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Synthetic peripherally-restricted cannabinoid suppresses chemotherapy-induced peripheral neuropathy pain symptoms by CB1 receptor activation[⋆]

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a severe and dose-limiting side effect of cancer treatment that affects millions of cancer survivors throughout the world and current treatment options are extremely limited by their side effects. Cannabinoids are highly effective in suppressing pain symptoms of chemotherapy-induced and other peripheral neuropathies but their widespread use is limited by central nervous system (CNS)-mediated side effects. Here, we tested one compound from a series of recently developed synthetic peripherally restricted cannabinoids (PRCBs) in a rat model of cisplatin-induced peripheral neuropathy. Results show that local or systemic administration of 4-{2-[-(1E)-1[(4-propylnaphthalen-1-yl)methylidene]-1H-inden-3-yl]ethyl}morpholine (PrNMI) dose-dependently suppressed CIPN mechanical and cold allodynia. Orally administered PrNMI also dose-dependently suppressed CIPN allodynia symptoms in both male and female rats without any CNS side effects. Co-administration with selective cannabinoid receptor subtype blockers revealed that PrNMI's anti-allodynic effects are mediated by CB1 receptor (CB1R) activation. Expression of CB2Rs was reduced in dorsal root ganglia from CIPN rats, whereas expression of CB1Rs and various endocannabinoid synthesizing and metabolizing enzymes was unaffected. Daily PrNMI treatment of CIPN rats for two weeks showed a lack of appreciable tolerance to PrNMI's anti-allodynic effects. In an operant task which reflects cerebral processing of pain, PrNMI also dose-dependently suppressed CIPN pain behaviors. Our results

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demonstrate that PRCBs exemplified by PrNMI may represent a viable option for the treatment of CIPN pain symptoms.

Keywords

Chemotherapy neuropathy; Allodynia; CB1 receptor; CB2 receptor; Endocannabinoid enzymes; Operant behavior; Tolerance

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a severe and dose limiting side-effect of cancer treatment by antineoplastic agents, causing significant disability among cancer survivors (Argyriou et al., 2014). Neuropathy starts distally with a characteristic stocking glove distribution presenting signs of numbness, paresthesia, dysesthesia and pain aggravated by physical activity. Symptoms are observed within a few days after initiation of the therapy and continue to progress for several months, especially in the case of platinum based compounds (van der Hoop et al., 1990). Medications recommended for other neuropathy types, although used for CIPN, show limited efficacy (Hammack et al., 2002; Majithia et al., 2016; Rao et al., 2007).

Mechanisms underlying CIPN development vary depending on the cytostatic agent used (Areti et al., 2014). However, lack of protection by the blood-nerve barrier make neurons within dorsal root ganglia (DRG) highly susceptible to the actions of anticancer agents (Abram et al., 2006). Thus, damage to mitochondria of long axons and somata of DRG neurons is considered the primary reason for neuropathy (Bennett et al., 2014; Sisignano et al., 2014). In addition to mitotoxicity, direct activation of ligand or voltage gated ion channels and intracellular signaling pathways of DRG neurons by cytostatic agents alter peripheral nerve excitability resulting in dysesthesia and pain (Bennett et al., 2014; Park et al., 2009; Sisignano et al., 2014).

Cannabinoids suppress allodynia and hyperalgesia associated with chronic inflammatory and neuropathic pain states in animal and human studies (Berman et al., 2004; Herzberg et al., 1997). In fact, activation of both cannabinoid 1 and cannabinoid 2 receptor (CB1R and CB2R) induces potent anti-allodynic effects in rodent models of CIPN (Deng et al., 2012; Vera et al., 2013). While the primary site of CB2R-mediated anti-allodynic effects is still unclear (Atwood and Mackie, 2010), both tissue-selective knockout and site-specific drug administration studies demonstrate that activation of CB1Rs on primary sensory neurons contributes to the majority of anti-allodynic effects of cannabinoids observed in rodent models of chronic pain (Agarwal et al., 2007; Fox et al., 2001), which eventually led to the development of several peripherally restricted cannabinoid receptor agonists (PRCBs) to harness the potential of CB1R-mediated peripheral analgesic effects and circumvent CNS side effects (Adam et al., 2012; Dziadulewicz et al., 2007; Seltzman et al., 2016; Yu et al., 2010). Despite being known for their potent anti-allodynic effects in models of peripheral nerve injury, and most recently in a model of cancer-induced bone pain (Zhang et al., 2018), the efficacy of PRCBs in alleviating CIPN symptoms is yet to be determined. Here, we use both conventional withdrawal reflex and operant task-based behavioral assays in a rat model

of CIPN to examine the anti-allodynic efficacy of 4-{2-[-(1E)-1[(4-propylnaphthalen-1-yl)methylidene]-1H-inden-3-yl]ethyl} morpholine (PrNMI), one of the compounds from our recently developed indene series of PRCBs which we showed to be full agonists at CB1Rs and partial agonists at CB2Rs (Seltzman et al., 2016). We also examine the effect of CIPN on expression of CB1Rs and CB2Rs, as well as the various endocannabinoid synthesizing and metabolizing enzymes. Furthermore, we address cannabinoid receptor selectivity, sex differences, and development of tolerance to repeated PrNMI administration.

2. Materials and methods

2.1. Subjects

Adult male and female Sprague–Dawley rats (Envigo, Placentia, CA) weighing 200–225 g are used throughout the study. Rats are housed in the vivarium under a 12 h light/dark cycle (lights on at 6AM) and have *ad libitum* access to food and water during the entire experiment. All experimental procedures are carried out in accordance with the National Institute of Health guidelines for the handling and use of laboratory animals and with approval from the Animal Research Committee of the University of California, Los Angeles.

2.2. Cisplatin induced peripheral neuropathy

Cisplatin (Sigma-Aldrich, St. Louis, MO) is dissolved in sterile bacteriostatic 0.9% NaCl solution (Hospira, Lake Forest, IL) at a concentration of 1 mg/mL and administered by intraperitoneal (i.p.) injection (27½ gauge needle) once a week at a dose of 3 mg/kg for 4 weeks. Control rats are injected similarly with equal amounts of saline. To minimize nephrotoxicity, all cisplatin-treated rats are pre-injected subcutaneously with 2 mL of 4% $NaHCO_3$ in sterile saline (Hospira).

2.3. Behavioral testing

All behavioral experiments are performed between 9 a.m. and 6 p.m. Investigators involved in behavioral testing and data analysis are blinded to the nature and dose of the experimental drugs administered.

2.3.1. Mechanical sensitivity testing—Rats are placed in a plastic-walled cage (10 × 20 × 13 cm) with a metal mesh floor (0.6 × 0.6 cm holes) and allowed to acclimate for 15 min. The hindpaw withdrawal thresholds are determined in response to pressure from a digital von Frey anesthesiometer (Model 1601C, IITC Instruments, Woodland Hills, CA). The amount of pressure (g) needed to produce a paw-withdrawal response is measured two times on each paw separated by 5 min intervals to prevent sensitization/desensitization of responses. The results of the tests are averaged across both hindpaws. For intraplantar administration and testing, 3 trials are averaged for each paw. Allodynia suppression is calculated using the formula:

$$\% \text{ Allodynia suppression} = \left(\frac{Post - drug\ response - Pre - drug\ response}{Pre - CIPN - Pre - drug\ response} \right) \times 100$$

2.3.2.   Cold sensitivity testing—The method to evaluate withdrawal responses to cold stimulation is adapted from Guindon et al. (2013). Briefly, rats are placed in an individual plastic cages on an elevated metal mesh floor similar to that described in mechanical hypersensitivity testing and allowed to acclimate for 15 min. A 1 mL syringe without the needle is loaded with acetone at room temperature and a single drop (~20 μL) is applied through the mesh platform by gently touching the plantar surface of the hindpaw. Care is taken to avoid mechanical stimulation by the syringe tip. A flick or brisk withdrawal is considered a positive response and the percent response is obtained by averaging a total of six trials (3 from each hindpaw). Each trial is performed at least 5 min apart. Data are expressed as percent withdrawal response or % allodynia suppression (calculated as described above).

2.3.3.   Rotarod—Rats are tested for motor function and the ataxic effects of drugs as described previously (Seltzman et al., 2016). Rats are trained 72 h before the test (3 sessions 24 h apart) to remain for at least 180 s on a rotarod revolving at an acceleration of 4–40 revs over 5 min. Rats are tested 1 h before vehicle or drug injections and tested again at 2, 6, 24, and 48 h after drug administration. The time for which the rats are able to remain on the rotarod is recorded up to a cutoff of 3 min.

2.3.4.   Hypothermia—Rats are acclimated to a plastic restrainer apparatus (Model RTV-180 Braintree Scientific Inc., Braintree, MA) on the day of testing by placing them in the restrainer twice for 5 min separated by 20 min. Core temperature measurements are obtained by inserting rectally (1 inch depth) a sterile lubricating jelly (First Priority, Elgin, IL)-coated thermometer probe (model RET-2, Physitemp Instruments, Clifton, NJ) and recording the digital temperature readout (model BAT-12, Physitemp Instruments) at 1 min after probe insertion. Baseline core temperature is taken before treatment, and again at 2, 6, 24 and 48 h after drug administration.

2.3.5.   Catalepsy (ring) test—Catalepsy is determined with a ring immobility test modified for rats (Fox et al., 2001). Rats are placed with their forepaws on a horizontal metal ring (12 cm diameter) at a height that allowed their hindpaws to just touch the bench surface. Immobility is recorded as the time for the rat to move off the ring with a 100s cutoff. Rats are tested before drug administration and again at 2, 6, 24, and 48 h after administration.

2.3.6.   Tail-flick test—(model 390, IITC Instr.) is also used to measure tail-flick latency (TFL). Radiant heat is directed to a point 3 cm from the tail tip and the TFL observed and timed with a photo cell counter. The intensity of the radiant heat is adjusted for a baseline TFL of approximately 5–7 s for naïve rats, with a 25 s cutoff set to avoid tissue damage.

2.3.7.   Operant behavior analysis—A mechanical conflict avoidance system (Stoelting, Wood Dale, IL) based on Harte-Morrow method is used to assess the operant behavior of CIPN and control rats (Harte et al., 2016). The apparatus is made of bright and dark compartments separated by 15-inch (38 cm) long middle compartment arranged with an array of height-adjustable probes (0.5–4.0 mm). Before baseline measurements, 4 training sessions are carried out over 4 days. Rats are allowed to explore all the compartments without nociceptive probes for 5 min and the bright compartment is illuminated with a low

heat generating light-emitting diode bulb delivering an average of 544 lm/ft^2 at floor level. On the day of baseline recordings, rats are tested for escape latencies with nociceptive probes set at variable heights (0.5, 1.0, 2.0 and 3.0 mm). For each trial, rats are placed individually into the light compartment with the light turned off and an escape door closed. Following 10 s of dark acclimatization, compartment light is turned on for the rest of the trial. The escape door is released 20 s after the light is turned on. The animal is considered inside the dark compartment when all four paws are placed completely inside that compartment. For rats that initiated walking on the nail bed but failed to reach the dark compartment during the 3-min testing period, a cutoff latency of 120 s is used. All trials are video recorded for offline analysis.

2.4. Quantitative PCR

L4-L5 DRG are harvested from CIPN and control rats ~1 week after the administration of the 4th cisplatin or saline injection. Total RNA is extracted with RNEasy mini kit (Qiagen, Valencia, CA) and concentration is determined by Nanophotometer (Implen, Munich, Germany). 1 μg of total RNA is used for cDNA synthesis (Superscript III, Life Technologies, Carlsbad, CA) and qPCR reaction in triplicates is carried out with Taqman gene expression master mix (Life Technologies) on a 7900 HT real time fast PCR system. Primers and probes (Life Technologies, and Integrated DNA Technologies, San Diego, CA) used for qPCR reaction are shown in Table 1. The mRNA levels of cannabinoid receptors and endocannabinoid metabolizing enzymes are normalized to the internal reference gene *Gapdh*. Fold change in mRNA is calculated using the 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.5. Western blotting

L4-L5 DRG samples are isolated from the same rats used in qPCR experiments. Tissue samples are homogenized with Dounce homogenizer in a RIPA lysis buffer (Sigma-Aldrich) with Halt protease inhibitor cocktail (Invitrogen, Carlsbad, CA) and total protein concentration is determined by DC protein assay (Biorad, Hercules, CA). Samples (25 μg) are mixed with a loading dye in a 1:1 ratio and heated to 65 °C for 10 min prior to running on 4–15% polyacrylamide stain free gels (Mini-protean TGX Stain free gels, Biorad). Proteins are segregated under denatured running conditions and transferred to 0.2 μ PVDF (Immun-blot, Biorad) using a wet transfer method. Blots are incubated with anti-L15 CB1R 1° antibody in 2% ECL prime blocking reagent (1:1000) overnight (16 h) at 4° C followed by an HRP-conjugated 2° antibody (2.5% NFDM, 1:2000, Millipore, Temecula, CA) at room temperature for 1 h. The CB1R 1° antibody has been extensively characterized in numerous publications (Coutts et al., 2002; Hajos et al., 2000). Stain-free images are taken prior to application of substrate (ECL Prime detection reagent, GE) on the blot using ChemiDoc MP imaging system (Biorad). CB1R bands are visualized by chemiluminescence detection by ChemiDoc MP imager (Biorad). Acquired images are then analyzed by ImageLab software (BioRad). Band volume intensity of each lane is normalized to the total lane protein.

2.6. Drugs

PrNMI is synthesized by Dr. Seltzman at Research Triangle Institute (RTI, Research Triangle Park, NC). SR141716 and SR144528 are also synthesized at RTI as part of the National Institute of Drug Abuse (NIDA) drug supply program. For intraperitoneal (i.p.) and intraplantar injections, PrNMI is first dissolved in a 50/50% mixture of pure DMSO and Tween 80 (Fisher Scientific), then appropriately diluted in sterile saline (1.5 mL/kg for i.p.) and administered using 29½ gauge sterile needles and 1mL syringes equipped with a 0.22 μm filter. For oral administration, PrNMI, selective CB1R antagonist, SR141716, and selective CB2R antagonist, SR144528, are dissolved in pure DMSO, appropriately diluted in 20% sweet condensed milk (16 mL/kg), and delivered intragastrically (i.g.) by oral intubation with the aid of a ball-tipped gavage needle and a 5-mL syringe. For vehicle administration, vehicles had the same composition but the active drug was omitted. In all rat cohorts, experimental drugs are administered 2–7 days after the last cisplatin injection and a minimum of 3-day interval is maintained for all the subsequent drug administrations except for tolerance studies.

2.7. Data analysis

Data are expressed as mean ± standard error of mean (S.E.M). Two group comparisons are made with unpaired *t*-test. Analysis of variance with appropriate post-hoc method is used for within and between group comparisons. Least squares non-linear regression is used to plot the dose-response curves and estimate ED_{50} values. Between group dose-response curves are compared by multiple sum of squares F test. A p value of <0.05 is considered statistically significant. All statistical analyses are performed using Prism 7 software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Cisplatin induced peripheral neuropathy

Before the start of cisplatin injections, the average mechanical withdrawal thresholds of male and female rats are 49.73 ± 2.76 g and 50.14 ± 3.06 g, respectively. Withdrawal responses to acetone application are $9.37 \pm 6.57\%$ for males and $25 \pm 8.18\%$ for females. Repeated injections of cisplatin (3 mg/kg, i.p., once weekly) result in significantly decreased withdrawal thresholds in male [$F_{treatment}$ (2.68, 18.78) = 25.62, p < 0.0001 vs baseline] and female rats [$F_{treatment}$ (2.71, 19.02) = 20.58, p < 0.0001 vs baseline], indicating mechanical allodynia (Fig. 1A–B). Similarly, withdrawal responses to acetone application become exaggerated after cisplatin injections in both cohorts [Male: $F_{treatment}$ (1.55, 10.9) = 24.15, p < 0.0001 vs baseline; Female: $F_{treatment}$ (2.48, 17.42) = 4.14; p < 0.05 vs baseline], indicating cold allodynia (Fig. 1C–D). However, a significant delay in the development of mechanical [F_{gender} (1, 70) = 9.23, p < 0.05] and cold allodynia [F_{gender} (1, 70) = 8.62, p < 0.05] is observed in female vs male cisplatin-treated rats.

3.2. PrNMI suppression of CIPN symptoms is more effective after local rather than systemic administration

Dose dependent anti-allodynic effects of PrNMI in male rats measured two hours after intraperitoneal administration are shown in Fig. 2. The 2-h time period is chosen based on our previous pharmacokinetic data from rats (Seltzman et al., 2016). Dose-response curves yielded an estimated ED_{50} of 0.49 ± 0.06 mg/kg for mechanical allodynia suppression and 0.15 ± 0.07 mg/kg for cold allodynia suppression (Fig. 2A–B). We also compared the effectiveness of local PrNMI administration at the site of allodynia testing with its systemic administration. Intraplantar administration of a vehicle or needle puncture alone has no significant effect on withdrawal thresholds to mechanical [$F_{treatment}$ (1.91, 11.48) = 0.27, p = 0.758 for vehicle; $F_{treatment}$ (1.92, 11.55) = 1.8, p = 0.209 for puncture] and cold stimuli [$F_{treatment}$ (1.35, 8.15) = 1.28, p = 0.308 for vehicle; $F_{treatment}$ (1.19, 7.16) = 1.32, p = 0.29 for puncture] (Fig. 3A–B). At 2 h post-intraplantar injection, PrNMI (0.25 mg/kg) completely suppresses cisplatin-induced mechanical and cold allodynia symptoms ipsilateral to injection [$F_{treatment}$ (2.05,12.32) = 21.95, p < 0.0001 for mechanical; $F_{treatment}$ (2.67,16.04) = 11.75, p < 0.001 for cold] with only a partial effect at the contralateral hindpaw [$F_{treatment}$ (2.1, 12.66) = 9.86, p = 0.002 for mechanical; $F_{treatment}$ (2.24, 13.46) = 10.7, p = 0.0013 for cold] (Fig. 3C–D). In contrast, systemic administration of the same dose produces only partial suppression of neuropathy symptoms at its peak effect [$F_{treatment}$ (2.23, 15.61) = 11.02, p = 0.0008 for mechanical; $F_{treatment}$ (2.49, 17.45) = 10.54, p = 0.0006 for cold] (Fig. 3E–F). One-way analysis of variance revealed that at 2 h post-PrNMI, mechanical but not cold allodynia suppression is significantly greater on the ipsilateral paw compared to contralateral paw or systemic administration [$F_{treatment}$ (2, 18) = 5.7, p = 0.012] (Fig. 3G–H).

3.3. PrNMI is equally effective in male and female rats after oral administration

Previous studies demonstrated sex differences in rodent responses to administration of centrally active cannabinoids (Cooper and Craft, 2018; Cooper and Haney, 2016; Craft et al., 2013b; Tseng and Craft, 2001). To address possible sex differences, we compared the dose response curves of PrNMI after oral administration. PrNMI at 3.0 mg/kg completely suppresses mechanical [p < 0.0001 vs pre-drug] and cold allodynia symptoms [p < 0.0001 vs pre-drug] in male and female rats with significant effects lasting >24 h after administration (Fig. 4A–B). Furthermore, oral PrNMI dose-dependently reduces CIPN symptoms in both male and female rats with no significant difference among their ED_{50} values (Fig. 4C–D). The estimated ED_{50} values in male and female rats after oral administration are 0.59 and 0.60 mg/kg for mechanical allodynia suppression and 0.47 mg/kg each for cold allodynia suppression. In addition, oral PrNMI does not elicit any CNS mediated side-effects in naïve female rats at doses 0.1, 0.3,1.0, 3.0 & 10.0 mg/kg (Fig. 5), similarly to our previously reported results in male rats (Seltzman et al., 2016). Tetrad testing of the same female rats after cisplatin treatments also shows no CNS side effects at 0.3, 1.0 & 3.0 mg/kg, i.g. PrNMI (Fig. 5), suggesting that the restriction of PrNMI to the periphery is unaffected by cisplatin treatments. However, tail flick latencies of these rats are significantly increased post-chemotherapy (p < 0.05 vs pre-CIPN) indicating cisplatin-induced heat hypoalgesia (Fig. 5D1 and D2) (Han et al., 2014; Hopkins et al., 2016; Seto et al., 2016). Since PrNMI is

equally effective in male and female CIPN rats, we continued all subsequent experiments with male rats.

3.4. Anti-allodynic effects of PrNMI in CIPN are mediated mainly by CB1Rs

PrNMI is a full agonist at CB1R and only a partial agonist at CB2R (Seltzman et al., 2016). To determine the relative contribution of CBR subtypes to the alleviation of CIPN symptoms, we co-administered PrNMI with either CB1R or CB2R antagonists. Oral administration of PrNMI at 1.0 mg/kg produces submaximal suppression of CIPN symptoms (Fig. 6A–B) which are blocked, albeit incompletely, by the selective CB1R antagonist SR141716 (Fig. 6E–F), but not the selective CB2R antagonist SR144528 (Fig. 6C–D), indicating that the anti-allodynic effects of PrNMI in CIPN are mediated mainly by CB1Rs.

3.5. Lack of appreciable tolerance after repeated PrNMI administration

Development of tolerance and dependence are of major concern with the repeated use of synthetic and naturally occurring cannabinoids. Tolerance to antinociceptive effects has been observed after 3–7 daily administrations in uninjured animals (Bass and Martin, 2000; Gonzalez et al., 2005). By contrast, no appreciable tolerance to the anti-allodynic effects of cannabinoids have been observed in clinical trials (Burstein, 2005; Russo, 2008) or to continuous delivery of cannabinoids using osmotic mini-pumps in a rodent CIPN model (Rahn et al., 2014). To address the potential development of tolerance to anti-allodynic effects of PrNMI in the CIPN model, we administered PrNMI daily (1 mg/kg, i.g.) for two weeks and compared the post-treatment responses to their pre-treatment baselines. Results show no significant tolerance to suppression of both mechanical (significant effect of treatment [$F_{treatment} (1,12) = 30.56$, p = 0.0001] and no interaction [$F_{treatment \times time} (14,168) = 1.1$, p = 0.354]) and cold allodynia (significant effect of treatment [$F_{treatment} (1,12) = 32.07$, p = 0.0001] and no interaction [$F_{treatment \times time} (14,168) = 0.71$, p = 0.761]) during the two-week testing period (Fig. 7A–B).

3.6. PrNMI suppresses CIPN symptoms in an operant-based mechanical conflict system

A significant portion of cancer survivors experience CIPN-induced functional disability often characterized by slow walking speed and increased risk of falls (Hile et al., 2010; Lichtman et al., 2012; Winters-Stone et al., 2017). To date there have been no studies that examined the efficacy of cannabinoids in improving CIPN-induced functional disability in rodent models. Mechanical conflict system is an operant task in which rats are given a choice to remain in an aversive bright light compartment or to escape to a dark compartment by crossing an array of nociceptive probes (Fig. 8A). Prior to saline/cisplatin treatment, the average escape latencies measured in control and CIPN groups combined at probe heights of 0.5, 1, 2 and 3 mm are 32.13 ± 6.54, 27.16 ± 5.07, 50.91 ± 8.96 and 58.94 ± 9.15 s respectively (Fig. 8B). These baseline escape latencies show a positive correlation between probe height and latency (r = 0.93, Pearson correlation; p = 0.03, one-tailed *t*-test; n =16 rats) suggesting that elevated probes are relatively more nociceptive. However, in subsequent tests the escape latencies measured at 3 mm probe height decreased significantly in control rats, most likely due to improved performance in navigating through the nail bed after repeated testing. In contrast, the mean escape latencies measured at the same probe height have remained significantly higher in cisplatin-treated rats indicating that CIPN impairs rat's

ability to cross the nail bed [$F_{treatment}$ (1, 56) = 20.77, p < 0.001 vs control; F_{time} (3, 56) = 2.96, p = 0.039; $F_{treatment \times time}$ (3, 56) = 1.04, p = 0.37]. (Fig. 8C). Administration of PrNMI (0.1 mg/kg, i.p.), but not vehicle, in CIPN rats significantly reduces escape latencies at 2, 6 and 24 h post-injection (Fig. 8D–E). However, higher doses of PrNMI at 0.3 and 0.5 mg/kg decrease escape latencies for up to 48 h post-injection indicating longer-lasting suppression of CIPN-induced pain behaviors (Fig. 8F–G). These results demonstrate that PrNMI can effectively improve performance of CIPN rats in a mechanical conflict system-based operant assay.

3.7. CB2R mRNA is downregulated in L4-L5 DRG of CIPN rats

The relative transcript levels of cannabinoid receptors and endocannabinoid metabolizing enzymes of control rats are shown in Fig. 9 (Fig. 9A). Compared to saline, cisplatin treatments have no significant effect on the relative mRNA and protein levels of CB1Rs in L4-L5 DRG (Fig. 9B–C). In addition, mRNAs of endocannabinoid synthesizing and degrading enzymes also remain statistically unaffected in individual comparisons although an overall treatment effect is observed on mRNA expression [$F_{treatment}$ (1,48) = 22.68, p < 0.0001 vs control]. By contrast, CB2R mRNA expression is significantly decreased in the DRG of cisplatin-treated rats (Fig. 9B).

4. Discussion

The current study demonstrates that PrNMI, one of our recently developed indene series of PRCBs (Seltzman et al., 2016), potently suppresses painful CIPN symptoms after local, systemic and oral administration. The anti-allodynic effects of PrNMI are demonstrated to be primarily mediated by CB1R and not CB2R activation, with minimal centrally-mediated side effects. Moreover, we demonstrate a lack of appreciable tolerance to repeated PrNMI administration, similar anti-allodynic potency and efficacy in males and females, and prolonged suppression of pain behaviors in the operant task.

Using intraplantar administration of PrNMI, we show a complete suppression of CIPN symptoms ipsilateral to injection with a partial effect on the contralateral paw, suggestive of drug entry into systemic circulation to reach the contralateral paw. Anti-allodynic efficacy of cannabinoids after local administration has been demonstrated previously in several models of peripheral nerve injury and inflammation (Agarwal et al., 2007; Amaya et al., 2006; Fox et al., 2001). Our results are consistent with previous findings that showed complete suppression of CIPN symptoms by a peripherally restricted fatty acid amide hydrolase inhibitor, URB937, in a rat model of cisplatin-induced neuropathy (Guindon et al., 2013). In contrast, intraplantar administration of WIN 55,212–2, a non-selective cannabinoid agonist, failed to produce anti-allodynic effects in rat models of paclitaxel and vincristine induced neuropathy (Pascual et al., 2005; Rahn et al., 2007). Thus, it remains to be investigated if PRCBs will be effective in other CIPN models.

The dose-dependent anti-allodynic effects of PrNMI revealed an ED_{50} of 0.49 and 0.15 mg/kg for inhibition of mechanical and cold hypersensitivity, respectively. To our knowledge, this is the first study to demonstrate differential effectiveness of peripheral CB1R activation on mechanical and cold allodynia suppression in a CIPN model. However,

suppression of cold allodynia by WIN 55,212–2 at doses without significant effects on mechanical allodynia has been shown previously in a rat model of spinal nerve ligation neuropathy (Bridges et al., 2001). Although the underlying mechanisms are still unclear, studies suggest that cannabinoids may antagonize the actions of a menthol and icilin sensitive transient receptor potential M8 (TRPM8) ion channel (De Petrocellis et al., 2007, 2008, 2011), which is activated by cold and is mostly expressed in a nociceptive subpopulation of DRG neurons (Dhaka et al., 2007, 2008). The role of TRPM8 in the development of cold hypersensitivity has been demonstrated previously in animal and human CIPN studies (Gauchan et al., 2009; Kono et al., 2012; Ta et al., 2010). A recent study showed that global CB1R deletion exaggerated the responses to hindpaw cold stimulation in naive and neuropathic mice suggesting that CB1R activation may protect against the development of cold allodynia (Sideris et al., 2016). In contrast, other studies have demonstrated a lack of such effects of CB1R deletion on cold sensitivity before and after induction of neuropathy (Castane et al., 2006; Deng et al., 2015). Future studies using tissue selective knockout mice should clarify the role of peripheral CB1Rs in cold allodynia development.

We show that orally administered PrNMI is equipotent in male and female rats as determined by their ED_{50} values. Previous studies of non-selective brain-permeant cannabinoids have demonstrated greater potency and efficacy of their analgesic effects in females than in males (Cooper and Craft, 2018). For example, peripheral or systemic administration of tetrahydrocannabinol was more effective in females than males in a rat model of persistent hindpaw inflammation and the anti-allodynic/anti-hyperalgesic effects of peripherally administered tetrahydrocannabinol were mediated by both CB1R and CB2Rin females, contrary to CB1R only in males (Craft et al., 2013a). In another study, selective activation of peripheral CB1Rs in a rat model of persistent orofacial inflammation showed robust anti-hyperalgesic effects in males and only a moderate effect in females (Niu et al., 2012) suggesting that peripheral CB1R activation mediates most of the analgesic effects of cannabinoids in male rats. Even though PrNMI is equipotent in male and female CIPN rats, we acknowledge that it will be important to determine the type(s) of cannabinoid receptor(s) involved in the anti-allodynic actions of PrNMI in female CIPN rats.

We also show that PrNMI inhibits CIPN behaviors in an operant task with greater efficacy and longer duration of action than in reflex withdrawal assays. The increased duration of action cannot be due to drug accumulation because the estimated plasma half-life of PrNMI after i.p. administration in rats is 7.2 h (Seltzman et al., 2016) and test doses are administered randomly at 3-day intervals (vehicle-0.3–0.1–0.5 mg/kg). Unlike operant assays, reflex measures may not mirror clinical states of pain as they don't necessarily address cerebral processing in animals (Vierck et al., 2008). In the mechanical conflict system operant assay, an animal's choice to escape aversive bright light by crossing an array of nociceptive probes measures a behavior that reflects affective and motivational components of pain (Harte et al., 2016). The linear relationship observed between the probe height and escape latency suggests that an escape pathway with elevated and relatively more painful probes increases the latency to reach the dark compartment. Moreover, longer escape latencies in CIPN rats as compared to control rats also suggests that neuropathy may amplify pain intensity or increase aversiveness to probes in CIPN rats. Impaired mobility as a

causative factor for increased latencies is unlikely as previous studies (Cata et al., 2008; Garcia et al., 2008) and our rotarod data indicates no significant motor effects of cisplatin at doses administered in this study. Greater efficacy and longer duration of action of PrNMI in the operant task versus reflex assays also indicates an involvement of affective and motivational components of pain. Previously, peripherally and spinally administered analgesics have been shown to induce motivated behavior in rats (LaBuda and Fuchs, 2001; Martin et al., 2006) and in humans (Duse et al., 2009) in chronic pain states. After PrNMI administration, CIPN rats may perceive nociceptive probes as less painful while escaping from the light compartment and exhibit greater motivational drive to escape resulting in shorter latencies. In contrast, we do not observe any significant effect of PrNMI treatment in control rats, unlike morphine which significantly increases escape latencies in the mechanical conflict system assay (Harte et al., 2016), presumably due to an anxiolytic effect and reduced aversiveness to bright light by morphine. Lack of significant anxiolytic effects by PrNMI in control rats underscores its peripherally mediated effects on CIPN behaviors.

Our qPCR data show that CB2R mRNA levels decrease in the DRG of CIPN rats. Previous studies in CIPN models reported either no change or were unable to detect CB2R transcript expression in the DRG (Guindon et al., 2013; Khasabova et al., 2014). It has been shown that mRNA of CB2R undergoes alternative splicing with differential expression of splice variants in various tissues (Zhang et al., 2015). In the current study, qPCR primers are designed to target exons 1–2 of rat cnr2 transcript variant NM_001164142, which is highly expressed in pure neuronal cultures of rat DRG as determined by high throughput RNA sequencing in our previous study (Hirai et al., 2017). This isoform has also been shown previously to be expressed in CNS neurons (Van Sickle et al., 2005; Zhang et al., 2017). Reduced levels of CB2R protein in the hindpaw skin has been reported in a murine model of cisplatin induced neuropathy (Khasabova et al., 2014). Despite decreases in peripheral CB2R expression, CB2R agonists effectively suppress allodynia in CIPN models, suggesting that these actions may be centrally mediated (Rahn et al., 2007, 2014). We also show lack of significant changes in CB1R mRNA and protein levels in CIPN which is in agreement with previous studies that reported lack of significant changes in CB1R mRNA in DRG (Guindon et al., 2013) and spinal cord (Guindon et al., 2013; Rahn et al., 2014) in CIPN models indicating that chemotherapy does not affect CB1R expression. By contrast, other peripheral neuropathy models do exhibit site-specific changes in CB1R expression. For example, increased CB1R expression is seen in rat L4-L5 DRG ipsilateral to L5 spinal nerve ligation (Mitrirattanakul et al., 2006). In the rat diabetic neuropathy model decreased CB1R expression is seen in the DRG (Zhang et al., 2007). In the rat sciatic nerve chronic constriction injury, CB1R expression is predominantly increased in the ipsilateral spinal dorsal horn (Lim et al., 2003). Interestingly, in the same model, there is a decrease in CB1R expression in the ventrolateral periaqueductal grey, consistent with decreased analgesic effectiveness of WIN 55,212–2 microinjections in this area in the injured rats (Palazzo et al., 2012). These studies underscore the site-specificity of changes in cannabinoid receptor expression in different neuropathy models.

Daily oral administration of PrNMI did not result in significant tolerance to either mechanical or cold allodynia suppression during the two-week testing period. In contrast, tolerance to anti-allodynic effects has been observed with a centrally acting cannabinoid in a

mouse CIPN model (Deng et al., 2015), suggesting that the site of CB1R activation might play a role in the development of tolerance to cannabinoids. Other factors such as receptor density, potency of the CB1R agonist, agonist bias for intracellular pathways activated, and disease-induced alterations in membrane trafficking of CB1Rs could also affect the development of tolerance to cannabinoids (Breivogel et al., 2003; Lichtman and Martin, 2005; Martin et al., 2004; Tappe-Theodor et al., 2007). Further studies would be needed to assess the possible development of tolerance to continuous administration of PRCBs in CIPN models.

In conclusion, the current study demonstrates effective suppression of CIPN pain symptoms by PrNMI without appreciable CNS side effects or tolerance to repetitive administration. Thus, PCRBs may represent a viable option for the treatment of CIPN pain symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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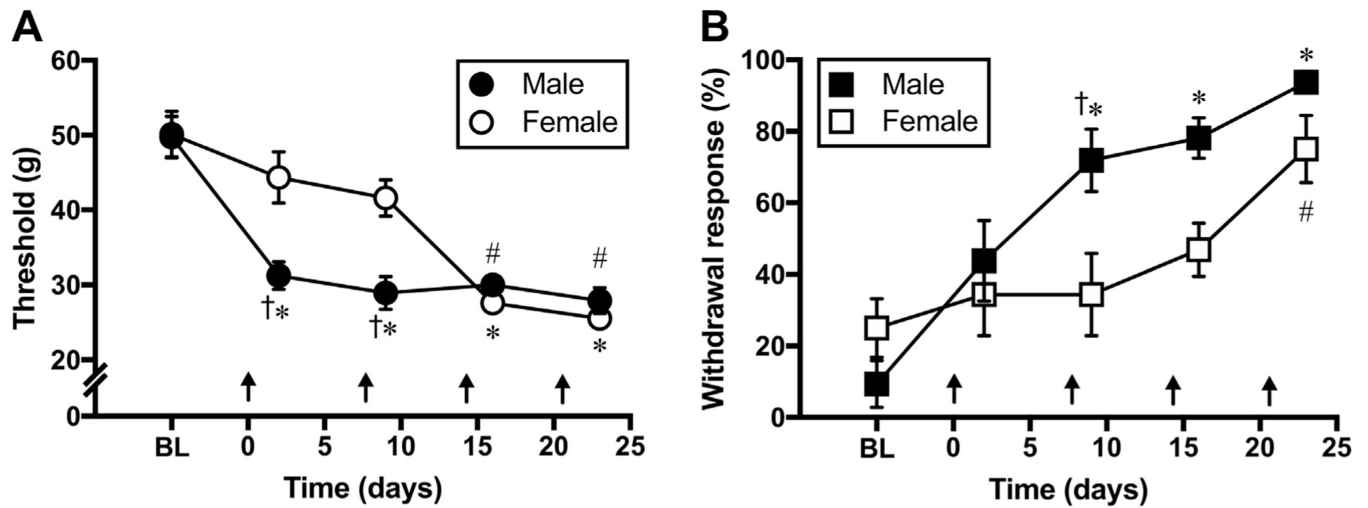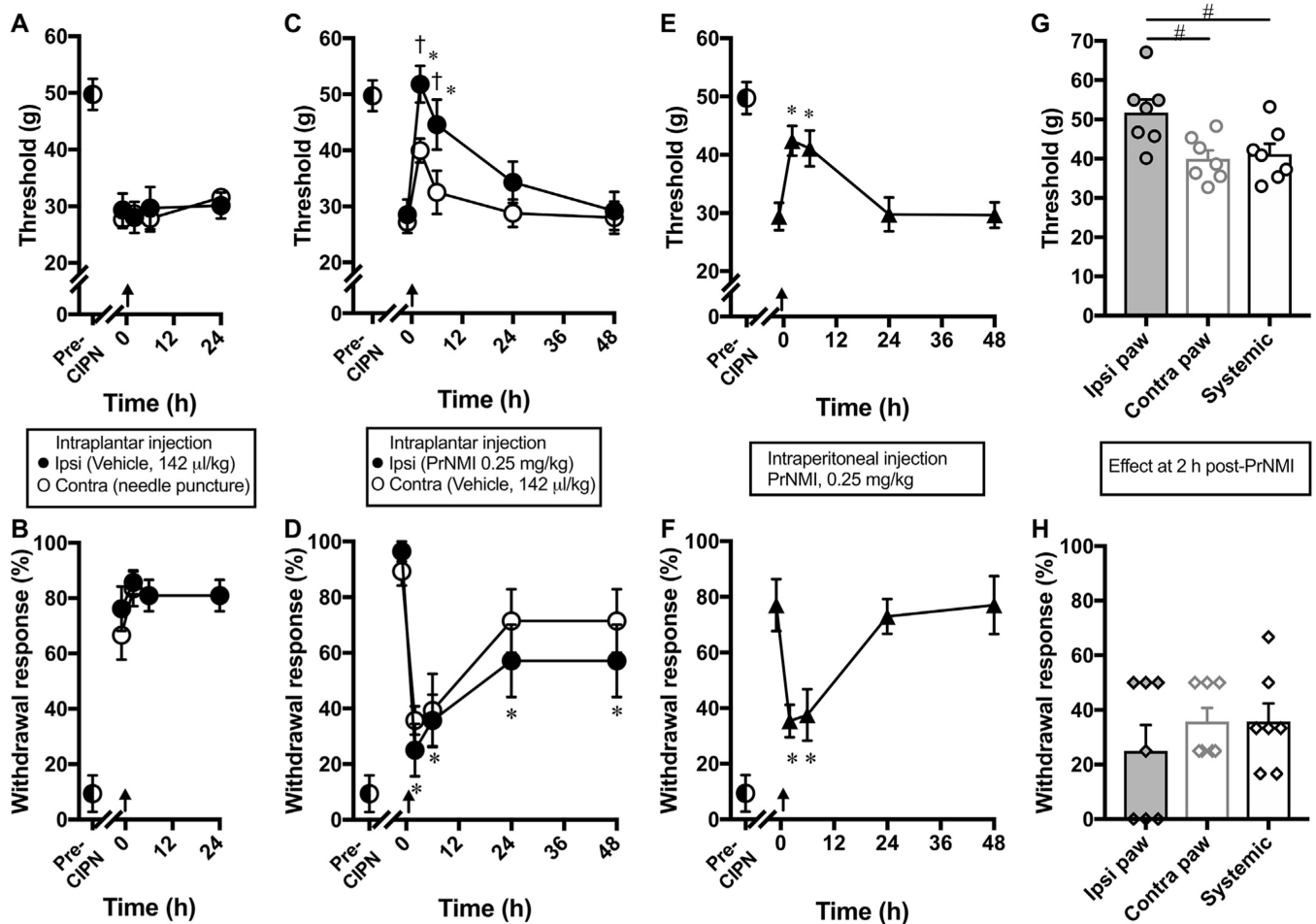
Fig. 1. Cisplatin-induced mechanical and cold allodynia in male and female rats.
(**A-B**) Time course of the development of cisplatin induced mechanical (**A**) and cold allodynia (**B**) after weekly injections of cisplatin (3 mg/kg, i.p.) in male and female rats. One-way analysis of variance shows that cisplatin treatments significantly reduce mechanical withdrawal thresholds and increase acetone withdrawal responses (%) in male (*, $p < 0.05$ vs baseline) and female rats (#, $p < 0.05$ vs baseline). However, a slight delay in the development of allodynia symptoms is noticed in female rats (†, $p < 0.05$ vs female; two-way analysis of variance, n = 8 rats/group). Arrows indicate timing of cisplatin injections.
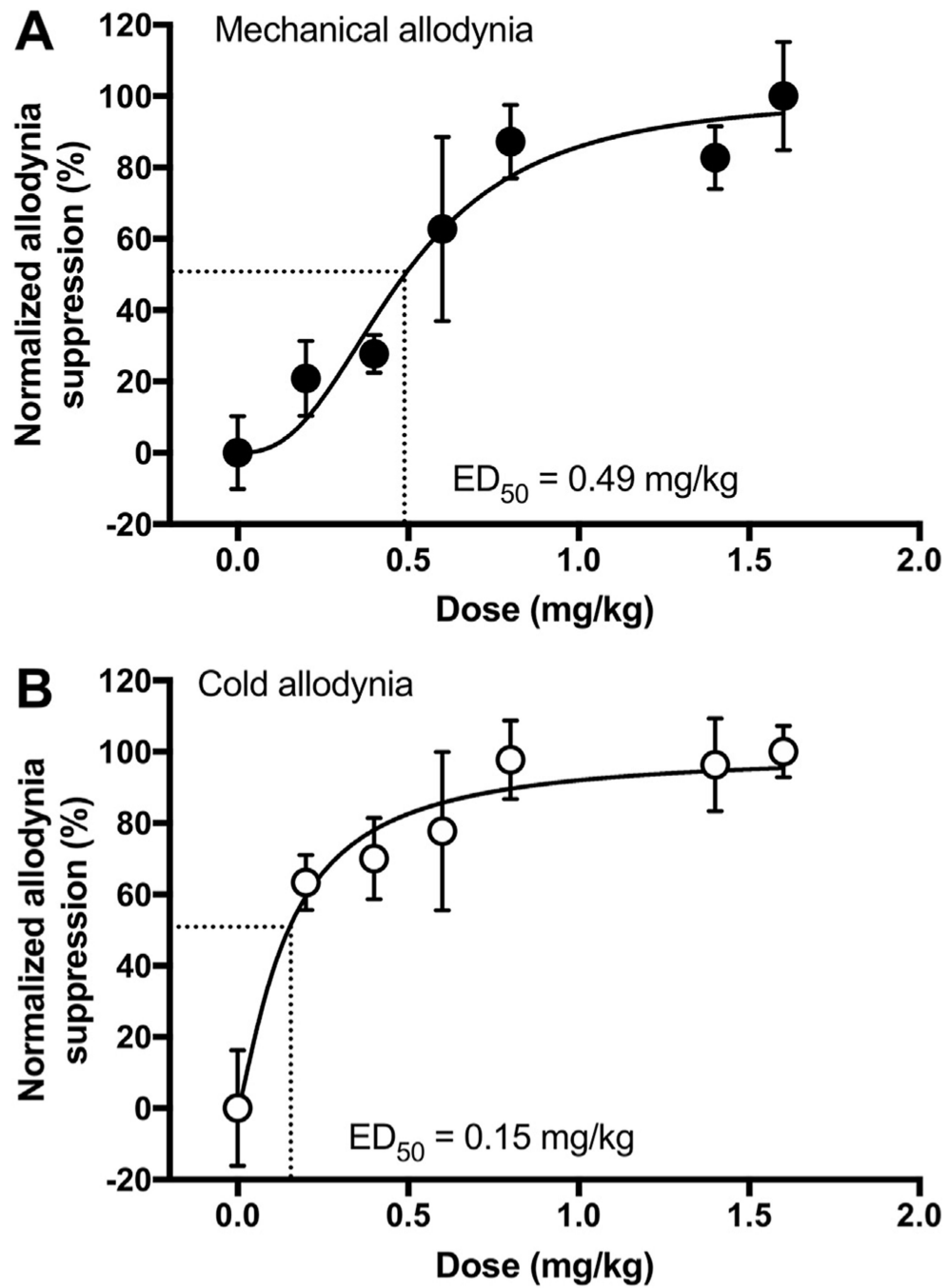
Fig. 2. PrNMI dose-dependently suppresses cisplatin-induced mechanical (A) and cold allodynia (B) in male rats.

PrNMI is administered by intraperitoneal injection. Data points are fit by least squares non-linear regression to estimate ED_{50} values (n = 6–7 rats).

Fig. 3. Suppression of CIPN-induced allodynia symptoms after local compared to systemic administration of PrNMI.

(**A–B**) Pre-CIPN (◐) responses to mechanical and cold stimuli become exaggerated after cisplatin treatment, indicative of allodynia development. Intraplantar ipsilateral vehicle (●) administration and contralateral puncture with a 29½ gauge needle (○) does not affect cisplatin-induced symptoms of mechanical (A) or cold (B) allodynia. (**C–D**) Intraplantar ipsilateral PrNMI (0.25 mg/kg) administration (●) and contralateral vehicle (○) administration results in complete suppression (at peak effect) of mechanical (C) and cold (D) allodynia symptoms on the ipsilateral side and a smaller effect on the contralateral side. (**E–F**) Systemic PrNMI (0.25 mg/kg, i.p.) administration (▲) also produces a significant but sub-maximal suppression of mechanical (E) and cold (F) allodynia symptoms. (**G–H**) Comparison of site-specific effects revealed that local PrNMI is more effective on the ipsi paw compared to contra paw or systemic dose at 2 h post-injection. Each data point represents mean ± SEM of 7 rats. Arrows indicate timing of puncture, or injection of vehicle or PrNMI. *, $p < 0.05$ vs. pre-drug values (one-way repeated measures analysis of variance). †, $p < 0.05$ vs. contralateral values (two-way analysis of variance). #, $p < 0.05$ (one-way analysis of variance).
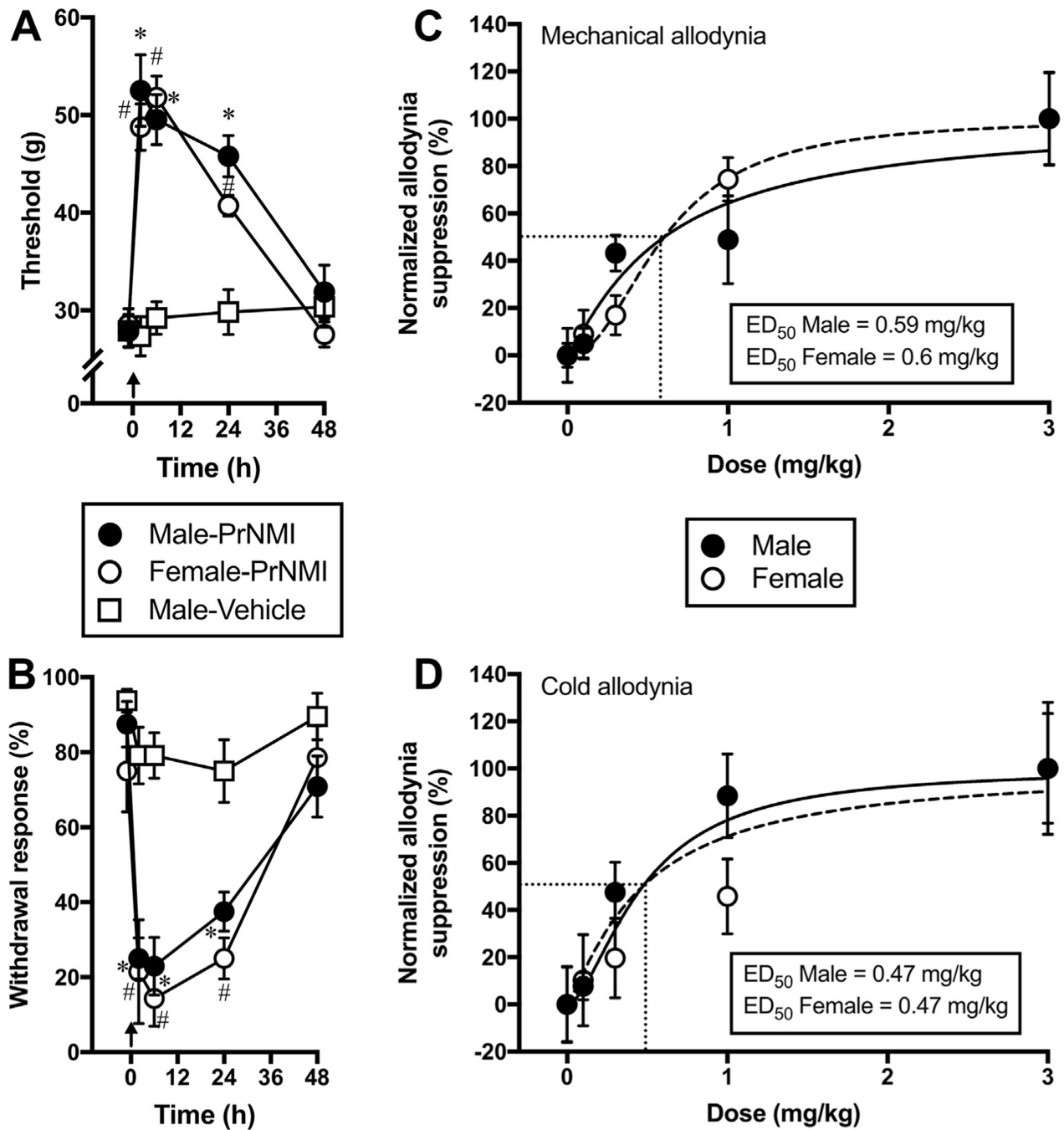
Fig. 4. Oral PrNMI dose-dependently suppresses CIPN symptoms in both male and female rats.
(**A-B**) Oral PrNMI at 3.0 mg/kg significantly reduces cisplatin induced mechanical (A) and cold (B) allodynia in male (●) and female (○) rats at 2, 6 and 24 h post-drug. No effect of vehicle (□) is observed in male CIPN rats. Arrows indicate timing of vehicle or PrNMI administrations. *(male), #(female), $p < 0.05$ vs pre-drug (one-way repeated measures analysis of variance; Šídák's multiple comparison test, n = 8 rats). (**C-D**) Cisplatin-induced mechanical (C) and cold (D) allodynia symptoms are dose-dependently reduced in male (●) and female rats (○) 2 h after oral administration of PrNMI at 0.1, 0.3,1.0 or 3.0 mg/kg. Data

points are fit by least squares non-linear regression to estimate ED_{50} values (n = 7–8 rats/ group).
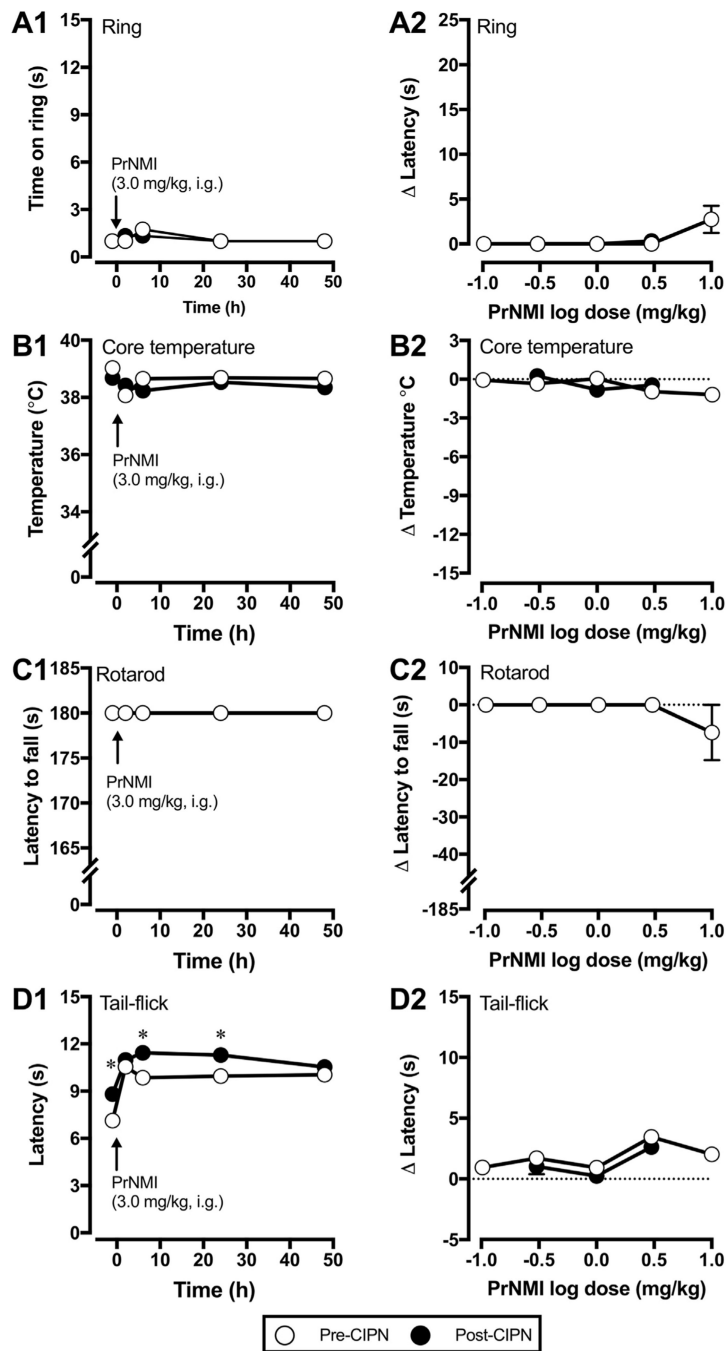
Fig. 5. Tetrad assays of oral PrNMI effects in female rats before and after CIPN.
(**A1-D1**) Comparison of time-dependent effects of PrNMI at 3.0 mg/kg, i.g. before (○) and
after (●) induction of CIPN in ring, core temperature, rotarod and tail-flick assays. Cisplatin
treatments do not affect peripheral restriction of PrNMI as observed by the lack of
significant effects in ring, core temperature and rotarod assays. However, tail-flick latencies
are significantly increased after CIPN when tested at 1 h before and 6 & 24 h after PrNMI
administration, indicative of cisplatin induced heat hypoalgesia (*,$p < 0.05$ vs. pre-CIPN,
paired t-test; Bonferroni-Šídák correction method, n = 6 rats). (**A2-D2**) At peak effect (2 h),

after oral administration of different doses of PrNMI, tetrad assays are compared before and after CIPN induction. Note the lack of significant effects at all doses tested.
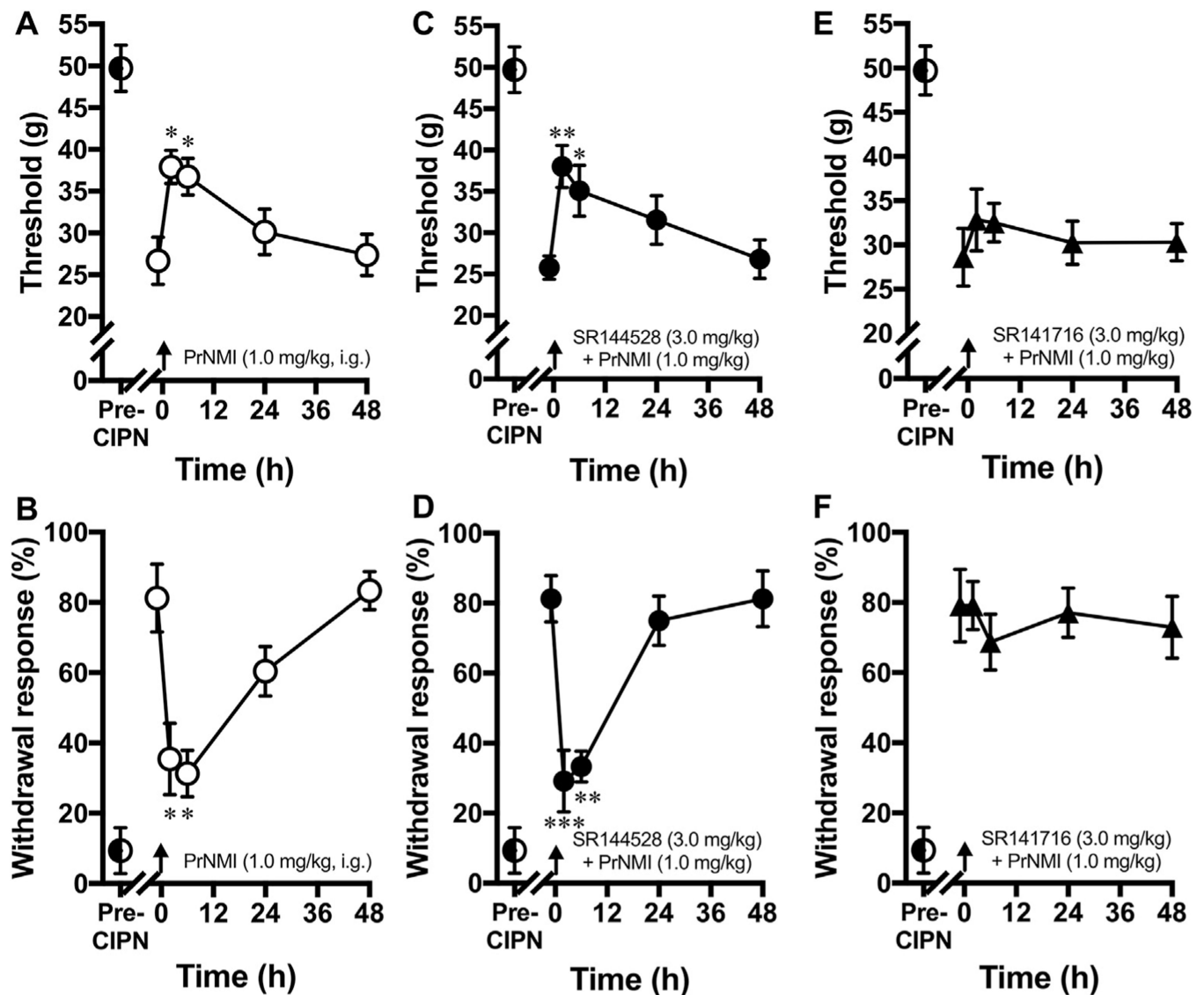
Fig. 6. Anti-allodynic effects of PrNMI in the CIPN are mediated mainly by CB1Rs.
(**A–B**) Suppression of cisplatin-induced mechanical (A) and cold (B) allodynia by PrNMI
(1.0 mg/kg, i.g., ○). (**C–D**) In the same rats, co-administration of the selective CB2R
blocker SR144528 (3 mg/kg, i.g., ●) does not affect the response to PrNMI. (**E–F**)
However, PrNMI co-administration with the selective CB1R blocker SR141716 (3 mg/kg,
i.g., ▲) blocks the response to PrNMI. Arrows indicate timing of drug administrations. *, p
< 0.05; **, p < 0.01; ***, p < 0.001 vs. pre-drug (one-way repeated measures analysis of
variance, Šídák's multiple comparison method, n = 8/group).
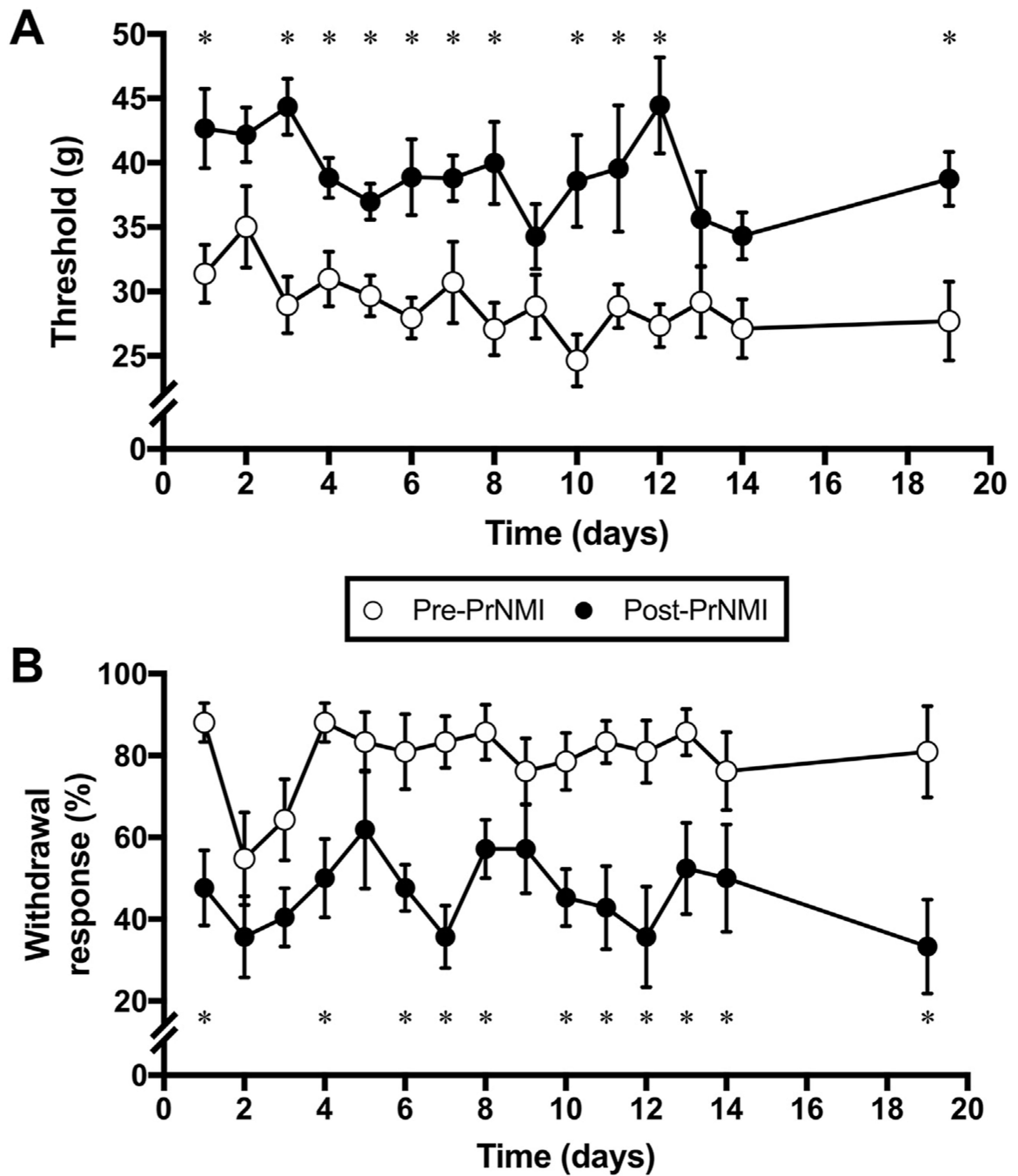
Fig. 7. Daily oral administration of PrNMI (1 mg/kg) for 2 weeks suppresses mechanical (A) and cold (B) allodynia CIPN symptoms.

*, $p < 0.05$ (two-way repeated measures analysis of variance with Fisher's least significant difference test, n = 8).
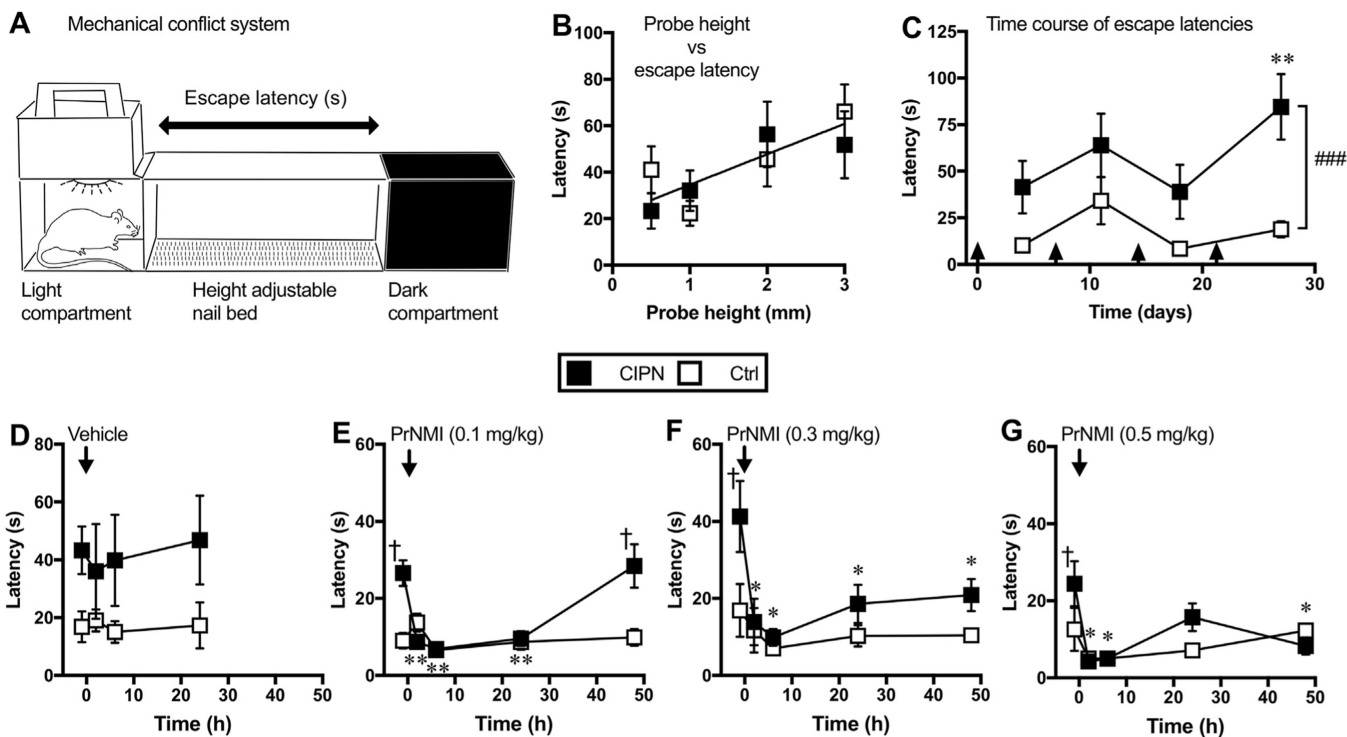
Fig. 8. CIPN behaviors are potently suppressed by PrNMI in the mechanical conflict system-based operant assay.

(**A**) Schematic view of the mechanical conflict system equipped with light and dark compartments separated by a 15-inch (38-cm) long middle compartment with a height adjustable nail bed. (**B**) XY scatter plot of baseline measurements showing a positive correlation between the probe height (mm) and escape latency (s). A single correlation coefficient is calculated for both CIPN (■) and control (□) groups. (r = 0.93, Pearson correlation; p = 0.03, one-tailed *t*-test; n = 8 rats/group). Each data point represents an average of three baseline measurements recorded before the start of cisplatin or saline injections. (**C**) Time course of escape latencies of CIPN and control rats measured with 3 mm probe height after weekly injections of cisplatin or saline. Arrows indicate time points of intraperitoneal injections. Analysis revealed significant treatment effect between the groups (###, p < 0.001, two-way analysis of variance). Tukey post hoc analysis revealed significant increase of escape latency in CIPN vs. control rats: **, p < 0.01. (**D-G**) Intraperitoneal administration of PrNMI at 0.1, 0.3 & 0.5 mg/kg, but not vehicle, significantly reduces escape latencies post-injection. *, p < 0.05; **, p < 0.01 vs. pre-injection (one-way repeated measures analysis of variance with Dunnett's post hoc method, n = 7 rats/group); †, p < 0.05 vs. control (two-way analysis of variance). Similar administration of PrNMI or vehicle in control rats has no significant effect on their escape latencies at all post-injection time points.
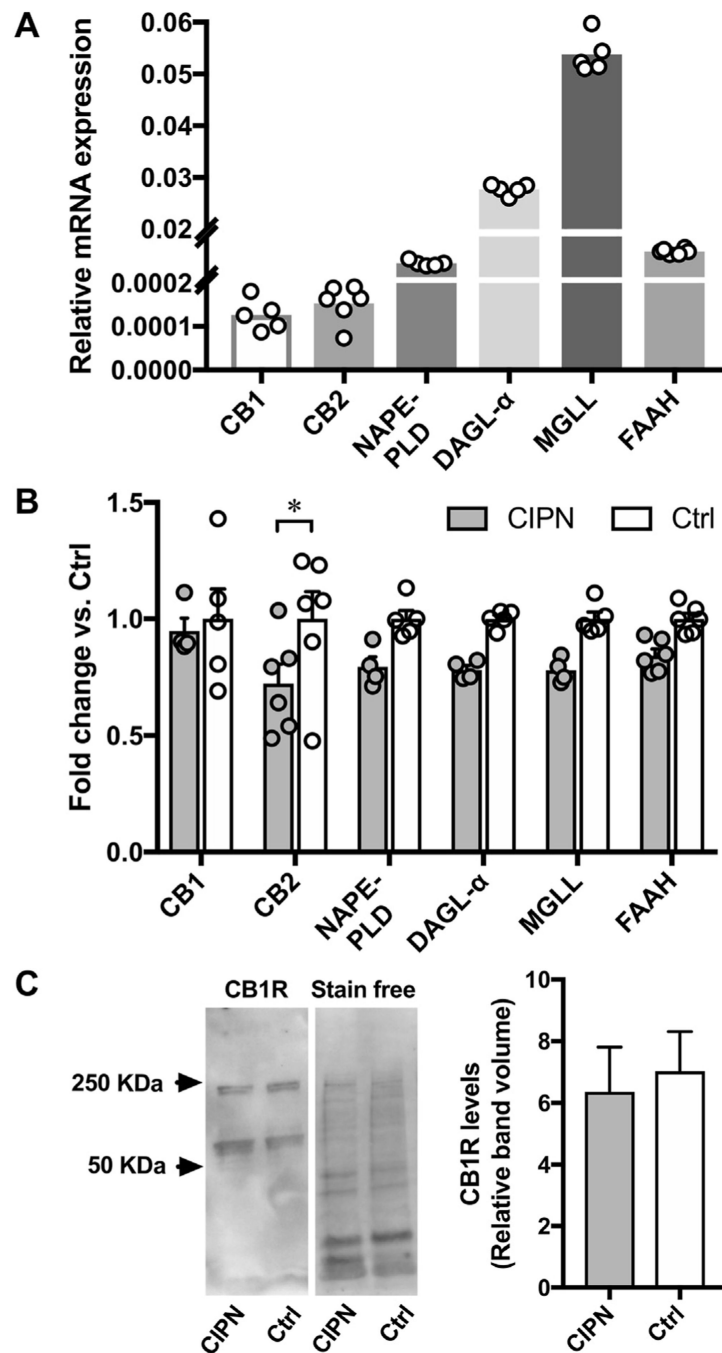
Fig. 9. Cisplatin treatments decrease CB2R mRNA without affecting CB1R mRNA, protein, and mRNAs of endocannabinoid metabolizing enzymes in L4-L5 DRG.
(**A**) Relative mRNA levels of cannabinoid receptors and endocannabinoid metabolizing enzymes after normalizing to the internal control GAPDH from the L4-L5 DRG of control rats (n = 5–6/assay). (**B**) CB2R mRNA levels are downregulated in the L4-L5 DRG of CIPN rats (*, p < 0.05; two-way analysis of variance with Šídák's multiple comparison test, n = 4–6/group). (**C**) Western blot analysis reveals no significant change in CB1R levels in L4-L5 DRG of CIPN rats (p = 0.739 vs. control group; unpaired *t*-test, n = 6/group). Note.

Additional bands at −220 KDa indicative of CB1R oligomers are also included in the quantitative analysis. NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; DAGL-α, diacyl glycerol lipase - alpha; MGLL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase.

Table 1

Primers and probes used for qPCR.

Cnr1	**Rn00562880_m1**
Cnr2	Primer 1: 5′-TCA TAG GGT TGA ACT CCA AGC-3′
	Primer 2: 5′-CAT CTA TGC TGA CTC TGA ATG GG-3′
	Probe: 5′-/56-FAM/ACT CCA GCT/ZEN/CCC GGC ATC C/3IABkFQ/-3′
Napepld	Primer 1: 5′-ACG GAT CTC TTG GCT TGA AC-3′
	Primer 2: 5′-TTT GAC CTC GCG GCT ATT C-3′
	Probe: 5′-/56-FAM/TGT GAA TCC/ZEN/TTA CAG CAT CCT CTG GG/3IABkFQ/-3′
Dagla	Primer 1: 5′-AGA GGA ACA CTT TTA GAC GGC-3′
	Primer 2: 5′-AAC CTG CGG ACT TAC AAC C-3′
	Probe: 5′-/56-FAM/CAT CGG TTA/ZEN/GAG GAG GGT CAG GC/3IABkFQ/-3′
Mgll	Primer 1: 5′-GGC AGG ACA AAA TTG AGC AG-3′
	Primer 2: 5′-AGA GAC CAA CCC ACT TTT CTG-3′
	Probe: 5′-/56-FAM/CCG GAT TGG/ZEN/CAA GGA TCA GAG GT/3IABkFQ/-3′
Faah	Primer 1: 5′-TCA CAG TCG GTC AGA TAG GAG-3′
	Primer 2: 5′-CAG AAG TTA CAG AGT GGA GAG C-3′
	Probe: 5′-/56-FAM/CCT GGG AAG/ZEN/TGA ACA AAG GGA CCA A/3IABkFQ/-3′
Gapdh	Primer 1: 5′-GTA ACC AGG CGT CCG ATA C-3′
	Primer 2: 5′-TCT CTG CTC CTC CCT GTT C-3′
	Probe: 5′-/56-FAM/CAC ACC GAC/ZEN/CTT CAC CAT CTT GTC T/3IABkFQ/-3′