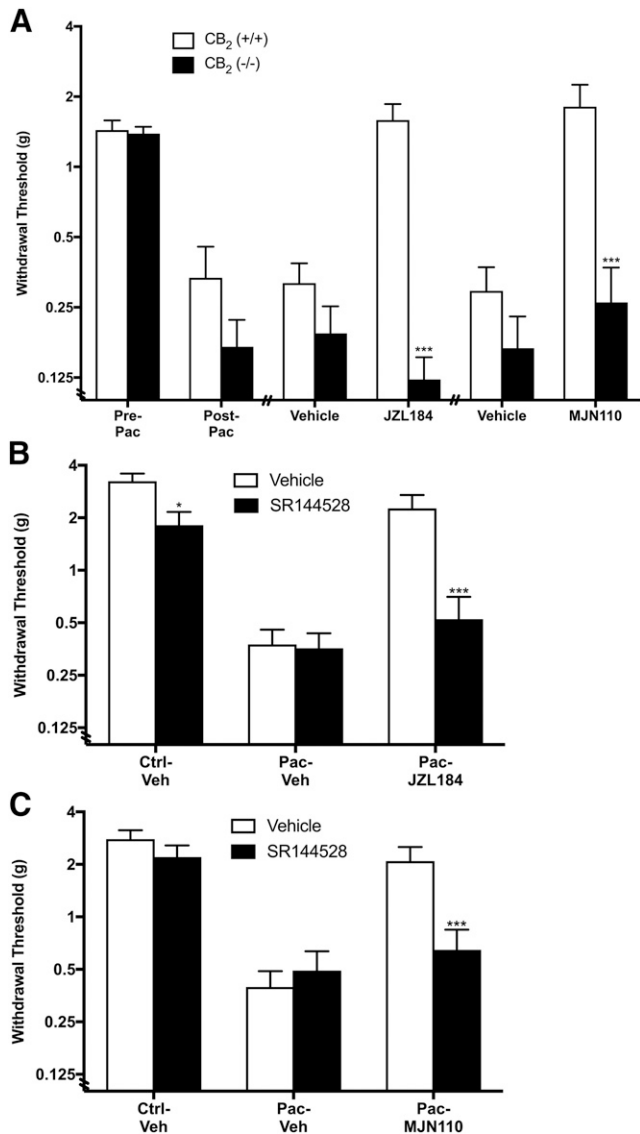
**Fig. 3.** Antiallodynic effects of MAGL inhibitors require $CB_2$ receptor activation. (A) A cycle of paclitaxel (Pac) leads to the development of mechanical allodynia in $CB_2$ (+/+) and (−/−) mice. JZL184 and MJN110 lack antiallodynic effects in $CB_2$ (−/−) mice, but fully reverse paclitaxel-induced mechanical allodynia in $CB_2$ (+/+) animals. The $CB_2$ receptor antagonist SR144528 blocks the antiallodynic effects of JZL184 and MJN110. Control (Ctrl) mice and vehicle (Veh) treatment groups are shown for comparison. Data are reported as mean ± S.E.M., $n$ = 8 mice/group. ***$P < 0.001$ vs. $CB_2$ (+/+) mice (A). *$P < 0.05$, ***$P < 0.001$ vs. vehicle pretreatment (B and C).

**Conditioned Place Preference.** An unbiased conditioned place preference paradigm was used to examine the effects of MJN110 in control mice and in paclitaxel-treated mice (Kota et al., 2007; Sanjakdar et al., 2015). Following at least 1 week of acclimation to the vivarium, mice were handled for 3 weeks leading up to CPP conditioning. A three-chamber design was used (two conditioning chambers with a central acclimation chamber; ENV3013; Med Associates, St Albans, VT). The outer chambers were 20 × 20 × 20 cm with differing flooring (white mesh or black rods) and wall coloring (white or black) to distinguish each. A small gray chamber in the middle connected to each outer chamber with a door.

This experiment consisted of four groups of mice in which each group received a cycle of paclitaxel or vehicle and then received conditioning sessions in which they were either administered vehicle

each conditioning day or administered MJN110 or vehicle on alternating days. Eight days after the final paclitaxel injection, mice were placed in the central chamber and allowed to acclimate for 5 minutes. The doors to both chambers were then opened, and the mouse was allowed to explore the apparatus in a drug-free state for 15 minutes to record baseline chamber preferences. Beginning on postpaclitaxel day 9, half the mice in each group received a single daily intraperitoneal injection of vehicle or MJN110 (5 mg/kg), alternating between these treatments each day for a total of eight conditioning sessions. MJN110 or vehicle was randomly assigned to either the black or white chamber at the start of the experiment to avoid potential bias. The other half of the mice received a single daily intraperitoneal injection of vehicle prior to each conditioning session. One hour after each injection, each mouse was placed in the appropriate conditioning chamber for 30 minutes. The day after the final conditioning session, each mouse was returned to the apparatus, but did not receive an injection and was allowed to move freely among the chambers for a 15-minute test period. Preference scores were calculated for MJN110 treatment based on the total amount of time spent in the MJN110-paired side (day 10, in seconds) minus the baseline preference (day 1, in seconds) for the same chamber. Paclitaxel- and vehicle-treated control mice (mice received vehicle paired to both chambers), the preference score was calculated as the average of the test preferences minus the baseline preference for each chamber.

**Cell Culture.** All lung cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FB22-500HI; Serum Source International, Charlotte, NC) and 1% (v/v) combination of 10,000 U/ml penicillin and 10,000 $\mu$g/ml streptomycin (15140-122, Pen/Strep; Thermo Fisher Scientific, Carlsbad, CA). Cells were incubated at 37°C under a humidified, 5% $CO_2$ atmosphere. The H460 NSCLC cell line was generously provided by the laboratory of Dr. Richard Moran at VCU, and the A549 NSCLC cell line was a gift from the laboratory of Dr. Charles Chalfant at VCU.

Paclitaxel (50 nM) and JZL184 (1 $\mu$M) were dissolved in DMSO, diluted with sterile PBS, and added to the medium to obtain the desired concentrations; less than 0.1% DMSO was present in the medium. This concentration of JZL184 was chosen as it inhibits MAGL in cancer cells (Nomura et al., 2010). All experiments using DMSO were performed in the dark.

**Assessment of Cell Viability.** The NSCLC cells were exposed to JZL184, paclitaxel, or a combination of JZL184 and paclitaxel for 24 hours, after which the drugs were removed and replaced with fresh medium. The number of viable cells was determined via trypan blue exclusion on days 1, 3, 5, and 7 posttreatment. Cells were incubated with trypsin (0.25% trypsin-EDTA) for 3 minutes and stained with trypan blue (15250; Invitrogen, Carlsbad, CA). The viable unstained cells were counted using a hemocytometer with bright-field microscopy.

**Assessment of Apoptosis.** Flow cytometry analyses were performed using BD FACSCanto II (BD Biosciences, San Jose, CA) and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core facility. For all studies, 10,000 cells per replicate within the gated region were analyzed. When collecting samples, both adherent and floating cells were harvested with 0.1% trypsin-EDTA and neutralized with medium after 48 hours of drug exposure. For quantification of apoptosis, cells were centrifuged and washed with PBS, then resuspended in 100 $\mu$l of 1× binding buffer with 5 $\mu$l of Annexin V and 5 $\mu$l of propidium iodide (556547, FITC Annexin V Apoptosis Detection Kit; BD Biosciences). The samples were then incubated at room temperature while protected from light for 15 minutes. The suspension solution was then brought up to 500 $\mu$l using the 1× binding buffer and analyzed by flow cytometry.

**Statistical Analyses.** Results are reported as the mean ± S.E.M. and were prepared using GraphPad Prism 7. $ED_{50}$ values were calculated as previously described (Grim et al., 2017). Briefly, data were converted to % maximal positive effect (average maximum and minimum values for drug treatment) for each cohort and plotted versus log dose values. $ED_{50}$ values and 95% confidence limits were

## A

**MCP-1, Dorsal Root Ganglia**



## B



Control-Vehicle

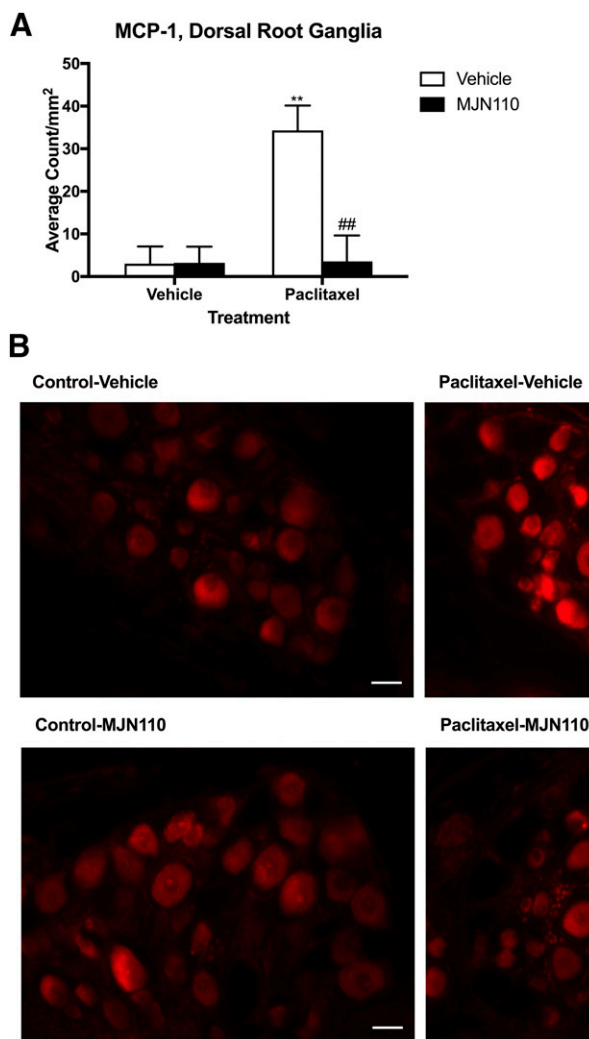Paclitaxel-Vehicle

Control-MJN110

Paclitaxel-MJN110

**Fig. 4.** MJN110 attenuates paclitaxel-induced MCP-1 expression in the dorsal root ganglia. (A) Nine days after a cycle of paclitaxel, vehicle-treated mice show a significant increase of MCP-1 expression in lumbosacral dorsal root ganglia compared with control mice that did not receive paclitaxel. MJN110 significantly blocks paclitaxel-induced increases of MCP-1 expression. (B) Representative dorsal root ganglia image for each of the four treatment conditions. All images are at 40×. Scale bar, 10 μm. Data are reported as mean ± S.E.M., $n = 4$ mice/group. **$P < 0.01$ vs. vehicle-treated mice that did not receive paclitaxel. ##$P < 0.01$ vs. vehicle-treated mice that received paclitaxel.

calculated using a linear regression analysis. Potency ratios were calculated based on the distance between the two dose-response curves with 95% confidence limits. Based on the study design, a one- or two-way analysis of variance (ANOVA) was used to identify statistical differences followed by Holm-Sidak post hoc testing of significant ANOVAs. Dunnett's test was used when comparing all treatments to a single control group in the dose-response studies. A within-subjects design was used for studies involving genetic knockouts, MJN110 time course, KT-195 time course, and MJN110 antagonism studies. A repeated-measures ANOVA was used specifically for time course studies and when comparing paw withdrawal thresholds pre- and postpaclitaxel. Unpaired $t$ tests were used to compare two groups when indicated. Unless otherwise noted, prepaclitaxel/vehicle baseline allodynia values were not included in statistical analysis ($n = 7$ to 8 for all allodynia studies; $n = 15$ to 16 per group for CPP studies; $n = 4$ for immunohistochemistry studies). Grubbs' test was used to remove any significant outliers from each group in the CPP studies only. For all experiments, the probability of a type I error ($\alpha$) was set to 5% with $P$ values of <0.05 considered significant.

## Results

**MAGL Inhibitors Reverse Paclitaxel-Induced Mechanical Allodynia in Dose- and Time-dependent Manners.** Mice undergoing a paclitaxel cycle regimen displayed significant mechanical allodynia relative to control

mice within 24 hours following the final injection [main interaction of drug × $F_{(1,13)} = 8.71$, $P < 0.05$; Supplemental Fig. 1]. JZL184 (40 mg/kg, i.p.) significantly reversed paclitaxel-induced mechanical allodynia [main interaction of drug × time, $F_{(7,98)} = 9.50$, $P < 0.001$] for up to 5 hours (Fig. 1A). Similarly, MJN110 (5 mg/kg, i.p.) reversed mechanical allodynia compared with vehicle treatment [main interaction of drug × time, $F_{(7,98)} = 6.83$, $P < 0.001$] for up to 5 hours postadministration (Fig. 1B). In mice that did not receive paclitaxel, JZL184 (40 mg/kg) altered paw withdrawal thresholds [main interaction of drug × time, $F_{(7,98)} = 2.204$; $P < 0.05$]; however, post hoc analysis did not reveal any significant differences compared with vehicle up to 24 hours posttreatment (Supplemental Fig. 2A). In vehicle control mice, MJN110 (5 mg/kg) did not significantly alter von Frey withdrawal thresholds (main effect of time, $P = 0.48$; main effect of drug, $P = 0.61$; interaction, $P = 0.96$; Supplemental Fig. 2B). To control for ABHD6 inhibition, an off-target effect of MJN110 (Niphakis et al., 2013), KT-195 (40 mg/kg) was administered to paclitaxel- and control vehicle-treated mice. KT195 did not alter paw withdrawal thresholds in either paclitaxel-treated ($P > 0.3$) or control animals ($P > 0.3$; Supplemental Fig. 3).

The mechanical antiallodynic dose-response relationships of JZL184 and MJN110 from separate cohorts of mice are
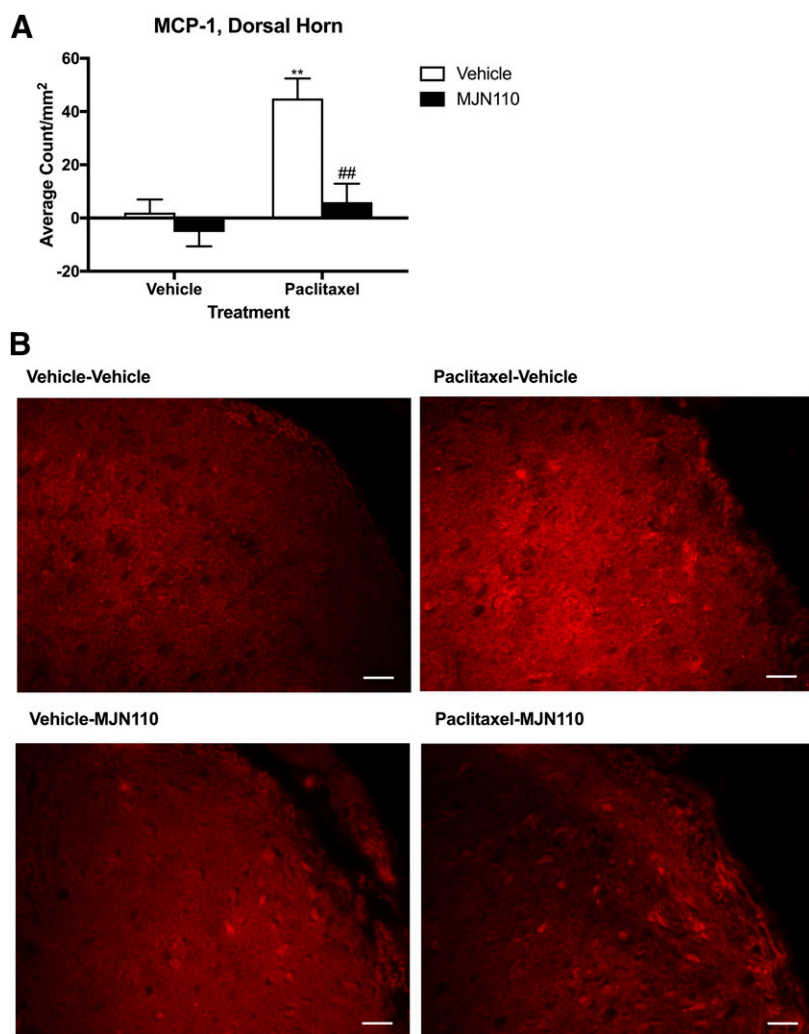
## A

**MCP-1, Dorsal Horn**



## B



Vehicle-Vehicle

Paclitaxel-Vehicle

Vehicle-MJN110

Paclitaxel-MJN110

**Fig. 5.** MJN110 attenuates paclitaxel-induced MCP-1 expression in the dorsal horn of the spinal cord. (A) A cycle of paclitaxel elicits a significant increase in MCP-1 expression in lumbosacral spinal cord compared with control mice that did not receive paclitaxel. MJN110 (5 mg/kg) reverses paclitaxel-induced elevations of MCP-1 expression. (B) Representative image for each of the four conditions. All images are at 40×. Scale bar, 10 $\mu$m. Data are reported as mean ± S.E.M., $n = 4$ mice/group. **$P < 0.01$ vs. vehicle-treated mice that did not receive paclitaxel. ##$P < 0.01$ vs. vehicle-treated mice that received paclitaxel.

depicted in Fig. 1C. JZL184 significantly reversed paclitaxel-induced allodynia [$F(5,42) = 5.74$, $P < 0.001$], with 16 and 40 mg/kg. JZL184 significantly differing from the vehicle condition. JZL184 (40 mg/kg) fully reversed paclitaxel-induced allodynia and elicited similar von Frey thresholds as those from mice that did not receive paclitaxel ($t$ test, $P > 0.05$). Similarly, MJN110 significantly reversed mechanical allodynia [$F(4,35) = 4.83$, $P < 0.01$]. MJN110 (3 and 5 mg/kg) significantly differed from vehicle. MJN110 (5 mg/kg) fully reversed paclitaxel-induced allodynia to withdrawal thresholds comparable to control mice not treated with paclitaxel ($t$ test, $P > 0.05$). The respective ED$_{50}$ values (95% confidence limits) for JZL184 and MJN110 were 8.4 (5.2–13.6) and 1.8 (1.0–3.3) mg/kg. MJN110 was 4.7 (2.0–10.6; 95% confidence limits) times more potent than JZL184.

**Antiallodynic Effects of MAGL Inhibitors Require CB$_1$ Receptors.** A cycle of paclitaxel elicited significant mechanical allodynia in both CB$_1$ (+/+) and (−/−) mice [main effect of paclitaxel, $F(1,14) = 144.2$, $P < 0.001$] with no significant main effects of genotype ($P = 0.06$) and no significant interaction between genotype and paclitaxel treatment ($P = 0.18$; Fig. 2A). Allodynia was stable during the 4 weeks of behavioral assessment. JZL184 significantly reversed paclitaxel-induced allodynia in CB$_1$ (+/+) mice, but not

in CB$_1$ (−/−) mice [interaction of drug × genotype, $F(1,26) = 8.316$, $P < 0.01$; Fig. 2A]. Similarly, MJN110 significantly reversed allodynia in CB$_1$ (+/+) mice, and not in CB$_1$ (−/−) mice [interaction of drug × genotype, $F(1,28) = 5.574$, $P < 0.05$; Fig. 2A].

By using a complementary pharmacologic approach, the CB$_1$ receptor antagonist rimonabant (3 mg/kg) or vehicle was administered 30 minutes before each respective MAGL inhibitor. As shown in Fig. 2B, rimonabant blocked the antiallodynic effects of JZL184 but had no effects on its own [interaction of antagonist × drug, $F(1,28) = 11.56$, $P < 0.01$]. Similarly, rimonabant blocked the antiallodynic effects of MJN110 [$F(1,28) = 40.0$, $P < 0.001$; Fig. 2C]. However, rimonabant did not affect von Frey threshold in control mice that did not receive a cycle of paclitaxel ($t$ test, $P > 0.05$) (Fig. 2, B and C).

**Antiallodynic Effects of MAGL Inhibitors Require CB$_2$ Receptors.** CB$_2$ (+/+) and (−/−) mice developed significant mechanical allodynia after a cycle of paclitaxel [main effect of paclitaxel, $F(1,14) = 113.8$, $P < 0.001$] with no difference between genotypes ($P = 0.35$) (Fig. 3A), and allodynic responses remained stable throughout the 5-week behavioral assessment period. JZL184 [interaction of drug × genotype, $F(1,28) = 21.8$, $P < 0.001$; Fig. 3A] as well as MJN110
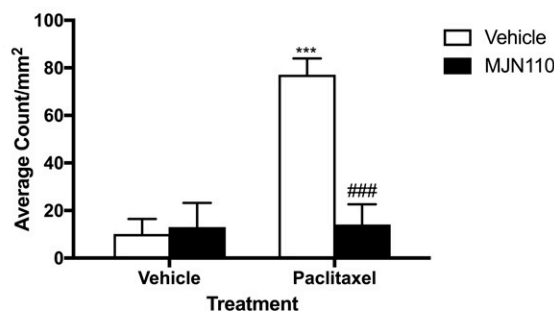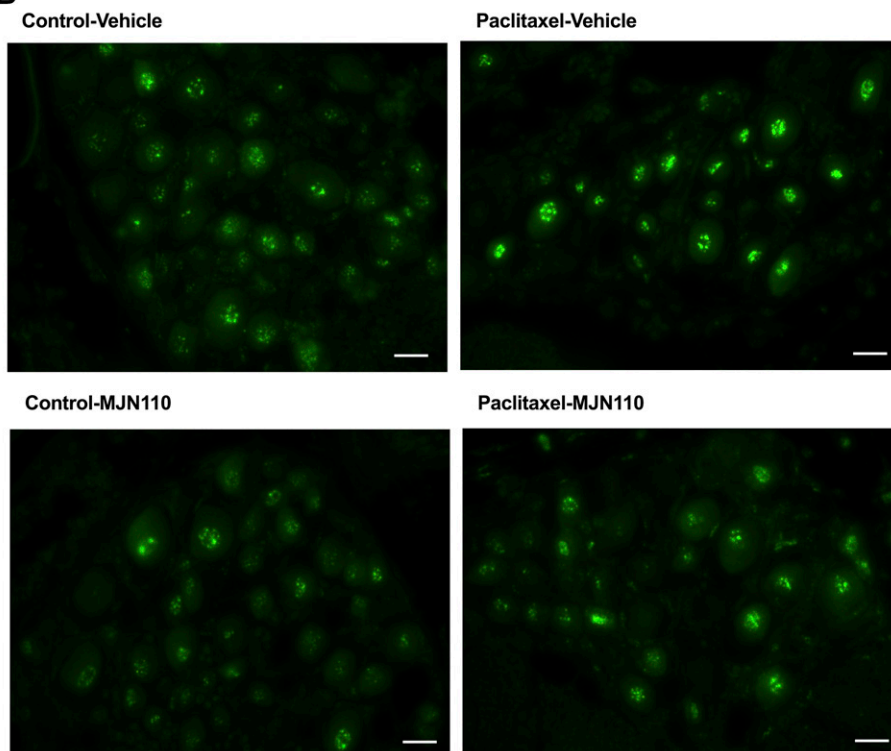
**A**   phospho-p38 MAPK, Dorsal Root Ganglia



**B**



Control-Vehicle

Paclitaxel-Vehicle

Control-MJN110

Paclitaxel-MJN110

**Fig. 6.** MJN110 attenuates paclitaxel-induced phospho-p38 MAPK expression in the dorsal root ganglia. (A) Following paclitaxel treatment, vehicle-treated mice show a significant increase of phospho-p38 expression in lumbosacral dorsal root ganglia compared with control mice that did not receive paclitaxel. MJN110 significantly decreased paclitaxel-induced expression of phospho-p38. (B) Representative dorsal root ganglia image for each of the four conditions. All images are at 40×. Scale bar, 10 $\mu$m. Data are reported as mean $\pm$ S.E.M., $n$ = 4 mice/group. ***$P < 0.001$ vs. vehicle-treated mice that did not receive paclitaxel. ###$P < 0.001$ vs. vehicle-treated mice that received paclitaxel.

[$F$ (1, 28) = 9.491, $P < 0.01$; Fig. 3A] significantly reversed paclitaxel-induced mechanical allodynia in CB$_2$ (+/+) and not in CB$_2$ (−/−) mice.

In the next experiment, we tested whether a CB$_2$ receptor antagonist would block the antinociceptive effects of these MAGL inhibitors. SR144528 (3 mg/kg, i.p.) or vehicle was administered 30 minutes prior to JZL184 or MJN110. As shown in Fig. 3B, SR144528 blocked the antiallodynic effects of JZL184 and lacked effects on its own [interaction of antagonist × drug, $F$(1,28) = 12.33, $P < 0.01$]. Similarly, SR144528 blocked the antiallodynic effects of MJN110 [interaction of antagonist × drug, $F$(1,28) = 9.43, $P < 0.01$; Fig. 3C]. Although SR144528 produced a significant but small reduction in paw withdrawal thresholds in control mice in the JZL184 experiment (Fig. 3B), it did not affect thresholds in control mice in the MJN110 experiment (Fig. 3C).

**MJN110 Attenuates MCP-1 and Phospho-p38 Expression in Paclitaxel-Treated Mice.** As paclitaxel causes inflammatory responses in DRG (Li et al., 2015; Zhang et al., 2016) and spinal cord (Pevida et al., 2013; Zhang

et al., 2013), we evaluated whether a MAGL inhibitor would attenuate this inflammation at 3 hours, a time point corresponding to allodynia reversal. A cycle of paclitaxel led to a significant increase in MCP-1 expression in the DRG compared with mice that did not receive paclitaxel [interaction of paclitaxel treatment × MJN110 treatment, $F$(1,12) = 9.426, $P < 0.05$; post hoc: $P < 0.01$; Fig. 4]. Treatment with MJN110 (5 mg/kg) significantly decreased MCP-1 expression in paclitaxel-treated mice [$P < 0.01$], but not in the control vehicle-treated mice [$P = 1.0$ for comparison between paclitaxel-MJN110 and control-vehicle mice]. Paclitaxel also increased MCP-1 expression in the spinal dorsal horn, an effect that was attenuated by MJN110 pretreatment versus vehicle [$F$(1,12) = 6.294, $P < 0.05$; post hoc $P < 0.01$; Fig. 5]. MJN110 significantly decreased spinal MCP-1 expression in paclitaxel-treated mice to levels comparable to control-vehicle mice ($P = 0.68$).

Mice given a cycle of paclitaxel also demonstrated significantly increased phospho-p38 expression in the DRG compared with control mice, and this effect was inhibited by
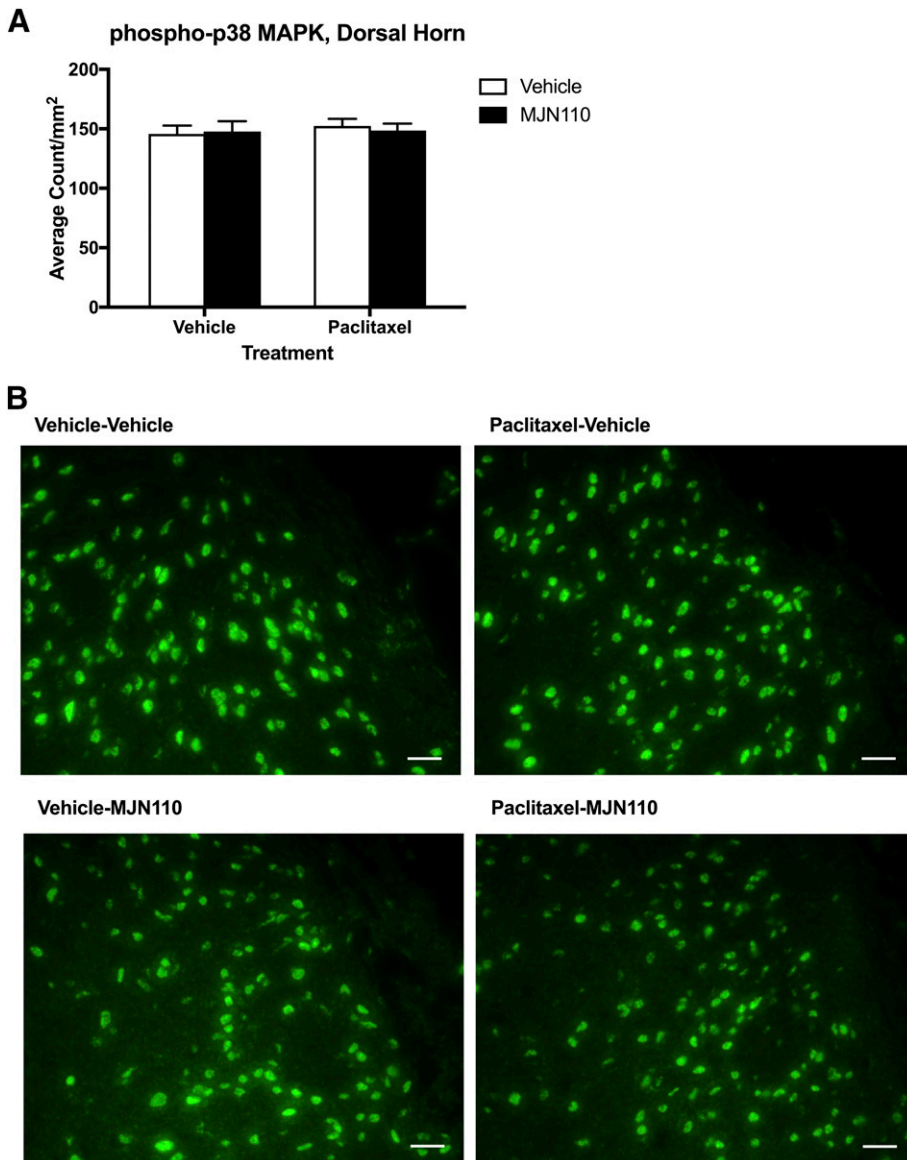
**A**

### phospho-p38 MAPK, Dorsal Horn



**B**



**Fig. 7.** Paclitaxel does not increase phospho-p38 expression in the spinal dorsal horn. (A) Neither paclitaxel nor MJN110 alters phosphorylated phospho-38 MAPK expression in the spinal dorsal horn. (B) Representative images for each condition. All images are at 40×. Scale bar, 10 $\mu$m. Data are reported as mean ± S.E.M., $n = 4$ mice/group.

MJN110 [interaction of paclitaxel treatment × MJN110 treatment, $F(1,12) = 16.56$, $P < 0.01$; Fig. 6]. MJN110 significantly decreased DRG phospho-p38 expression in paclitaxel-treated mice to levels comparable to control-vehicle mice ($P = 0.98$). However, there were no significant changes in the expression of phospho-p38 MAPK in the spinal dorsal horn (no main effect of paclitaxel, $P = 0.60$; no main effect of MJN110, $P = 0.90$; Fig. 7). Qualitative confocal microscopy of DRG showed that MCP-1 and phospho-p38 expression colocalizes in neurons and cells consistent with the location of satellite cells as indicated by the nuclear marker DAPI (Fig. 8).

**Repeated Administration of Low-dose JZL184 Produces Sustained Antiallodynic Effects and Increases 2-AG Levels in the Lumbar Spinal Cord.** Figure 9A outlines the treatment procedure depicting the consequences of acute and repeated administration of 4 mg/kg (threshold dose) and 40 mg/kg (high dose) JZL184. These treatments produced statistically significant differential effects on paclitaxel-induced allodynia [$F(5,42) = 8.38$, $P < 0.001$;

Fig. 9B]. Acute administration of 40 mg/kg JZL184 fully reversed paclitaxel-induced allodynia ($P < 0.01$), but this effect underwent tolerance following 6 days of daily administration ($P = 1.0$). In contrast, whereas acutely administered 4 mg/kg JZL184 did not significantly attenuate paclitaxel-induced allodynia ($P = 1.0$), repeated administration of this dose fully reversed the allodynia ($P < 0.05$).

Following mechanical allodynia assessment, mice were euthanized and the L4-L6 level of the lumbar spinal cord was procured for lipid quantification. As depicted in Fig. 10A, JZL184 significantly elevated 2-AG levels [$F(5,42) = 81.37$, $P < 0.001$]. While acute ($P < 0.001$) and repeated ($P < 0.001$) administration of 40 mg/kg JZL184 elevated 2-AG levels, 4 mg/kg JZL184 significantly increased 2-AG spinal levels following repeated produced administration ($P < 0.001$), but not acute administration ($P = 0.09$). Repeated administration of 40 mg/kg JZL184 led to higher 2-AG levels than acute treatment ($P < 0.001$). A significant effect was also found for spinal AEA levels [$F(5,42) = 19.25$, $P < 0.001$]. Repeated administration of either 4 mg/kg ($P < 0.05$) or 40 mg/kg
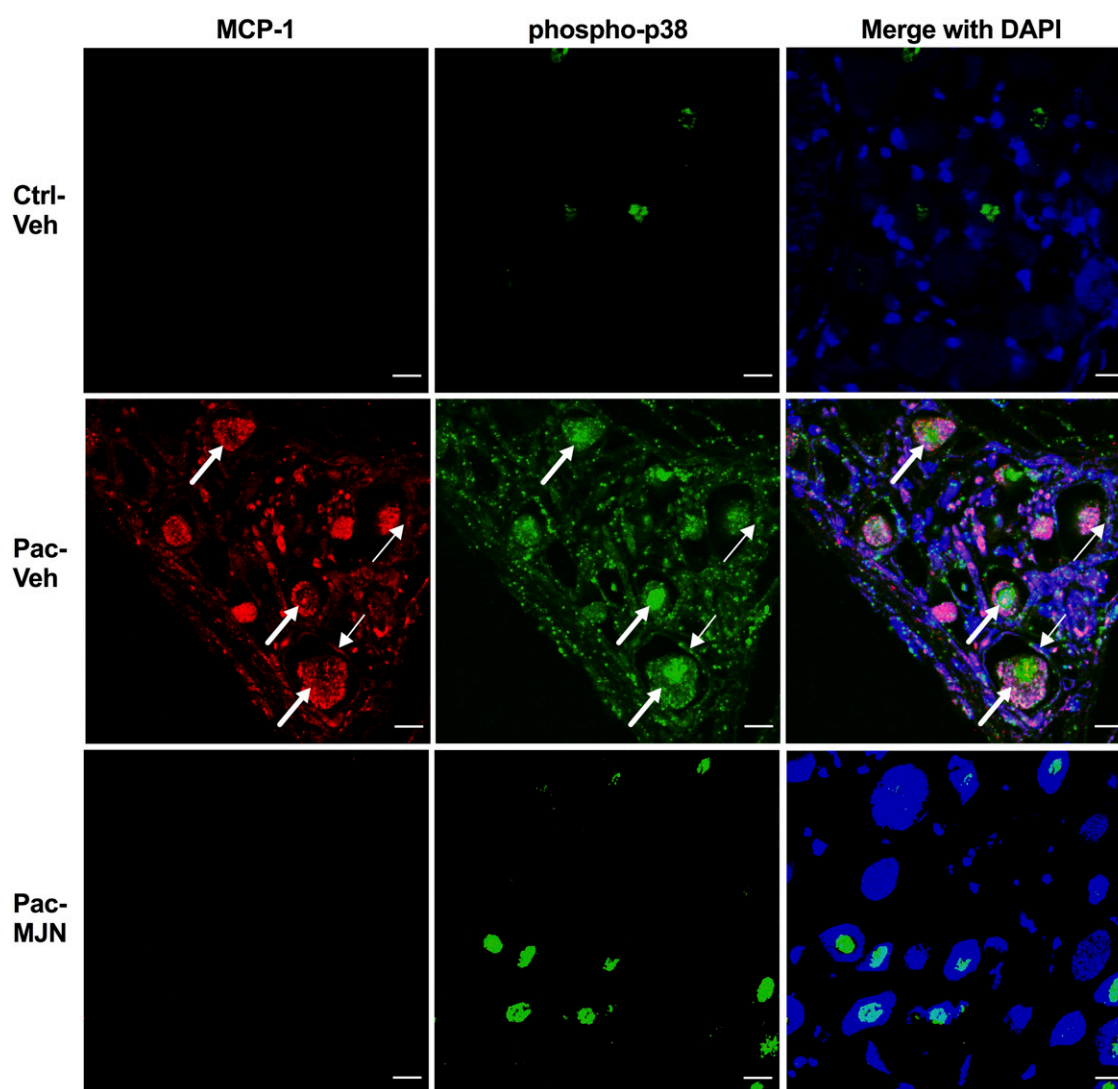
**Fig. 8.** Qualitative confocal microscopy of MCP-1 and phospho-p38 MAPK colocalization in dorsal root ganglia cells. A cycle of paclitaxel (Pac) increased MCP-1 (left) and phospho-p38 (middle) expression compared with control vehicle (Ctrl) treatment. MCP-1 and phospho-p38 colocalize in cells stained with the nuclear marker DAPI (right). Thick arrows indicate neurons, and thin arrows indicate cells consistent with the location of satellite cells. This colocalization is not observed in sections from MJN110 (MJN)-treated mice compared with vehicle (Veh) treatment. All images are at 63×. Scale bar, 10 $\mu$m.

($P < 0.001$) JZL184 (Fig. 10B) produced significant increased spinal AEA levels. As shown in Fig. 10C, a significant effect was found for arachidonic acid spinal levels [$F(5,42) = 6.238$, $P < 0.001$]. However, repeated administration of 40 mg/kg JZL184 was the only condition significantly different from the control condition ($P < 0.01$). Finally, a significant effect was found for PGD$_2$ [$F(5,42) = 4.591$, $P < 0.01$; Fig. 10D]. Post hoc analysis revealed that paclitaxel-treated mice given repeated administration of 40 mg/kg JZL184 possessed lower spinal PGD$_2$ levels in mice given repeated administration of 40 mg/kg JZL184 than vehicle control mice that did not receive paclitaxel ($P < 0.001$) and mice given an acute injection of 4 mg/kg JZL184 [$P = 0.03$]. Compared with vehicle control mice, paclitaxel treatment did not alter 2-AG ($P = 0.98$), AEA ($P = 0.95$), arachidonic acid ($P = 0.81$), or PGD$_2$ ($P = 0.52$) levels in the lumbar spinal cord.

**MJN110 Produces a Conditioned Place Preference in Paclitaxel-Treated Mice.** In the next study, we examined whether a MAGL inhibitor produces a CPP in vehicle-treated or paclitaxel-treated mice (see Fig. 11A for a schematic depicting the experimental procedure). MJN110 produced a significant place preference in paclitaxel-treated mice, but did not affect place conditioning in control mice [interaction of paclitaxel treatment × MJN110 treatment, $F(1,59) = 4.338$, $P < 0.05$; Fig. 11B]. As confirmed in Supplemental Fig. 4, paclitaxel-treated mice had significantly lower withdrawal thresholds than vehicle-treated mice both before and after CPP training and testing ([interaction of paclitaxel treatment × time, $F(2,122) = 61.54$, $P < 0.001$].

**JZL184 Does Not Interfere with Paclitaxel-Induced Growth Arrest or Apoptosis in Non-Small Cell Lung Cancer Cells.** The final experiment examined whether JZL184 interferes with paclitaxel-induced growth arrest of two human NSCLC lines or has effects on its own. Consistent with its known antiproliferative actions, paclitaxel decreased the number of viable A549 cells [interaction of treatment ×
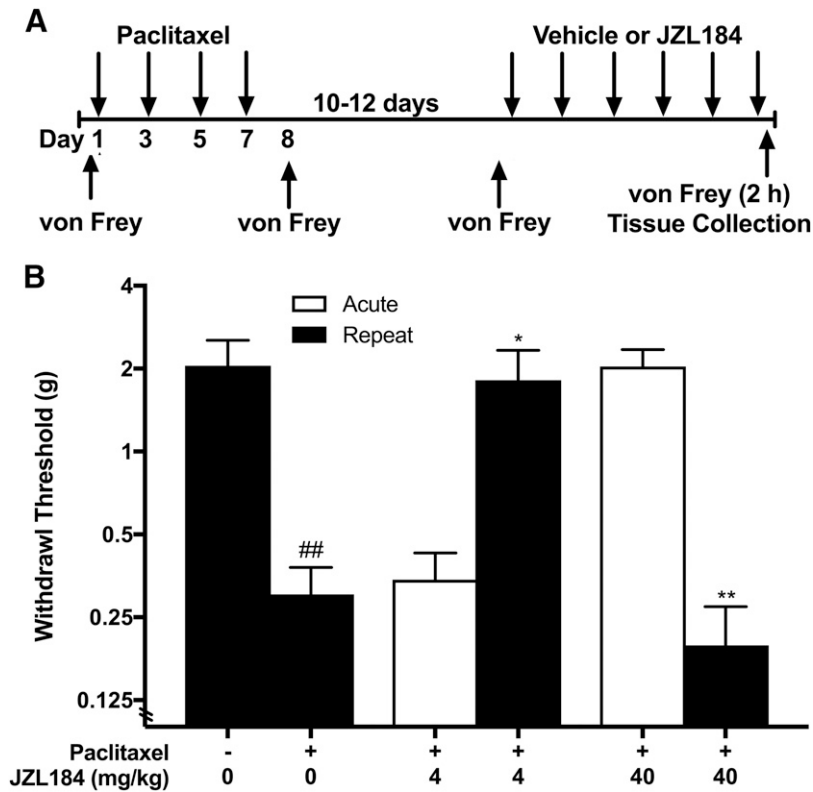
**Fig. 9.** Acute and repeated administration of 4 and 40 mg/kg JZL184 differentially affect paclitaxel-induced allodynia. (A) Experimental procedural timeline. (B) Although acute administration of high-dose JZL184 fully reverses paclitaxel-induced allodynia, this antinociceptive effect undergoes tolerance following 6 days of daily JZL184. Whereas acute administration of low-dose JZL184 does not attenuate paclitaxel-induced allodynia, it completely reverses allodynia after repeated administration. All data were recorded 2 hours after the last treatment. Data are reported as mean $\pm$ S.E.M., $n = 8$ mice/group. $*P < 0.05$; $**P < 0.01$ vs. the corresponding acute condition; $\#\#P < 0.01$ vs. no paclitaxel.

day, $F(12,32) = 10.4$, $P < 0.001$; Fig. 12A]. JZL184 did not affect viable cell number alone ($P = 0.94$ compared with control, day 7) or in combination with paclitaxel ($P = 0.98$ compared with paclitaxel alone; Fig. 12A). Paclitaxel also decreased the number of viable H460 cells [interaction of treatment $\times$ day, $F(12,32) = 115$, $P < 0.001$; Fig. 12B], and

JZL184 did not affect viable cell number alone [$P = 0.93$ compared with control, day 7] or in combination with paclitaxel [$P = 0.93$ compared with paclitaxel alone]. Likewise, JZL184 did not affect apoptotic cell population. Paclitaxel produced apoptosis in both A549 cells [main effect of paclitaxel, $F(1,8) = 194.4$, $P < 0.001$; Fig. 13A] and H460 cells
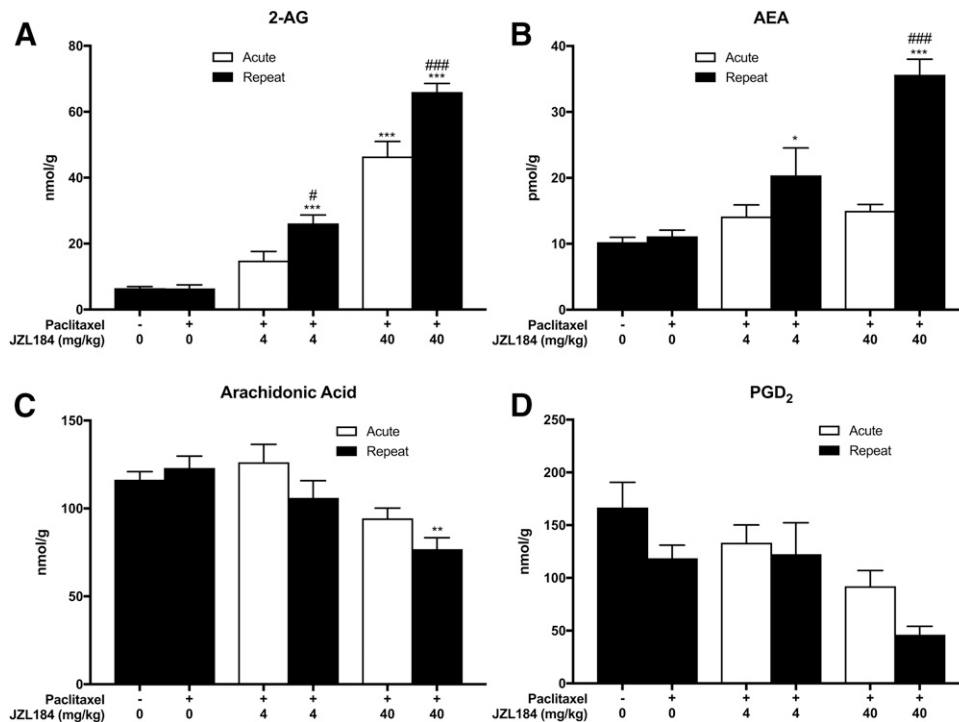


**Fig. 10.** Acute vs. repeated administration of JZL184 (4 or 40 mg/kg) on 2-AG (A), anandamide (AEA) (B), arachidonic acid (C), and prostaglandin D2 (PGD$_2$) (D) in spinal cord. Data are reported as mean $\pm$ S.E.M., $n = 8$ mice/group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. paclitaxel-vehicle. $\#P < 0.05$; $\#\#\#P < 0.001$ vs. the corresponding acute treatment.
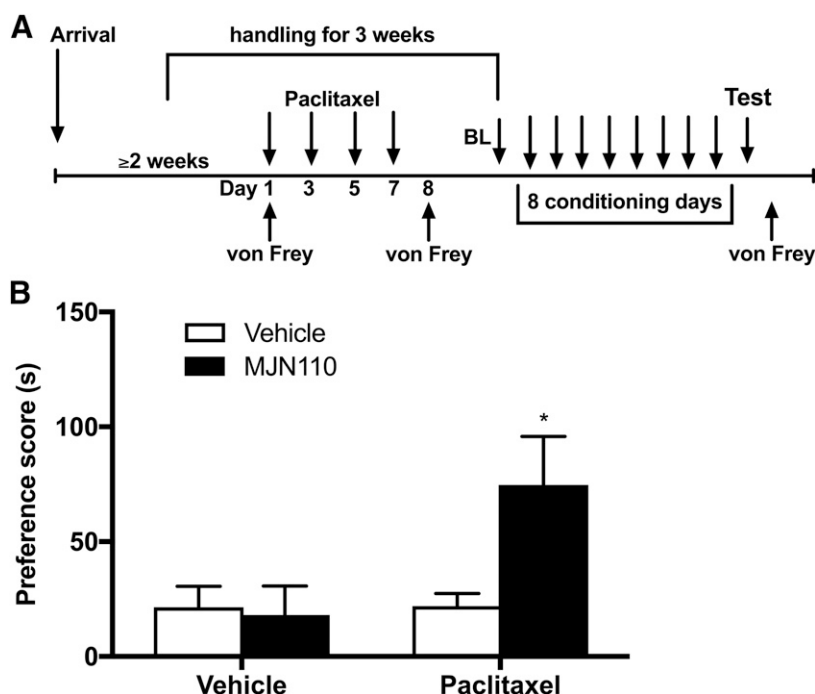
**A**



**B**



Fig. 11. A MAGL inhibitor produces a conditioned place preference in paclitaxel-treated mice. (A) Conditioned place preference procedure. (B) MJN110 (5 mg/kg) leads to the development of a CPP in paclitaxel-treated mice, but not in mice that received a cycle of vehicle a CPP in paclitaxel-treated. BL, baseline preference test prior to conditioning. Test, drug-free preference test after conditioning. Data are reported as mean ± S.E.M., $n$ = 15 or 16 mice/group. *$P < 0.05$ MJN110 vs. vehicle control in paclitaxel mice.

[main effect of paclitaxel, $F(1,8) = 7.549$, $P < 0.03$; Fig. 13B]. JZL184 did not interfere with paclitaxel-induced apoptosis and had no effect on its own in either A549 (no main effect of JZL184 treatment, $P = 0.59$) or H460 (no main effect of JZL184 treatment, $P = 0.60$) cells.

## Discussion

The present study replicates and extends the results of other studies showing that the MAGL inhibitor JZL184 reverses paclitaxel-induced (Slivicki et al., 2017) and cisplatin-induced (Guindon et al., 2013) allodynia. Here, we demonstrate that JZL184 and MJN110 fully reverse paclitaxel-induced mechanical allodynia in a manner consistent with their respective MAGL inhibitory constant estimates of 8 and 2.1 nM (Long et al., 2009; Niphakis et al., 2013). Notably, neither MAGL inhibitor produced enhanced or depressed paw withdrawal thresholds in control mice not given paclitaxel. To control for MJN110 inhibition of ABHD6, the ABHD6 inhibitor KT195 did not affect the allodynic effects of paclitaxel. The antiallodynic effects of each MAGL inhibitor required both CB$_1$ and CB$_2$ receptors, as demonstrated by genetic and pharmacologic approaches. MJN110 also attenuated paclitaxel-induced increases in phospho-p38 and MCP-1/CCL2 expression, suggesting a concomitant decrease of inflammatory responses with allodynia reversal. Tolerance to the antiallodynic effects of repeated administration, noted after a high dose of JZL184, was not observed with a subthreshold dose regimen that produced antiallodynic effects and increased spinal 2-AG and anandamide levels following repeated administration. Furthermore, MJN110 produced a CPP in paclitaxel-treated mice, but not in control animals. Finally, we show that JZL184 treatment, at a concentration that inhibits MAGL (Nomura et al., 2010), does not interfere with the antiproliferative and apoptotic effects of paclitaxel in human cell lines of NSCLC.

Here we report that the antiallodynic effects of MJN110 and JZL184 in the paclitaxel neuropathic pain model requires activation of both CB$_1$ and CB$_2$ receptors. Likewise, both receptors are required for the antinociceptive effects of MAGL inhibitors in chronic constriction injury of the sciatic nerve (Ignatowska-Jankowska et al., 2015), carrageenan (Ghosh et al., 2013), and cisplatin (Guindon et al., 2013) models of pain. In contrast, selective CB$_2$ receptor agonists fully reversed paclitaxel-induced allodynia (Rahn et al., 2008; Deng et al., 2015b). Similarly, pan CB$_1$/CB$_2$ receptor agonists (CP55,940; WIN55-212; $\Delta^9$-tetrahydrocannabinol) reverse paclitaxel-induced allodynia in rodents (Pascual et al., 2005; Deng et al., 2015a,b). The antiallodynic effects of CP55,940 in paclitaxel-treated mice show increased potency in CB$_2$ ($-/-$) mice compared with CB$_1$ ($-/-$) mice (Deng et al., 2015), demonstrating that sufficient stimulation of either receptor alone can elicit antinociceptive effects. Thus, MAGL inhibitors may require activation of both receptors because the degree to which they elevate 2-AG may be insufficient to drive antiallodynic responses at either receptor alone.

Paclitaxel elicits neuronal damage in DRG followed later by satellite cell hypertrophy and macrophage infiltration (Peters et al., 2007). MCP-1 promotes macrophage recruitment (Zhang et al., 2016), and its expression in small nociceptive neurons sensitizes large- and medium-sized neurons by increasing intracellular calcium (Zhang et al., 2013). Paclitaxel also increases MCP-1 expression in the spinal dorsal horn from astrocytes (Zhang et al., 2013) as well as phosphorylates p38 through toll-like receptor 4 activation in DRG neurons, but not in the spinal cord (Li et al., 2015). Phospho-p38 expression on small IB4- and CGRP-positive neurons (Li et al., 2015) leads to sodium channel activation and hyperexcitability of nociceptive neurons (Hudmon et al., 2008). Here, we confirmed that a cycle of paclitaxel induces MCP-1 expression in both the lumbosacral DRG and lumbar dorsal horn, whereas phospho-p38 is increased in the DRG,
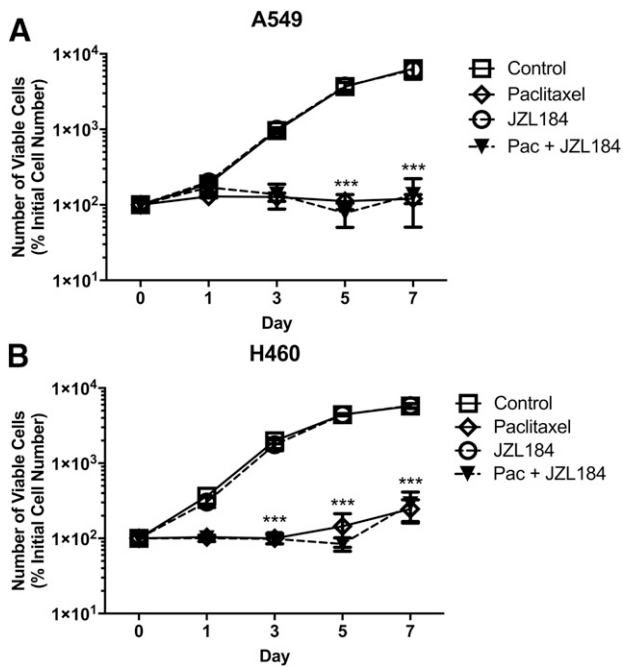
**Fig. 12.** JZL184 does not stimulate non-small cell lung cancer (NSCLC) cell proliferation alone or interfere with paclitaxel (Pac, 50 nM)-induced growth inhibition of A549 (A) or H460 (B) cells. Day 0 represents the initial number of cells after seeding. JZL184 (1 $\mu$M), paclitaxel, or the combination of paclitaxel and JZL184 was added to the cultures on day 0 and was replaced with drug-free medium 24 hours later. The number of viable cells was determined via trypan blue exclusion. Data are expressed as the mean $\pm$ S.E.M. of three independent experiments. ***$P < 0.001$ vs. control.
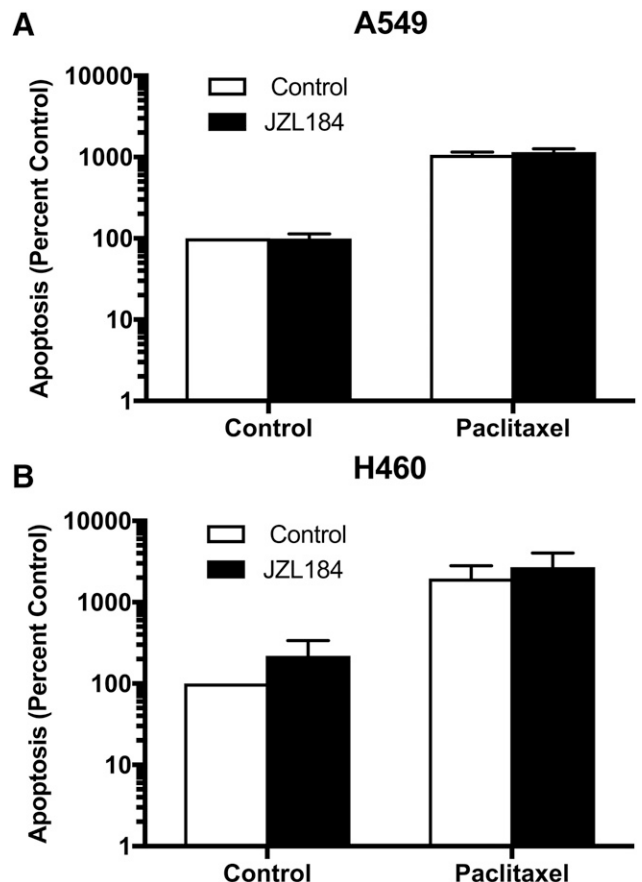


**Fig. 13.** JZL184 does not affect paclitaxel-induced apoptosis of non-small cell lung cancer (NSCLC) cells. A549 (A) and H460 (B) cells were exposed to JZL184 (1 $\mu$M), paclitaxel (100 nM), or the combination of paclitaxel and JZL184 for 48 hours. Quantification of apoptotic cells was determined using the Annexin V/PI assay, followed by flow cytometry analysis. Data are expressed as mean $\pm$ S.E.M. of three independent experiments.

but not in the dorsal horn, as previously reported (Zhang et al., 2013; Li et al., 2015). As shown in Fig. 8, phospho-p38 and MCP-1 coexpression occurs in the same DRG neurons and cells consistent with the location of satellite cells. The findings that MJN110 attenuates paclitaxel-induced expression of phospho-p38 and MCP-1 suggest an anti-inflammatory action. However, it remains to be determined whether $CB_1$ and/or $CB_2$ receptors mediate these actions.

Whereas prolonged and complete blockade of MAGL leads to high brain levels of endocannabinoids, $CB_1$ receptor down-regulation and desensitization, and tolerance to the antinociceptive effects of MAGL inhibitors (Schlosburg et al., 2010), repeated administration of a low dose JZL184 produces elevated endocannabinoid brain levels without $CB_1$ receptor functional tolerance (Kinsey et al., 2013). Similarly, the present study demonstrates that antiallodynic effects of high-dose JZL184 (40 mg/kg) in paclitaxel-treated mice undergo tolerance following repeated administration, but repeated administration of a subthreshold dose of JZL184 (4 mg/kg), which given acutely did not reverse allodynia, significantly elevated 2-AG and AEA in the L4–L6 region and fully reversed paclitaxel-induced allodynia. MAGL also contributes to the production of proinflammatory lipid mediators, such as arachidonic acid, prostanoids (Nomura et al., 2011b), and phosphatidic acids (Nomura et al., 2010), which could contribute to the antiallodynic actions of MAGL inhibitors. However, only repeated administration of high-dose JZL814 reduced arachidonic acid in the L4-L6 region of the spinal cord, and none of the JZL184 treatments significantly affected $PGD_2$ compared with vehicle. Also, as paclitaxel leads to

increased levels of lysophosphatidic acid in the spinal cord dorsal horn and lysophosphatidic acid receptor 1 ($-/-$) and 3 ($-/-$) mice show a phenotypic resistance to the development of paclitaxel-induced mechanical allodynia (Uchida et al., 2014), it is possible that MAGL inhibitors reduce this lipid as well as affect other mediators.

Although thermal, chemical, or mechanical stimuli are widely used to assess analgesia in rodents, these outcomes may lack clinical predictive value (Mogil, 2009). Alternatively, the CPP paradigm is used to infer potential affective aspects of pain relief into rodent pain models (King et al., 2009; Navratilova and Porreca, 2014; Havelin et al., 2016). Here, we make the unique observation that the MAGL inhibitor MJN110 produces a significant place preference in paclitaxel-treated mice, but not in control mice. This pattern of findings suggests that MJN110 lacks intrinsically rewarding or aversive effects, but is rewarding in paclitaxel-treated mice. The MJN110 data from control mice are consistent with the failure of JZL184 to produce a conditioned place preference or aversion (Gamage et al., 2015). The MJN110-induced CPP in paclitaxel-treated mice may represent a relief from affective or sensory aspects of nociception, as described in rodent models of cisplatin neuropathy (Park et al., 2013; Krukowski et al., 2017). However, MAGL inhibition may also relieve other

aversive states in paclitaxel-treated mice, such as a preference for the dark chamber in the light/dark box test and increased immobility time in the forced swim test (Toma et al., 2017). As MAGL inhibitors produce pharmacological effects in laboratory animal assays used to screen antidepressant and anxiolytic drugs (Kinsey et al., 2011; Sciolino et al., 2011; Zhong et al., 2014), MJN110 chamber preference in paclitaxel-treated mice may represent relief from an aversive state distinct from neuropathy.

Because treatments for CIPN may be given while patients are still receiving chemotherapy, we tested if JZL184, at concentrations that inhibit MAGL (Nomura et al., 2010), interferes with paclitaxel-induced cell death or growth arrest. We found that paclitaxel decreases cell viability and induces apoptosis in two human cell lines of NSCLC, which were not altered by JZL184. In other cancer types (i.e., prostate, melanoma, ovarian), decreased MAGL expression or activity inhibited cell proliferation and transformation (Nomura et al., 2010, 2011a), although it is unclear whether A549 and H460 cells express MAGL. Taken together, these results suggest that inhibition of MAGL neither affects cancer growth alone nor interferes with the antiproliferative or antiapoptotic effects of paclitaxel in these in vitro models of NSCLC.

The results of the present study do not support the idea that the endogenous cannabinoid system contributes to the development of paclitaxel-induced allodynia. Consistent with previous findings (Deng et al., 2015a), a cycle of paclitaxel elicited sustained mechanical allodynia in both $CB_1$ (−/−) and $CB_2$ (−/−) mice and receptor antagonists of these receptors did not alter paclitaxel-induced allodynia. The finding that a cycle of paclitaxel did not alter 2-AG and AEA spinal levels approximately 2 weeks later is consistent with a previous study in which paclitaxel did not alter expression of $CB_1$ receptor, $CB_2$ receptor, MAGL, or FAAH mRNA levels in spinal cord (Deng et al., 2015b). In contrast, cisplatin-induced CIPN leads to increased spinal 2-AG and AEA levels (Guindon et al., 2013), suggesting that the endogenous cannabinoid system differentially responds to these chemotherapeutic agents. Nonetheless, as the present study examined only a single time point more than 2 weeks following paclitaxel treatment, a full-time course evaluation of spinal and DRG endocannabinoid levels would be of value.

The goal of this work was to test whether MAGL inhibition reverses paclitaxel-induced allodynia as well as markers of DRG neuroinflammation. As paclitaxel neuropathies are long lasting (Tanabe et al., 2013), substantially harm quality of life, and are difficult to treat (Kim et al., 2015), novel analgesic strategies are needed. The present study demonstrates that MAGL inhibitors attenuate paclitaxel nociceptive-related behaviors using both mechanical allodynia and the conditioned place preference paradigm. We also show that MAGL inhibition ameliorates MCP-1 and phospho-p38 expression in the DRG. Taken together, the findings of the present study suggest that MAGL represents a viable target for possible treatment of CIPN.

## Authorship Contributions

*Participated in research design:* Curry, Wilkerson, Bagdas, Kyte, Patel, Donvito, Gewirtz, Damaj, Lichtman Cravatt.

*Conducted experiments:* Curry, Wilkerson, Bagdas, Mustafa, Kyte, Patel, Donvito, Poklis.

*Contributed new reagents or analytic tools:* Niphakis, Hsu, Cravatt.

*Performed data analysis:* Curry, Wilkerson, Bagdas, Mustafa, Kyte, Patel, Poklis.

*Wrote or contributed to the writing of the manuscript:* Curry, Wilkerson, Bagdas, Kyte, Donvito, Gewirtz, Damaj, Lichtman.

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**Address correspondence to:** Aron H. Lichtman, Department of Pharmacology and Toxicology, Virginia Commonwealth University, P.O. Box 980613, Richmond, VA 23298-0613. E-mail: aron.lichtman@vcuhealth.org
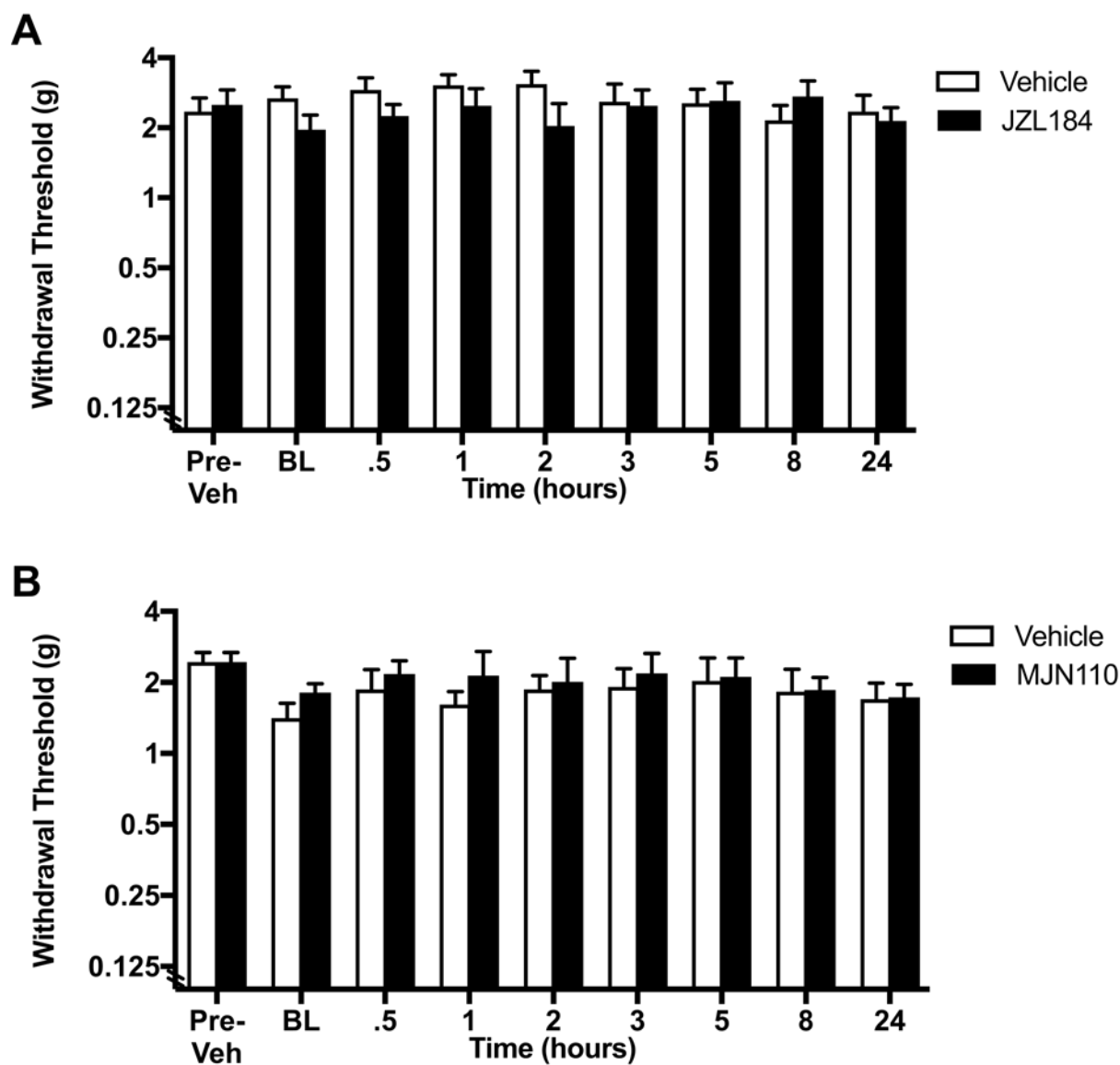
**Supplemental Data**

Monoacylglycerol lipase inhibitors reverse paclitaxel-induced nociceptive behavior and proinflammatory markers in a mouse model of chemotherapy-induced neuropathy

Zachary A Curry, Jenny L Wilkerson, Deniz Bagdas, S Lauren Kyte, Nipa Patel, Giulia Donvito, Mohammed A Mustafa, Justin L Poklis, Micah J Niphakis, Ku-Lung Hsu, Benjamin F Cravatt, David A Gewirtz, M Imad Damaj, Aron H Lichtman
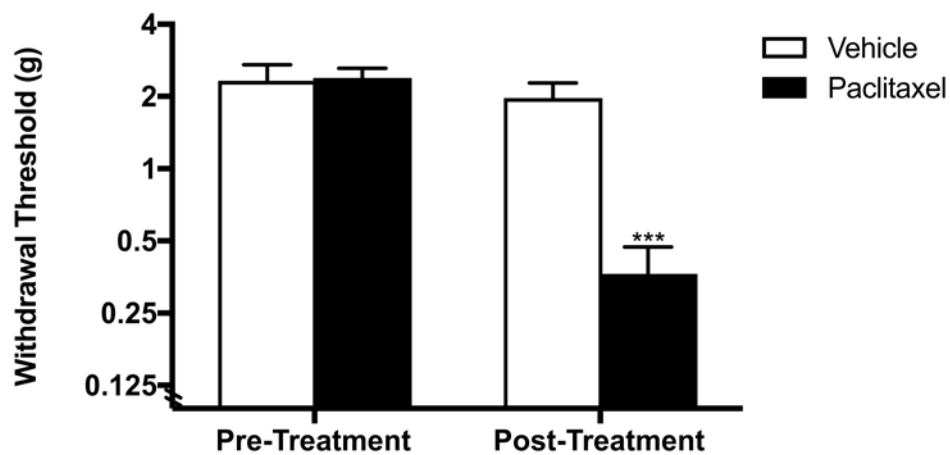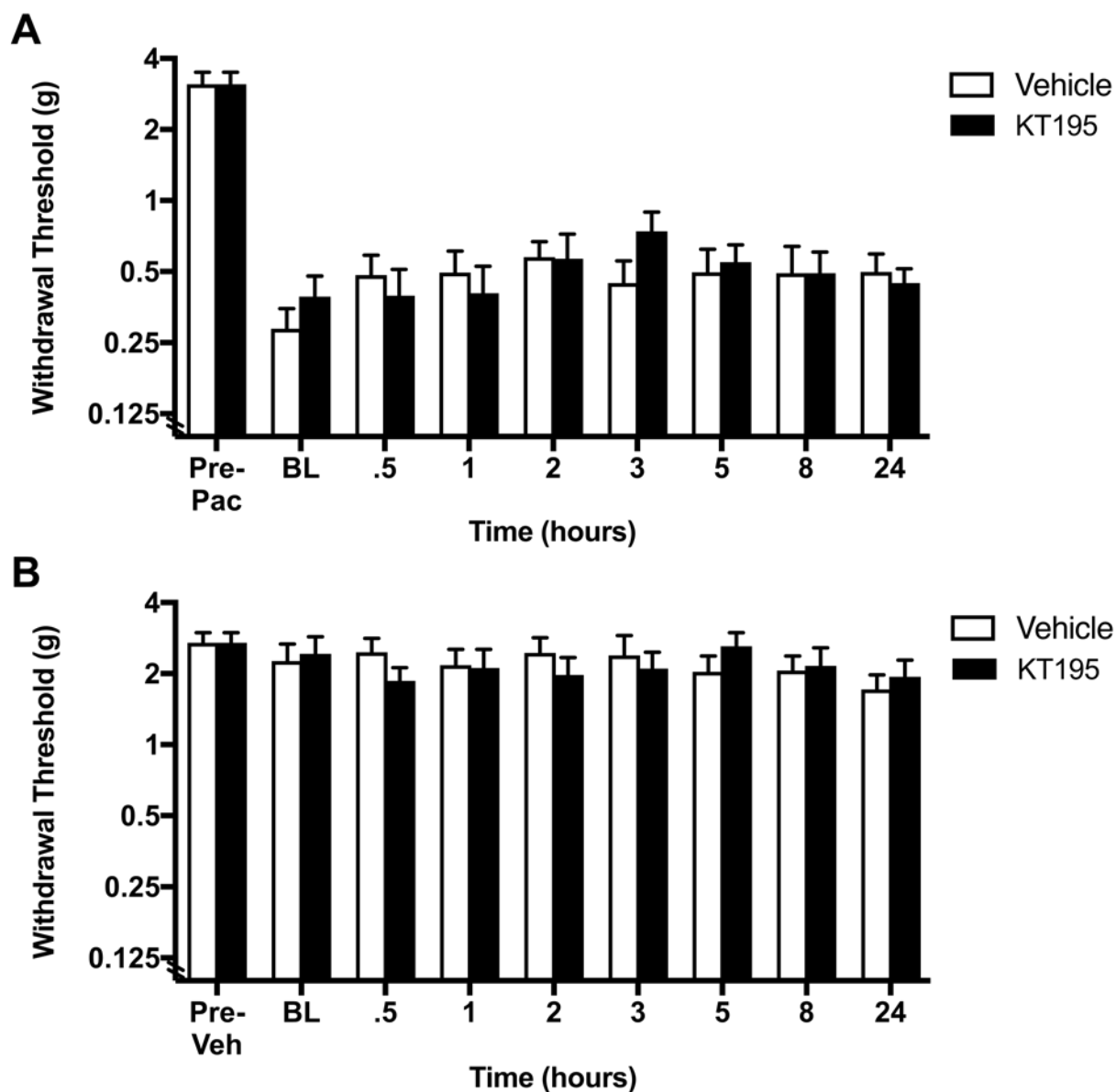
Supplemental Figure 1. A cycle of paclitaxel induces a significant mechanical allodynia. Prior to treatment with paclitaxel or vehicle, mice were assessed for paw withdrawal using von Frey filaments. After testing, 8 mg/kg of paclitaxel (or equivalent volume of vehicle) was administered i.p. on alternate days for a total of four injections (cumulative dose: 32 mg/kg). On the day after the last injection, paw withdrawal thresholds were assessed. Data are reported as mean ± S.E.M., $n$ = 7-8 mice/group. ***P<0.001 vs control (vehicle-treated) mice.

Supplemental Figure 2. MAGL inhibitors do alter paw withdrawal thresholds in control mice that did not receive paclitaxel. Neither (**A**) 40 mg/kg JZL184 nor (**B**) 5 mg/kg MJN110 altered paw withdrawal thresholds compared with vehicle. Pre-Veh= baseline prior to vehicle-treatment. BL = baseline prior to drug administration. Data are reported as mean ± S.E.M., $n$ = 8 mice/group.

Supplemental Figure 3. The ABHD6 inhibitor KT195 (40 mg/kg) did not alter paw withdrawal thresholds in either (**A**) paclitaxel (pac)-treated or (**B**) vehicle (veh) control mice. BL = baseline prior to drug administration. Data are reported as mean ± S.E.M., $n$ = 8 mice/group.
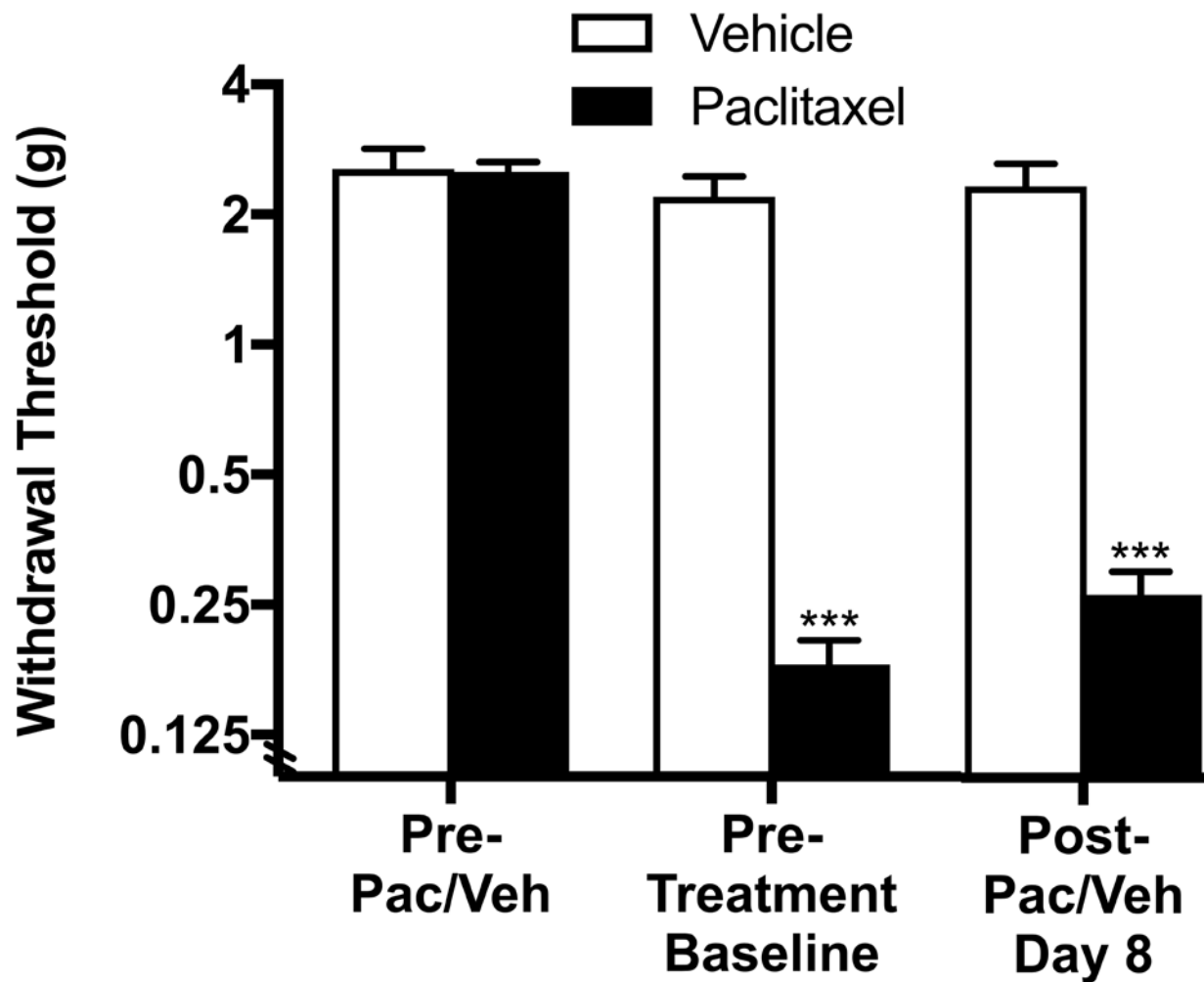
Supplemental Figure 4. Paclitaxel-treated mice from the conditioned place preference (CPP) experiment developed a significant mechanical allodynia compared to the control group that was not treated with paclitaxel. Allodynia was observed before commencing the first CPP sessions and following the conclusion of the experiment. Data are reported as mean ± S.E.M., $n = 31$-$32$ mice/group. ***$P<0.001$ vs vehicle control mice.