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Tolerance to WIN55,212-2 is delayed in desensitization-resistant S426A/S430A mice

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Abstract

Tolerance to cannabinoid agonists can develop through desensitization of the cannabinoid receptor 1 (CB₁) following prolonged administration. Desensitization results from phosphorylation of CB₁ by a G protein-coupled receptor kinase (GRK), and subsequent association of the receptor with arrestin. Mice expressing a mutant form of CB₁, in which the serine residues at two putative phosphorylation sites necessary for desensitization have been replaced by non-phosphorylatable alanines (S426A/S430A), display reduced tolerance to ⁹-tetrahydrocannabinol (⁹-THC). Tolerance to the antinociceptive effects of WIN55,212-2 was delayed in S426A/S430A mutants using the tail-flick and formalin tests. However, tolerance to the antinociceptive effects of once daily CP55,940 injections was not significantly delayed in S426A/S430A mutant mice using either of these tests. Interestingly, the dose response curve shifts for the hypothermic and antinociceptive effects of CP55,940 that were induced by chronic treatment with this agonist in wild-type mice were blocked in S426A/S430A mutant mice. Assessment of mechanical allodynia in mice exhibiting chronic cisplatin-evoked neuropathic pain found that tolerance to the anti-allodynic effects WIN55,212-2 but not CP55,940 was delayed in S426A/S430A mice compared to wild-type littermates. Despite these deficits in tolerance, S426A/S430A mutant mice eventually developed tolerance to both WIN55,212-2 and CP55,940 for all pain assays that were examined, suggesting that other mechanisms likely contribute to tolerance for these cannabinoid agonists. These findings suggest that GRK- and β arrestin2-mediated desensitization of CB₁ may strongly contribute to the rate of tolerance to the antinociceptive effects of WIN55,212-2, and raises the possibility of agonist-specific mechanisms of cannabinoid tolerance.

Keywords

Cannabinoid; Pain; Tolerance; Antinociception; Desensitization; CB₁; Mice

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1. Introduction

Cannabinoid-based therapeutics have shown efficacy in clinical studies for the treatment of chronic and neuropathic pain (Whiting et al., 2016). Cannabinoid drugs act through cannabinoid receptors including the cannabinoid receptor 1 (CB₁) to exert a wide range of effects in the brain. The antinociceptive effects of cannabinoids have been demonstrated in a wide range of preclinical models of acute (Lichtman and Martin, 1991; Morgan et al., 2014), chronic neuropathic (Deng et al., 2015; Fox et al., 2001), and inflammatory pain (Walker et al., 1999). Despite the potential use of cannabinoids as clinical analgesics, there are limitations to their therapeutic use, including dependence (Degenhardt et al., 2013), tolerance (D'Souza et al., 2008), and adverse side effects (Hall, 2015). Therefore, a greater understanding of the mechanisms responsible for these effects is important to facilitate the development of better cannabinoid-based therapies.

The therapeutic actions of cannabinoids are limited, in part, by the development of tolerance during chronic administration (Bedi et al., 2010; D'Souza et al., 2008). Prolonged agonist exposure leads to receptor desensitization and has been implicated as one possible mechanism underlying tolerance (Martin et al., 2004; Morgan et al., 2014; Selley et al., 2004). Phosphorylation of CB₁ by G protein-coupled receptor kinases (GRKs) leads to desensitization (Gainetdinov et al., 2004; Sim et al., 2006) and endocytosis (Hsieh et al., 1999; Wu et al., 2008) of the receptor. Agonist-bound CB₁ can be phosphorylated at two serine residues (426 and 430) on the intracellular tail of CB₁ facilitating interaction with an arrestin protein, such as β -arrestin2 (Breivogel et al., 2008; Daigle et al., 2008; DeWire et al., 2008; Jin et al., 1999). Mutation of these putative phosphorylation sites (S426A/S430A) leads to decreased desensitization of CB₁ (Daigle et al., 2008; Delgado-Peraza et al., 2016; Jin et al., 1999; Morgan et al., 2014). We have previously demonstrated that mice expressing this desensitization-resistant form of CB₁ are slower to develop tolerance to both the antinociceptive and hypothermic effects of delta-9-tetrahydrocannabinol (Δ^9 -THC) (Morgan et al., 2014). Similarly, delayed tolerance to Δ^9 -THC due to disrupted desensitization and down-regulation of CB₁ has also been observed in β -arrestin2 knock-out mice (Nguyen et al., 2012).

Cannabinoid agonists exhibit substantial structural diversity (Pertwee, 2008), which may lead to distinct differences in the responses activated by their action at CB₁ (Ahn et al., 2013; Georgieva et al., 2008). The cannabinoids R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl-methyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-maphthalenyl)methanone mesylate (WIN55,212-2) and (1 α ,2 β)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl-cyclohexyl)phenyl] (CP55,940) are synthetic cannabinoids that are structurally distinct. Cannabinoid receptor agonists, like many other G protein-coupled receptor (GPCR) agonists, are able to selectively activate different intracellular signaling responses, a concept known as biased agonism or functional selectivity (Laprairie et al., 2014; Priestley et al., 2017). Δ^9 -THC and WIN55,212-2 induce different regional patterns of CB₁ desensitization and downregulation in the mouse brain with Δ^9 -THC treatment generally causing a more robust effect on these neuroadaptations (Sim-Selley and Martin, 2002) raising the possibility of agonist-specific cannabinoid tolerance. The acute antinociceptive effects of CP55,940 were found to be attenuated in β -arrestin1^{-/-} mice,

while the effects of Δ^9 -THC were not altered (Breivogel and Vaghela, 2015). Interestingly, no differences in the development of tolerance to either agonist were observed in β -arrestin1^{-/-} mice. Likewise, the S426A/S430A mutant form of CB₁ has been demonstrated to induce biased signaling (Delgado-Peraza et al., 2016).

There is good evidence to support a role for GRK/ β -arrestin2-mediated desensitization as a potential mechanism for the development of tolerance to the behavioral effects of cannabinoid agonists. Previous studies have shown that disrupted desensitization and down-regulation of the CB₁ receptor in β -arrestin2 knock-out mice (Nguyen et al., 2012) delays cannabinoid tolerance and that GRK3/ β -arrestin2 mediated desensitization of CB₁ requires phosphorylation of serines 426 and 430 (Jin et al., 1999). However, whether biased agonism through this pathway plays a role in agonist-specific cannabinoid tolerance has not been widely investigated. Therefore, we utilized models of acute nociceptive pain (tail-flick, formalin test) and chronic neuropathic pain to investigate agonist differences in the development of tolerance to the synthetic, high-potency, full cannabinoid agonists WIN55,212-2 and CP55,940 in S426A/S430A mutant mice resistant to GRK/ β -arrestin2-mediated desensitization of CB₁.

2. Methods

2.1. Animals

This study was performed using 235 experimentally naïve male S426A/S430A mutant (n = 115) and wild-type (n = 120), 8-12-week-old, littermate-controlled mice on a C57BL/6 background. The generation of S426A/S430A mutant mice has been described previously (Morgan et al., 2014). After weaning, mice were group housed, with a standard 12:12h light/dark cycle (lights on at 07:00, lights off at 19:00). Mice were provided with *ad libitum* access to standard rodent chow (Teklad 2018, Envigo, Indianapolis, IN) and water. All animal care procedures were performed in accordance with the *Guidelines of the National Institutes of Health on the Care and Use of Animals* and with approval from the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (IACUC).

2.2. Drugs

(+)-WIN55,212-2 and (-)-CP55,940 were purchased from Cayman Chemical (Ann Arbor, MI). CP55,940 was prepared in 100% ethanol and subsequently diluted 20-fold in vehicle containing 0.9% saline and 5% Cremaphor EL (Sigma-Aldrich, St Louis, MO) (18:1 vehicle v/v). WIN55,212-2 was prepared in 100% DMSO and diluted in vehicle containing 0.9% saline, 5% Cremaphor EL, and 5% ethanol (18:1:1 vehicle v/v/v) for injection. The total amount of DMSO contained in all WIN55,212-2 injections was 4% v/v. Cisplatin was purchased from Tocris (Minneapolis, MN) and subsequently dissolved in 0.9% saline. Sodium bicarbonate (Fisher Scientific, Pittsburgh, PA) was dissolved in 0.9% saline to prepare a 4% w/v solution for administration. Drugs were administered via intraperitoneal (i.p.) injection (CP55,940, WIN55,212-2, cisplatin) in a single volume of 10ml/kg of body weight or via subcutaneous (s.c.) injection (sodium bicarbonate) in a single volume of 1 ml.

Formalin (2.5% v/v) was prepared by diluting formaldehyde (37%, Fisher Scientific) in water.

2.3. Procedures

2.3.1. Cumulative dose-responses—Male wild-type and S426A/S430A mutant mice were assessed for antinociceptive and hypothermic responses to vehicle and cumulative doses of 0.3, 1, 3, 10, and 30 mg/kg WIN55,212-2 or 0.01, 0.03, 0.1, 0.3, and 1 mg/kg CP55,940. Mice were administered doses of 0.3, 0.7, 2, 7, and 20 mg/kg WIN55,212-2 or 0.01, 0.02, 0.07, 0.2, and 0.7 mg/kg CP55,940 to achieve final cumulative doses. Following baseline assessments of nociceptive responses, all vehicle and drug measurements were taken 1h apart, or 60 min following each injection of either vehicle or drug. Antinociception was measured using a Columbus Instruments (Columbus, OH) TF-1 tail-flick analgesia meter with the radiant heat source set to an intensity level of 5 as described previously (Nealon et al., 2017). Exposure of the tail to the heat source was limited to 10s to avoid tissue damage. Tail-flick latencies were measured immediately prior to drug administration, and at 60 min after treatment with either vehicle or each dose of drug to calculate the percent maximal possible efficacy (% MPE) where $\% \text{ MPE} = [(\text{post drug latency} - \text{pre-drug latency}) / (10 - \text{pre-drug latency})] * 100$. Hypothermia was measured hourly by taking body temperatures with a mouse rectal thermometer (Physitemp Instruments, Clifton, NJ). Body temperature was calculated as percent change in body temperature, where $\% \text{ BT} = [(\text{post-drug temperature}) - (\text{pre-drug temperature}) / (\text{pre-drug temperature})] * 100$.

Cumulative dose-response curves to assess tolerance to the antinociceptive and hypothermic effects of WIN55,212-2 and CP55,940 were determined in two groups of mice for each drug. Acute cumulative dose-response curves (pre; Day 0) were determined in drug naïve wild-type and S426A/S430A mice. An additional group of wild-type and S426A/S430A mice were administered either 10 mg/kg WIN55,212-2 or 0.3 mg/kg CP55,940 (i.p.) once-daily for nine consecutive days and assessed for cumulative dose responses (post) on the tenth day as described above to assess changes in drug response following chronic treatment.

2.3.2. WIN55,212-2 and CP55,940 tolerance—To identify any tolerance to repeated drug treatment over time, wild-type and S426A/S430A mutant mice were injected (i.p.) once-daily with either 10 mg/kg WIN55,212-2 for 20 consecutive days or 0.3 mg/kg CP55,940 for 15 consecutive days. Mice were tested for responses to drug effects until nociceptive tail-flick responses reached a plateau of 20–30% MPE in mice of both genotypes, resulting in different durations in the length of treatment for CP55,940 and WIN55,212-2. The doses for each drug selected for chronic administration (10 mg/kg WIN55,212-2 and 0.3 mg/kg CP55,940) were chosen based on the ability to produce antinociceptive responses of approximately 80% MPE in drug naïve mice as assessed in the cumulative dose-response curves (see Fig. 1). Another factor in the selection of 0.3 mg/kg CP55,940 was consistency with previous work from our group (LeFleur et al., 2018) and other laboratories (Deng et al., 2015). Tail-flick antinociception and hypothermia were measured each day immediately prior to and 60 min after drug administration. Drug responses (% MPE and % BT) were calculated as described above.

2.3.3. Formalin test—Wild-type and S426A/S430A mutant mice were assessed for inflammatory pain responses using the formalin test, which produces a characteristic transient, biphasic pattern of pain behavior (Tjølsen et al., 1992). One hour prior to testing, mice were injected (i.p.) with either 3 mg/kg WIN55,212-2, 0.3 mg/kg CP55,940, or the appropriate vehicle. Previous work from our group found that 0.3 but not 0.1 mg/kg CP55,940 was able to attenuate antinociception in the formalin test. In addition to our previous work, other laboratories have demonstrated the ability of 0.3 mg/kg CP55,940 to reverse paclitaxel-evoked neuropathic pain (Deng et al., 2015). The dose of WIN55,212-2 used in the formalin assay (3 mg/kg) was selected as the highest dose that did not preclude the assessment of pain behavior by causing substantial motor effects including catalepsy.

Mice were acclimated in a Plexiglass chamber (5"x5"x5"), on an elevated transparent platform, for 20 min before testing. Prior to and during testing, a mirror placed at a 45° angle underneath the platform was used to view the paws of the mouse, and a high-definition camera (Logitech, Newark, CA) placed underneath the platform was used to record the behavior of the mice in the chamber. Following acclimation (20 min), 10 µl of a 2.5% formalin solution was injected into the plantar surface of a single hind paw, using a 0.5 ml syringe with a 28 ½ gauge needle (Becton Dickson, Franklin Lakes, NJ). Mice were returned to the observation chamber immediately following formalin administration. Nociceptive behavior was measured for 60 min and quantified within twelve 5 min time bins throughout that observation period. Pain behaviors that were quantified included: the injected paw has little weight placed on it (0); the injected paw is held above the surface of the platform (1); or the injected paw is bitten, shaken, or licked (2). The amount of time the mouse spent engaged in each behavior was recorded and weighted using the composite pain score-weighted scores technique (CPS-WST₀₋₂), which produced composite pain scores (CPS) between 0 (no pain behavior) and 2 (continuous pain behavior) for each 5 min bin (Guindon et al., 2011; Marcus et al., 2015; Watson et al., 1997). The area under the curve (AUC) was calculated for the acute phase (0–15 min; Phase I) and the inflammatory phase (15–60min, Phase II).

Wild-type and S426A/S430A mutant mice were assessed for tolerance to the antinociceptive effects of CP55,940 and WIN 55,212-2 using the formalin test after treatment with once-daily injections of 3 mg/kg WIN55,212-2 and 0.3mg/kg CP55,940 (i.p.) for up to 21 days. Mice were assessed for pain responses in the acute (Phase I) and inflammatory (Phase II) phases following 1, 7, 14, and 21 consecutive days of administration of WIN55,212-2 and following 7, 14, and 21 consecutive days of administration of 0.3 mg/kg CP55,940 (LeFleur et al., 2018). As mice were euthanized immediately after formalin testing, separate groups of mice were used to determine drug responses for each time point. Mice were not assessed for inflammatory pain responses to 0.3 mg/kg CP55,940 on the first day of drug administration due to profound motor effects, including catalepsy, which prevented assessment of pain behaviors.

2.3.4. Cisplatin-induced neuropathy and von Frey testing—Chemotherapy-evoked neuropathic pain was induced in male wild-type and S426A/S430A mutant mice following four weeks of once-weekly i.p. injections of 5 mg/kg cisplatin. Mice were co-administered 1 ml 4% sodium bicarbonate via s.c. injection concurrent with cisplatin

administration to prevent toxicity due to renal damage (Guindon et al., 2014). Neuropathic pain was assessed using the von Frey test to measure mechanical allodynia. Mice were assayed for mechanical allodynia using an electronic von Frey anesthesiometer equipped with a semi-flexible polypropylene super-tip (IITC Life Science Inc, Woodland Hills, CA). Mice were administered vehicle and assayed for allodynia prior to the first cisplatin injection and also one week following the final cisplatin administration to determine baseline and neuropathic allodynic responses. Anti-allodynic responses to once-daily treatment with 3 mg/kg WIN55,212-2 or 0.3 mg/kg CP55,940 were assessed in male wild-type and S426A/S430A mice 1 h following drug administration on alternating days (1, 3, 5, 7, etc.) until von Frey measurements returned to the pre-drug neuropathic baseline. Mice were administered drug (i.p.) once daily for up to 19 (CP55,940) or 25 (WIN55,212-2) days. These durations of drug treatment and testing were based on the amount of time for the responses of mice to return to their baseline neuropathic values. The doses of WIN55,212-2 (3 mg/kg) and CP55,940 (0.3 mg/kg) used for the formalin test were also selected based on the absence of motor effects to be consistent with previous work from other laboratories that used this dose to evaluate paclitaxel-evoked neuropathic pain (Deng et al., 2015).

2.4. Data analysis

For all dose-response data, three-way mixed ANOVAs were performed with genotype and time as the between-subjects factor and dose as the repeated measure. For all three-way ANOVAs, Mauchly's test of sphericity was determined, and when sphericity was violated, the Greenhouse-Geisser correction was used with degrees of freedom and error rounded off to the nearest whole number. Additionally, nonlinear regression analyses were performed for each dose-response curve to calculate ED_{50} values which were used to assess shifts in dose-response curves. For all formalin analyses, two-way (genotype and time/number of injections) between subjects ANOVAs were used to assess differences in tolerance across days (excluding vehicle). The half-time to tolerance was calculated using Prism 6 by performing non-linear regression of the hypothermic and antinociceptive effects of WIN55,212-2 and CP55,940 using a single-phase exponential decay curve. For analyses of cisplatin-evoked chronic pain, data were analyzed using mixed two-way ANOVAs where genotype was the between-subjects factor and time/number of injections served as the repeated measure. For both formalin and cisplatin analyses, S426A/S430A and wild-type mice were also assessed against their own vehicle to assess differences in tolerance development. Data were analyzed using Prism 5 (GraphPad, La Jolla, CA) for all two-way ANOVAs and using SPSS version 25.0 (IBM SPSS Statistics, Armonk, New York) for all three-way ANOVAs. For all tests, Bonferroni post-hoc analyses were performed where appropriate, and for all analyses, significance was set at $p < 0.05$.

3. Results

3.1. WIN55,212-2 and CP55,940 dose-response curve shifts

3.1.1. Tolerance to the antinociceptive effects of WIN55,212-2 and CP55,940

—Treatment with WIN55,212-2 dose-dependently increased antinociception in the tail-flick assay ($F_{2,79} = 166.76$, $p < 0.001$; Fig. 1A). There were also main effects of time ($F_{1,38} = 5.39$, $p = 0.026$), genotype ($F_{1,38} = 4.49$, $p = 0.041$), and significant dose x genotype ($F_{2,79} =$

4.94, $p = 0.009$), dose $\times$ time ($F_{2,79} = 8.03$, $p = 0.001$), and genotype $\times$ time interaction ($F_{1,38} = 7.12$, $p = 0.011$), but no dose $\times$ genotype $\times$ time ($p > 0.05$) interaction effect. Post-hoc analyses revealed that S426A/S430A mice were more sensitive to the antinociceptive effects of 10 ($p = 0.037$) and 30 ($p = 0.016$) mg/kg WIN55,212-2 compared to wild-type mice. Post-hoc analyses also showed that following nine days of once-daily treatment with 10 mg/kg WIN55,212-2, that wild-type (but not S426A/S430A mutant) mice showed evidence of tolerance due to lower antinociceptive responses to 10 ($p = 0.002$) and 30 ($p < 0.001$) mg/kg WIN55,212-2 (Fig. 1A). Wild-type ($p < 0.0001$) but not S426A/S430A ($p = 0.8734$) mice demonstrated a significant increase in the calculated ED₅₀ values following nine consecutive days of CP55,940 treatment (Table 1), suggesting that only wild-type mice demonstrate tolerance to the antinociceptive effects of WIN55,212-2.

Acute treatment with CP55,940 dose-dependently increased antinociception using the tail-flick assay ($F_{3,104} = 113.78$, $p < 0.001$; Fig. 1B). While there was not an effect of time ($p = 0.725$) or a dose $\times$ genotype $\times$ time interaction effect ($p = 0.449$), there was a main effect of genotype ($F_{1,41} = 5.82$, $p = 0.020$) and significant dose $\times$ genotype ($F_{3,104} = 4.09$, $p = 0.013$) and dose $\times$ time ($F_{3,104} = 14.57$, $p < 0.001$) interaction effects. Post-hoc analyses showed that the antinociceptive response on day 10 was attenuated following treatment with cumulative doses of 0.3 ($p = 0.024$), and 1.0 ($p = 0.002$) mg/kg CP55,940 compared to day 0. However, for both time points, S426A/S430A mice showed a greater antinociceptive response compared to their wild-type littermates at doses of 0.3 ($p = 0.022$) and 1.0 ($p = 0.005$) mg/kg of CP55,940. Wild-type ($p < 0.0001$), but not S426A/S430A ($p = 0.3576$) mice, demonstrated a significant increase in ED₅₀ following chronic CP55,940 treatment (Table 1), suggesting that only wild-type mice demonstrated tolerance to the antinociceptive effects of CP55,940.

3.1.2. Tolerance to the hypothermic effects of WIN55,212-2 and CP55,940—

Dose-dependent decreases in body temperature were observed following treatment with WIN55,212-2 (0.3, 1.0, 3.0, 10.0, and 30.0 mg/kg) ($F_{2,73} = 197.04$, $p < 0.001$; Fig. 1C). There was no effect of genotype ($p = 0.183$) nor a dose $\times$ genotype ($p = 0.207$) or a dose $\times$ time $\times$ genotype ($p = 0.817$) interaction effect. However, there was an effect of time ($F_{1,38} = 38.49$, $p < 0.001$) and a dose $\times$ day ($F_{2,73} = 36.49$, $p < 0.001$) interaction effect. Post-hoc analysis of the dose $\times$ day interaction effect indicated that mice displayed lower hypothermic responses to 10 ($p = 0.003$) and 30 ($p = 0.001$) mg/kg WIN55,212-2 on day 10 compared to day 0. Analysis of the ED₅₀ values for the hypothermic effects of WIN55,212-2 indicate that the shifts in ED₅₀ values were not significant for either wild-type ($p = 0.2742$) or S426A/S430A mice ($p = 0.3339$) (Table 2).

Dose-dependent hypothermia was observed following treatment with CP55,940 (0.01, 0.03, 0.1, 0.3 and 1mg/kg) ($F_{2,73} = 184.68$, $p < 0.001$; Fig. 1D). There was not an effect of genotype ($p = 0.308$) or either a dose $\times$ genotype ($p = 0.151$) or a dose $\times$ time $\times$ genotype ($p = 0.230$) interaction effect. There was an effect of time ($F_{1,41} = 18.73$, $p < 0.001$) as well as a dose $\times$ time ($F_{2,73} = 8.72$, $p = 0.001$) interaction effect. Post-hoc analyses revealed that mice display less hypothermia to 0.01 ($p = 0.002$), 0.03 ($p = 0.012$), 0.1 ($p = 0.005$), 0.3 ($p = 0.003$), and 1.0 ($p < 0.001$) mg/kg CP55,940 on day 10 compared to day 0. The ED₅₀ values for the hypothermic effect of CP55,940 differed between drug naive and chronically treated

wildtype ($p = 0.0260$) but not naïve and chronically treated S426A/S430A mice ($p = 0.7678$) (Table 2). These results suggest that wild-type mice develop tolerance to the hypothermic effects of WIN55,212-2 and CP55,940, while tolerance to these drugs is not complete in S426A/S430A mutants.

3.2. Tolerance to once-daily injections of WIN55,212-2 and CP55,940

3.2.1. Tolerance to the antinociceptive effects of WIN55,212-2 and CP55,940

—Tolerance to once-daily treatment with either 10 mg/kg WIN55,212-2 or 0.3 mg/kg CP55,940 was also assessed in wild-type and S426A/S430A mutant mice (Fig. 2). There was an effect of time (number of WIN55,212-2 injections) ($F_{19,627} = 10.03$, $p < 0.0001$), genotype ($F_{1,33} = 95.89$, $p < 0.0001$), and a significant time $\times$ genotype interaction effect ($F_{19,627} = 1.73$, $p = 0.0275$). Post-hoc analyses revealed that tolerance to the antinociceptive effects of 10 mg/kg WIN55,212-2 was altered in S426A/S420A mutant mice compared to wild-type littermates after 3–11 (3, 7, 8, $p < 0.01$; 4, 5, 6, 9, 10, 11, $p < 0.001$) and 13–17 (13, $p < 0.01$; 14–17, $p < 0.001$) days of WIN55,212-2 treatment (Fig. 2A). The half-time for tolerance to the antinociceptive effects of 10 mg/kg WIN55,212-2 was longer (16.62 d, 95%CI 4.5954–27.83 d) in S426A/S430A mutant mice compared to wild-type controls (1.746 d, 95%CI 1.187–3.301 d).

Analysis of antinociception in mice treated daily with 0.3 mg/kg CP55,940 resulted in an effect of time (number of CP55,940 injections) ($F_{14,280} = 15.93$, $p < 0.0001$) and genotype ($F_{1,20} = 23.07$, $p = 0.0001$), but not a time $\times$ genotype interaction ($p = 0.0517$) effect. While the half-time for tolerance to antinociceptive effect of 0.3 mg/kg CP55,940 in wild-type mice was 12.32 days (95%CI 4.246-infinity), the half-time for tolerance in S426A/S430A mice could not be calculated using our curve fitting analysis. Therefore, although S426A/S430A mutants were more sensitive to the antinociceptive effects of CP55,940 compared to their wild-type littermates, there was no evidence of a significant difference in tolerance to 0.3 mg/kg CP55,940 (Fig. 2B).

3.2.2. Tolerance to the hypothermic effects to WIN55,212-2 and CP55,940—

Analysis of hypothermia in mice treated once-daily with 10 mg/kg WIN55,212-2 indicated an effect of time (number of WIN55,212-2 injections) ($F_{19,627} = 27.60$, $p < 0.0001$) and genotype ($F_{1,33} = 58.23$, $p < 0.0001$) but not a significant time $\times$ genotype interaction effect ($p = 0.2267$) (Fig. 2C). This data analysis indicates that although S426A/S430A mutant mice were more sensitive to the hypothermic effects of WIN55,212-2 than their wild-type littermates, there was no difference in tolerance to the hypothermic effects for this agonist. In contrast, analysis of hypothermia in mice treated once-daily with 0.3 mg/kg CP55,940 indicated an effect of time (number of CP55,940 injections) ($F_{14,280} = 40.46$, $p < 0.0001$), genotype ($F_{1,20} = 16.18$, $p = 0.0007$), and a time $\times$ genotype interaction effect ($F_{14,280} = 1.98$, $p = 0.0191$) (Fig. 2D). Post-hoc analyses revealed that tolerance to the hypothermic effects of 0.3 mg/kg CP55,940 was different in S426A/S430A mutants compared to wild-type littermates on days 3 ($p < 0.01$), 7 ($p < 0.05$), and 10 ($p < 0.05$) of treatment.

3.3. Antinociceptive effects of WIN55,212-2 and CP55,940 in the formalin test

3.3.1. Acute phase (phase I) of the formalin test—Tolerance to the antinociceptive effects of WIN55,212-2 and CP55,940 in the acute and inflammatory phases of the formalin test was also assessed in wild-type and S426A/S430A mutant mice. Analysis of antinociception in mice treated once-daily with 3 mg/kg WIN55,212-2 found an effect of time (number of WIN55,212-2 injections) ($F_{4,31} = 52.25$, $p < 0.0001$) in the acute phase of the formalin test (Fig. 3A). The main effect of time was driven by the differences in pain responses following vehicle compared to WIN55,212-2 as pre-treatment with 3 mg/kg WIN55,212-2 significantly attenuated pain responses on all days tested (1, 7, 14, and 21; $p < 0.0001$) compared to vehicle. There was not a main effect of genotype ($p = 0.8740$) or a genotype $\times$ time interaction ($p = 0.8716$), suggesting that the S426A/S430A mutation did not affect either tolerance or the acute antinociceptive response to WIN55,212-2 in phase I of the formalin test.

Analysis of antinociception in mice treated once-daily with 0.3 mg/kg CP55,940 did not reveal either a main effect of genotype ($p = 0.0584$) or a time $\times$ genotype interaction effect ($p = 0.8610$), but did indicate a main effect of time (number of CP55,940 injections) ($F_{3,27} = 37.61$, $p < 0.0001$) in the acute phase (Phase I; Fig. 3B) of the formalin test. This main effect was due to differences in baseline pain following pre-treatment with vehicle versus pre-treatment with 0.3 mg/kg CP55,940 on days 7 ($p < 0.0001$), 14 ($p < 0.0001$), and 21 ($p < 0.01$) and the onset of partial tolerance following 21 days of once-daily CP55,940 treatment compared to 7 days of once-daily treatment ($p < 0.05$; Fig. 3B).

3.3.2. Inflammatory phase (phase II) of the formalin test—Tolerance to the antinociceptive effects of once-daily 3 mg/kg WIN55,212-2 and 0.3 mg/kg CP55,940 were also assessed in the inflammatory phase (Phase II) of the formalin test (Fig. 3C and D). For the inflammatory phase of the formalin test, there was an effect of time (number of WIN55,212-2 injections) ($F_{4,30} = 70.66$, $p < 0.0001$), genotype ($F_{1,31} = 18.46$, $p = 0.0002$), and a time $\times$ genotype interaction effect ($F_{4,31} = 4.20$, $p = 0.0081$; Fig. 3C). Post-hoc analyses revealed that wild-type mice were quicker to develop tolerance to WIN 55,212-2 than S426A/S430A mice. Wild-type mice showed significantly greater pain responses following 14 ($p < 0.01$) and 21 ($p < 0.001$) days of once-daily treatment with WIN55,212-2 than S426A/S430A mice.

There was also an effect of time ($F_{3,3027} = 32.63$, $p < 0.0001$) and genotype ($F_{1,27} = 4.39$, $p = 0.0457$) but not a time $\times$ genotype interaction ($p = 0.4102$) on the antinociceptive effects of once-daily injections of 0.3 mg/kg CP55,940 in the inflammatory phase of the formalin test. While the lack of a significant interaction indicates there was no difference in tolerance between wild-type and S426A/S430A mice, overall, wild-type mice were less sensitive to the anti-inflammatory effects of CP55,940 compared to S426A/S430A mice. Taken together, these data suggest that the S426A/S430A mutation might selectively alter tolerance to the antinociceptive effects of WIN55,212-2 specifically in the inflammatory phase of the formalin test.

3.4. Tolerance to WIN55,212-2 and CP55,940 in cisplatin-evoked chronic neuropathic pain

Tolerance to the anti-allodynic effects of 3 mg/kg WIN55,212-2 and 0.3 mg/kg CP55,940 was examined in wild-type and S426A/S430A mutant mice with cisplatin-induced neuropathic pain. We found no difference in baseline paw withdrawal thresholds between untreated wild-type and S426A/S430A mutant mice ($p = 0.8094$). Neuropathic pain, as evidenced by a decrease in von Frey paw pressure thresholds, was induced by four weeks of cisplatin treatment [$t(2) = 6.478$, $p = 0.0230$] and this effect was reversed by 3 mg/kg WIN55,212-2 [$t(2) = 4.549$, $p = 0.0451$; Fig. 4A]. Mice developed tolerance to the anti-allodynic effects of WIN55,212-2 ($F_{12,252} = 14.97$, $p < 0.0001$) across 25 days of once-daily treatment. There was not an effect of genotype ($p = 0.1225$) but there was a significant genotype $\times$ time interaction effect ($F_{12,252} = 4.60$, $p < 0.0001$) indicating that tolerance to WIN55,212-2 was different between mutant and wild-type mice. Post-hoc analysis of this interaction effect indicates that tolerance to the anti-allodynic effects of WIN55,212-2 was altered in S426A/S430A mutants compared to wild-type mice following 15 ($p < 0.05$), 17 ($p < 0.05$), and 19 ($p < 0.01$) days of WIN55,212-2 injections. Tolerance was confirmed by determining when the anti-allodynic effects of WIN55,212-2 were no longer different from their neuropathic pain baseline. Wild-type mice displayed tolerance to WIN55,212-2 by day 15 ($p > 0.05$) while S426A/S430A mutants exhibited tolerance by day 21 ($p > 0.05$) (Fig. 4A), providing further evidence that tolerance for the anti-allodynic effects of WIN55,21202 were disrupted in S426A/S430A mutants compared to wild-type littermates.

Tolerance to the anti-allodynic effects of 0.3 mg/kg CP55,940 in mice with cisplatin-induced neuropathic pain was also assessed in wild-type and S426A/S430A mutant mice (Fig. 4B). Mice demonstrated significant neuropathic pain as evidenced by a decrease in von Frey thresholds following cisplatin treatment [$t(2) = 10.25$, $p = 0.0094$] that was reversed by treatment with 0.3 mg/kg CP55,940 [$t(2) = 18.19$, $p = 0.0030$]. Tolerance to the anti-allodynic effects of CP55,940 ($F_{9,225} = 16.57$, $p < 0.0001$) developed across 19 consecutive days of treatment. Subsequent analyses indicated that wild-type and S426A/S430A mutant mice developed tolerance to the anti-allodynic effects after 15 days of once-daily 0.3 mg/kg CP55,940 treatment (no difference from neuropathic baseline; $p > 0.05$). Interestingly, S426A/S430A mice did show a slight difference from neuropathic baseline following day 19 ($p < 0.05$). There was no effect of genotype ($p = 0.1637$) nor a genotype $\times$ time interaction effect ($F_{9,225} = 1.07$, $p = 0.3878$), suggesting that tolerance to the anti-allodynic effects of CP55,940 was not altered in S426A/S430A mutants.

4. Discussion

Tolerance to the antinociceptive effects of cannabinoid agonists has been described in both rodents (Morgan et al., 2014; Sim-Selley and Martin, 2002) and humans (Clark et al., 1981). However, the molecular mechanisms underlying tolerance to cannabinoids are still being elucidated. Previous work demonstrated that phosphorylation of two serines at residues 426 and 430 by GRK3 and subsequent association with β -arrestin2 (Daigle et al., 2008; Jin et al., 1999) was sufficient for desensitization of cannabinoid receptor 1 (CB₁) *in vitro*. Previous work has shown that mice expressing a mutant, desensitization-resistant form of CB₁ (S426A/S430A) develop tolerance to Δ^9 -tetrahydrocannabinol (Δ^9 -THC) more slowly than

wild-type littermates (Morgan et al., 2014). There is growing evidence to suggest that some cannabinoid agonists, including WIN55,212-2, can induce biased signaling through β -arrestin pathways (Flores-Otero et al., 2014). Evidence from cell culture models also suggests that the S426A/S430A mutant form of CB₁ exhibits biased signaling through β -arrestin1 versus G-protein-mediated signaling mechanisms (Delgado-Peraza et al., 2016). Evidence of biased signaling at CB₁ highlights the need to test whether cannabinoid tolerance occurs through agonist-specific mechanisms. The current study evaluates this possibility by assessing the role of GRK/ β -arrestin2-mediated desensitization of CB₁ in tolerance to CP55,940, and WIN55,212-2, two structurally diverse synthetic cannabinoid agonists with known signaling biases.

Tolerance to the effects of a drug often develops following prolonged administration, where a constant dose of drug produces a reduced effect (Martin et al., 2004; Taylor and Fleming, 2001). In this study, we used two models of acute nociceptive pain as well as a model of chronic neuropathic pain to assess tolerance to the antinociceptive, hypothermic, and anti-allodynic effects of WIN55,212-2 and CP55,950 in desensitization-deficient S426A/S430A mutant mice. We found that tolerance to the antinociceptive and anti-allodynic effects of WIN55,212-2 was attenuated in S426A/S430A mutant mice consistent with previous studies examining tolerance to ⁹-THC (Morgan et al., 2014). S426A/S430A mutant mice demonstrated a longer half-time to tolerance for WIN55,212-2 than wild-type littermates. Tolerance to the antinociceptive effects of WIN55,212-2 was also disrupted in S426A/S430A mice using the formalin test. Tolerance to the antinociceptive effects of once-daily injections of CP55,940 was not disrupted in S426A/S430A mutants while tolerance to the hypothermic effects of these injections was modestly reduced in S426A/S430A mutants. However, the dose-response curve shifts for the antinociceptive and hypothermic effects of CP55,940 following 10 days of once daily CP55,940 administration was absent in S426A/S430A mutant mice. These results suggest that CP55,940 tolerance is mediated through complex mechanisms that are likely dose and response dependent and not well-understood. Collectively, our current results, as well as those from previous work, demonstrate that disruption of β -arrestin2-mediated desensitization, either through removal of the protein or its binding sites on CB₁, might alter tolerance to cannabinoid agonists.

In this study, tolerance to specific cannabinoid agonists was differentially affected by blockade of GRK/ β -arrestin2-mediated CB₁ desensitization. For example, tolerance to the antinociceptive and anti-allodynic effects of WIN55,212-2 was significantly disrupted and delayed in S426A/S430A mutant mice. In contrast, tolerance to CP55,940 was altered in S426A/S430A mutant mice under certain experimental conditions but not others. Previous *in vitro* studies demonstrated that CP55,940 and ⁹-THC binding induced greater association of β -arrestin2 with CB₁ compared to WIN55,212-2 (Laprairie et al., 2014), suggesting that interfering with β -arrestin2-mediated desensitization might have a greater effect on tolerance to CP55,940 and ⁹-THC. Instead, we observed an opposite effect on tolerance to the antinociceptive effects of these agonists *in vivo*, highlighting the necessity of investigating agonist biases in whole-animal models. Further studies are needed to fully elucidate the other pathways that may contribute to tolerance for the effects of these agonists.

The results we observed for tolerance to these agonists in acute pain assays did not fully manifest in our assay for chronic chemotherapy-evoked neuropathic pain. We found that disruption of GRK/ β -arrestin-mediated CB₁ desensitization produced only a modest effect on tolerance to the anti-allodynic effects of WIN55,212-2 in mice experiencing chronic cisplatin-induced neuropathy. The effect of the S426A/S430A mutation in CB₁ on cannabinoid tolerance may be masked in pathological chronic pain conditions where CB₂ signaling pathways play a more important role in modulating antinociception. This possibility is supported by an upregulation of CB₂ expression that occurs in chronic pain states following peripheral nerve injury (Wotherspoon et al., 2005; Zhang et al., 2003). WIN55,212-2 and CP55,940 are agonists for both CB₁ and CB₂ (Howlett, 2002) and multiple studies demonstrate a lack of tolerance for CB₂ agonists (Deng et al., 2015; Yuill et al., 2017). Therefore, it is possible that upregulation of CB₂ in cisplatin-treated mice leads to a sufficient level of CB₂-mediated nociceptive signaling to mask the effects of the S426A/S430A mutation on cannabinoid tolerance. Future studies could investigate this possibility by using a CB₁selective agonist such as arachidonyl-2-chloroethylamide (ACEA) (Hillard et al., 1999) or by co-administering these mixed agonists to S426A/S430A double mutant mice that lack CB₂ to isolate the specific contribution of the S426A/S430A mutation to tolerance for these agonists in this chronic pain model. Further, the use of single doses of WIN55,212-2 and CP55,940 for evaluating the effect of the S426A/S430A mutation on cannabinoid tolerance is a limitation to our study, and it is possible that more pronounced genotype effects might be detected using other doses. Nonetheless, our findings suggest that GRK/ β -arrestin-mediated desensitization plays a more profound role in tolerance to WIN55,212-2, while this pathway plays only a minor role in tolerance to CP55,940 and Δ^9 -THC (Morgan et al., 2014).

We report that tolerance to the antinociceptive effect of WIN55,212-2 was profoundly delayed but not entirely blocked in S426/S430A mutant mice. One advantage of this study is the relatively long duration of daily drug administration. This allowed us to look at the development of robust tolerance and to better attempt to determine the half-time to tolerance for each drug and assess differences in those half-times as a function of the mutation. Mice lacking GPCR-associated sorting protein 1 (GASP-1), a protein involved in CB₁ trafficking and degradation, exhibit delayed tolerance to the antinociceptive but not the hypothermic effects of WIN55,212-2 (Martini et al., 2010). GASP-1 does not appear to interact with CB₁ in the distal regions of the C-terminal tail responsible for internalization (Hsieh et al., 1999; Tappe-Theodor et al., 2007), but it is unknown if GASP-1 interacts with CB₁ in the separate more proximal arrestin-binding regions responsible for CB₁ desensitization. Additionally, mice lacking protein kinase C epsilon (PKC ϵ) demonstrate accelerated tolerance to WIN55,212-2 (Wallace et al., 2009). Similar to our findings, an agonist bias was observed for the effect of PKC ϵ in cannabinoid tolerance as tolerance to CP55,940 was not affected by PKC ϵ deletion. Either one of these proteins could be involved in delayed tolerance to WIN55,212-2 in the S426A/S430A mutant mice through some unknown interaction or mechanism. Therefore, a better understanding of how these two proteins might interact with the arrestin-binding region that is mutated in the S426A/S430A mice would provide valuable insight into this possibility.

Previous work from our laboratory has demonstrated enhanced acute antinociceptive and hypothermic responses to Δ^9 -THC, N-arachidonylethanolamine (AEA) in combination with the fatty acid amide hydrolase (FAAH) inhibitor URB597, and the dual monoacylglycerol lipase (MAGL)/FAAH inhibitor JZL195 (Morgan et al., 2014). These findings demonstrate an increased acute response to endocannabinoid and cannabinoid agonists in S426A/S430A mutant mice that is also supported by the current work showing enhanced responses to WIN55,212-2 and CP55,940. Since most of the previous work assessed the acute response to partial cannabinoid agonists such as Δ^9 -THC and AEA, the current work showing that this trend is maintained for potent full agonists, such as WIN55,212-2 and CP55,940, augments this important effect of the S426A/S430A mutation. Taken together, our current data and previously published work demonstrate the crucial role of GRK/ β -arrestin2-mediated CB₁ desensitization in regulating the magnitude of acute responses to a diverse range of cannabinoid agonists.

Although we observed enhanced acute antinociceptive responses to WIN55,212-2 and CP55,940 in S426A/S430A mutants, we did not observe similar changes in the acute hypothermic response to these agonists. Such an enhancement of the acute antinociceptive but not hypothermic response to these high potency full agonists represents a significant difference compared to what we have previously observed for the partial agonists, Δ^9 -THC and AEA. This result raises the possibility of response and/or tissue specific differences in the effect of the S426/S430A mutation on the enhancement of specific cannabinoid-induced responses. Another possible explanation for these full agonists is the loss of body temperature in excess of 20% represents a basement or floor effect where further enhancement of cannabinoid-induced hypothermia is not possible.

S426A/S430A mutant mice demonstrated delayed tolerance to the hypothermic effects of CP55,940 but not WIN55,212-2, suggesting that the effects of interference with GRK/ β -arrestin2-mediated desensitization may modulate tolerance in ways that are also response and/or tissue-specific. This possibility is supported by previous work showing that tolerance develops more slowly for the motor effects of cannabinoids due to resistance of the neuroadaptations responsible for CB₁ desensitization and down-regulation in brain regions, such as the striatum that are involved in these motor responses (Breivogel et al., 1999; McKinney et al., 2008). Further work is needed to understand the potential signaling and regional mechanisms involved in tolerance to specific cannabinoid responses.

In summary, the findings from our study suggest that tolerance to WIN55,212-2 and CP55,940 may develop through different mechanisms, where tolerance to WIN55,212-2 is more highly dependent on a signaling mechanism involving GRK/ β -arrestin-mediated desensitization of CB₁. However, disruption of GRK/ β -arrestin-mediated CB₁ desensitization had a limited effect on tolerance to CP55,940. S426A/S430A mutants displayed an enhanced anti-nociceptive response to both WIN55,212-2 and CP55,940. This study provides behavioral and physiological evidence that the mechanisms involved in tolerance may differ for specific agonists. However, additional future work is needed using additional doses of these agonists to examine tolerance and also neuroadaptations at CB₁ that mediate the effects on cannabinoid tolerance.

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Abbreviations:

AUC	area under the curve
CB₁	cannabinoid receptor 1
CP55,940	(1 α ,2 β)-R-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl-cyclohexyl)]phenyl
CPS	composite pain score
GASP-1	G protein coupled receptor associated sorting protein 1
GPCR	G protein-coupled receptor
GRK	G protein-related kinase
i.p.	intraperitoneal
%MPE	percent maximal possible effect
PKCϵ	epsilon isoform of protein kinase c
s.c.	subcutaneous
;⁹-THC	delta-9-tetrahydrocannabinol
WIN55,212-2	R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3- <i>de</i>]-1,4-benzoxazinyl]-(1-maphthalenyl)methanone mesylate (WIN55,212-2).

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Fig. 1. S426A/S430A mutant mice demonstrate smaller shifts in the antinociceptive and hypothermic dose-response curves following chronic treatment with WIN55,212-2 and CP55,940.

Wild-type and S426A/S430A mutant mice were tested for antinociceptive (A,B) and hypothermic (C,D) responses across a cumulative dose range of either 0.3, 1, 3, 10, and 30 mg/kg WIN55,212-2 or 0.01, 0.03, 0.1, 0.3, and 1 mg/kg CP55,940 as drug naïve (Pre; filled symbols and solid lines) or following nine days of once-daily treatment with either 10 mg/kg WIN55,212-2 or 0.3 mg/kg CP55,940 (Post; open symbols and dashed lines). Means represent [%MPE (A,B) and % BT (C,D)] and error bars represent the standard error of the mean (SEM). Data were analyzed with three-way ANOVA and Bonferroni multiple comparisons test. The number of mice in each group are shown in parentheses.

Fig. 2. Antinociceptive tolerance to WIN55,212-2 but not CP55,940 is significantly delayed in S426A/S430A mice.

Wild-type and S426A/S430A mice were assessed for the development of tolerance to the antinociceptive (A,B) and hypothermic (C,D) effects of once-daily 10 mg/kg WIN55,212-2 (A,C) or 0.3 mg/kg CP55,940 (B,D) in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Means represent [(%MPE (A,B) and % BT (C,D)] and error bars represent the standard error of the mean (SEM). Data were analyzed with two-way ANOVA and Bonferroni multiple comparisons test. The number of mice in each group are shown in parentheses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. The S426A/S430A mutation delays the rate of antinociceptive tolerance development to WIN55,212-2 (but not CP55,940) in a model of acute inflammatory pain.

Wild-type (black bars; $n = 4-5/\text{dose}$) and S426A/S430A mutants (red bars; $n = 4-6/\text{dose}$) were assessed for tolerance to the antinociceptive effects of either 3 mg/kg WIN55,212 (A,C) or 0.3 mg/kg CP55,940 (B,D) in the acute (phase I; A,B) and inflammatory (phase II; C,D) phases of the formalin test. Means represent the AUC of the composite pain score and error bars represent the standard error of the mean (SEM). Data were analyzed with two-way ANOVA and Bonferroni multiple comparisons test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. The S426A/S430A mutation delays tolerance to the anti-allodynic effects of WIN55,212-2 (but not CP55,940) in a model of chronic neuropathic pain.

The von Frey test was used to assess the anti-allodynic effects of once-daily treatment with either 3mg/kg WIN55,212-2 (A) or 0.3 mg/kg CP55,940 (B) in wild-type (black lines, squares) and desensitization-resistant S426A/S430A (red lines, circles) mice. Baseline (BL) and cisplatin-induced neuropathic (Cis) allodynic responses and anti-allodynic responses to 3 mg/kg WIN55,212-2 (Days 1–25) or 0.3 mg/kg CP55,940 (Days 1–19) are shown. The mean represents the amount of force (g) required for paw withdrawal from a noxious mechanical stimulus (A,B) and error bars represent the standard error of the mean (SEM). The number of mice in each group is indicated within the parentheses. Data were analyzed with two-way RM ANOVA and Bonferroni multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Calculated ED₅₀ values (mg/kg) from tail-flick tests following treatment with WIN55,212-2 and CP55,940. ED₅₀ values were calculated from dose-response curves generated by non-linear regression analysis. Values shown are mean and 95% confidence interval, and 10–12 mice were tested for each group. Potency ratios were calculated as the ratio between the ED₅₀ after chronic drug dosing and the ED₅₀ prior to chronic dosing. Confidence intervals that could not be accurately calculated from curve fitting are listed as not able to determine (ND). Data were analyzed using F tests.

	WIN55,212-2		CP55,940	
	WT	S426A/S430A	WT	S426A/S430A
Pre drug	7.668 (4.647–12.65)	8.624 (ND)	0.341 (0.251–0.462)	0.213 (0.177–0.256)
Post drug	28.30 (19.10–41.91)	7.899 (6.403–9.743)	2.206 (0.324–15.02)	0.252 (0.163–0.391)
F statistic	39.29 ($p < 0.0001$)	0.22555 ($p = 0.8734$)	14.74 ($p = 0.0002$)	0.5822 ($p = 0.4471$)
Potency Ratio	3.70	0.92	6.47	1.18

Calculated ED₅₀ values (mg/kg) from body temperature measures following treatment with WIN55,212-2 and CP55,940. ED₅₀ values were calculated from dose-response curves generated by non-linear regression analysis. Values shown are the means with the 95% confidence intervals in parentheses. Potency ratios were calculated as the ratio between the ED₅₀ after chronic drug dosing and the ED₅₀ prior to chronic dosing. A sample size of 10–13 mice were tested for each group. Confidence intervals that could not be accurately calculated from curve fitting are listed as not able to determine (ND). Data were analyzed using F tests.

Table 2

	WIN55,212-2		CP55,940	
	WT	S426A/S430A	WT	S426A/S430A
Pre drug	5.878 (4.148–8.330)	6.396 (5.041–8.115)	0.267 (0.045–1.598)	0.341 (0.218–0.534)
Post drug	9.643 (ND)	9.577 (ND)	1.221 (0.031–47.73)	0.386 (0.179–0.893)
F statistic	0.1209 ($p = 0.2742$)	0.9436 ($p = 0.3339$)	4.464 ($p = 0.037$)	0.0855 ($p = 0.7706$)
Potency Ratio	1.64	1.59	4.57	1.13