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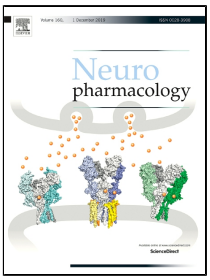

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c-Jun N terminal kinase signaling pathways mediate cannabinoid tolerance in an agonist-specific manner

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Abstract:

Tolerance to the antinociceptive effects of cannabinoids represents a significant limitation to their clinical use in managing chronic pain. Tolerance likely results from desensitization and down-regulation of the cannabinoid type 1 receptor (CB_1R), with CB_1R desensitization occurring via phosphorylation of CB_1Rs by a G protein-coupled receptor kinase and subsequent association with an arrestin protein. Previous studies have shown that (1) desensitization-resistant S426A/S430A mice exhibit a modest delay in tolerance for Δ^9-THC and CP55,940 but a more pronounced disruption in tolerance for WIN 55,212-2 and (2) that c-Jun N-terminal kinase (JNK) signaling may selectively mediate antinociceptive tolerance to morphine compared to other opioid analgesics. In the current study, we found that pretreatment with the JNK inhibitor SP600125 (3 mg/kg) attenuates tolerance to the antinociceptive and anti-allodynic effects of Δ^9-THC (6 mg/kg) in wild-type mice using the formalin test and in mice with cisplatin-evoked neuropathic pain, respectively. We also find that SP600125 causes an especially robust reduction in tolerance to the antinociceptive effects of Δ^9-THC (30 mg/kg) but not WIN 55,212-2 (10 mg/kg) in the tail-flick assay using S426A/S430A mice. Interestingly, SP600125 pretreatment accelerated tolerance to the antinociceptive and anti-allodynic effects of CP55,940 (0.3 mg/kg) in mice with acute and neuropathic pain. These results demonstrate that inhibition of JNK signaling pathways delay tolerance to Δ^9-THC, but not to CP55,940 or WIN55,212-2, demonstrating that the mechanisms of cannabinoid tolerance are agonist-specific.

Keywords: cannabinoid, CB_1R, THC, pain, JNK, tolerance, mouse, arrestin, GPCR

1.1 Introduction:

Delta-9-tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive constituent in cannabis and is used to alleviate chronic neuropathic pain associated with neurodegenerative diseases, chemotherapy, and acquired immunodeficiency syndrome (for a review, see Pacher et al., 2006). However, tolerance represents a significant disadvantage for cannabinoid therapies and has been demonstrated clinically in cancer patients receiving high doses of cannabis for pain treatment (MacCallum and Russo, 2018; Turgeman and Bar-Sela, 2019).

The effects of Δ^9-THC are mediated through the activation of two G protein-coupled receptors (GPCRs), cannabinoid receptor type-1 (CB_1R) and cannabinoid receptor type-2 (CB_2R). Recent work demonstrates that c-Jun N-terminal kinases (JNK) might play a crucial role in GPCR desensitization and tolerance for GPCR-directed ligands. JNK has been shown to be involved in tolerance to the μ-opioid receptor agonist morphine such that inactivation of JNK signaling prevented tolerance to the antinociceptive and anti-allodynic effects of morphine (Chen et al., 2008; Melief et al., 2010; Hervera et al., 2012; Marcus et al., 2015; Yuill et al., 2016) while morphine treatment increased levels of the activated phospho-JNK in transfected HEK-293 cells and rat embryonic cortical neurons (Melief et al., 2010; Cao et al., 2013; Kuhar et al., 2015). In addition, substantial evidence demonstrates that inhibition of JNK signaling attenuates multiple forms of pathological pain (Marcus et al., 2015; Gao et al., 2009; Gao et al., 2010; Zhuang et al., 2006). Similar to morphine, Δ^9-THC can also activate JNK signaling via CB_1R (Rueda et al., 2000; Downer et al., 2003; Bosier et al., 2008), raising the possibility that JNK activation may also mediate tolerance to cannabinoid drugs.

Receptor desensitization and down-regulation have been proposed as putative biological mechanisms driving behavioral tolerance to a wide variety of GPCR agonists (Gainetdinov et al.,

2004). Desensitization involves uncoupling of the GPCR from its cognate G protein(s) via phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) and the interaction of the phosphorylated receptor with arrestin protein(s) (Gainetdinov et al., 2004; DeWire et al., 2007; Moore et al., 2007). Mutant mice lacking β-arrestin2 exhibit an enhanced acute response to Δ^9-THC and show reduced tolerance and CB_1R desensitization following repeated administration of cannabinoid agonists (Breivogel et al., 2008; Nguyen et al., 2012). Desensitization of CB_1R signaling in transfected cells and oocytes requires serines 426 and 430 (S426 and S430), two putative intracellular GRK phosphorylation sites in the intracellular C-terminal tail of CB_1R (Jin et al., 1999; Daigle et al., 2008a; Daigle et al., 2008b). S426A/S430A mutant mice exhibit exaggerated responses to Δ^9-THC (Morgan et al., 2014), WIN 55,212-2 (Nealon et al., 2019), and CP55,940 (Nealon et al., 2019; LaFleur et al, 2018). S426A/S430A mutant mice also exhibit a modest (1-2 day) delay in tolerance to once-daily Δ^9-THC injections (Morgan et al., 2014) and a more pronounced delay in tolerance to once-daily WIN55,212-2 injections (Nealon et al., 2019). These data demonstrate that although GRK-mediated desensitization of CB_1R contributes to tolerance to Δ^9-THC, additional mechanisms also participate.

Our group tested the hypothesis that JNK signaling is partially responsible for the residual tolerance to Δ^9-THC observed in S426A/S430A mutant mice. We found that pretreatment of S426A/S430A mutants with the selective JNK-inhibitor, SP600125, severely disrupts tolerance to the antinociceptive, but not hypothermic effects of Δ^9-THC in an acute model of thermal pain. In wild-type mice, pretreatment with SP600125 delays tolerance to the antinociceptive effects of Δ^9-THC in the tail-flick and formalin tests, models of acute thermal and inflammatory pain, respectively. Interestingly, SP600125 pretreatment alone strongly attenuates tolerance to the anti-allodynic effects of Δ^9-THC in wild-type mice with neuropathic pain, demonstrating that JNK-

mediated tolerance to Δ^9-THC varies depending on the type of pain. Additionally, we report a ligand bias for the involvement of JNK in tolerance to different cannabinoid agonists. Interestingly, tolerance to WIN55,212-2 was unaffected by SP600125 pretreatment while tolerance to (-)-CP55,940 (CP55,940) was enhanced in the tail-flick assay, highlighting that cannabinoid tolerance most likely occurs through agonist-specific mechanisms.

2.1 **Methods**

2.2 Animals

Subjects included 520 8-16 week old male age-matched S426A/S430A mutant and wild-type mice. S426A/S430A mutant mice were generated as previously described by substituting alanines for serines at residues 426 and 430 in the carboxy terminal of the CB_1 receptor (Morgan et al., 2014). S426A/S430A mutant and wild-type littermate controls were group housed together and used for the experiments in Figures 3-8 S426A/S430A mutant and wild-type littermate mice used in these studies were backcrossed onto a C57Bl6 background for 10 generations. All work in this study using S426A/S430A mutant mice was performed using mice bred at Indiana and Penn State Universities. To be noted, the S426A/S430A mutant mouse strain is now commercially available from Jackson Laboratories as cryo-preserved embryos (JAX Stock No: 026849). Wild-type C57Bl6/J mice were ordered from Jackson Labs and used for the experiments in Figures 1-2 and 9. Mice of both genotypes were group-housed on a 12:12 hour light/dark cycle with *ad libitum* access to food and water. All animal care and experimental procedures used were approved and conducted in accordance with NIH accepted guidelines (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011) and with approval from the Institutional Animal Care and Use Committees (IACUC) at Indiana University Bloomington, Penn State University, and/or Texas Tech University Health Sciences Center.

2.3 Drugs

SP600125 was obtained from Sigma (St. Louis, MO). Δ^9-THC was obtained from the National Institute on Drug Abuse Drug Supply (Baltimore, MD). (-)-CP55,940 (referred in the text as

CP55,940), WIN55,212-2, SR141716A (referred in the text as SR1), and SR144528 (referred in the text as SR2) were obtained from Cayman Chemical (Ann Arbor, MI). Δ^9-THC, CP55,940, SR141716A, and SR144528 were dissolved in 5% ethanol and WIN55,212-2 and SP600125 in 4% DMSO. All drugs were then diluted in a 0.9% physiological saline and 5% Cremaphor EL (18:1 v/v) vehicle and prepared fresh on the day of the experiment prior to being administered intraperitoneally (IP) in an injection volume of 10 ml/kg.

2.4 Formalin Testing

The formalin test is a widely used model of inflammatory pain that elicits a biphasic pattern of pain behavior, with an initial phase (phase I) of acute pain followed by a secondary phase (phase II) of inflammatory pain (Tjolsen et al., 1992). The formalin test was used to assess the effect of 3 mg/kg SP600125 pretreatment on tolerance to the antinociceptive effects of 6 mg/kg Δ^9-THC in a model of pathological inflammatory pain. The formalin test was performed one hour after the last Δ^9-THC injection as described below. To determine the effect of pretreatment with SP600125 on the development of tolerance to Δ^9-THC, wild-type mice were injected (IP) once-daily for 1, 4, 8, or 12 days with either vehicle or 3 mg/kg SP600125 60 minutes prior to receiving a second injection (IP) of either vehicle or 6 mg/kg Δ^9-THC, resulting in the following treatment combinations: vehicle/vehicle, 3 mg/kg SP600125/vehicle, vehicle/6 mg/kg Δ^9-THC, and 3 mg/kg SP600125/6 mg/kg Δ^9-THC. Each time point was assessed using an independent cohort of mice. Formalin testing was performed and video recorded 60 minutes after the last injection of vehicle or Δ^9-THC.

Prior to testing, mice were acclimated for 20 minutes in a Plexiglas (5”x5”x5”) observation chamber placed on a transparent elevated platform. A mirror angled at 45° was placed below the

platform to allow for constant observation of the animal's paws. Following habituation, mice were administered 10μL of a 2.5% formalin solution into the plantar surface of a single hind paw. Mice were returned to the chamber immediately following formalin injection and video recorded using a high-definition digital camera (Logitech, Newark, CA) placed underneath the transparent platform. Pain behaviors were scored and quantified by a trained, blind observer within 12 consecutive 5-minute bins based on the amount of time that mice exhibited one of three behavioral categories regarding the injected paw; no behavior; the injected paw has little or no weight on it; the injected paw is raised; or, licking, shaking, biting, or rapid lifting of the injected paw. These pain-induced behaviors were assessed visually by analog scoring. Behaviors were weighted and quantified with the composite pain score-weighted scores technique, resulting in composite scores ranging from 0 (no pain) to 2 (maximal pain response) for each 5-minute bin (Watson et al., 1997; Henderson-Redmond et al., 2016; Nealon et al., 2017; LaFleur et al., 2018; Nealon et al., 2019). The area under the curve (AUC) was calculated for the acute phase (0–15 minutes; phase I) and the inflammatory phase (15–60 minutes; phase II) using the trapezoidal rule.

2.5 Cisplatin Treatment

A separate cohort of mice were made neuropathic by injecting (IP) 5 mg/kg cisplatin once-weekly for 4 consecutive weeks. Injections were done by diluting cisplatin in sterile 0.9% saline and injecting a volume of 10 mL/kg of body weight. Prior to cisplatin treatment, each mouse was treated subcutaneously (SC) with 1 mL of 4% sodium bicarbonate to prevent nephrotoxicity-induced lethality (Guindon et al., 2014).

2.6 Von Frey Testing

Mechanical allodynia was tested using the von Frey test as previously described (Deng et al., 2015a; Deng et al., 2015b; Marcus et al., 2015; Nealon et al., 2019). Prior to testing, mice were allowed to habituate for thirty minutes in individual plexiglass containment chambers placed on a wire mesh table. After habituation, mice were given the following treatment combinations to assess the effect of 3 mg/kg SP600125 on tolerance to 6 mg/kg Δ^9-THC or 0.3 mg/kg CP55,940: vehicle only (days 8-25), SP600125 only (days 8-25), vehicle (days 8-25) plus Δ^9-THC (days 11-25), vehicle (days 8-25) plus CP55,940 (days 11-25), SP600125 (days 8-25) plus Δ^9-THC (days 11-25), and SP600125 (days 8-25) plus CP55,940 (days 10-25). Pretreatment with either vehicle or 3 mg/kg SP600125 was always given 60 minutes prior to administration of 6 mg/kg Δ^9-THC or 0.3 mg/kg CP55,940. Mice were tested by a trained, blind experimenter 60 minutes after the last IP injection of: vehicle, 3 mg/kg SP600125, vehicle + 6 mg/kg Δ^9-THC, 0.3 CP55,940, vehicle (days 8-25) and Δ^9-THC (days 11-25). Testing was performed using a digital electronic von Frey anesthesiometer (IITC Life Sciences, Woodland Hills, CA) equipped with a semi-flexible plastic tip that was used to apply force to animal's mid-plantar hindpaw. Paw withdrawal thresholds were evaluated in duplicate for each hindpaw.

2.7 Acute Tolerance

Immediately following baseline assessments of body temperature and tail-flick, mice were administered (IP) either 3 mg/kg SP600125 or vehicle. One hour following pretreatment with either vehicle or 3 mg/kg SP600125, all mice were administered 30 mg/kg Δ^9-THC. All injections were given in a volume of 10 mL/kg of body weight. Tail-flick testing was measured using a Columbus Instruments TF-1 tail-flick analgesia meter (Columbus, OH). The radiant heat source

was calibrated to elicit a tail-flick latency of ~3 seconds in untreated wild-type control mice. A 10 second cutoff was used for all testing sessions to avoid tissue damage to the tail. The tail-flick latency was measured before drug treatment and also 60 minutes after drug treatment to calculate the antinociceptive response as the percent of the maximal possible effect (%MPE) with %MPE= (post-drug latency–pre-drug latency)/(10-pre-drug latency). Drug-induced hypothermia was measured by taking body temperatures using a mouse rectal thermometer (Physitemp, Clifton, NJ) at baseline and at 30, 60, 120, 180, 240, 300, 360, 420 and 480 minutes after the first injection of 30 mg/kg Δ^9-THC and calculated as % change in body temperature (%ΔBT). Approximately 420 minutes (7 hours) following the first round of injections, the same mice were given a second injection of 3 mg/kg SP600125 followed by an injection of 30 mg/kg Δ^9-THC 60 minutes later. Tail-flick latency was again assessed at 60 (t=540) minutes and body temperatures at 30 (t=510), 60 (t=540), 120 (t=600), 180 (t=660), 240 (t=720), 300 (t=780), 360 (t=840), 420 (t=900) and 480 (t=960) minutes after the second injection of 30 mg/kg Δ^9-THC in these same mice. Tail-flick and body temperature measurements were made by an experimenter blind to both treatment conditions and genotype.

2.8 Chronic Tolerance

Tolerance was assessed by measuring tail-flick antinociception and hypothermia once-daily for seven (Δ^9-THC), 10 (CP55,940, or 20 (WIN55,212-2) consecutive days in separate cohorts of mice pretreated with either vehicle or 3 mg/kg SP600125 60 minutes prior to daily IP injections of: 30 mg/kg Δ^9-THC, 0.3 mg/kg CP55,940, or 10 mg/kg WIN55,212-2. Immediately following baseline assessments of tail-flick latency and body temperature (as described above), mice were injected (IP) with either vehicle or 3 mg/kg SP600125. One hour following vehicle or SP600125 injections,

mice were given an (IP) injection of drug (30 mg/kg of Δ^9-THC, 0.3 mg/kg CP55,940, or 10 mg/kg WIN55,212-2). Tail-flick antinociception and hypothermia were reassessed 60 minutes following drug administered and reported as %MPE (antinociception) and %ΔBT (hypothermia) as previously described. Tail-flick and body temperature measurements were made by an experimenter blind to both treatment conditions and genotype.

2.9 Contribution of CB_1R and CB_2R to the Acute Effects of WIN 55,212-2, CP55,940, and Δ^9-THC

Wild-type and S426A/S430A mice (N=10; 5/genotype) were assessed once per week for 12 weeks in a within-subjects design to determine the degree to which agonist-induced antinociception and/or hypothermia are mediated through CB_1R and CB_2R. Mice were first pretreated (IP) with either vehicle (Veh), the selective CB_1R inverse agonist rimonabant (SR1; SR141716A; 10 mg/kg) or the selective CB_2R antagonist (SR2) SR144528 (10 mg/kg) 30 minutes prior to undergoing baseline assessment of tail-flick antinociception and body temperature. Immediately after baseline testing, mice were injected with either vehicle (Veh) or drug [Δ^9-THC (30 mg/kg), CP55,940 (0.3 mg/kg), or WIN55,212-2 (10 mg/kg)] and reassessed 60 minutes later. Each group of mice received a different drug combination (veh/veh; SR1/veh; SR2/veh; veh/Δ^9-THC, SR1/Δ^9-THC; SR2/Δ^9-THC; veh/CP55,940; SR1/CP55,940; SR2/CP55,940; veh/WIN55,212-2; SR1/WIN55,212-2; SR2/WIN55,212-2) each day of testing, and no group of mice was tested in the same order. Tail-flick and body temperature measurements were made by an experimenter blind to both pretreatment and genotype.

2.10 Data Analysis

Data were analyzed using Prism 6 (GraphPad, La Jolla, CA). All analyses involving formalin testing were run as two-way between measures analyses of variance (ANOVAs) with either genotype or drug treatment as one factor and day of treatment/number of injections as the second factor. All once-daily tolerance studies, the acute Δ^9-THC tolerance testing, the SR1/SR2 study, and daily cisplatin studies were assessed as two-way mixed ANOVAs where genotype or drug treatment was the between subjects factor and time/number of injections served as the repeated measure. Bonferroni post-hoc tests were performed where appropriate, and in all analyses, significance was set at $p < 0.05$.

3.1 **Results**

3.2 JNK inhibition reduces tolerance to the antinociceptive effect of Δ^9-THC in the formalin test in wild-type mice

This experiment sought to determine whether pretreatment with 3 mg/kg SP600125 could delay tolerance to 6 mg/kg Δ^9-THC in wild-type mice in the formalin test. In the acute phase (phase I) of the formalin test, we found significant main effects of drug treatment ($F_{3,39}$=32.29, p<0.0001), time/number of drug injections ($F_{3,39}$=6.479, p=0.0012), and a treatment x time interaction ($F_{9,39}$=3.040, p=0.0075) in wild-type mice. Post-hoc analyses revealed that treatment with 6 mg/kg Δ^9-THC alone resulted in a significant attenuation of pain responses relative to vehicle on days 1 (p<0.001) and 4 (p<0.05) (but not days 8 and 12), indicating that wild-type mice were tolerant to the antinociceptive effects of 6 mg/kg of Δ^9-THC by day 8. In contrast, pretreatment with 3 mg/kg SP600125 one hour prior to treatment with 6 mg/kg Δ^9-THC, produced a robust attenuation of pain responses relative to vehicle on days 1 (p<0.0001), 4 (p<0.001), 8 (p<0.0001), and 12 (p<0.01). A separate analysis revealed a significant difference between wild-type mice pretreated with 3 mg/kg SP600125 alone and SP600125 in combination with 6 mg/kg of Δ^9-THC ($F_{1,22}$=26.86, p<0.0001) with post-hoc analyses revealing that treatment with SP600125 in combination with Δ^9-THC further attenuated pain on days 1 (p<0.01) and 8 (p<0.05) compared to pretreatment with SP600125 alone. Thus, pretreatment of wild-type mice with 3 mg/kg SP600125 was able to delay tolerance development to the antinociceptive effects of 6 mg/kg Δ^9-THC in the acute phase (phase I) of the formalin model with mice failing to develop tolerance after 12 days of treatment. Of note, pretreatment with SP600125 alone induced a significant antinociceptive response relative to vehicle on days 1 (p<0.01), 4 (p<0.05), and 8 (p<0.05) (Fig 1A).

In the inflammatory phase (phase II) of the formalin test, we found main effects of drug treatment ($F_{3,39}$=49.13, p<0.0001) and time/number of drug injections ($F_{3,39}$=6.067, p=0.0017) and a treatment x time interaction ($F_{9,39}$=3.376, p=0.0038) in wild-type mice (Fig 1B). Post-hoc analyses revealed that treatment with 6 mg/kg of Δ^9-THC alone resulted in a significant attenuation of pain responses relative to vehicle on days 1 (p<0.0001) and 4 (p<0.05) (but not days 8 and 12), indicating that wild-type mice were tolerant to the antinociceptive effects of 6 mg/kg of Δ^9-THC by day 8. As in the acute phase, pretreatment with 3 mg/kg SP600125 one hour prior to treatment with 6 mg/kg of Δ^9-THC delayed the development of tolerance to Δ^9-THC, with mice failing to develop tolerance even after 12 days of combined treatment (Fig 1B). Further, pretreatment with SP600125 did not result in a greater attenuation of pain responses compared to treatment with Δ^9-THC alone on days 1 or 4. As in the acute phase (phase I), pretreatment with SP600125 alone induced significant antinociception relative to vehicle on days 1 (p<0.001), 4 (p<0.05), 8 (p<0.01) and 12 (p<0.01). Pretreatment with SP600125 in combination with Δ^9-THC further attenuated pain responses compared to treatment with SP600125 alone on days 1 (p<0.0001), 4 (p<0.0001), 8 (p<0.0001) and 12 (p<0.01).

3.3 JNK inhibition reduces tolerance to the anti-allodynic effect of Δ^9-THC in wild-type mice with cisplatin-induced peripheral neuropathy

We also examined the effect of SP600125 pretreatment on tolerance to the anti-allodynic effects of once-daily, repeated injections of 6 mg/kg Δ^9-THC in wild-type mice in a cisplatin-induced model of chronic neuropathic pain. We found no differences in baseline pain scores prior to the induction of cisplatin-induced neuropathy (p=0.978). Following cisplatin-induced neuropathy, we found significant main effects of drug treatment ($F_{3,24}$=286.1, p<0.0001) and day of treatment

($F_{14,336}$=59.46, p<0.0001) and a treatment x time interaction ($F_{42,336}$=23.69, p<0.0001). Post-hoc analyses revealed that the introduction of once-daily injections of 6 mg/kg of Δ^9-THC alone starting on day 11 significantly attenuated allodynia on days 11-13 (p<0.001), but that mice became fully tolerant to the anti-allodynic effects of 6 mg/kg Δ^9-THC by day 14 (that is, after 4 days of Δ^9-THC treatment). In contrast, mice that were pretreated with 3 mg/kg SP600125 one hour prior to the administration of Δ^9-THC starting on day 11 showed a significant attenuation of allodynia on days 11-20 (p<0.001). Furthermore, SP600125 pretreatment delayed tolerance to the anti-allodynic effects of once-daily Δ^9-THC until day 21 (following 11 days of treatment). As in the formalin test, pretreatment with 3 mg/kg SP600125 alone induced a modest, yet significant (p<0.0001) anti-allodynic effect, losing its effectiveness on day 21 (Fig 2).

3.4 Inhibition of JNK reduces acute tolerance to the antinociceptive effect of Δ^9-THC

This experiment sought to determine whether pretreatment with 3 mg/kg of the JNK inhibitor SP600125 could attenuate the development of tolerance to the acute antinociceptive or hypothermic effects that develop following two sequential injections of 30 mg/kg Δ^9-THC in both wild-type and S426A/S430A mutants. Analyses revealed main effects of SP600125 pretreatment ($F_{1,32}$=4.10, p=0.051) and time/Δ^9-THC injection ($F_{1,32}$=6.49, p=0.016), but not a treatment x time interaction (p=0.539) in wild-type mice, such that pretreatment with 3 mg/kg SP600125 blunted the development of tolerance to the acute effects of 30 mg/kg Δ^9-THC (Fig 3A). Likewise, there were significant main effects of both SP600125 pretreatment ($F_{1,45}$=5.76, p=0.021) and time/Δ^9-THC injection ($F_{1,45}$=6.12, p=0.017) as well as a significant treatment x time interaction ($F_{1,45}$=5.76, p=0.021) in S426A/S430A mutant mice. Post-hoc analyses found that pretreatment with 3 mg/kg SP600125 was able to completely block the development of acute tolerance to the

antinociceptive effects of 30 mg/kg Δ^9-THC in the tail-flick assay in S426A/S430A mice (Fig 3B). Interestingly, there was not a main effect of SP600125 pretreatment on the attenuation of acute tolerance to the hypothermic effects of two sequential injections of 30 mg/kg of Δ^9-THC in either wild-type (Fig 4A; p=0.084) or S426A/S430A (Fig 4B; p=0.796) mice. There was, however, a significant treatment x time interaction ($F_{18,288}$=1.97, p=0.011) in body temperature in wild-type but not S426A/S430A (p=0.408) mice with post-hoc analyses revealing that SP600125 attenuated the hypothermic effects of Δ^9-THC at 120 minutes (p<0.05) following initial treatment with 30 mg/kg Δ^9-THC.

3.5 JNK inhibition reduces tolerance to the antinociceptive effect of Δ^9-THC in the tail-flick test

This experiment determined whether pretreatment with 3 mg/kg SP600125 delayed tolerance to the antinociceptive and hypothermic effects of once-daily administration of 30 mg/kg Δ^9-THC in wild-type and S426A/S430A mutant mice. We saw significant main effects of both SP600125 pretreatment ($F_{1,24}$=17.11, p<0.001) and days/number of Δ^9-THC injections ($F_{6,144}$=16.52, p<0.001), but not a treatment x time interaction (p=0.881) in wild-type mice. Thus, while pretreatment with SP600125 resulted in wild-type mice being, overall, more sensitive to the antinociceptive effects of 30 mg/kg of Δ^9-THC, it did not alter the rate at which tolerance develops to Δ^9-THC compared to vehicle (Fig 5A). In S426A/S430A mutants, there were main effects of SP600125 pretreatment ($F_{1,39}$=45.92, p<0.001), day/number of Δ^9-THC injections ($F_{6,234}$=23.48, p<0.001), and a treatment x day interaction ($F_{6,234}$=9.84, p<0.001). Post-hoc analyses showed that pretreatment with 3 mg/kg SP600125 drastically delayed tolerance development to the antinociceptive effects following once-daily injections 30 mg/kg Δ^9-THC in S426A/S430A

mutants compared to mutants pretreated with vehicle, and that this difference was especially pronounced on days 3-7 (p<0.0001) of the experiment (Fig 5B).

In contrast, we failed to see an effect of SP600125 pretreatment (p=0.146) on tolerance to hypothermia following once-daily administration of 30 mg/kg of Δ^9-THC in wild-type (Fig 5C) or S426A/S430A (p=0.066) mice (Fig 5D). Interestingly, however, we found a main effect of number of Δ^9-THC injections ($F_{6,186}$=111.6, p<0.001) and a treatment x day interaction ($F_{6,186}$=4.52, p=0.005) with post-hoc analyses revealing significant differences in Δ^9-THC tolerance between vehicle and SP600125-pretreated mice on days 3 (p<0.01) and 4 (p<0.05), suggesting that pretreatment with SP600125 may briefly delay the rate at which tolerance develops to the hypothermic effects of once-daily 30 Δ^9-THC mg/kg without altering the fact that complete tolerance to hypothermia occurs in both S426A/S430A mice pretreated with or without SP600125 by Day 5 (Fig 5D).

3.6 JNK inhibition does not alter tolerance for WIN55,212-2

These next experiments determined whether JNK inhibition could also alter cannabinoid tolerance for two structurally distinct, synthetic, high potency, high efficacy cannabinoid agonists, WIN55,212-2 and CP55,940. In this first experiment, we determined whether pretreatment with 3 mg/kg SP600125 could alter tolerance to the antinociceptive and/or hypothermic effects of once-daily injections of 10 mg/kg WIN55,212-2 in wild-type and/or S426A/S430A mice. We saw a significant main effect of day/number of WIN55,212-2 injections for antinociceptive tolerance in both wild-type ($F_{19,285}$=6.55, p<0.001) and S426A/S430A ($F_{19,304}$=6.59, p<0.001) mice. However, we failed to find a main effect of SP600125 pretreatment (SP6) or a treatment x day interaction (INT) for either wild-type (SP6: p=0.113; INT: p=0.884) or S426A/S430A (SP6: p=0.687; INT:

p=0.510) mice (Fig 6A-B). Likewise, we found significant main effects of both day/number of WIN55,212-2 injections in both wild-type ($F_{(19,285)}$=16.08, p<0.0001) and S426A/S430A ($F_{(19,304)}$=14.72, p<0.0001) mice in the development of hypothermic tolerance. However, once again, we failed to see an effect of SP600125 pretreatment or a treatment x day interaction in either wild-type (SP6: p=0.209; INT: p=0.998) or S426A/S430A (SP6: p=0.010; INT: p=0.501) mice (Fig 6C-D). Taken together, these results indicate that, unlike with Δ^9-THC, SP600125 pretreatment fails to alter tolerance to the antinociceptive or hypothermic effects of once-daily administration of 10 mg/kg of WIN55,212-2 in either wild-type or S426A/S430A mutant mice.

3.7 JNK inhibition accelerates tolerance to the antinociceptive effects of CP55,940 in S426A/S430A mice

When determining whether pretreatment with 3 mg/kg SP600125 could alter tolerance development to once-daily administration of 0.3 mg/kg of CP55,940, we found main effects of SP610025 pretreatment ($F_{1,23}$=4.81, p=0.039) and day/number of CP55,940 injections ($F_{9,207}$=5.96, p<0.001, but not a treatment x day interaction (p=0.760) in wild-type mice. Though pretreatment of wild-type mice with 3 mg/kg of SP600125 decreased their antinociceptive response to 0.3 mg/kg CP55,940, it did not alter the rate at which tolerance to CP55,940 developed (Fig 7A). However, S426A/S430A mutant mice showed a main effect of SP600125 pretreatment ($F_{1,21}$=79.82, p<0.0001), an effect of day ($F_{9,189}$=10.82, p<0.001), and a treatment x day interaction ($F_{9,189}$=2.09, p=0.033). Post-hoc analyses revealed that pretreatment of S426A/S430A mutant mice with SP600125 not only decreased their sensitivity to 0.3 mg/kg CP55,940 [noted by the differences in response on days 1 and 3(p<0.05), 4-5 (p<0.001), 6-7 (p<0.0001), 8 and 10 (p<0.01)] but also

accelerated the rate at which antinociceptive tolerance to CP55,940 developed in mutant mice compared to those pretreated with vehicle (Fig 78B).

In contrast, we failed to find an effect of SP600125 pretreatment (p=0.053) on tolerance to hypothermia following once-daily administration of 0.3 mg/kg of CP55,940 in wild-type mice (Fig 7C). Interestingly, we did find main effects of both SP600125 pretreatment ($F_{1,21}$=7.94, p=0.010) and day/number of CP55,940 injections ($F_{9,189}$=54.69, p<0.001), but not a significant treatment x day interaction (p=0.432) on tolerance to hypothermia in S426A/S430A mutants. Thus, while pretreatment with SP600125 results in mutant mice being, overall, less sensitive to the hypothermic effects of 0.3 mg/kg CP55,940, it does not alter the rate at which hypothermic tolerance develops to 0.3 mg/kg CP55,940 compared to vehicle (Fig 7D).

In addition to assessing the effects of JNK on tolerance development to the synthetic cannabinoids, WIN55,212-2 and CP55,940, we also investigated the effect of JNK signaling on tolerance following the elevation of endocannabinoids using the dual fatty-acid amide hydrolase and monoacylglycerol lipase inhibitor, JZL195. Interestingly, we saw little evidence of tolerance to the antinociceptive effects of 10 mg/kg JZL195 and no indication that pretreatment with 3 mg/kg SP600125 either attenuates or accelerates tolerance for JZL195-induced enhancement of endocannabinoids (data not shown).

3.8 The antinociceptive and hypothermia effects of cannabinoid agonists are mediated primarily by CB_1R

The next set of experiments determined the extent to which the acute antinociceptive and hypothermic effects of Δ^9-THC, CP55,940, and WIN55,212-2 are mediated by CB_1R and/or CB_2R. We found a significant main effects of treatment for all three drugs for antinociception [Δ^9-THC

($F_{5,40}$=13.49, p<0.001); CP55,940 ($F_{5,40}$=17.90, p<0.001); WIN55,212-2 ($F_{5,40}$=2.91, p=0.025)] and hypothermia [Δ^9-THC ($F_{5,40}$=88.05, p<0.001); CP55,940 ($F_{5,40}$=80.64, p<0.001); WIN55,212-2 ($F_{5,40}$=30.52, p<0.001)]. Post-hoc analyses revealed that the treatment differences in hypothermic responses for Δ^9-THC, CP55,940, and WIN55,212-2 were due to differences in hypothermic responses between the veh/veh group and the groups getting veh/drug (for all drugs, p<0.001) and the SR2/drug groups (for all drugs, p<0.001; Fig 8B,D,F). In contrast, mice getting SR1 prior to cannabinoid drug did not differ from mice getting veh/veh. This result indicates that pretreatment with SR1 (but not SR2) blocked the hypothermic effects of Δ^9-THC, CP55,940, and WIN55,212-2. Similarly, treatment differences in the antinociceptive effects of Δ^9-THC and CP55,940 were caused by differences between mice receiving veh/veh and mice getting veh/drug and SR2/drug for Δ^9-THC (veh/veh vs veh/drug, p.<0.001; veh/veh vs SR2/drug, p<0.01; Fig 8A) and CP55,940 (veh/veh vs veh/drug, p.<0.001; veh/veh vs SR2/drug, p<0.001; Fig 8C). In contrast, none of the treatment groups differed from the veh/veh group in mice assessed for WIN55,212-2 mediated antinociception (Fig 8E).

3.9 JNK inhibition accelerates tolerance to the anti-allodynic effect of daily 0.3 mg/kg CP55,940 in wild-type mice following cisplatin-induced neuropathy

To determine whether the effect of JNK inhibition on once-daily treatment with CP55,940 extended to other pain models, we examined the effect of SP600125 pretreatment on tolerance to the anti-allodynic effects of once-daily, repeated injections of 0.3 mg/kg CP55,940 in wild-type mice in a cisplatin-induced model of chronic neuropathic pain. We found no differences in baseline pain scores prior to the induction of cisplatin-induced neuropathy (p=0.225). Following cisplatin-induced neuropathy, we found significant main effects of drug treatment ($F_{3,17}$=370.5, p<0.001)

and day of treatment ($F_{14,238}$=155.7, p<0.001) and a treatment x time interaction ($F_{42,238}$=44.96, p<0.001). Post-hoc analyses revealed that the introduction of once-daily injections of 0.3 mg/kg of CP55,940 alone starting on day 11 significantly attenuated allodynia on days 11-20 (p<0.001), but that mice became fully tolerant to the anti-allodynic effects of 0.3 mg/kg CP55,940 by day 21 (following 11 days of CP55,940 treatment). Interestingly, mice pretreated with 3 mg/kg SP600125 one hour prior to the administration of CP55,940, starting on day 11, showed a significant attenuation of allodynia on days 11-18 (p<0.001). However, SP600125 pretreatment actually accelerated tolerance to the anti-allodynic effects of once-daily CP55,940 with mice fully tolerant by day 18 (following only 8 days of treatment). Pretreatment with SP600125 also made mice less sensitive to the anti-allodynic effects of CP55,940, with mice showing significantly attenuated pain thresholds on days 12 (p<0.01), 15 (p<0.01), 16 (p<0.001), 18 (p<0.05), 19 (p<0.001), and 20 (p<0,001) compared to mice given CP55,940 alone. As noted previously, pretreatment with 3 mg/kg SP600125 alone induced a modest, yet statistically significant (p<0.0001; days 10-20) anti-allodynic effect, losing its effectiveness on day 20. (Fig 9).

4.1 Discussion

Previous work demonstrated that desensitization of CB_1R signaling requires phosphorylation at serine residues 426 and 430 by GRK and interaction with β-arrestin2 (Jin et al., 1999; Daigle et al., 2008a). Mice expressing the CB_1R S426A/S430A point mutations exhibit an exaggerated response to cannabinoids and a modest delay in tolerance to Δ^9-THC (Morgan et al., 2014). Although tolerance to Δ^9-THC was delayed by 1-2 days in S426A/S430A mutant mice, these mutants did eventually develop complete tolerance to Δ^9-THC. Thus, the goal of this current work was three-fold. The first objective was to test the hypothesis that a recently identified mechanisms of JNK-mediated opioid tolerance might mediate tolerance to Δ^9-THC in models of acute (tail-

flick), inflammatory (formalin), and chronic, neuropathic (cisplatin-evoked) pain models. The second objective was to test whether the effects of JNK in an acute pain model (tail-flick) could be responsible for the residual Δ^9-THC tolerance observed in S426A/S430A mice and, finally, whether the effects of JNK on tolerance are agonist-specific for Δ^9-THC or extend to other cannabinoid agonists (i.e., CP55,940 and WIN55,212-2).

In this study, we used SP600125, a selective inhibitor of JNK, to assess the role of this signaling pathway in cannabinoid tolerance. Previous work has characterized the potency, efficacy, and selectivity of SP600125 and found that it exhibits greater than 20-fold selectivity versus a range of kinases, including more than 100-fold selectivity compared to related mitogen activated kinases such as ERK and p38 (Bennett et al, 2001). Previous studies investigating the effect of this kinase inhibitor *in vivo* have used doses ranging from 10-30 mg/kg SP600125 (Bennett et al., 2001; Melief et al., 2010; Marcus et al., 2015). Interestingly, previous work found that SP600125 pretreatment blocked acute and chronic tolerance to morphine while pretreatment with the selective MEK 1/2 inhibitor, SL327, had no effect on morphine tolerance (Melief et al., 2010). In this current study, as well as our previous work investigating the role of SP600125 on morphine tolerance, we used a much lower dose of 3 mg/kg to help reduce potential off-target effects (Marcus et al., 2015).

In this study, we find that pretreatment with the JNK inhibitor, SP600125, modestly attenuates tolerance to the acute antinociceptive effects of Δ^9-THC in wild-type mice using the tail-flick test. Thus, the effect of inhibiting either the GRK/β-arrestin-2 (using the S426A/S430A mutant mice) or the non-canonical JNK (using SP600125) pathways individually caused partial, incomplete reductions in Δ^9-THC tolerance. However, the effect of SP600125 pretreatment on tolerance to the antinociceptive effects of Δ^9-THC using the tail-flick test was much more pronounced in S426A/S430A mutant mice, with these mice showing an ~85% reduction in Δ^9-THC tolerance.

This result suggests that both mechanisms coordinate to mediate Δ^9-THC tolerance, at least in the context of an acute, spinally-mediated, thermal, antinociceptive pain response.

Interestingly, tolerance to the hypothermic effects of Δ^9-THC is not significantly affected by SP600125 pretreatment in either wild-type or mutant mice. The antinociceptive response to Δ^9-THC is mediated through CB_1R signaling within neurons of the spinal cord, primary afferent nociceptive neurons, and periaqueductal gray (Smith and Martin, 1992; Lichtman et al., 1996). However, the hypothermic effects of Δ^9-THC are mediated within neurons of the hypothalamus (Rawls et al., 2002) and rostroventral medulla (Martin et al., 1998; Meng et al., 1998). Thus, our finding that JNK signaling is involved in tolerance for the antinociceptive effects of Δ^9-THC, but not the hypothermic effects, suggests the possibility of cell type and/or region-specific mechanisms of Δ^9-THC tolerance. Our results suggesting cell type/region-specific mechanisms of cannabinoid tolerance complement previous studies demonstrating brain-region differences in CB_1R down-regulation and desensitization following chronic Δ^9-THC administration (Sim et al., 1996; Nguyen et al., 2012).

This study also demonstrates a more pronounced effect of SP600125 on tolerance for the antinociceptive and anti-allodynic effects of Δ^9-THC in both the inflammatory and chemotherapy-induced models of pathological pain that were examined. Pretreatment of wild-type mice with 3 mg/kg SP600125 caused a substantial disruption, but not a complete block, in tolerance for the antinociceptive effects of 6 mg/kg Δ^9-THC in the formalin test. Other studies have demonstrated the anti-allodynic and antinociceptive effects of SP600125 in surgically-induced nerve ligation (Zhuang et al., 2006; Sanna et al., 2017) and inflammatory (Gao et al., 2010; Marcus et al., 2015) pain models. Studies have demonstrated that inhibition of JNK signaling in spinal astrocytes

attenuates neuropathic pain and mechanical allodynia through an inhibition of excitatory glutamatergic in dorsal horn neurons (Gao et al., 2009; Zhuang et al., 2006). The mechanism responsible for altered excitatory neuronal signaling involves JNK-mediated upregulation of the cytokine CCL2/monocyte chemoattractant protein-1 in spinal astroglia (Gao et al., 2009).

When we examined the role of JNK signaling in tolerance to Δ^9-THC in wild-type mice with cisplatin-evoked neuropathic pain, we observed an even more dramatic effect of SP600125 pretreatment. Pretreatment with SP600125 alone was able to elicit an anti-allodynic effect until about day 20. In this experiment, there was no evidence of tolerance to the anti-allodynic effects of 6 mg/kg Δ^9-THC (following daily pretreatment with SP600125) until approximately day 20, coinciding with the time point at which SP600125 on its own, ceased to elicit anti-allodynic effects, at which point we see the development of rapid and complete tolerance to Δ^9-THC. The loss of the antinociceptive effects elicited by JNK inhibition after 21 days is an interesting finding. Indeed, it is suggesting that the mechanism by which SP600125 induces its own, independent anti-allodynic effect likely mediates, in part, how SP600125 induces tolerance to Δ^9-THC. This response could be explained by other mechanisms coming into play, such as cAMP-dependent protein kinase (PKA) and phosphatidylinositol-3 kinase (PI3-K). Indeed, reversal of Δ^9-THC tolerance has been associated with PKA (Lee et al., 2003; Bass et al., 2004) and PI3-K (Lee et al., 2003) inhibition. Another possible explanation could be that increased IL-6 and/or IL-1β levels, triggered after several days of chemotherapy-induced neuropathy, might act by reversing the antinociceptive effects of SP600125 following chronic treatment. An increase in cytokines, such as IL-6 (Al-Mazidi et al., 2017; Ding et al., 2018) and IL-1 β (Al-Mazidi et al., 2017) has been demonstrated to be associated with the maintenance of neuropathic pain.

Therefore, the independent role of JNK signaling in models of pathological pain represents an important finding of our study that needs to be investigated further in the context of cannabinoid tolerance in different pain models. Indeed, the effect of SP600125 pretreatment on Δ^9-THC tolerance was more profound in the formalin and cisplatin models. Alternatively, this finding that JNK signaling plays a more pronounced role in tolerance for Δ^9-THC in pathological pain, supports the possibility of cell-type/tissue/region-specific mechanisms of tolerance. For example, tolerance to Δ^9-THC is only partially attenuated by SP600125 pretreatment in wild-type mice for the tail-flick test, a pain response that is mediated primarily at the level of the spinal cord. However, tolerance to Δ^9-THC is severely disrupted by SP600125 pretreatment in wild-type mice using the formalin and von Frey test tests (cisplatin-evoked neuropathy), two pain responses that require significant modulation by supra-spinal pain pathways. Indeed, it has been shown that cannabinoids modulate rostroventral medulla neuronal activity in pain models (Martin et al., 1998; Meng et al., 1998). Thus, perhaps, JNK signaling pathways play a more pronounced role in Δ^9-THC tolerance occurring at supra-spinal pain processing centers such as the rostroventral medulla and periaqueductal gray compared to tolerance occurring at the level of the spinal cord and dorsal root ganglia.

Another mechanism that has not been explored, but warrants additional study, is the possibility that JNK signaling, either directly or indirectly, might cause phosphorylation of CB_1R. Recently, it has been shown that JNK-mediated acute morphine tolerance occurs through an intriguing novel mechanism that involves phosphorylation of peroxiredoxin 6, reactive oxygen species generation, and G protein palmitoylation (Schattauer et al., 2017). Future work is needed to investigate whether this pathway is also involved in JNK-mediated Δ^9-THC tolerance.

The mechanism responsible for JNK-mediated Δ^9-THC tolerance is not well understood in comparison to its role in morphine tolerance. Indeed, work investigating the mechanism of JNK-mediated tolerance for morphine, another GPCR-directed agonist that acts at the mu opioid receptor (MOR), has been investigated more thoroughly and offers clues regarding the possible mechanisms of JNK-mediated Δ^9-THC tolerance. Previous studies, including our own, have demonstrated that JNK signaling mechanisms mediate tolerance for morphine, but not fentanyl, in an agonist-specific, functionally selective manner (Melief et al., 2010; Marcus et al., 2015).

Acute tolerance to the antinociceptive effects of morphine was found to occur through JNK2-mediated desensitization of the MOR (Melief et al., 2010). Thus, desensitization of CB_1R represents one possible mechanism of JNK-mediated Δ^9-THC tolerance. However, chronic treatment with Δ^9-THC can also cause tolerance through down-regulation of CB_1R (Sim et al., 1996). It is important to point out that a significant limitation to the current study is that all of the cannabinoid agonists used are mixed CB_1R and CB_2R agonists. Our previous work has found that the selective CB_2R agonist JWH133 elicits potent antinociceptive effects in the formalin test despite no measurable effects in either the tail-flick or hotplate tests (Yuill et al., 2017). Likewise, our own findings suggest that acute thermal antinociception in the tail-flick model and hypothermia are mediated primarily through CB_1Rs (Figure 8). However, we did not assess the extent to which JNK could differentially mediate its actions via CB_1R or CB_2R. Other studies have demonstrated good efficacy, without the development of tolerance, for CB_2R selective agonists in models of chemotherapy-evoked neuropathic pain (Deng et al., 2015b). Thus, the effects of JNK signaling on tolerance for the effects of cannabinoids in pathological forms of pain, such as the formalin test and cisplatin-evoked neuropathic pain, could also be due to action and/or receptor adaptations at CB_2R.

Our study is the first to address the role of JNK signaling in agonist-specific cannabinoid tolerance in acute, inflammatory and chronic (cisplatin-evoked neuropathy) pain models. Similar to our findings in the opioid system (Marcus et al., 2015; Yuill et al., 2016), we found that the role of JNK signaling in cannabinoid tolerance was agonist-specific. Tolerance to WIN55,212-2 was not affected by SP600125 pretreatment while inhibition of JNK signaling drastically accelerated tolerance to CP55,940 in mice in both acute and chronic, neuropathic pain models. The mechanism underlying the varying effects of SP600125 in mutant mice is unknown. However, previous work has shown that a S426A/S430A mutant form of CB_1R differentially couples to βarrestin1 instead of βarrestin2, and such a switch in arrestin coupling could impact the dynamics of JNK modulation of cannabinoid signaling for certain agonists (Delgado-Peraza et al., 2016).

Our data demonstrate the antinociceptive and anti-allodynic efficacy of multiple cannabinoid drugs, including Δ^9-THC, CP55,940, and WIN55,212-2 across a wide range of acute (tail-flick), inflammatory (formalin test) and chronic (cisplatin-evoked neuropathy) pain models. These findings are consistent with an extensive body of literature from preclinical studies that cannabinoids possess substantial antinociceptive and analgesic properties. Preclinical data (Guindon and Hohmann, 2011) demonstrating the efficacy of cannabinoid drugs is supported by a rapidly expanding number of clinical studies (Savage et al., 2016) showing the effectiveness of cannabinoid-based therapies for certain types of human pain. In addition, this work provides the first *in vivo* report, at least to our knowledge, of agonist-specific mechanisms of cannabinoid tolerance. This study highlights the importance of gaining a better understanding of the diverse mechanisms that mediate GPCR and cannabinoid tolerance in order to identify novel drugs and/or drug targets that might enhance clinical pain management, including biased cannabinoid agonists that might have reduced liabilities for tolerance and dependence.

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Figure Legends

Figure 1. Tolerance to the antinociceptive effect of Δ^9-THC in the formalin test is attenuated by SP600125 in wild-type mice.

Wild-type mice were given once-daily injections of either: vehicle alone (black bars), 3 mg/kg SP600125 (SP6) alone (red bars), 6 mg/kg Δ^9-THC alone (white striped bars), or 3 mg/kg SP6 in combination with 6 mg/kg Δ^9-THC (white bars). The formalin model was used to assess the effect of 3 mg/kg SP6 on tolerance to the antinociceptive effects of 6 mg/kg Δ^9-THC in both the acute, phase I (**A**) and inflammatory, phase II (**B**) pain. Error bars represent the SEM and data analyses were performed using two-way ANOVAs with Bonferroni post-hoc tests (^p<0.05; #p<0.01; \$$p$<0.001; *$p$<0.0001).

Figure 2. Tolerance to the anti-allodynic effects of Δ^9-THC is prevented by SP600125.

Male wild-type mice were treated with either vehicle alone (Days 8-22; open black circles and line), 3 mg/kg SP600125 alone (Days 8-22; red circles and line) or pretreated with either vehicle (open black triangles and line) or SP600125 (open black diamonds and line) 60 min prior to IP administration of 6 mg/kg Δ^9-THC (Days 11-22). Arrows on days 8-10 indicate days when only vehicle or SP600125 were administered prior to any Δ^9-THC on board. Paw withdrawal thresholds were measured using the von Frey test each day 30 minutes after administration of vehicle, SP600125, or Δ^9-THC. Error bars represent the SEM and data analysis was performed using two-way ANOVAs with Bonferroni post-hoc tests (****p<0.0001 SP600125 + Δ^9-THC and vehicle; #p<0.0001 Δ^9-THC only and vehicle; ^p<0.0001 SP600125 alone and vehicle). Sample sizes for each group are in parentheses.

Figure 3. SP600125 blunts tolerance to the acute antinociceptive effect of Δ^9-THC.

3 mg/kg SP600125 (black bars) or vehicle (white bars) was administered via IP injection one hour prior to two IP injections of 30 mg/kg Δ^9-THC at t=0 and t=480 minutes in wild-type (**A**) and S426A/S430A (**B**) mice. Tail-flick antinociception was assessed 1 hour after each injection of 30 mg/kg Δ^9-THC. Error bars represent the SEM and data analyses were performed using two-way ANOVAs with Bonferroni post-hoc tests (**p<0.01). Sample sizes for each group are in parentheses.

Figure 4. SP600125 did not delay tolerance to the hypothermic effect of Δ^9-THC.

The effect of 3 mg/kg SP600125 (SP6; red circles and red solid line) or vehicle (black squares and black solid line) on acute tolerance for the hypothermic effects of two injections of 30 mg/kg of Δ^9-THC at t=0 and t=480 minutes was assessed in wild-type (**A**) and S426A/S430A (**B**) mice. Error bars represent the SEM and data analyses were performed using two-way ANOVA with Bonferroni post-hoc tests (*p<0.05). Sample sizes for each group are in parentheses.

Figure 5. Effect of 3 mg/kg SP600125 on tolerance to daily Δ^9-THC injections in wild-type and S426A/S430A mice. SP600125 (SP6; 3 mg/kg IP, red line and circles) or vehicle (black line and squares) was administered one hour prior to IP injection of 30 mg/kg Δ^9-THC for seven days. Tail-flick antinociception (%MPE; **A,B**) and hypothermia (%ΔBT; **C,D**) were measured one hour following the injection of 30 mg/kg Δ^9-THC for 7 consecutive days in wild-type (**A,C**) and S426A/S430A (**B,D**) mutant mice. Error bars represent the SEM and data analyses were performed using two-way ANOVAs with Bonferroni post-hoc tests (**p<0.01). Sample sizes for each group are in parentheses.

Figure 6. Tolerance to the antinociceptive and hypothermic effects of WIN 55,212-2 is not altered by SP600125.

Wild-type and S426A/S430A mutant mice were pretreated with vehicle (black line with squares) or 3 mg/kg SP600125 (SP6; red line with circles) one hour prior to administration of 10 mg/kg WIN55,212-2 for 20 consecutive days. Tail-flick antinociception (%MPE; **A,B**) and hypothermia (%ΔBT; **C,D**) were assessed in both wild-type (**A,C**) and S426A/S430A mutant (**B,D**) mice one hour after WIN55,212-2 treatment. Error bars represent the SEM and data analysis was performed using two-way ANOVAs. Sample sizes for each group are in parentheses.

Figure 7. Tolerance to the hypothermic and antinociceptive effects of 0.3 mg/kg CP55,940 is accelerated following pretreatment with 3 mg/kg of SP600125.

Wild-type and S426A/S430A mutant mice were pretreated with vehicle (black line with squares) or 3 mg/kg SP600125 (red line with circles) one hour prior to administration of 0.3 mg/kg CP55,940 for 10 consecutive days. Tail-flick antinociception (%MPE; **A,B**) and hypothermia (%ΔBT; **C,D**) were assessed in both wild-type (**A, C**) and S426A/S430A mutant (**B, D**) mice one hour after CP55,940 treatment. Error bars represent the SEM and data analysis was performed using two way ANOVAs with Bonferroni post-hoc tests (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Sample sizes for each group are in parentheses.

Figure 8. Tolerance to the antinociceptive and hypothermic effect of Δ^9-THC, CP55,940, and WIN55,212-2 are mediated by CB_1R

Wild-type (black bars) and S426A/S430A (red bars) mice were assessed for the degree to which the antinociceptive (**A,C,E**) and hypothermic (**B,D,F**) effects of 30 mg/kg Δ^9-THC (**A,B**), 0.3 mg/kg CP55,940 (**C,D**), and 10 mg/kg WIN55,212-2 (**E,F**) are mediated by CB_1R and/or CB_2R. Mice (N=5/genotype) were randomly administered either vehicle, the inverse CB_1R agonist SR141716A (SR1; 10 mg/kg), or the selective CB_2R antagonist SR144528 (SR2; 10 mg/kg) 30 minutes prior to treatment with drug (vehicle, Δ^9-THC, CP55,940, or WIN55,212-2) and assessed for antinociception via the tail-flick and alterations in body temperature immediately prior to- and 60 minutes post drug administration.once weekly across 12 weeks in a within-subjects design. Error bars represent the SEM and data analyses were performed using two-way ANOVAs with Bonferroni post-hoc tests (*p<0.05; **p<0.01; ***<0.001; ****p<0.0001 compared to Veh/Veh).

Figure 9. Tolerance to the anti-allodynic effects of CP55,940 in cisplatin-evoked chronic neuropathic pain is accelerated by SP600125 in wild-type mice.

Male wild-type mice were treated with either vehicle alone (Days 8-22; open black circles and line), 3 mg/kg SP600125 alone (Days 8-22; red circles and line), or pretreated with either vehicle (open black triangles and line) or SP600125 (open black diamonds and line) one hour prior to IP injection of 0.3 mg/kg CP55,940 (days 11-22). Arrows on days 8-10 indicate days when only vehicle or SP600125 were administered prior to any CP55,940 on board. Paw withdrawal thresholds were measured using the Von Frey test each day 30 minutes after administration of vehicle, SP600125 (SP6) or CP55,940 (CP55). Error bars represent the SEM and data analysis was performed using two-way ANOVAs with Bonferroni post-hoc tests (****p<0.0001 denotes SP6+CP55 from vehicle; ****p<0.0001 denotes both SP6+CP55 and CP55 alone from vehicle; ^p<0,0001 denotes SP6 alone from vehicle). Sample sizes for each group are in parentheses.

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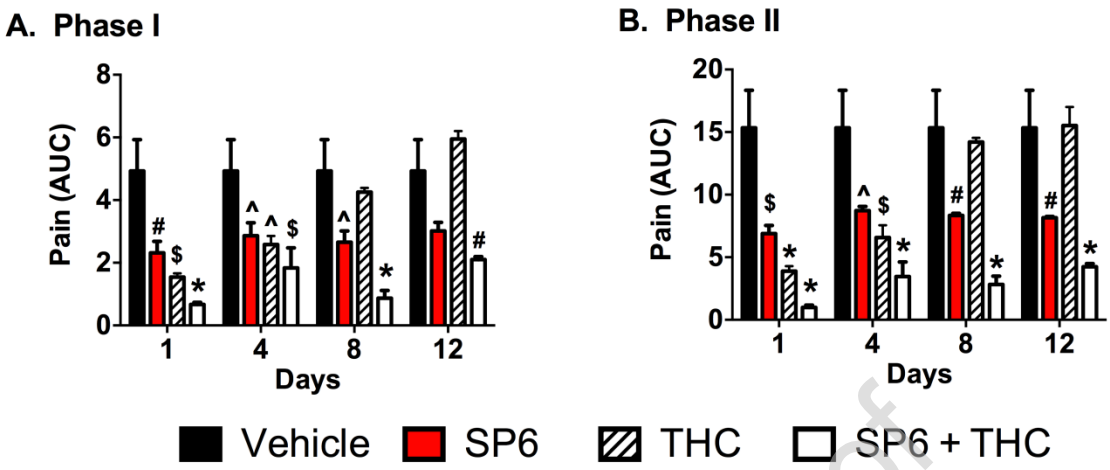
Figure 1.



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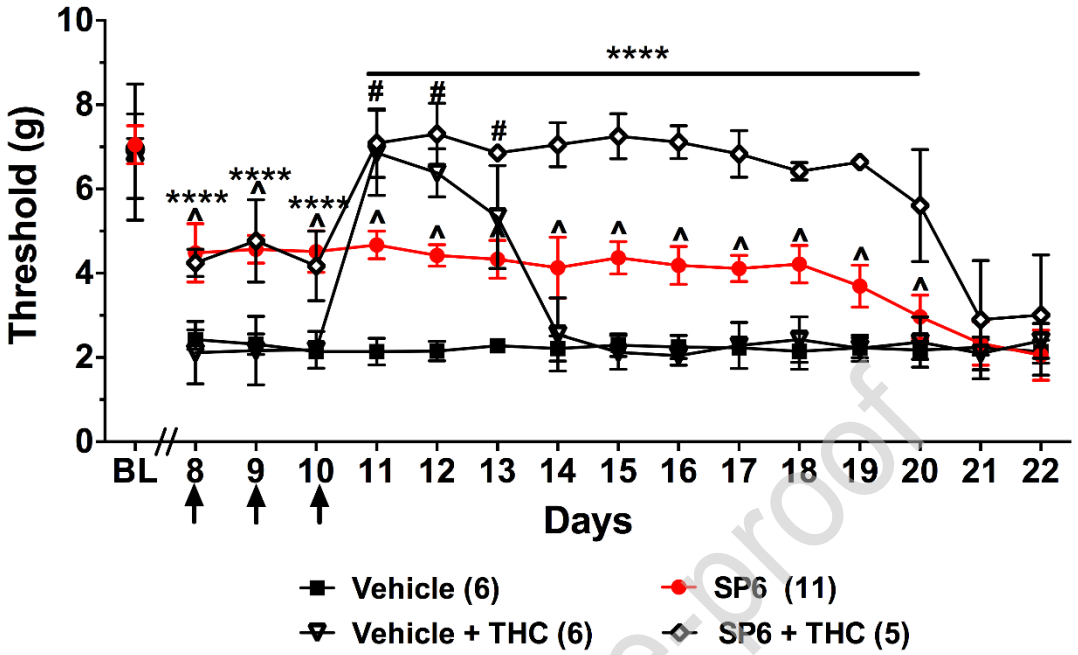

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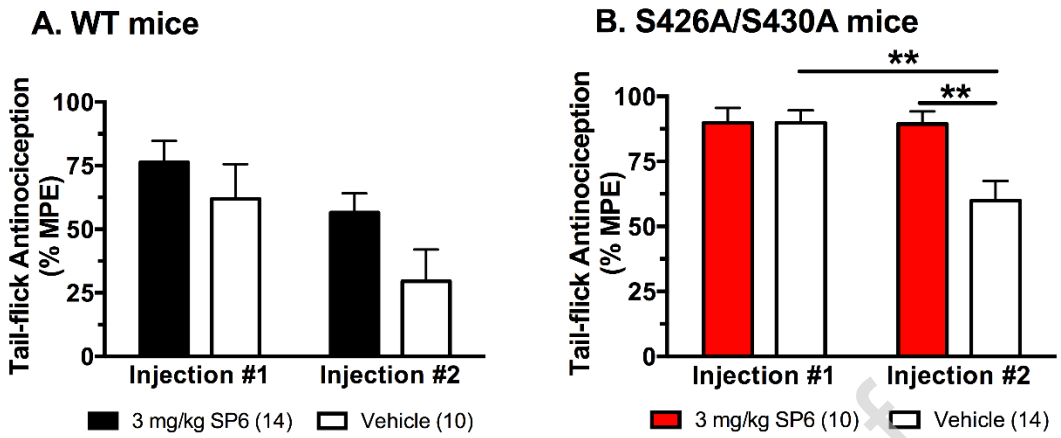

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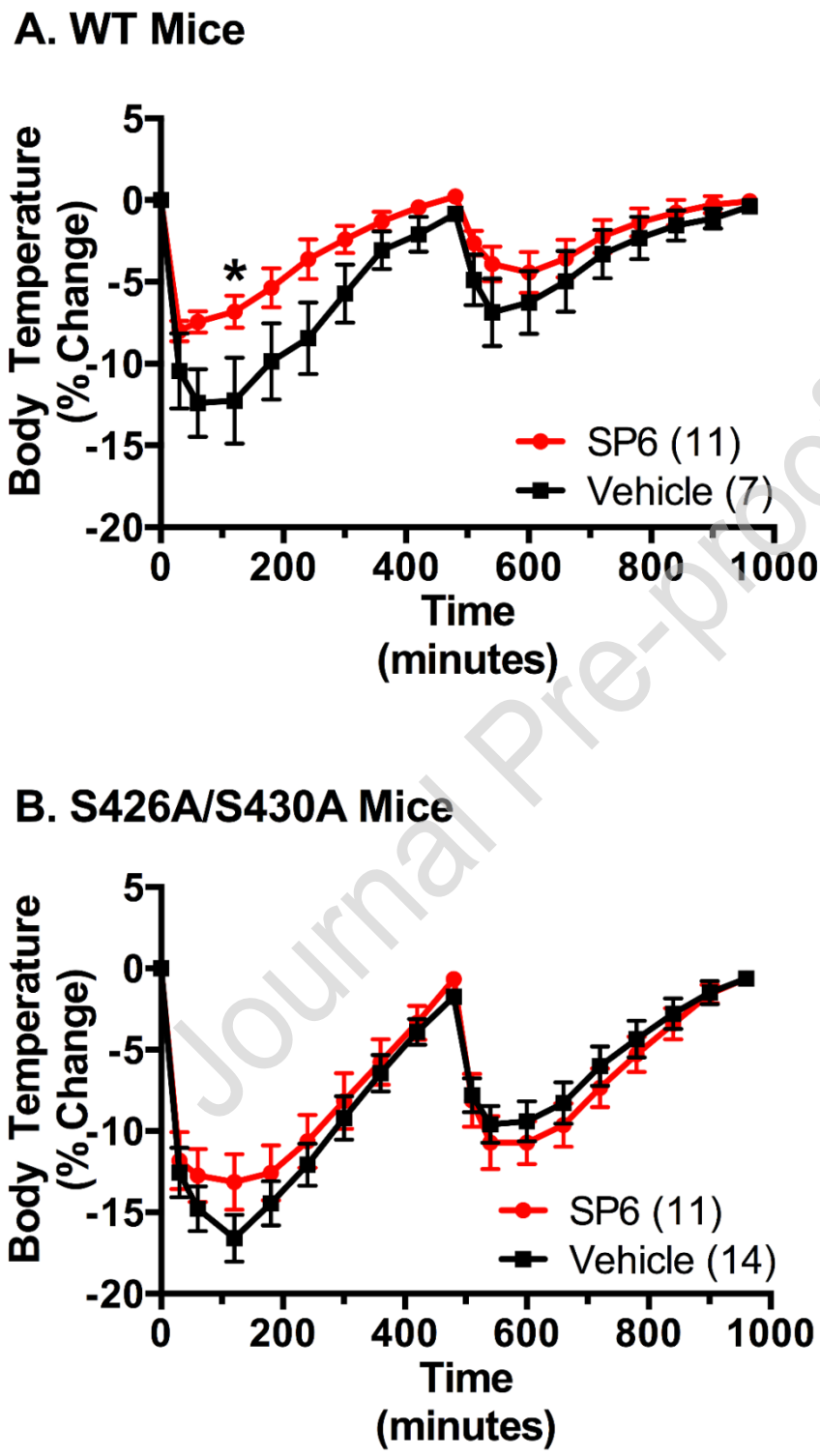

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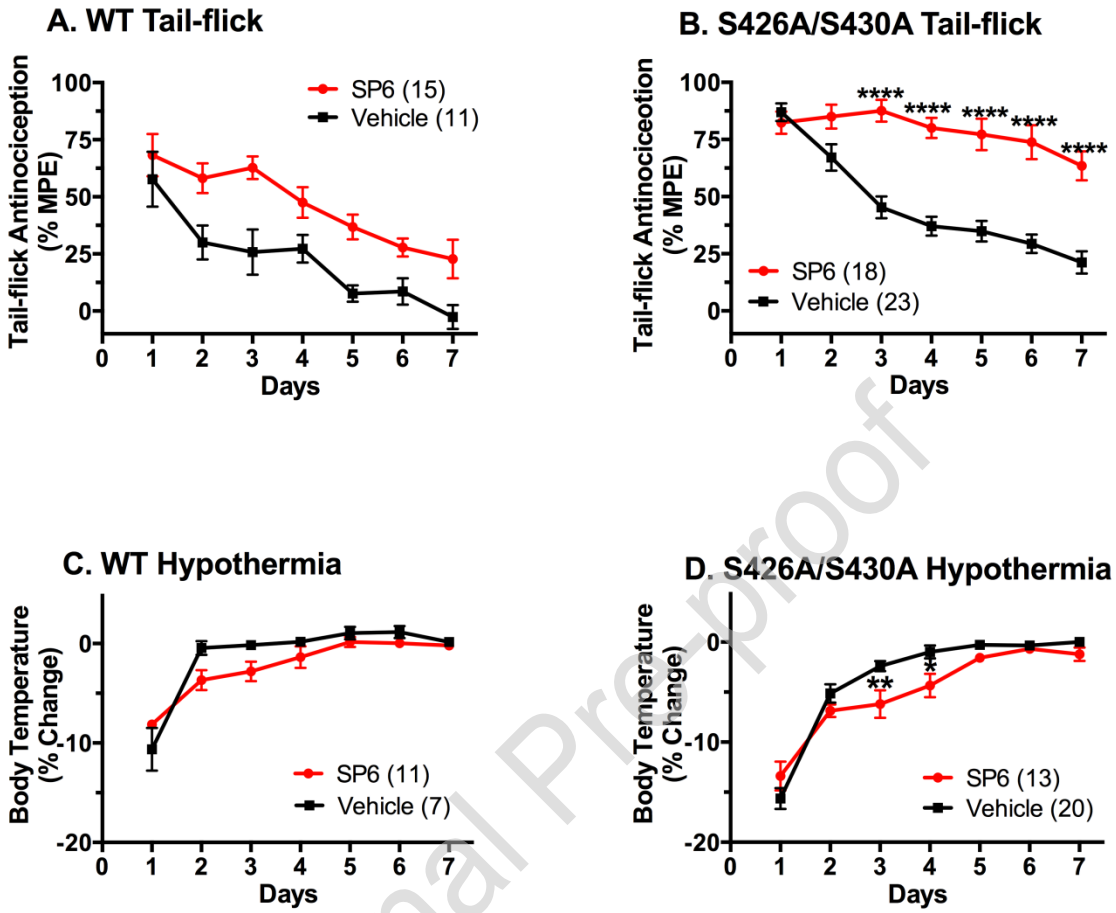

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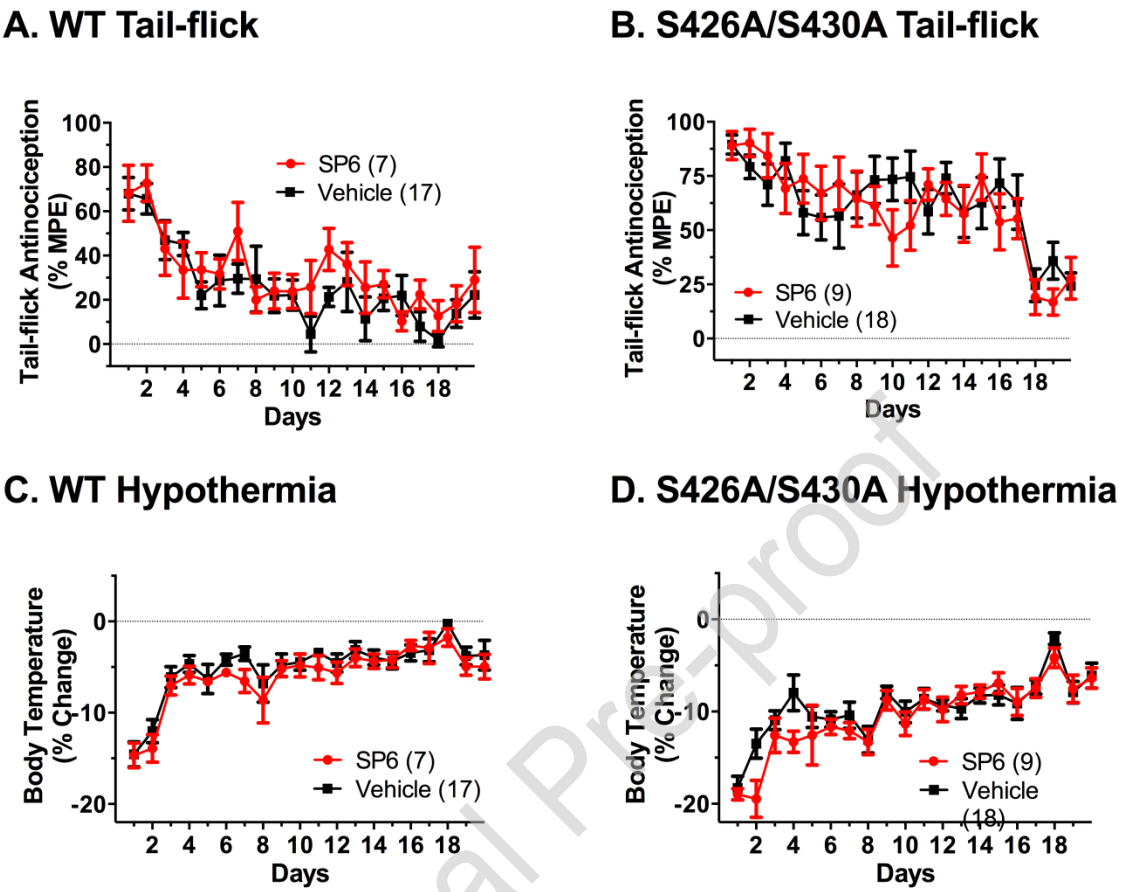

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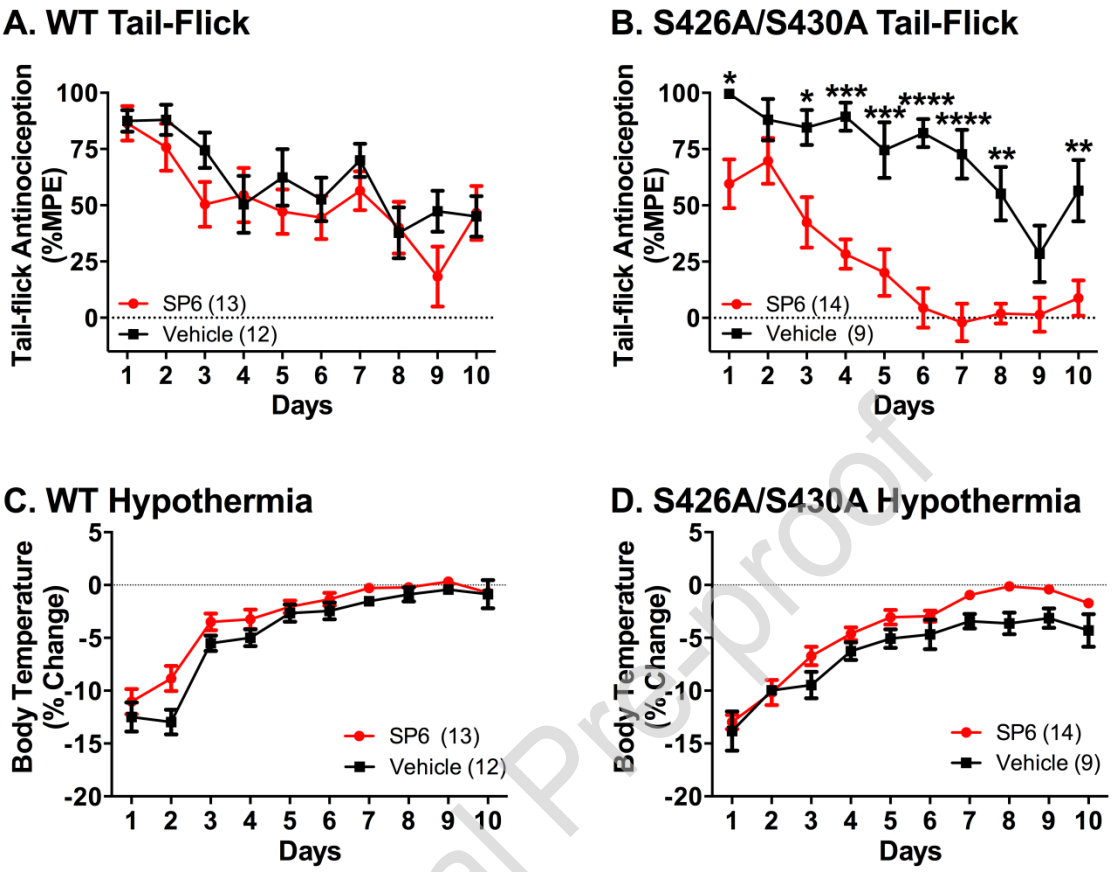

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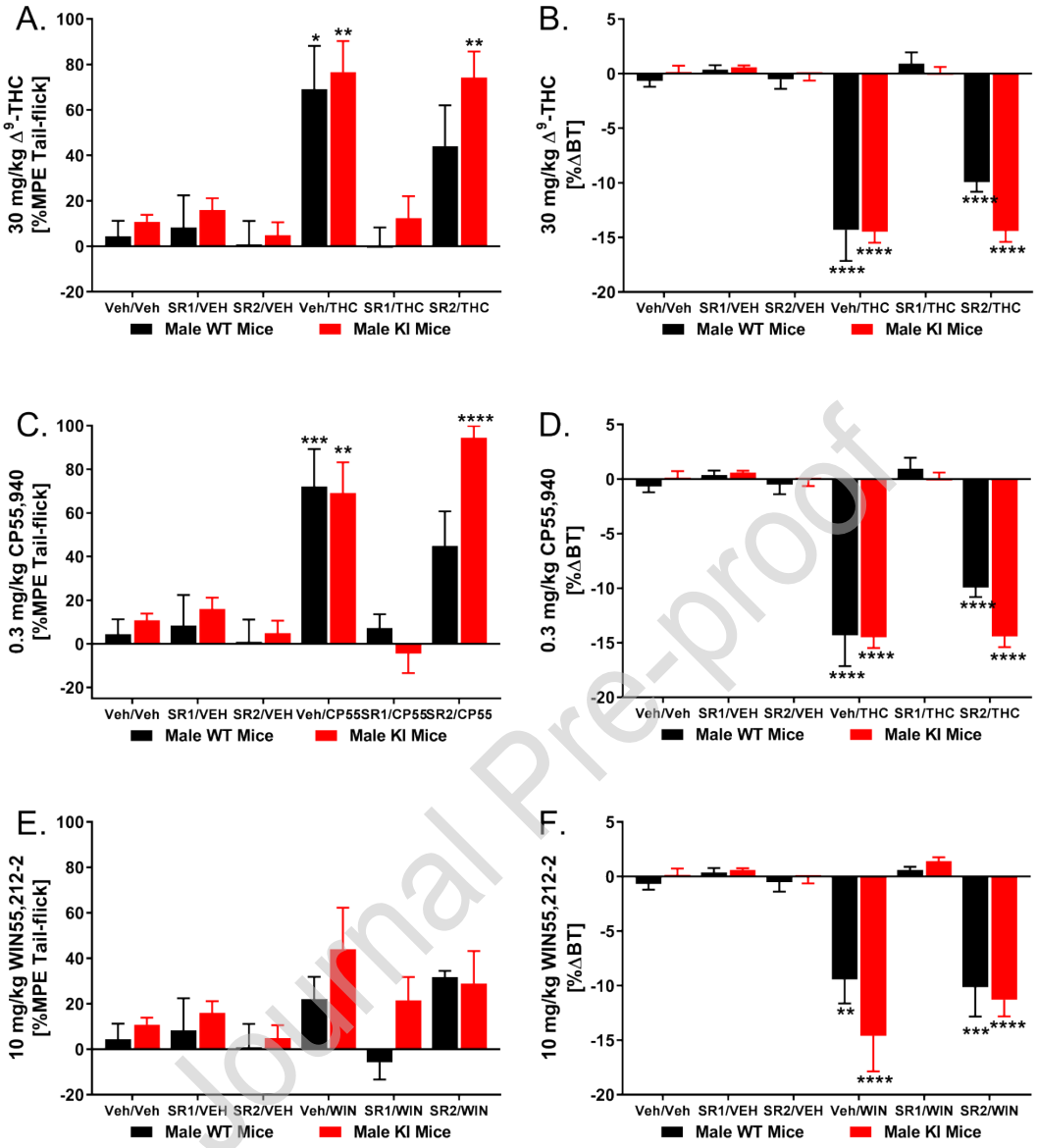

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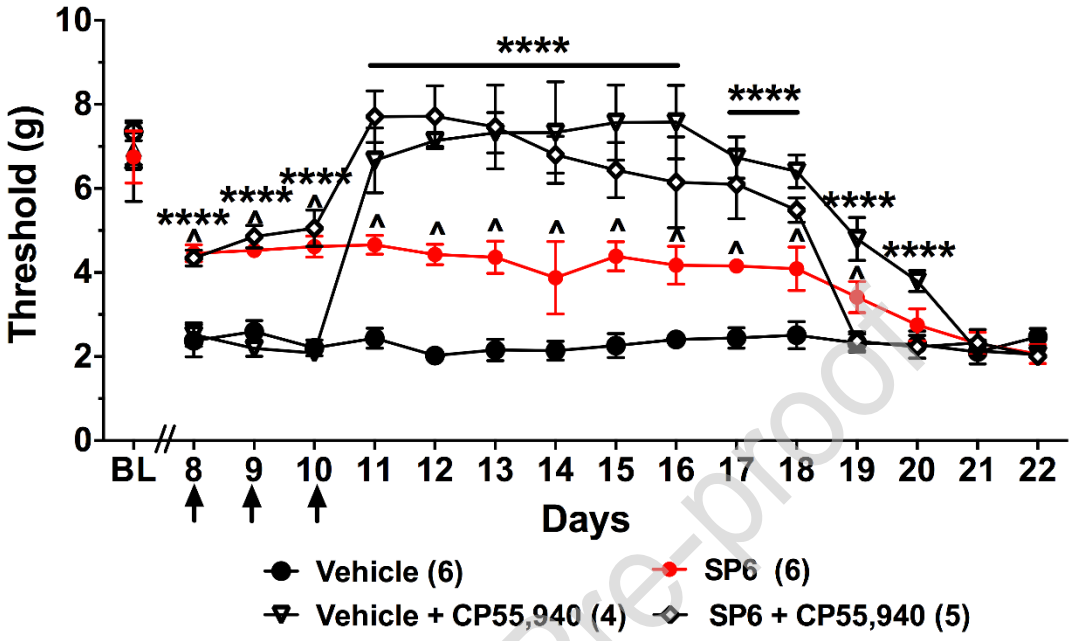

Highlights

- c-Jun N-terminal Kinase (JNK) signaling is required for tolerance to Δ^9-THC
- Serine residues 426 and 430 in CB1R are also required Δ^9-THC tolerance
- JNK signaling is not involved in tolerance to WIN55,212-2
- Inhibition of JNK signaling accelerates tolerance to CP55,940