Chronic Cannabinoid CB_2 Activation Reverses Paclitaxel Neuropathy without Tolerance or CB_1-Dependent Withdrawal

Supplementary Information

Supplemental Methods and Materials

Assessment of Mechanical Allodynia

Paw withdrawal thresholds (g) to mechanical stimulation were measured using an electronic von Frey anesthesiometer supplied with a 90-gram range probe (IITC Life Science Inc., CA, USA) as described previously (1). Mice were individually placed in transparent plastic chambers on an elevated metal mesh table and were habituated to the testing apparatus for 30 min prior to testing. When animals ceased exploratory behaviors, a force was applied to the midplantar region of the hind paw by a semi-flexible tip connected to the anesthesiometer. Mechanical stimulation was terminated upon paw withdrawal. Mechanical paw withdrawal thresholds (g) were measured in duplicate for each paw and reported as the mean of duplicate determinations averaged across paws.

Assessment of Cold Allodynia

The duration of time (s) spent attending to acetone-stimulated paws was used to assess cold allodynia (2). Mice were individually placed underneath transparent plastic chambers on an elevated metal mesh table. After a 30-min habituation period, an acetone bubble (approximately 20 μl) that formed at the end of a blunt one ml syringe was gently presented to the plantar surface of the hind paw with care taken to avoid inadvertent mechanical stimulation of the paw through contact with the syringe hub. The time that the animal spent responding (i.e. elevating, licking, biting or shaking) to the stimulated paw was measured over a 60 s observation period. Three measurements were taken for each paw alternately with a 5 min interval between applications.

Cold response time (s) for each animal was determined by averaging the duration of time spent responding to acetone across the six acetone-applications.

Rotarod Test

Motor performance and balance were assessed using an accelerating rotarod (IITC), as previously described (4 to 40 rpm for a cut-off time of 300 s) as previousely described (3). Mice were subjected to three training trails on two consecutive days prior to baseline testing. On the testing day, mice were subjected to three trials with 20 min intervals. Latency (s) to fall off the rotarod was recorded.

RNA Extraction and qRT-PCR

We explored the impact of paclitaxel as well as AM1710 on spinal mRNA levels of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and a chemokine (MCP-1) in paclitaxel treated WT (C57BL/6J) mice. To evaluate the impact of paclitaxel on cytokine and chemokine levels, lumbar spinal cords were harvested on day 15 following initial paclitaxel/cremophor injection from paclitaxel- and cremophor-treated WT (C57BL/6J) mice. These spinal cords are also used to characterize the effects of paclitaxel on spinal mRNA levels of proteins in the endocannabinoid systems (i.e. CB_1, CB_2, FAAH, MGL). Lumbar spinal cords (obtained 30 min following the last injection) were also harvested from paclitaxel-treated WT mice that received injections of vehicle (i.e. once daily x 8 days), acute AM1710 (i.e. once daily injections of vehicle x 7 days followed by a terminal injection of AM1710 (5 mg/kg i.p.) on the 8^{th} day) or chronic AM1710 (5 mg/kg/day i.p. x 8 days) to quantify the impact of AM1710 on cytokine and chemokine mRNA levels.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify mRNA levels from lumbar spinal cord samples as previously described (4). Lumbar

spinal cord from each subject was homogenized in TRI Reagent Solution (Ambion, CA, USA) and total RNA was extracted using RNeasy Mini Kit (Qiagen, CA, USA) following manufacturer's protocol as described previously (5). Purified RNA from each sample was then treated with DNase 1 (New England BioLabs, MA, USA). One step RT-PCR was performed using a Mastercycler ep realplex RT-PCR machine (Eppendorf, NY, USA) using PowerSYBR green PCR kit (Applied Biosystems, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard to normalize mRNA levels. The quantified mRNA levels were expressed as fold induction relative to control (i.e. cremophor vehicle in lieu of paclitaxel or vehicle in lieu of AM1710, as appropriate). Mouse primer sequences are shown in Table S1.

Table S1. Mouse primers used for RT-PCR

Primers		Sequences
GAPDH	sense	5’-GGGAAGCTCACTGGCATGGC-3’
	anti-sense	5’-GGTCCACCACCCTGTTGCT-3’
IL-1β	sense	5’-CGTGGACCTTCCAGGATGAG-3’
	anti-sense	5’-CATCTCGGAGCCTGTAGTGC-3’
IL-6	sense	5’-GCCTTCTTGGGACTGATGCT-3’
	anti-sense	5’-TGCCATTGCACAACTCTTTTC-3’
TNFα	sense	5’-CGTCGTAGCAAACCACCAAG-3’
	anti-sense	5’-TAGCAAATCGGCTGACGGTG-3’
MCP-1 (6)	sense	5’-GAAGGAATGGGTCCAGACAT-3’
	anti-sense	5’-ACGGGTCAACTTCACATTCA-3’
CB_1	sense	5’-CTGATCCTGGTGGTGTTGATCATCTG-3’
	anti-sense	5’-CGTGTCTGTGGACACAGACATGGT-3’
CB_2	sense	5’- CCTGGGATAGCTCGGATGCG-3’
	anti-sense	5’-GTGGTTTTCACATCAGCCTCTGTTTC-3’
FAAH (7)	sense	5’-GCTGTGCTCTTTACCTACCTG-3’
	anti-sense	5’-GAAGCATTCCTTGAGGCTCAC-3’
MGL	sense	5’-TCTTCCTCCTGGGCCACT-3’
	anti-sense	5’-AAAGTAGGTTGGCCTCTCTGC-3’

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1β, interleukin-1 beta; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; FAAH, fatty acid amide hydrolase; MGL, monoacylglycerol lipase.

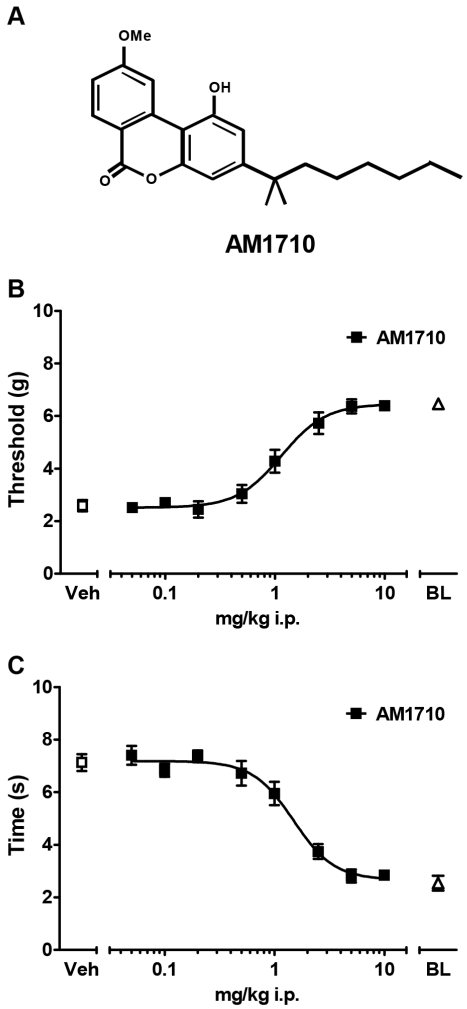

Figure S1. Acute treatment with the CB_2 agonist AM1710 produced dose-dependent anti-allodynic effects in paclitaxel-treated WT mice. (**A**) Chemical structure of AM1710. (**B**, **C**) Dose response of AM1710 (0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10 mg/kg i.p.), administered systemically, on the maintenance of (**B**) mechanical and (**C**) cold allodynia in paclitaxel-treated WT (C57BL/6J) mice. Veh, vehicle in lieu of AM1710; BL, pre-paclitaxel baseline. Data are expressed as mean ± SEM (n = 5-8 per group).

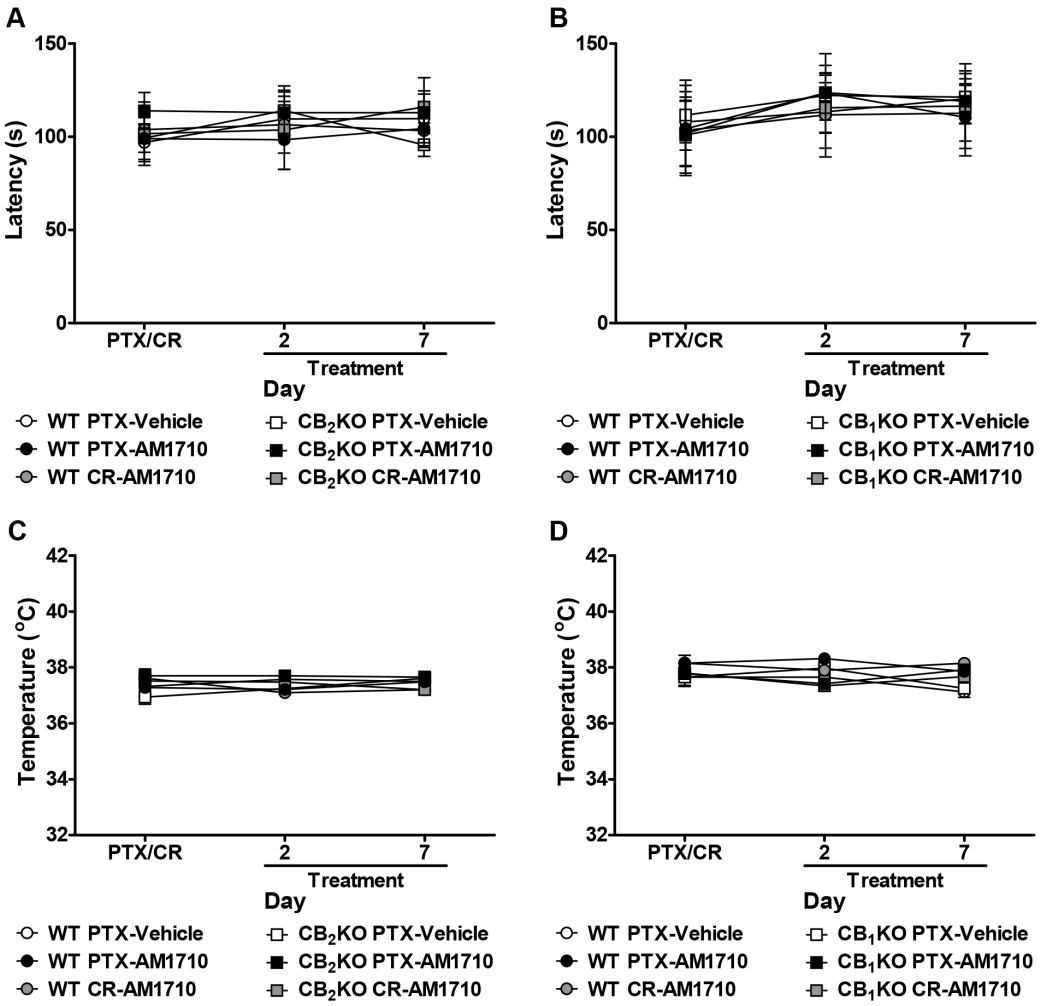

Figure S2. Chronic systemic AM1710 did not produce motor ataxia or hypothermia. (**A**, **B**) AM1710 (5 mg/kg/day i.p.) did not produce motor ataxia in the rotarod test in (**A**) CB_2KO, (**B**) CB_1KO, or respective WT littermates. (**C**, **D**) AM1710 (5 mg/kg/day i.p.) did produce hypothermia in (**C**) CB_2KO, (**D**) CB_1KO, or respective WT littermates. PTX/CR, post-paclitaxel/cremophor baseline. Data are expressed as mean ± SEM (*n* = 4-7 per group).

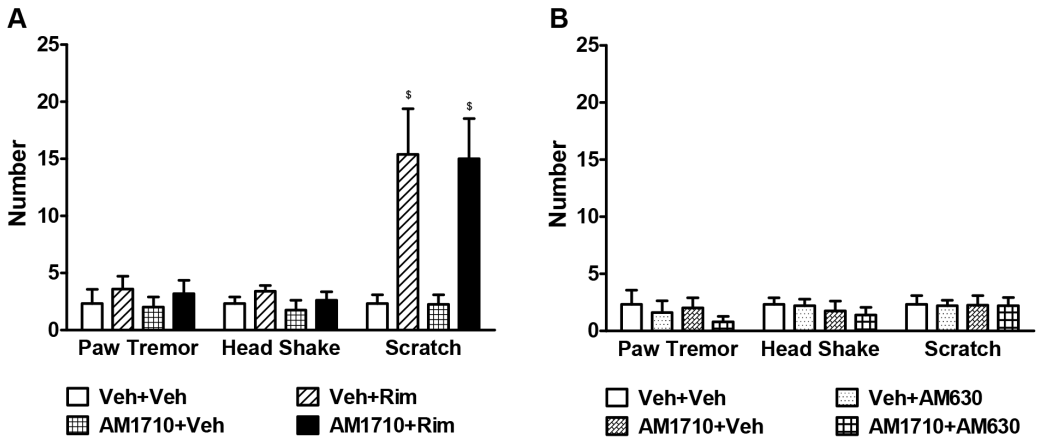

Figure S3. Effects of challenge with CB_1 antagonist, CB_2 antagonist, or vehicle on putative withdrawal behaviors in paclitaxel-treated WT (C57BL/6J) mice receiving chronic vehicle or AM1710. (**A**) Quantification of paw tremors, headshakes, and scratching behaviors in paclitaxel-treated WT mice receiving vehicle or AM1710 (5 mg/kg/day i.p. x 9 days) that were challenged with vehicle or rimonabant (10 mg/kg i.p.). (**B**) No difference between vehicle and AM630 (5 mg/kg i.p.) challenge on paw tremors, headshakes, scratching behaviors were detected in paclitaxel-treated WT mice receiving vehicle or AM1710 (5 mg/kg/day i.p. x 9 days). Data are expressed as mean ± SEM (n = 4-5 per group). Rim, rimonabant; Veh, vehicle. $^{\$}P < 0.05$ vs. Veh+Veh (chronic vehicle and challenge by vehicle), one-way analysis of variance followed by Bonferroni *post hoc* test.

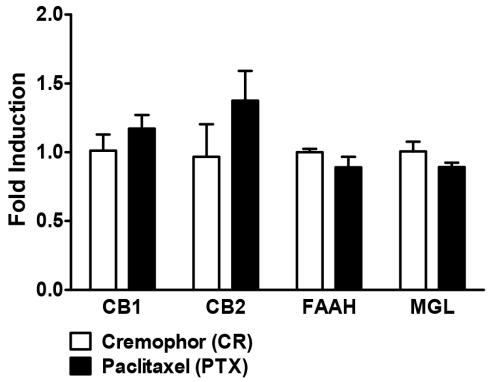

Figure S4. Impact of paclitaxel on CB_1, CB_2, FAAH, and MGL mRNA levels in lumbar spinal cord. Paclitaxel did not alter mRNA levels of CB_1, CB_2, FAAH, or MGL relative to cremophor in lumbar spinal cords of WT mice (day 15 post initial paclitaxel dosing). CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; FAAH, fatty acid amide hydrolase; MGL, monoacylglycerol lipase. Data are expressed as mean ± SEM (*n* = 3-4 per group).

Supplemental References

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5. Okada S, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, *et al.* (2006): Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med.* 12:829-834.

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