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The CB_1 Receptor Positive Allosteric Modulator, ZCZ011, Attenuates Naloxone-Precipitated Diarrhea and Weight Loss in Oxycodone-Dependent Mice

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Running Title Page:

a) CB_1 Receptor PAMs Attenuate Opioid Withdrawal Signs in Mice

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4 figures

4 tables

77 references

Abstract: 245 words

Introduction: 693 words

Discussion: 1453 words

d) THC, Δ^9-tetrahydrocannabinol; 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonylethanolamine (anandamide); CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; GAT211, 3-(2-nitro-1-phenylethyl)-2-phenyl-1H-indole; MAGL, monoacylglycerol lipase; Naloxone hydrochloride, (1S,5R,13R,17S)-10,17-dihydroxy-4-(prop-2-en-1-yl)-12-oxa-4-azapentacyclo[9.6.1.01,13.05,17.07,18]octadeca-7(18),8,10-trien-14-one; Oxycodone hydrochloride, (5α)-4,5-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one; Rimonabant, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; SR144528, *N*-[(1*S*)-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; ZCZ011, 6-methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1H-indole

e) Behavioral Pharmacology

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Abstract

Opioid use disorder reflects a major public health crisis of morbidity and mortality where opioid withdrawal often contributes to continued use. However, current medications that treat opioid withdrawal symptoms are limited by their abuse liability or lack of efficacy. Although cannabinoid 1 (CB_1) receptor agonists, including Δ9-tetrahydrocannabinol (THC), ameliorate opioid withdrawal in both clinical and pre-clinical studies of opioid dependence, this strategy elicits cannabimimetic side effects as well as tolerance and dependence following repeated administration. Alternatively, CB_1 receptor positive allosteric modulators (PAMs) enhance CB_1 receptor signaling and show efficacy in rodent models of pain and cannabinoid dependence but lack cannabimimetic side effects. We hypothesize that the CB_1 receptor PAM, ZCZ011, attenuates naloxone-precipitated withdrawal signs in opioid-dependent mice. Accordingly, male and female mice given an escalating dosing regimen of oxycodone, a widely prescribed opioid, and challenged with naloxone displayed withdrawal signs that included diarrhea, weight loss, jumping, paw flutters, and head shakes. ZCZ011 fully attenuated naloxone-precipitated withdrawal-induced diarrhea and weight loss and reduced paw flutters by approximately half, but its effects on head shakes were unreliable and it did not affect jumping behavior. The anti-diarrheal and anti-weight loss effects of ZCZ0111 were reversed by a CB_1, not a CB_2, receptor antagonist and were absent in CB_1 (-/-) mice, suggesting a necessary role of CB_1 receptors. Collectively, these results indicate that ZCZ011 completely blocked naloxone-precipitated diarrhea and weight loss in oxycodone-dependent mice and suggest that CB_1 receptor PAMs may offer a novel strategy to treat opioid dependence.

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Significance Statement.

Opioid use disorder represents a serious public health crisis in which current medications used to treat withdrawal symptoms are limited by abuse liability and/or side effects. The CB_1 receptor positive allosteric modulator (PAM), ZCZ011, which lacks overt cannabimimetic behavioral effects, ameliorated naloxone-precipitated withdrawal signs through a CB_1 receptor mechanism of action in a mouse model of oxycodone dependence. These results suggest that CB_1 receptor PAMs may represent a viable strategy to treat opioid withdrawal.

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Introduction

Misuse of opioids and the prevalence of individuals with opioid use disorder (OUD) remains a significant public health problem in the United States. Recent 2019 estimates are that 9.7 and 1.4 million individuals aged 12 or older misused opioids and developed OUD, respectively (Substance Abuse and Mental Health Services Administration, 2020). Although current medications used to treat opioid dependence attenuate withdrawal symptoms such as diarrhea, emesis, hand tremors, and anxiety (Farrell, 1994; Wesson and Ling, 2003), they also possess abuse liability (e.g., methadone and buprenorphine) (Cicero and Inciardi, 2005) and are not fully effective for all patients (e.g., lofexidine/clonidine) (Kuhlman *et al.*, 1998; Stolbach and Hoffman, 2020). Thus, a great need exists to develop new efficacious pharmacotherapies, which lack abuse potential, to alleviate opioid withdrawal.

A case report from the 19th century describing an opium-dependent patient treated with cannabis extract (Birch, 1889) suggested that cannabinoid-based medications may effectively treat opioid dependence. A clinical trial conducted more than a century later demonstrated that the primary active constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), alleviates withdrawal symptoms in opioid-dependent patients (Bisaga *et al.*, 2015; Lofwall *et al.*, 2016). Nevertheless, unwanted side effects of THC, including tachycardia, somnolence, and intoxication (Jicha *et al.*, 2015; Lofwall *et al.*, 2016), hinders its application to treat opioid use disorder. THC produces most of its pharmacological effects through the activation of two G-protein coupled receptors, cannabinoid receptor type 1 (CB_1; Matsuda *et al.*, 1990) and type 2 (CB_2; Munro *et al.*, 1993). THC and other CB_1 receptor agonists effectively attenuate withdrawal signs in opioid-dependent rodents (Hine, Friedman, *et al.*, 1975; Hine, Torrelio, *et al.*, 1975; Bhargava, 1976a,b; Vela *et al.*, 1995; Lichtman *et al.*, 2001; Cichewicz and Welch, 2003; Ramesh *et al.*, 2011; Gamage *et al.*, 2015), though this pharmacological approach elicits acute cannabimimetic side effects and results in tolerance and physical dependence after repeated administration (Wiley and Martin, 2003; Grim *et al.*, 2016; Trexler *et al.*, 2018, 2019). Alternatively, inhibitors of monoacylglycerol lipase (MAGL; Dinh *et al.*, 2002) and fatty acid amide hydrolase (FAAH; Cravatt *et al.*, 1996), which hydrolyze the respective endogenous cannabinoids, 2-arachidonoylglycerol

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(2-AG; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) and anandamide (AEA; Devane *et al.*, 1992), attenuate naloxone-precipitated and spontaneous withdrawal signs in morphine-dependent mice with reduced cannabimimetic side effects (Ramesh *et al.*, 2011, 2013; Gamage *et al.*, 2015; Wills *et al.*, 2016). However, CB_1 receptor downregulation and desensitization, and physical dependence following repeated administration of high doses of MAGL inhibitors (Schlosburg *et al.*, 2010, 2014; Ramesh *et al.*, 2011) represents a challenge for this approach.

CB_1 receptor positive allosteric modulators (PAMs) offer therapeutic potential to treat opioid dependence and other conditions, without eliciting side effects associated with THC. Indeed, the CB_1 receptor PAMs ZCZ011 and GAT211 lack overt behavioral cannabimimetic effects, but produce antinociceptive effects in multiple rodent models of pain (Ignatowska-Jankowska, Baillie, *et al.*, 2015; Slivicki *et al.*, 2017, 2020; Thapa *et al.*, 2020) as well as reduce withdrawal signs in cannabinoid-dependent mice (Trexler *et al.*, 2019). These molecules are believed to bind at allosteric site(s) on the CB_1 receptor that results in a conformational change of the orthosteric site to enhance the binding and efficacy of endogenous cannabinoids and/or elicit allosteric agonist effects on their own (Dopart *et al.*, 2018; Tseng *et al.*, 2019), and may also be classified as a positive allosteric agonist (ago-PAMs) (Kenakin, 2013).

Here we tested whether the CB_1 receptor PAM, ZCZ011, attenuates naloxone-precipitated withdrawal signs using an established mouse model of oxycodone-dependence (Enga *et al.*, 2016; Carper *et al.*, 2021). Oxycodone represents a widely used prescription opioid misused by 3.2 million people aged 12 or older in the United States (Substance Abuse and Mental Health Services Administration, 2020). Oxycodone-dependent male and female mice were challenged with naloxone to precipitate somatic withdrawal signs (i.e., jumps, paw flutters, and head shakes), diarrhea, and body weight loss. We compared the actions of ZCZ011 on naloxone-precipitated withdrawal signs to those of oxycodone, which served as a positive control. Lastly, we employed the CB_1 receptor inverse agonist/antagonist, rimonabant, the CB_2 receptor antagonist, SR144528, and constitutive CB_1 (-/-) mice (Zimmer *et al.*, 1999) to assess cannabinoid receptor involvement of the anti-withdrawal effects of ZCZ011.

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Materials and Methods.

Animals. Male and female ICR mice (minimum 8 weeks of age; Envigo, Indianapolis, IN, USA) with respective body weights of 35-45 g and 30-40 g served as subjects. ICR mice were delivered at 7 weeks followed by one week of habituation in VCU's vivarium before testing began. Age-matched male and female CB_1 (+/+) and CB_1 (-/-) mice, derived from CB_1 (+/-) breeding pairs bred within the Mutant Mouse Core at Virginia Commonwealth University, also served as subjects. These transgenic mice were created by Zimmer and colleagues (1999) and have been backcrossed onto a C57BL/6J background for at least 15 generations. The subjects were housed up to four mice per cage, in a light- (12 h light/dark cycle; lights on at 0600), temperature- (20–22°C) and humidity-controlled (55% ± 10%) AAALAC–approved facility at Virginia Commonwealth University. Mice received water and standard rodent chow *ad libitum*. Animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs. Oxycodone hydrochloride [(5α)-4,5-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one], naloxone hydrochloride [(1S,5R,13R,17S)-10,17-dihydroxy-4-(prop-2-en-1-yl)-12-oxa-4-azapentacyclo[9.6.1.01,13.05,17.07,18]octadeca-7(18),8,10-trien-14-one], rimonabant, the CB_1 receptor inverse agonist/antagonist [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl], and SR144528, the CB_2 receptor antagonist, [*N*-[(1*S*)-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide], were obtained from the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). ZCZ011, the CB_1 receptor PAM [6-methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1H-indole], was synthesized based on Ignatowska-Jankowska and colleagues (2015) and improved upon according to an established protocol in the Lu laboratory (see Supplemental information for revised synthesis). ZCZ011, rimonabant, and SR144528 were dissolved in a vehicle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9% NaCl; sterile) in a 1:1:18 ratio. Oxycodone and naloxone were dissolved in sterile 0.9% saline. All injections were administered in

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a volume of 10 ml/kg of body weight. Oxycodone and naloxone were administered via the subcutaneous (s.c.) route of administration, whereas ZCZ011, rimonabant, and SR144528 were given via intraperitoneal (i.p.) injection.

Naloxone-Precipitated Oxycodone Withdrawal Model. To induce oxycodone dependence, counter-balanced groups of male and female mice were administered escalating doses of oxycodone over an eight day period as previously described (Enga *et al.*, 2016; Carper *et al.*, 2021). In brief, mice were administered s.c. injections of 9.0, 17.8, 23.7, then 33.0 mg/kg oxycodone (or saline) twice a day separated by approximately 7 h on days 1-2, 3-4, 5-6, and 7-8, respectively. On day nine, mice were administered 33 mg/kg oxycodone (or saline) followed by 1 mg/kg naloxone 2 h later to precipitate withdrawal (Enga *et al.*, 2016; Carper *et al.*, 2021) and withdrawal signs were assessed as previously described with some modifications (Ramesh *et al.*, 2011).

Plexiglas chambers were used to house mice during withdrawal assessment, with each chamber constructed of white sides and white bottom panels, a clear perforated top panel (eight ¼ inch diameter holes for ventilation), a sliding clear front panel (23 cm H), and a sliding mirrored back panel (23 cm H). The chambers were enclosed in sound-attenuating cabinets that contained an indirect filtered LED light source and fans for air circulation and white noise. Each cabinet contained a mounted 2.8-12.0mm varifocal lens mini-USB camera (Ailipu Technology Co., Ltd, Guangdong, China) that recorded mice through the clear front panel and were saved using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL). All recorded videos were randomized and scored by one primary trained observer who was blinded to treatment condition or genotype using ANY-maze software and ODLog (v2.7.2 for Windows; Macropod), respectively. A subset of sixteen videos of oxycodone-dependent mice were scored by a second trained observer to ensure inter-rater reliability (Supp. Fig. 1).

On the morning of day 9 (i.e., 0900 h), mice were administered 33 mg/kg oxycodone and were placed in their respective Plexiglas chambers after 90 min for a 30-min acclimation period. At 120 min, the mice were removed from the chambers, weighed, administered naloxone (1 mg/kg s.c.), and returned to the chambers for a 30-min test session after the chambers were cleaned using a paper towel moistened

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with water. The mice were weighed again immediately after the test session. Chambers were changed between tests and cleaned with 10% ethanol to mitigate accumulation of residual odor between cohorts of mice.

To reduce the number of mice (Kirk, 2013) used for the acute oxycodone and ZCZ011 dose-response experiments (Fig. 1 and 2), preliminary experiments demonstrated that 75 mg/kg oxycodone and 40 mg/kg ZCZ011 did not exhibit behavioral effects (i.e., the expression of diarrhea, weight loss, jumps, paw flutters, or head shakes) in mice repeatedly administered saline and receiving a naloxone injection on day 9. In the dose-response experiment testing whether acute oxycodone administration will attenuate withdrawal responses, counter-balanced groups of male and female oxycodone-dependent ICR mice received an acute oxycodone injection (17, 33, or 75 mg/kg s.c.) or saline 30 min before naloxone administration with doses based on experiments conducted in C57BL/6J male mice (Carper *et al.*, 2021). Male and female ICR mice injected repeatedly with saline received an acute saline injection 30 min before naloxone to serve as a negative control. The two experimental factors of repeated oxycodone and acute oxycodone were treated as a single statistical factor and were analyzed as a 2-way ANOVA of oxycodone treatment by sex. In the experiment evaluating the dose-response relationship of ZCZ011, counter-balanced groups of male and female oxycodone-dependent ICR mice received ZCZ011 (5, 10, 20, or 40 mg/kg i.p.) or vehicle 75 min before naloxone. The pretreatment time and doses of ZCZ011 were based upon previous literature coinciding with its peak antinociceptive effects (Ignatowska-Jankowska, Baillie, *et al.*, 2015). An additional group of male and female oxycodone-dependent ICR mice were administered oxycodone (75 mg/kg s.c.) 30 min before naloxone to serve as a positive control. Male and female ICR mice repeatedly administered saline received vehicle 75 min before naloxone administration to serve as a negative control. The two experimental drug factors of oxycodone treatment and ZCZ011 treatment were also collapsed and treated as a single statistical factor.

We employed pharmacological and genetic approaches to determine the involvement of the cannabinoid receptor and the anti-withdrawal effects of ZCZ011. Employing pharmacological and genetic approaches mitigates associated pitfalls when investigating cannabinoid receptor involvement for CB_1

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receptor PAMs *in vivo*; ZCZ011 decreases equilibrium binding of rimonabant, the CB_1 receptor inverse agonist/antagonist, *in vitro* (Ignatowska-Jankowska, Baillie, *et al.*, 2015) and constitutive CB_1 (-/-) mice exhibit a lower frequency of opioid withdrawal signs *in vivo* (Ledent *et al.*, 1999; Lichtman *et al.*, 2001), which provides potential alternative explanations for cannabinoid receptor involvement when examining CB_1 receptor PAMs in animal models of opioid dependence. In the rimonabant experiment, counter-balanced groups of male and female oxycodone-dependent ICR mice received two i.p. injections before naloxone administration. The first injection consisted of an i.p. injection of vehicle, rimonabant (3 mg/kg), or SR144528 (3 mg/kg), the CB_2 receptor antagonist, and the second injection consisted of vehicle or ZCZ011 (40 mg/kg), with the respective injections given 85 and 75 min before naloxone administration. A dose of 3 mg/kg for rimonabant and SR144528 was chosen based upon previous literature demonstrating reversal of the effects of CB_1 and CB_2 receptor agonists in models of inflammatory and neuropathic pain (Kinsey *et al.*, 2009, 2011; Ignatowska-Jankowska, Wilkerson, *et al.*, 2015) as well as cannabinoid and opioid withdrawal (Long, Li, *et al.*, 2009; Schlosburg *et al.*, 2009; Ramesh *et al.*, 2011, 2013). In the experiment using CB_1 (-/-) mice, we used age-matched male and female oxycodone-dependent CB_1 (+/+) and CB_1 (-/-) mice that were administered vehicle or ZCZ011 (40 mg/kg i.p.) 75 min before undergoing naloxone-precipitated withdrawal. Each figure contains schematics outlining the timeline of treatments for each experiment.

Somatic signs of withdrawal were measured as previously described (Ramesh *et al.*, 2011), which included the number of jumps, front paw flutters (including single and double flutters), head shakes, the occurrence of diarrhea, and the amount of body weight loss. Jumps were recorded as every incident when the mouse jumped from its hind legs or all four legs. Paw flutters were recorded as single or double flutters separated by at least 1 s or interrupted by any other behavior, such as jumps, head shakes, or grooming. Head shakes were recorded as every incident when the mouse quickly rotated the head clockwise and counter-clockwise. The occurrence of diarrhea was assessed by the presence of increased fluid content in the fecal pellets, discoloration of the pellet (e.g., dark brown to light brown), or

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fragmentation of fecal pellets. Mice were also weighed before and immediately after the 30-min test session to assess body weight loss (%).

Statistical Analyses. A power analysis from two preliminary experiments indicated that a sample size of 12 and 16 mice per group was required to detect significant effects of acute oxycodone and ZCZ011, respectively. Data for the percentage of mice present with diarrhea are reported as mean bar graphs, whereas data pertaining to other withdrawal signs are individual data points with the mean ± standard deviation (SD). The occurrence of diarrhea during the 30-min test session was scored as a quantal measure. Body weight loss (%) was calculated using the following formula:

$$BWL(\%) = (100 - \left(\frac{postT}{preT}\right)) * 100\%$$

In which *postT* represents the body weight of mice after the 30-min test session and *preT* represents body weight before the 30-min test session. The Fisher exact test was used to analyze occurrence of diarrhea. Data were analyzed using two- and three-way between-measures analysis of variance (ANOVA) and Student's *t*-test for planned comparisons. Dunnett's post-hoc test was used to compare drug treatments with oxycodone-dependent saline-treated or vehicle-treated mice and Tukey's post-hoc test was used to compare between various treatments. Three-way ANOVAs were used to verify whether there were relevant sex differences for the latter studies investigating the anti-withdrawal mechanism of ZCZ011 (Fig. 3 and 4). If no significant three-way interaction is observed and a two-way interaction occurs for the respective factors when excluding sex, subsequent two-way ANOVAs were conducted by pooling data across sex to discern cannabinoid receptor involvement for the effects of ZCZ011 (Fig. 3 and 4) (Kirk, 2013). To determine inter-rater reliability, a Pearson correlation was conducted between the two observers for each measure scored in the sixteen videos of oxycodone-dependent mice undergoing precipitated withdrawal. The criterion of significance for all statistical tests was $p < 0.05$. Experimental protocols were designed to test working hypotheses with planned statistical analyses.

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Results

Supplementary Fig.1 compares data between a second trained observer, who scored a subset of sixteen videos of oxycodone-dependent mice, and the primary observer to ensure inter-rater reliability. Pearson correlations demonstrated a high correlation between observers for naloxone-precipitated jumps ($r = 0.9995$; $p < 0.0001$; Fig. S1A), paw flutters ($r = 0.9953$; $p < 0.0001$; Fig. S1B), and head shakes ($r = 0.9180$; $p < 0.0001$; Fig. S1C).

Oxycodone Dose-Dependently Attenuated Naloxone-Precipitated Withdrawal Signs in Oxycodone-Dependent ICR Mice. In the first experiment, we tested whether a positive control, acute oxycodone (17, 33, and 75 mg/kg s.c.), attenuate naloxone-precipitated diarrhea, weight loss, jumps, paw flutters, or head shakes. Table 1 summarizes Fisher exact test results for naloxone-precipitated diarrhea and two-way ANOVAs (Sex x Oxycodone treatment) for each of the other withdrawal signs. A schematic of treatment conditions for this experiment is shown in Fig. 1A. As illustrated in Fig. 1B, 33 mg/kg oxycodone (3 of 12 mice) and 75 mg/kg oxycodone (0 of 12 mice) elicited a lower incidence of naloxone-precipitated diarrhea than oxycodone-dependent saline-treated (12 of 12) mice. Moreover, mice repeatedly administered saline (3 of 12) exhibited a lower incidence of naloxone-precipitated diarrhea than oxycodone-dependent saline-treated (12 of 12) mice. Furthermore, 75 mg/kg oxycodone completely blocked naloxone-precipitated body weight loss, while 33 mg/kg oxycodone blocked half the amount of body weight loss compared to oxycodone-dependent saline-treated mice (Fig. 1C). Notably, while micturition was not quantified, weight loss often occurred in mice whose withdrawal chamber was saturated with substantial volumes of urine following the 30-min test period. In addition, 33 mg/kg and 75 mg/kg oxycodone completely lowered the frequency of naloxone-precipitated jumps compared to oxycodone-dependent saline-treated mice. Furthermore, 75 mg/kg oxycodone completely lowered the frequency of naloxone-precipitated paw flutters, whereas 33 mg/kg oxycodone lowered the frequency by approximately half compared to oxycodone-dependent saline-treated mice (Fig. 1E). Lastly, 33 mg/kg and 75 mg/kg oxycodone lowered the frequency of naloxone-precipitated head

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shakes compared to oxycodone-dependent saline-treated mice. No endpoint showed a significant sex by oxycodone treatment interaction or main effect of sex (Fig. 1F). Because there were no significant interactions between oxycodone and sex in this experiment or ZCZ011 and sex throughout the study, the variable of sex is collasped in all figures.

ZCZ011 Attenuates Naloxone-Precipitated Withdrawal Signs in Oxycodone-Dependent Mice. Figure 2 illustrates the effectiveness of ZCZ011 (5, 10, 20, and 40 mg/kg i.p.) in attenuating naloxone-precipitated diarrhea, weight loss, jumps, paw flutters, and head shakes. Table 2 summarizes Fisher exact test results for naloxone-precipitated diarrhea and two-way ANOVAs (sex x drug treatment) for each of the other withdrawal signs. A schematic of the treatment conditions for this experiment in which all mice were challenged with 1 mg/kg naloxone before behavioral observations is shown in Fig. 2A. As illustrated in Fig. 2B, 40 mg/kg ZCZ011 (3 of 16 mice) and 75 mg/kg oxycodone (0 of 16 mice) elicited a lower incidence of naloxone-precipitated diarrhea than oxycodone-dependent vehicle-treated (15 of 16) mice. Furthermore, naloxone did not elicit diarrhea in control mice that received repeated injections of saline (0 of 16 mice). In addition, 40 mg/kg ZCZ011 and 75 mg/kg oxycodone completely blocked naloxone-precipitated body weight loss compared to oxycodone-dependent vehicle-treated mice (Fig. 2C). As illustrated in Fig. 2D, 75 mg/kg oxycodone completely lowered the frequency of naloxone-precipitated jumps compared to oxycodone-dependent vehicle-treated mice, whereas no dose of ZCZ011 affected jumping behavior. Although a sex by ZCZ011 treatment interaction failed to achieve significance, a main effect of sex was observed with male mice averaging 61 jumps and female mice averaging 29 jumps. As shown in Fig. 2E, 20 mg/kg and 40 mg/kg ZCZ011 lowered the frequency of naloxone-precipitated paw flutters by about half while 75 mg/kg oxycodone completely lowered the frequency of paw flutters compared to oxycodone-dependent vehicle-treated mice. Lastly, 20 mg/kg ZCZ011, 40 mg/kg ZCZ011, and 75 mg/kg oxycodone lowered the frequency of naloxone-precipitated head shakes compared to oxycodone-dependent vehicle-treated mice (Fig. 2F).

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ZCZ011 Attenuates Naloxone-Precipitated Diarrhea and Weight Loss through the Activation of CB_1 Receptors. These experiments investigated whether CB_1 or CB_2 receptors mediate the effects of ZCZ011 on withdrawal-induced diarrhea, weight loss, and paw flutters (see schematic of the treatment conditions in Fig. 3A and 4A). Subjects were administered rimonabant, the CB_1 receptor inverse agonist/antagonist, SR144528, the CB_2 receptor antagonist, or vehicle prior to either vehicle or 40 mg/kg ZCZ011, and were then evaluated for naloxone-precipitated diarrhea, weight loss, paw flutters, and head shakes. Table 3 summarizes Fisher exact test results for naloxone-precipitated diarrhea and three-way ANOVAs (Sex x CB antagonist x ZCZ011 treatment) for each of the other withdrawal signs. As shown in Fig. 3B, oxycodone-dependent vehicle-ZCZ011-treated (4 of 16) mice and SR144528-ZCZ011-treated (3 of 15) mice exhibited a lower incidence of naloxone-precipitated diarrhea than oxycodone-dependent vehicle-vehicle-treated (15 of 15) mice and oxycodone-dependent SR144528-vehicle-treated (15 of 16) mice, respectively. Moreover, rimonabant, but not SR144528, blocked the protective effects of ZCZ011 on naloxone-precipitated body weight loss suggesting a CB_1-mediated effect. Again, ZCZ011 did not significantly affect naloxone-precipitated jumps (Fig. 3D); though, a main effect of sex revealed male mice averaged 84 jumps whereas female mice averaged 46 jumps. Significant main effects were found for ZCZ011 treatment and sex for naloxone-precipitated paw fluttering behavior (Fig. 3E). As seen in the figure, mice in the vehicle-ZCZ011 group averaged 27 paw flutters, whereas mice in the vehicle-vehicle group averaged 46 paw flutters. Moreover, male mice averaged 34 paw flutters whereas female mice averaged 44 paw flutters. However, no significant interactions of cannabinoid antagonists and ZCZ011 treatment were found, suggesting lack of CB_1 or CB_2 receptor involvement. Finally, neither ZCZ011 nor the cannabinoid receptor antagonists produced significant effects on naloxone-precipitated head shakes (Fig. 3F). On the contrary, a significant main effect of sex was found in which male mice averaged 5 head shakes whereas female mice averaged 3 head shakes.

Figure 4 shows the effects of ZCZ011 (40 mg/kg i.p.) on naloxone-precipitated diarrhea, weight loss, paw flutters, and head shakes in CB_1 (+/+) and CB_1 (-/-) mice (see Fig. 4A for the schematic of the

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experimental design). Table 4 summarizes Fisher exact test results for naloxone-precipitated diarrhea and three-way ANOVAs (Sex x Genotype x ZCZ011 treatment) for each of the other withdrawal signs. As shown in Fig. 4B, CB_1 deletion annihilated the effects of ZCZ011 in reducing the incidence of naloxone-precipitated diarrhea in oxycodone-dependent mice. Specifically, 0 of 16 CB_1 (+/+)-ZCZ011-treated oxycodone-dependent mice elicited a lower incidence of naloxone-precipitated diarrhea than 10 of 16 CB_1 (+/+)-vehicle-treated oxycodone-dependent mice and 13 of 15 CB_1 (-/-)-ZCZ011-treated oxycodone-dependent mice. A significant 2-way interaction between genotype and ZCZ011 treatment on naloxone-precipitated body weight loss indicates that CB_1 receptors mediate the protective effects of ZCZ011 for this measure (Fig. 4C). Specifically, CB_1 (+/+)-ZCZ011-treated mice elicited less body weight loss than CB_1 (-/-)-ZCZ011-treated mice. ZCZ011 did not affect naloxone-precipitated jumping behavior, though a significant main effect for genotype revealed that CB_1 (+/+) mice jumped significantly more than CB_1 (-/-) mice, which respectively averaged 69 and 37 jumps (Fig. 4D). As shown in Fig. 4E, a significant 2-way interaction between genotype and ZCZ011 treatment for naloxone-precipitated paw fluttering behavior revealed CB_1 (+/+)-ZCZ011-treated mice elicited little to no paw flutters compared to CB_1 (+/+)-vehicle-treated mice. Furthermore, regardless of treatment, CB_1 (-/-) mice elicited less paw flutters than CB_1 (+/+)-vehicle-treated mice. Notably, no difference was observed between CB_1 (+/+)-ZCZ011-treated and CB_1 (-/-)-ZCZ011-treated mice signifying a lack of a CB_1 receptor mediated effects. Lastly, Fig. 4F illustrates no 3-way interaction, relevant 2-way interactions, or main effects for naloxone-precipitated head shakes.

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Discussion

Here we report that the CB_1 receptor PAM, ZCZ011, attenuates a subset of naloxone-precipitated withdrawal signs in oxycodone-dependent mice. Notably, ZCZ011 fully blocked withdrawal-induced diarrhea and weight loss and attenuated paw flutters. In contrast, ZCZ011 did not impact naloxone-precipitated jumping in oxycodone-dependent mice and its attenuation of naloxone-precipitated head shakes was inconsistent. The anti-diarrheal and anti-weight loss effects of ZCZ011 occurred predominantly through a CB_1 receptor dependent mechanism in both male and female mice. Thus, a CB_1 receptor PAM redcues a subset pf withdrawal signs in both male and female opioid-dependent mice.

Complementary pharmacological and genetic approaches used to elucidate cannabinoid receptor mediation of the anti-withdrawal effects of ZCZ011 demonstrate that ZCZ011 attenuates diarrhea and body weight loss through CB_1, not CB_2, receptors. However, it is difficult to draw conclusions about CB_1 receptor involvement in the paw flutters measure because rimonabant did not significantly block the reduction of paw flutters produced by ZCZ011, and since CB_1 (-/-) mice exhibited an overall lower frequency of paw flutters than CB_1 (+/+) mice regardless of drug treatment, determining CB_1 receptor involvement was hindered. The unreliability of ZCZ011 to reduce naloxone-precipitated head shakes in oxycodone-dependent mice precluded discerning the role of CB_1 receptors in this measure. Consistent with previous reports (Ledent *et al.*, 1999; Lichtman *et al.*, 2001; Maldonado *et al.*, 2002), CB_1 (-/-) mice showed less jumps and paw flutters compared to (+/+) mice. Nonetheless, both genetic and pharmacological data reveal that ZCZ011 attenuates naloxone-precipitated diarrhea and weight loss in oxycodone-dependent mice through CB_1 receptors.

CB_1 receptor orthosteric agonists and endocannabinoid catabolic enzyme inhibitors effectively attenuate opioid withdrawal; however, they also produce unwanted cannabimimetic effects, and antinociceptive tolerance and dependence upon repeated administration, which limits their potential clinical utility. Moreover, while clonidine has shown to reduce the frequency of withdrawal-induced jumps, paw flutters, and weight loss (Carper *et al.*, 2021), its hypotensive effects limit its clinical use (Kuhlman *et al.*, 1998; Stolbach and Hoffman, 2020). In contrast to combined FAAH/MAGL blockade,

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which substitutes for THC in the drug discrimination paradigm (Long, Nomura, *et al.*, 2009), ZCZ011 does not substitute for CB_1 receptor orthosteric agonists (Ignatowska-Jankowska, Baillie, *et al.*, 2015), indicating that CB_1 receptor PAMs do not elicit cannabimimetic subjective effects. Thus, CB_1 receptor PAMs may offer a favorable strategy to target CB_1 receptors for therapeutic gain, with a reduced side effect profile, compared with orthosteric CB_1 receptor agonists or combined FAAH/MAGL blockade. Repeated administration of THC or MAGL inhibitors leads to antinociceptive tolerance, whereas repeated administration of ZCZ011 or GAT211 produces sustained antinociceptive effects in mouse models of inflammatory and neuropathic pain (Ignatowska-Jankowska, Baillie, *et al.*, 2015; Slivicki *et al.*, 2017). Finally, repeated administration of THC or MAGL inhibitors leads to cannabinoid dependence (Schlosburg *et al.*, 2014; Trexler *et al.*, 2019); which is not seen with repeated administration of GAT211 (Slivicki *et al.*, 2017). It is also noteworthy that ZCZ011 alone does not produce a conditioned place preference (CPP) (Ignatowska-Jankowska, Baillie, *et al.*, 2015; Trexler *et al.*, 2019), suggesting that it lacks rewarding effects. The present study showing that ZCZ011 attenuates oxycodone withdrawal signs and other work showing that it attenuates rimonabant-precipitated and spontaneous withdrawal signs in THC-dependent mice (Trexler *et al.*, 2019) without cannabimimetic side effects or tolerance supports the possibility that CB_1 receptor PAMs may offer a novel strategy to treat opioid and cannabinoid dependence.

The present study employed two strains (i.e., ICR and CB_1 (+/+) and (-/-) mice on a C57BL/6J background) of male and female mice, and showed that ZCZ011 reliably diminished naloxone-precipitated diarrhea and weight loss in oxycodone-dependent mice regardless of strain or sex. While female Wistar rats self-administer more oxycodone than males (Kimbrough *et al.*, 2020), we did not observe withdrawal sex differences with respect to drug treatment. However, independent of sex, we found strain differences in which the variability in jumps and paw flutters was high in ICR mice compared with CB_1 (+/+) mice or the C57BL/6J mice used in the study done by Carper and colleagues (2021). Additionally CB_1 (+/+) and (-/-) mice on a C57BL/6J background show less weight loss than the

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C57BL/6J mice used in the Carper and colleagues (2021) study. This solitary difference in the magnitude of weight loss is likely due to procedural differences between the studies. For example, the mice in the present study were exposed to the withdrawal chambers for an additional 30 min acclimation period before naloxone challenge, while the mice in the study by Carper *et al.* (2021) were placed in the chamber immediately after naloxone administration.

In contrast to the present study in which ZCZ011 modestly lowered the frequency of paw flutters, Slivicki and colleagues (2020) found that GAT211 did not attenuate naloxone-precipitated paw flutters in morphine-dependent mice. They also found that GAT211 did not reduce naloxone-precipitated jumps; however, they did not report other measures of withdrawal. Thus, it will be of value to evaluate multiple CB_1 receptor PAMs on a full complement of withdrawal signs in several rodent models of opioid dependence.

ZCZ011 and related compounds (i.e., GAT211) show mixed allosteric agonistic and PAM properties, which have been termed CB_1 ago-PAMs (Kenakin, 2013). These compounds enhance the effects of CB_1 receptor orthosteric agonists (i.e., CP55,940, WIN55,212-2, or AEA) in a variety of functional assays, including [^{35}S] GTPγS-binding, β-arrestin recruitment and, inhibition of cAMP production, but also activate β-arrestin recruitment and inhibit cAMP production in the absence of CB_1 receptor orthosteric agonists (Ignatowska-Jankowska, Baillie, *et al.*, 2015; Slivicki *et al.*, 2017; Saleh *et al.*, 2018; Tseng *et al.*, 2019; Garai *et al.*, 2021). Thus, additional studies (e.g., site directed mutagenesis) will be required to address the receptor mechanism(s) by which ZCZ011 ameliorates withdrawal signs in opioid-dependent mice.

The differential effectiveness of ZCZ011 in ameliorating diarrhea and weight loss compared with somatic withdrawal signs merits consideration of CNS- and peripherally-mediated processes. Somatic withdrawal signs in opioid-dependent mice have been mapped to various brain regions (Koob *et al.*, 1992; Maldonado *et al.*, 1992, 1996) including the locus coeruleus (LC; Maldonado and Koob, 1993) the periaqueductal grey (PAG; Laschka *et al.*, 1976), and the medial habenula (MHb; Boulos *et al.*, 2019). It

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would be of value to examine whether CB_1 receptors expressed in the LC, PAG, and MHb play a role in the attenuation of opioid somatic withdrawal signs by drugs targeting the endocannabinoid system, such as CB_1 receptor PAMs (Kenakin, 2013). Withdrawal-induced weight loss results from a combination of diarrhea and increased micturition. Thus, activation of CB_1 receptors, which are expressed in the kidneys (Shire *et al.*, 1995; Silva *et al.*, 2013; Lin *et al.*, 2014) and throughout the enteric nervous system (Massa *et al.*, 2005), particularly on neurons in the myenteric and submucosal plexuses (Storr *et al.*, 2010; Trautmann and Sharkey, 2015; Hasenoehrl *et al.*, 2016), may prevent withdrawal-induced weight loss. Notably, cannabinoid receptor agonists evoke diuresis (Sofia *et al.*, 1977). Interestingly, CB_1 receptor agonists produce biphasic effects on diuresis in which low doses increase urine output, while high doses decrease urine output (Chopda *et al.*, 2013). Lastly, naloxone-precipitated diarrhea is heavily associated with the ileum of the small intestine (Maguma *et al.*, 2010), where the coordination of motility and secretion occur (Ramesh *et al.*, 2011, 2013; Smith *et al.*, 2012), and CB_1 receptor activation leads to decreased motility and secretion (Storr *et al.*, 2010; Ramesh *et al.*, 2011, 2013; Hasenoehrl *et al.*, 2016). Accordingly, ZCZ011 may prevent withdrawal-induced diarrhea and weight loss through CB_1 receptor activation in peripheral organs mediating gastrointestinal motility and secretion as well as urine output.

Current medications (i.e., methadone and buprenorphine) employed to treat opioid withdrawal symptoms (Bell and Strang, 2020) possess abuse liability (Cicero and Inciardi, 2005) and do not effectively ameliorate all withdrawal symptoms. Alternative adjunct medications offer benefit such as clonidine for anxiety and loperamide for diarrhea and stomach cramps (Stolbach and Hoffman, 2020) yet also exhibit hypotension and abuse liability, respectively (Miller et al., 2017; Wu and Juurlink, 2017; Toce et al., 2018). As such, a need exists to identify alternative medications to treat the most severe withdrawal symptoms, such as diarrhea (Stolbach and Hoffman, 2020). The finding that ZCZ011 fully attenuates naloxone-precipitated diarrhea to a similar extent as 75 mg/kg oxycodone (figure 2), suggests that a CB_1 receptor PAM may serve as a potential alternative to substitution therapy.

In conclusion, the CB_1 receptor PAM, ZCZ011, attenuates a subset of naloxone-precipitated withdrawal signs in oxycodone-dependent male and female mice through a CB_1 receptor-dependent

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mechanism. As diarrhea is a severe withdrawal symptom in opioid-dependent humans and current medications possess side effect profiles, future directions will explore the antidiarrheal mechanisms of ZCZ011. Additionally, it would be of interest to investigate ZCZ011 in other preclinical models of bowel dysfunction. Overall, the results of the present study suggest that CB_1 receptor PAMs may represent an alternative strategy to treat selective and severe opioid withdrawal effects.

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Authorship Contributions

Participated in research design: Dodu, Schlosburg, Damaj, Akbarali, and Lichtman

Conducted experiments: Dodu, Moncayo

Contributed reagents: Schlosburg and Lu

Performed data analysis: Dodu and Moncayo

Statistical analysis: Dodu, O'Brien, and Lichtman

Wrote or contributed to the writing of the manuscript: Dodu, Moncayo, Schlosburg, Damaj, Akbarali, O'Brien, Kendall, Lu, and Lichtman.

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Legends for Figures

Fig. 1. Effects of acute oxycodone (17-75 mg/kg s.c.) on naloxone-precipitated withdrawal signs in oxycodone-dependent male and female mice. Panel A illustrates the timeline of treatments mice received prior to naloxone-precipitated withdrawal. The withdrawal signs measured include the occurrence of diarrhea (B), body weight loss (C), number of jumps (D), number of paw flutters (E), and number of head shakes (F). Data are expressed as percentage scores for panel B and individual data points with mean ± SD for panels C-F. Numbers at the bottom of bar graphs in panel B indicate the number of mice that presented with diarrhea. Fisher exact test was used to analyze the occurrence of diarrhea (B). Between-measures two-way ANOVA and Dunnett's *post hoc* test was used to analyze all other withdrawal signs (C, D, E, and F). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ versus oxycodone-saline mice. $n = 12$ mice/group ($n = 6$ male and $n = 6$ female mice/group).

Fig. 2. Effects of ZCZ011 (5-40 mg/kg i.p.) on naloxone-precipitated withdrawal signs in oxycodone-dependent male and female mice. Panel A illustrates the timeline of treatments mice received prior to naloxone-precipitated withdrawal. The withdrawal signs measured include the occurrence of diarrhea (B), body weight loss (C), number of jumps (D), number of paw flutters (E), and number of head shakes (F). Data are expressed as percentage scores for panel B and individual data points with mean ± SD for panels C-F. Numbers at the bottom of bar graphs in panel B indicate the number of mice that presented with diarrhea. Fisher exact test was used to analyze the occurrence of diarrhea (B). Between-measures two-way ANOVA and Dunnett's *post hoc* test was used to analyze all other withdrawal signs (C, D, E, and F). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ versus oxycodone-vehicle mice. $n = 15$-16 mice/group ($n = 8$ male and $n = 7$-8 female mice/group due to lost video footage).

Fig. 3. Effects of cannabinoid receptor antagonism (rimonabant, SR1 3 mg/kg i.p.; SR144528, SR2 3mg/kg i.p.) on ZCZ011 (40 mg/kg i.p.) attenuation of naloxone-precipitated withdrawal signs in oxycodone-dependent male and female mice. Panel A illustrates the timeline of treatments mice received prior to naloxone-precipitated withdrawal. The withdrawal signs measured include the occurrence of diarrhea (B), body weight loss (C), number of jumps (D), number of paw flutters (E), and number of head

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shakes (F). Data are expressed as percentage scores for panel B and individual data points with mean ± SD for panels C-F. Numbers at the bottom of bar graphs in panel B indicate the number of mice that presented with diarrhea. Fisher exact test was used to analyze the occurrence of diarrhea (B). Between-measures three-way ANOVA and Tukey's *post hoc* test was used to analyze all other withdrawal signs (C, D, E, and F). A pre-planned comparison Student's unpaired *t*-test was used to analyze whether ZCZ011 reliably attenuated paw flutters (E). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ vehicle-ZCZ011 or SR144528-ZCZ011 mice versus vehicle-vehicle or SR144528-vehicle mice, respectively. ##, $p < 0.01$; ####, $p < 0.0001$ vehicle-ZCZ011 mice versus rimonabant-ZCZ011 mice. n = 15-16 mice/group (n = 7-8 male and n = 8 female mice/group due to incorrect injection).

Fig. 4. Effects of ZCZ011 (40 mg/kg i.p.) on oxycodone-dependent male and female CB_1 (+/+) and CB_1 (-/-) mice undergoing naloxone-precipitated withdrawal. Panel A illustrates the timeline of treatments mice received prior to naloxone-precipitated withdrawal. The withdrawal signs measured include the occurrence of diarrhea (B), body weight loss (C), number of jumps (D), number of paw flutters (E), and number of head shakes (F). Data are expressed as percentage scores for panel B and individual data points with mean ± SD for panels C-F. Numbers at the bottom of bar graphs in panel B indicate the number of mice that presented with diarrhea. Between-measures three-way ANOVA and Tukey's *post hoc* test was used to analyze all other withdrawal signs (C, D, E, and F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ CB_1 (+/+)-vehicle mice versus CB_1 (+/+)-ZCZ011, CB_1 (-/-)-vehicle, or CB_1 (-/-)-ZCZ011 mice. ##, $p < 0.01$; ####, $p < 0.0001$ CB_1 (+/+)-ZCZ011 mice versus CB_1 (-/-)-ZCZ011 mice. The $$$ indicates a main effect of genotype for jumping behavior between CB_1 (+/+) mice versus CB_1 (-/-) mice. n = 15-16 mice/group (n = 8 male and n = 7-8 female mice/group due to one death during repeated oxycodone administration).

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TABLE 1
Summary of statistical results from Fisher exact tests and two-way ANOVA analyses for Figure 1

Withdrawal Signs	F Statistic, *P* value	Dunnett's	Fisher
Diarrhea			
Oxy-Sal vs Sal-Sal	—	—	*P* = 0.0003***
Oxy-Sal vs Oxy-Oxy (17)	—	—	*P* = 0.48
Oxy-Sal vs Oxy-Oxy (33)	—	—	*P* = 0.0003***
Oxy-Sal vs Oxy-Oxy (75)	—	—	*P* < 0.0001****
Body Weight Loss			
2-way ANOVA: Sex x Oxy	F (4, 50) = 0.271; *P* = 0.90	—	—
Main effect: Sex	F (1, 50) = 0.169; *P* = 0.68	—	—
Main effect: Oxy	F (4, 50) = 21.9; *P* < 0.0001****	—	—
Planned Comparisons:			
Oxy-Sal vs Sal-Sal	—	*P* < 0.0001****	—
Oxy-Sal vs Oxy-Oxy (17)	—	*P* = 0.15	—
Oxy-Sal vs Oxy-Oxy (33)	—	*P* = 0.0002***	—
Oxy-Sal vs Oxy-Oxy (75)	—	*P* < 0.0001****	—
Jumps			
2-way ANOVA: Sex x Oxy	F (4, 50) = 0.853; *P* = 0.50	—	—
Main effect: Sex	F (1, 50) = 1.69; *P* = 0.20	—	—
Main effect: Oxy	F (4, 50) = 6.38; *P* = 0.0003***	—	—
Planned Comparisons:			
Oxy-Sal vs Sal-Sal	—	*P* = 0.0017**	—
Oxy-Sal vs Oxy-Oxy (17)	—	*P* = 0.53	—
Oxy-Sal vs Oxy-Oxy (33)	—	*P* = 0.0022**	—
Oxy-Sal vs Oxy-Oxy (75)	—	*P* = 0.0010**	—
Paw Flutters			
2-way ANOVA: Sex x Oxy	F (4, 50) = 2.52; *P* = 0.0530	—	—
Main effect: Sex	F (1, 50) = 0.0865; *P* = 0.77	—	—
Main effect: Oxy	F (4, 50) = 36.5; *P* < 0.0001****	—	—
Planned Comparisons:			
Oxy-Sal vs Sal-Sal	—	*P* < 0.0001****	—
Oxy-Sal vs Oxy-Oxy (17)	—	*P* = 0.99	—
Oxy-Sal vs Oxy-Oxy (33)	—	*P* < 0.0001****	—
Oxy-Sal vs Oxy-Oxy (75)	—	*P* < 0.0001****	—
Head Shakes			
2-way ANOVA: Sex x Oxy	F (4, 50) = 0.507; *P* = 0.73	—	—
Main effect: Sex	F (1, 50) = 0.00; *P* > 0.99	—	—
Main effect: Oxy	F (4, 50) = 7.74; *P* < 0.0001****	—	—
Planned Comparisons:			
Oxy-Sal vs Sal-Sal	—	*P* = 0.0004***	—
Oxy-Sal vs Oxy-Oxy (17)	—	*P* = 0.22	—
Oxy-Sal vs Oxy-Oxy (33)	—	*P* = 0.0004***	—
Oxy-Sal vs Oxy-Oxy (75)	—	*P* = 0.0002***	—

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TABLE 2
Summary of statistical results from Fisher exact tests and two-way ANOVA analyses for Figure 2

Withdrawal Signs	F Statistic, *P* value	Dunnett's	Fisher
Diarrhea			
Oxy-Veh vs Sal-Veh	—	—	$P < 0.0001$****
Oxy-Veh vs Oxy-Oxy (75)	—	—	$P < 0.0001$****
Oxy-Veh vs Oxy-ZCZ (5)	—	—	$P > 0.99$
Oxy-Veh vs Oxy-ZCZ (10)	—	—	$P > 0.99$
Oxy-Veh vs Oxy-ZCZ (20)	—	—	$P = 0.083$
Oxy-Veh vs Oxy-ZCZ (40)	—	—	$P < 0.0001$****
Body Weight Loss			
2-way ANOVA: Sex x Drug tx	F (6, 90) = 0.413; $P = 0.87$	—	—
Main effect: Sex	F (1, 90) = 2.05; $P = 0.16$	—	—
Main effect: Drug tx	F (6, 90) = 11.3; $P < 0.0001$****	—	—
Planned Comparisons:			
Oxy-Veh vs Sal-Veh	—	$P < 0.0001$****	—
Oxy-Veh vs Oxy-Oxy (75)	—	$P < 0.0001$****	—
Oxy-Veh vs Oxy-ZCZ (5)	—	$P = 0.90$	—
Oxy-Veh vs Oxy-ZCZ (10)	—	$P = 0.13$	—
Oxy-Veh vs Oxy-ZCZ (20)	—	$P = 0.098$	—
Oxy-Veh vs Oxy-ZCZ (40)	—	$P < 0.0001$****	—
Jumps			
2-way ANOVA: Sex x Drug tx	F (6, 88) = 1.17; $P = 0.33$	—	—
Main effect: Sex	F (1, 88) = 9.49; $P = 0.0028$**	—	—
Main effect: Drug tx	F (6, 88) = 4.64; $P = 0.0004$***	—	—
Planned Comparisons:			
Oxy-Veh vs Sal-Veh	—	$P = 0.0045$**	—
Oxy-Veh vs Oxy-Oxy (75)	—	$P = 0.0004$***	—
Oxy-Veh vs Oxy-ZCZ (5)	—	$P = 0.78$	—
Oxy-Veh vs Oxy-ZCZ (10)	—	$P = 0.96$	—
Oxy-Veh vs Oxy-ZCZ (20)	—	$P = 0.87$	—
Oxy-Veh vs Oxy-ZCZ (40)	—	$P = 0.44$	—
Paw Flutters			
2-way ANOVA: Sex x Drug tx	F (6, 88) = 0.535; $P = 0.78$	—	—
Main effect: Sex	F (1, 88) = 0.964; $P = 0.33$	—	—
Main effect: Drug tx	F (6, 88) = 21.7; $P < 0.0001$****	—	—
Planned Comparisons:			
Oxy-Veh vs Sal-Veh	—	$P < 0.0001$****	—
Oxy-Veh vs Oxy-Oxy (75)	—	$P < 0.0001$****	—
Oxy-Veh vs Oxy-ZCZ (5)	—	$P = 0.99$	—
Oxy-Veh vs Oxy-ZCZ (10)	—	$P = 0.85$	—
Oxy-Veh vs Oxy-ZCZ (20)	—	$P = 0.0006$***	—
Oxy-Veh vs Oxy-ZCZ (40)	—	$P = 0.0002$***	—
Head Shakes			
2-way ANOVA: Sex x Drug tx	F (6, 88) = 1.14; $P = 0.35$	—	—
Main effect: Sex	F (1, 88) = 0.899; $P = 0.35$	—	—

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Main effect: Drug tx	F (6, 88) = 7.39; *P* < 0.0001****	—	—
Planned Comparisons:			
Oxy-Veh vs Sal-Veh	—	*P* = 0.0004***	—
Oxy-Veh vs Oxy-Oxy (75)	—	*P* < 0.0001****	—
Oxy-Veh vs Oxy-ZCZ (5)	—	*P* = 0.61	—
Oxy-Veh vs Oxy-ZCZ (10)	—	*P* = 0.24	—
Oxy-Veh vs Oxy-ZCZ (20)	—	*P* = 0.025*	—
Oxy-Veh vs Oxy-ZCZ (40)	—	*P* = 0.0004***	—

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TABLE 3
Summary of statistical results from Fisher exact tests and three-way ANOVA analyses for Figure 3

Withdrawal Signs	F Statistic, *P* value	Tukey's	Fisher
Diarrhea			
Veh-Veh vs Veh-ZCZ	—	—	$P < 0.0001$****
SR1-Veh vs SR1-ZCZ	—	—	$P > 0.99$
SR2-Veh vs SR2-ZCZ	—	—	$P < 0.0001$****
Veh-ZCZ vs SR1-ZCZ	—	—	$P < 0.0001$****
Veh-ZCZ vs SR2-ZCZ	—	—	$P > 0.99$
Body Weight Loss			
3-way ANOVA: Sex x CB Ant x ZCZ tx	F (2, 82) = 0.219; $P = 0.80$	—	—
2-way Interaction: CB Ant x ZCZ tx	F (2, 82) = 3.57; $P = 0.033$*	—	—
Planned Comparisons:			
Veh-Veh vs Veh-ZCZ	—	$P < 0.0001$****	—
SR1-Veh vs SR1-ZCZ	—	$P = 0.63$	—
SR2-Veh vs SR2-ZCZ	—	$P = 0.018$*	—
Veh-ZCZ vs SR1-ZCZ	—	$P = 0.0040$**	—
Veh-ZCZ vs SR2-ZCZ	—	$P = 0.98$	—
2-way Interaction: Sex x ZCZ tx	F (1, 82) = 0.280; P = 0.60	—	—
2-way Interaction: Sex x CB Ant	F (2, 82) = 0.338; P = 0.71	—	—
Main Effect: Sex	F (1, 82) = 0.447; P = 0.51	—	—
Main Effect: CB Ant	F (2, 82) = 12.5; P < 0.0001****	—	—
Main Effect: ZCZ tx	F (1, 82) = 33.6; P < 0.0001****	—	—
Jumps			
3-way ANOVA: Sex x CB Ant x ZCZ tx	F (2, 82) = 0.219; $P = 0.80$	—	—
2-way Interaction: CB Ant x ZCZ tx	F (2, 82) = 3.45; $P = 0.036$*	—	—
Planned Comparisons:			
Veh-Veh vs Veh-ZCZ	—	$P = 0.96$	—
SR1-Veh vs SR1-ZCZ	—	$P = 0.67$	—
SR2-Veh vs SR2-ZCZ	—	$P = 0.37$	—
Veh-ZCZ vs SR1-ZCZ	—	$P = 0.84$	—
Veh-ZCZ vs SR2-ZCZ	—	$P = 0.71$	—
2-way Interaction: Sex x ZCZ tx	F (1, 82) = 3.26; P = 0.074	—	—
2-way Interaction: Sex x CB Ant	F (2, 82) = 3.45; P = 0.036*	—	—
Main Effect: Sex	F (1, 82) = 10.2; P = 0.0020**	—	—
Main Effect: CB Ant	F (2, 82) = 1.17; P = 0.3147	—	—
Main Effect: ZCZ tx	F (1, 82) = 0.735; P = 0.39	—	—
Paw Flutters			
3-way ANOVA: Sex x CB Ant x ZCZ tx	F (2, 82) = 0.545; $P = 0.58$	—	—
2-way Interaction: CB Ant	F (2, 82) = 1.06; $P = 0.35$	—	—

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x ZCZ tx			
2-way Interaction: Sex x ZCZ tx	F (1, 82) = 0.135; P = 0.71	—	—
2-way Interaction: Sex x CB Ant	F (2, 82) = 0.943; P = 0.39	—	—
Main Effect: Sex	F (1, 82) = 4.19 P=0.044*	—	—
Main Effect: CB Ant	F (2, 82) = 5.09; P = 0.0083**	—	—
Main Effect: ZCZ tx	F (1, 82) = 6.04; P = 0.016*	—	—
Head Shakes			
3-way ANOVA: Sex x CB Ant x ZCZ tx	F (2, 82) = 0.403; *P* = 0.67	—	—
2-way Interaction: CB Ant x ZCZ tx	F (2, 82) = 0.489; *P* = 0.61	—	—
2-way Interaction: Sex x ZCZ tx	F (1, 82) = 1.56; P = 0.21	—	—
2-way Interaction: Sex x CB Ant	F (2, 82) = 0.0834; P = 0.92	—	—
Main Effect: Sex	F (1, 82) = 4.82; P = 0.031*	—	—
Main Effect: CB Ant	F (2, 82) = 2.73; P = 0.071	—	—
Main Effect: ZCZ tx	F (1, 82) = 2.73; P = 0.10	—	—

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TABLE 4
Summary of statistical results from Fisher exact tests and three-way ANOVA analyses for Figure 4

Withdrawal Signs	F Statistic, *P* value	Tukey's	Fisher
Diarrhea			
CB_1(+/+)-Veh vs CB_1(+/+)-ZCZ	—	—	*P* < 0.0001****
CB_1(-/-)-Veh vs CB_1(-/-)-ZCZ	—	—	*P* > 0.99
CB_1(+/+)-ZCZ vs CB_1(-/-)-ZCZ	—	—	*P* < 0.0001****
Body Weight Loss			
3-way ANOVA: Sex x Geno x ZCZ tx	F (1, 55) = 0.0866; *P* = 0.77	—	—
2-way Interaction: Geno x ZCZ tx	F (1, 55) = 6.16; *P* = 0.016*	—	—
Planned Comparisons:			
CB_1(+/+)-Veh vs CB_1(+/+)-ZCZ	—	*P* = 0.0055**	—
CB_1(-/-)-Veh vs CB_1(-/-)-ZCZ	—	*P* = 0.99	—
CB_1(+/+)-ZCZ vs CB_1(-/-)-ZCZ	—	*P* = 0.0013**	—
2-way Interaction: Sex x ZCZ tx	F (1, 55) = 1.27; P = 0.27	—	—
2-way Interaction: Sex x Geno	F (1, 55) = 0.215; P = 0.64	—	—
Main Effect: Sex	F (1, 55) = 1.04; P = 0.31	—	—
Main Effect: Genotype	F (1, 55) = 9.40; P = 0.0034**	—	—
Main Effect: ZCZ tx	F (1, 55) = 5.31; P = 0.025*	—	—
Jumps			
3-way ANOVA: Sex x Geno x ZCZ tx	F (1, 55) = 0.894; *P* = 0.35	—	—
2-way Interaction: Geno x ZCZ tx	F (1, 55) = 0.464; *P* = 0.50	—	—
2-way Interaction: Sex x ZCZ tx	F (1, 55) = 0.464; P = 0.50	—	—
2-way Interaction: Sex x Geno	F (1, 55) = 0.0307; P = 0.86	—	—
Main Effect: Sex	F (1, 55) = 0.0833; P = 0.77	—	—
Main Effect: Genotype	F (1, 55) = 15.1; *P* = 0.0003***	—	—
Main Effect: ZCZ tx	F (1, 55) = 0.0422; P = 0.84	—	—
Paw Flutters			
3-way ANOVA: Sex x Geno x ZCZ tx	F (1, 55) = 0.00293; *P* = 0.96	—	—
2-way Interaction: Geno x ZCZ tx	F (1, 55) = 9.63; *P* = 0.0030**	—	—
Planned Comparisons:			
CB_1(+/+)-Veh vs CB_1(+/+)-ZCZ	—	*P* = 0.0002***	—
CB_1(+/+)-Veh vs CB_1(-/-)-Veh	—	*P* = 0.020*	—
CB_1(+/+)-Veh vs CB_1(-/-)-ZCZ	—	*P* = 0.017*	—
CB_1(-/-)-Veh vs CB_1(-/-)-ZCZ	—	*P* = 0.99	—
CB_1(+/+)-ZCZ vs CB_1(-/-)-ZCZ	—	*P* = 0.51	—
2-way Interaction: Sex x ZCZ tx	F (1, 55) = 1.12; P = 0.30	—	—
2-way Interaction: Sex x Geno	F (1, 55) = 1.65; P = 0.20	—	—
Main Effect: Sex	F (1, 55) = 0.0150; P = 0.90	—	—
Main Effect: Genotype	F (1, 55) = 1.13; P = 0.29	—	—
Main Effect: ZCZ tx	F (1, 55) = 9.99; P = 0.0026**	—	—

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Head Shakes			
3-way ANOVA: Sex x Geno x ZCZ tx	F (1, 55) = 1.67; *P* = 0.20	—	—
2-way Interaction: Geno x ZCZ tx	F (1, 55) = 1.34; P = 0.25	—	—
2-way Interaction: Sex x ZCZ tx	F (1, 55) = 0.0755; P = 0.78	—	—
2-way Interaction: Sex x Geno	F (1, 55) = 4.18; P = 0.046*	—	—
Main Effect: Sex	F (1, 55) = 0.246; P = 0.62	—	—
Main Effect: Genotype	F (1, 55) = 0.0152; P = 0.90	—	—
Main Effect: ZCZ tx	F (1, 55) = 0.102; P = 0.75	—	—

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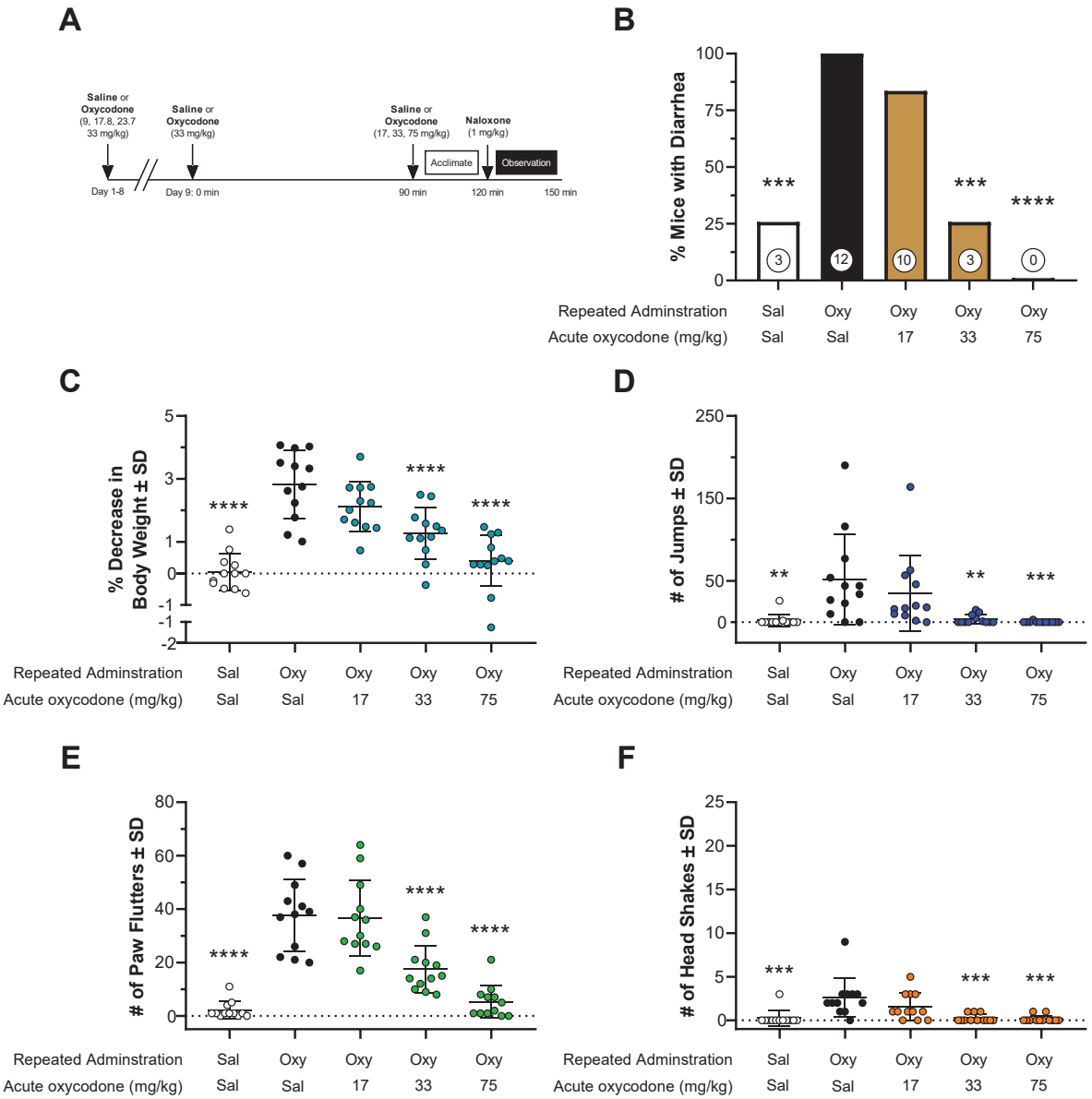

Fig. 1

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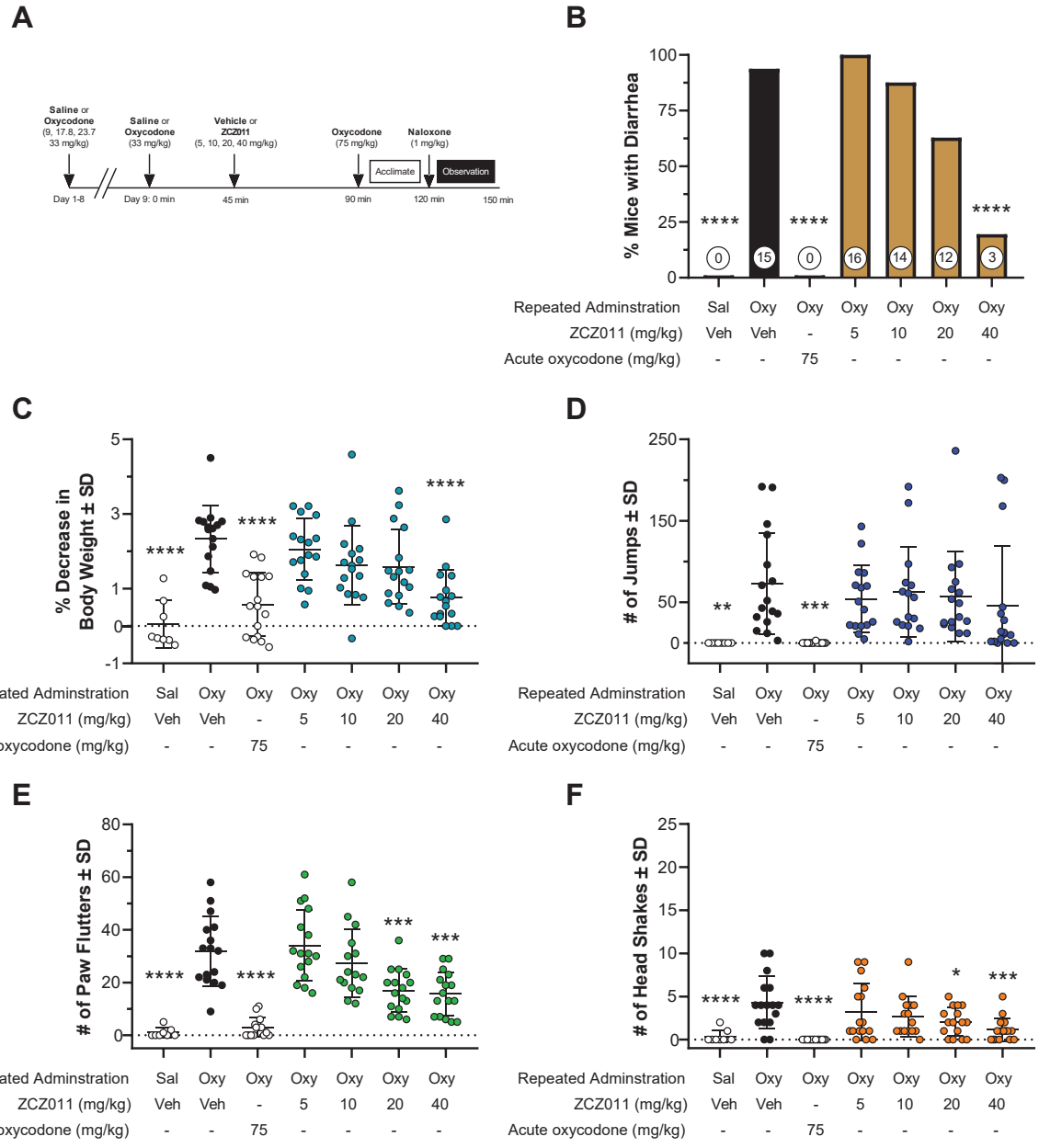

Fig. 2

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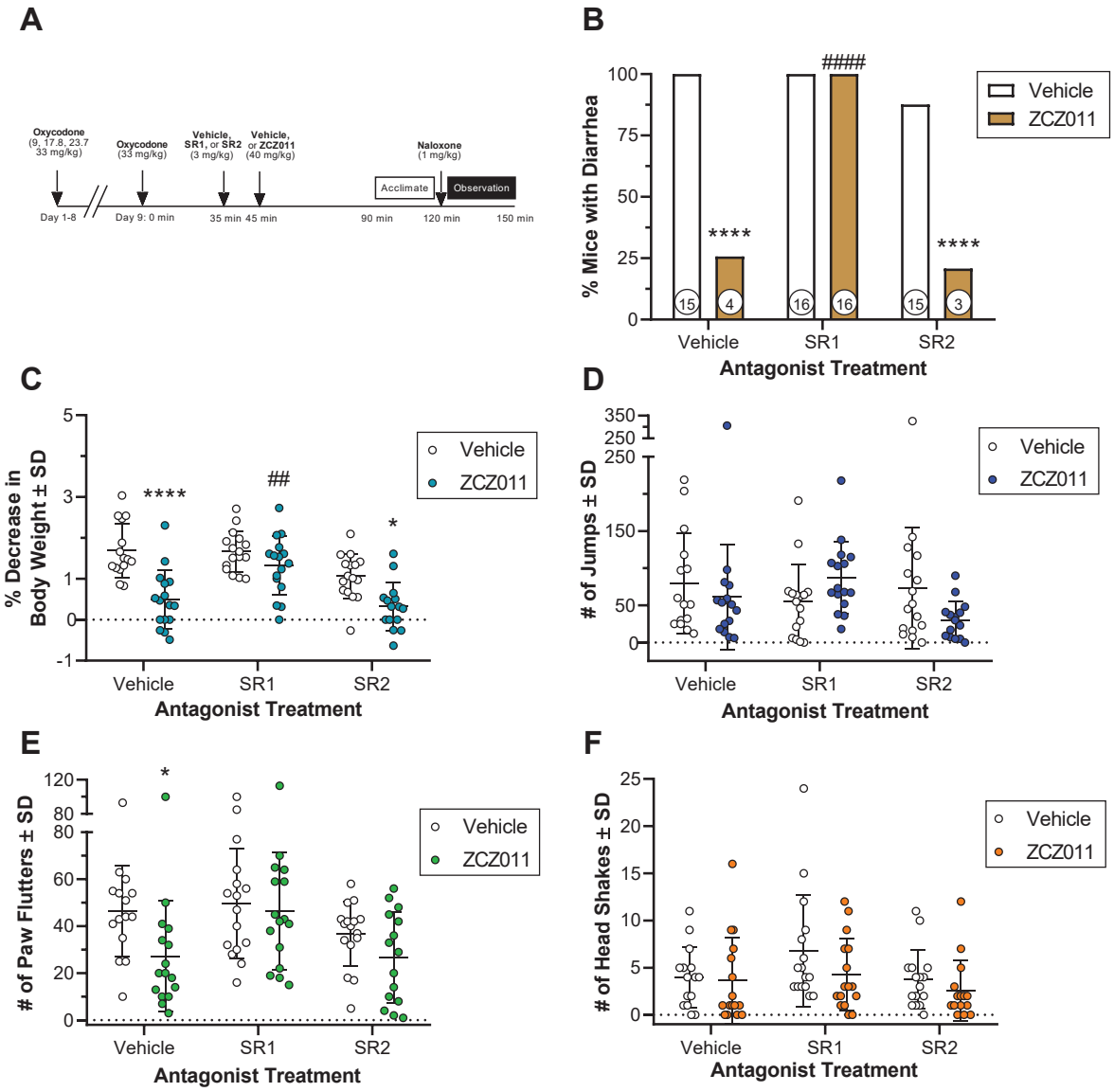

Fig. 3

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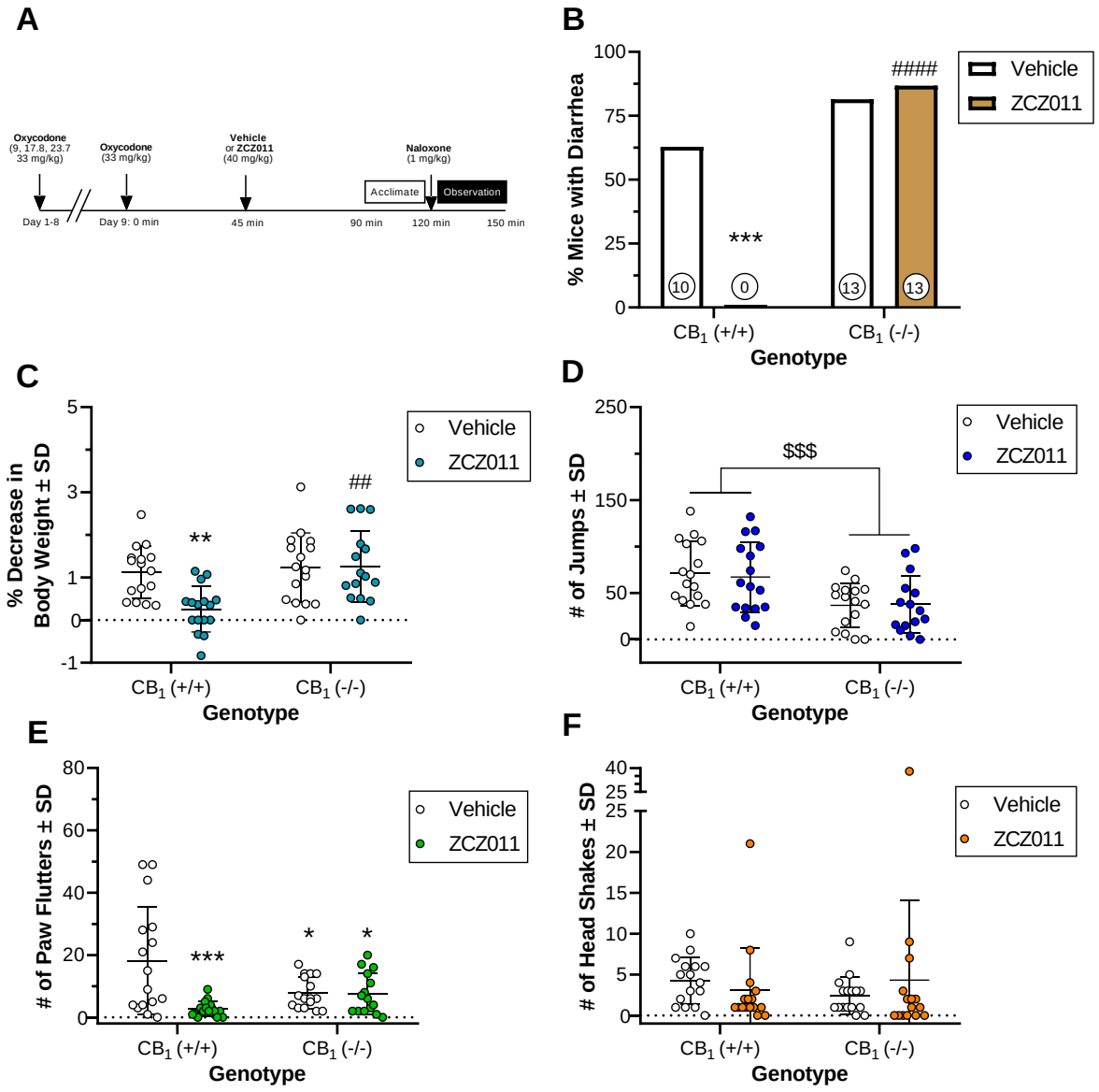

Fig. 4