A Cannabinoid CB_1 Receptor Positive Allosteric Modulator Reduces Neuropathic Pain in the Mouse with no Psychoactive Effects

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ABSTRACT

The CB_1 receptor represents a promising target for the treatment of several disorders including pain-related disease states. However, therapeutic applications of Δ^9-tetrahydrocannabinol (THC) and other CB_1 orthosteric receptor agonists remain limited because of psychoactive side effects. Positive allosteric modulators (PAMs) offer an alternative approach to enhance CB_1 receptor function for therapeutic gain with the promise of reduced side effects. Here we describe the development of the novel synthetic CB_1 PAM, 6-methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1*H*-indole (ZCZ011), which augments the *in vitro* and *in vivo* pharmacological actions of the CB_1 orthosteric agonists CP55,940 and *N*-arachidonoylethanolamine (AEA). ZCZ011 potentiated binding of [^{3}H]CP55,940 to the CB_1 receptor as well as enhancing AEA-stimulated [^{35}S]GTPγS binding in mouse brain membranes and β-arrestin recruitment and ERK phosphorylation in hCB_1 cells. In the whole animal, ZCZ011 is brain penetrant, increased the potency of these orthosteric agonists in mouse behavioral assays indicative of cannabimimetic activity, including antinociception, hypothermia, catalepsy, locomotor activity and in the drug discrimination paradigm. Administration of ZCZ011 alone was devoid of activity in these assays and did not produce a conditioned place preference or aversion, but elicited CB_1 receptor mediated antinociceptive effects in the chronic constriction nerve injury (CCI) model of neuropathic pain and carrageenan model of inflammatory pain. These data suggest that ZCZ011 acts as a CB_1 PAM and provide the first proof of principle that CB_1 PAMs offer a promising strategy to treat neuropathic and inflammatory pain with minimal or no cannabimimetic side effects.

Key words: CB_1 receptor; positive allosteric modulator; neuropathic pain; cannabinoid; inflammatory pain; allodynia; antinociception; drug discrimination

INTRODUCTION

Endocannabinoids (*N*-arachidonoylethanolamine (anandamide); AEA and 2-arachidoloylglycerol; 2-AG) are released on demand in response to various stimuli, including pain. Through their stimulation of CB_1 receptors, they inhibit pain transmission at central, spinal and peripheral synapses and may serve an auto protective role (Walker *et al*, 1999). While preclinical data indicate that Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of *Cannabis* (Gaoni and Mechoulam, 1964), and other direct CB_1 receptor agonists are also effective antinociceptive agents in laboratory animal models of neurodegenerative, neuroinflammatory and pain-related disease states (Fagan and Campbell, 2014; Guindon and Hohmann, 2009; Pryce and Baker, 2012), their distinct cannabimimetic side effect profile, which includes abuse, dependence, and memory impairment (Cooper and Haney, 2009; Hampson and Deadwyler, 2000; Hutcheson *et al*, 1998; Justinova *et al*, 2003; Lichtman *et al*, 1995), limits therapeutic use and further development. Direct agonists, including THC, target the orthosteric binding pocket on the CB_1 receptor and initiate global activation of the receptor, which is heterogeneously expressed in brain, spinal cord and periphery. While endocannabinoids also bind orthosterically (Devane *et al*, 1992; Mechoulam *et al*, 1995; Sugiura *et al*, 1995), they are released on demand where needed and are quickly metabolized (Di Marzo *et al*, 1999); hence, their actions are more transient and selective with highly specific temporal and spatial regulation. Allosteric modulators may offer a similarly selective approach for alteration of CB_1 receptor signaling, presumably with reduced pharmacodynamic-related side effects. Allosteric modulators bind to a distinct, non-orthosteric site on the receptor, and elicit conformational changes that alter ligand potency and/or efficacy (Kenakin, 2004, 2013). Accordingly, it has been hypothesized that CB_1 positive allosteric modulators (PAMs) should enhance antinociceptive and other functional effects of endogenously released cannabinoids, but with limited cannabimimetic side effects (Pertwee, 2005; Ross, 2007a, b).

Initially reported CB_1 receptor allosteric modulators were based on a series of Organon compounds, which enhanced orthosteric binding in a ligand-dependent manner, but paradoxically, inhibited signal transduction (Price *et al*, 2005). These, and other allosteric modulators of the CB_1 receptor, have been characterized on the basis of their actions in radioligand binding assays and other functional *in vitro* assays of CB_1 receptor signal transduction (Ahn *et al*, 2013; Baillie *et al*, 2013; Horswill *et al*, 2007; Navarro *et al*, 2009; Pamplona *et al*, 2012; Piscitelli *et al*, 2012). *In vivo*, the purported negative allosteric modulator (NAM), PSNCBAM-1, reduced food intake (Horswill *et al*, 2007), an action consistent with CB_1 orthosteric antagonism (Di Marzo *et al*, 2001), although CB_1 receptor mediation of this anorectic effect was not ascertained. Another purported CB_1 NAM, Org27569, reduced food intake, but this effect was CB_1 receptor independent (Gamage *et al*, 2014). Moreover, this compound generally failed to modify the pharmacological effects of CB_1 orthosteric agonists in common rodent models indicative of CB_1 receptor activity (Gamage *et al*, 2014). Likewise, ORG27569 generally did not perform as a CB1 receptor allosteric modulator in rats (Ding et al, 2014). Although it attenuated both cue- and drug-induced reinstatement of cocaine and methamphetamine-seeking behavior in rats, CB_1 receptor involvement was not determined (Jing et al, 2014). The first compelling pharmacological evidence demonstrating the effectiveness of a CB_1 receptor allosteric modulator in whole animals came from Pamplona *et al* (2012). They found that the endogenous anti-inflammatory mediator, lipoxin A4, enhanced the pharmacological effects of AEA at the CB_1 receptor both *in vitro* and *in vivo*, as well as protected against beta-amyloid (1-40)-induced performance deficits in the Morris water maze in mice (Pamplona *et al*, 2012).

In the present study, we examined a novel small molecule CB_1 PAM, ZCZ011 (Figure 1a), in *in vitro* and *in vivo* assays to evaluate whether it behaves as a CB1 PAM. *In vitro*, ZCZ011 increased the CB_1 receptor agonist receptor binding and potentiated AEA-stimulated signaling in $[^{35}S]GTP\gamma S$ binding, β arrestin recruitment, and ERK phosphorylation assays. As there remains a tremendous need for new medications to treat chronic pain conditions (Nightingale, 2012), we tested whether this compound would reduce nociceptive behavior in the chronic constriction injury (CCI) model of neuropathic pain, as well as in the carrageenan model of inflammatory pain. Each of these assays is highly sensitive to the antinociceptive effects of orthosteric CB_1 agonists and inhibitors of endocannabinoid catabolic enzymes (Ghosh *et al*, 2013; Kinsey *et al*, 2009; Lichtman *et al*, 2004; Russo *et al*, 2007). In addition, we examined ZCZ011 by itself or in combination with orthosteric CB_1 receptor agonists in a range of common assays sensitive to cannabimimetic activity, including the tetrad tests (locomotor activity, antinociception, catalepsy, and hypothermia; (Little *et al*, 1988)) and drug discrimination (Jarbe *et al*, 1981).

Finally, we tested whether systemically administered ZCZ011 was brain penetrant and whether it altered endocannabinoid levels in brain. Here, we demonstrate the first evidence of a CB_1 PAM that exhibits antinociceptive effects in neuropathic and inflammatory pain models with no associated cannabimimetic effects.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) and male FAAH (−/−) mice, backcrossed onto a C57BL/6J background for at least 13 generations served as subjects. FAAH (-/-) mice were employed in all experiments examining the *in vivo* actions of exogenously administered AEA to prevent its rapid hydrolysis to arachidonic acid, which is known to produce CB1 receptor independent effects (Wiley et al., 2006). While constitutively elevated levels of AEA and other lipids in FAAH (-/-) mice might complicate interpretation, it should be noted that these mice display normal CB1 receptor expression and function (Cravatt et al., 2001; Lichtman et al., 2002; Falenski et al., 2010). All animal protocols were approved by the respective Institutional Animal Care and Use Committees at Virginia Commonwealth University and West Virginia University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).Other details are included in the Supplementary Information.

Materials

ZCZ011 (6-Methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1*H*-indole) was synthesized at University of Aberdeen (see synthesis below). AEA was provided by Organix Inc. (Woburn, MA). The pan CB_1/CB_2 receptor agonist CP55,940, the CB_1 receptor antagonist rimonabant [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide-HCl] (SR141716A), and the CB_2 receptor antagonist SR144528 [5 - (4-chloro- 3-methylphenyl) - 1- [(4-methylphenyl)methyl] – N - [(1S,2S,4R) - 1,3,3- rimethylbicyclo [2.2.1] hept-2-yl]-1H-pyrazole-3- carboxamide] were obtained from the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Drugs were dissolved in a vehicle consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9 % NaCl) in a ratio of 1:1:18. Each drug was given via the intraperitoneal (i.p.) route of administration with exception of the discrimination studies, in which drugs were injected via subcutaneous (s.c.) route of administration. All drugs were administered at a volume of 10 μl/g body mass.

Synthesis of ZCZ011 (see Supplementary Information)

Mouse brain membrane preparation

Whole brains from adult male MF1 mice were suspended in centrifugation buffer (320 mM sucrose, 2 mM EDTA, 5 mM $MgCl_2$) and the tissues were homogenized with an Ultra-Turrex homogenizer. Tissue homogenates were centrifuged at 1600 g for 10 min and the resulting supernatant collected. This pellet was resuspended in centrifugation buffer centrifuged as before and the supernatant collected. Supernatants were combined before undergoing further centrifugation at 28,000 g for 20 min. The supernatant was discarded and the pellet resuspended in buffer A (50 mM Tris, 2 mM EDTA, 5 mM $MgCl_2$ at pH 7.0) and incubated at 37°C for 10 minutes. Following the incubation, the suspension was centrifuged for 20 min at 23,000 g. After resuspending the pellet in buffer A, the suspension was incubated for 40 min at room temperature before a final centrifugation for 15 min at 11,000 g. The final pellet was resuspended in buffer B (50 mM Tris, 1 mM EDTA, 3 mM $MgCl_2$) and the final protein concentration, determined by Bio-Rad Dc kit, was 1 mg ml^{-1}. All centrifugation procedures were carried out at 4°C. Prepared brain membranes were stored at -80°C and defrosted on the day of the experiment.

CHO-hCB1R cells

CHO cells stably transfected with cDNA encoding human cannabinoid CB_1 receptors (see Baillie et al., 2013) were maintained in Dulbecco's modified Eagles's medium (DMEM) nutrient mixture F-12 HAM, supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS), 0.6% penicillin–streptomycin, hygromycin B (300µgml^{-1}) and geneticin (600µgml^{-1}). All cells were maintained at 37°C and 5% CO_2 in their respective media and were passage twice a week using non-enzymatic cell dissociation solution. The CHO-hCB_1R transfected cell line was used for cAMP and pERK1/2.

Equilibrium Binding Assays

Equilibrium binding assays were carried out using [^{3}H]CP55,940, [^{3}H]WIN55212 and [^{3}H]SR141716A concentrations of 0.7 nM, 1.2 nM and 1 nM respectively. BSA (1 mg/ml) and 50 mM Tris buffer was used in a total assay volume of 500 µl containing 0.01% DMSO. Binding was initiated by adding 30 µg of mouse brain membranes, as previously described (Baillie *et al*, 2013). Assays were incubated at 37°C for 60 min, and then the reaction was stopped by the addition of ice cold wash buffer that contained 50 mM Tris buffer and 1 mg/ml BSA and vacuum filtration using a 24-well sampling manifold Brandel cell harvester (Gaithersburg, MD). Specific binding is defined as the difference between the binding that occurred in the presence and absence of 1 µM unlabelled ligand and varied between 70 and 90 % of the total binding.

[^{35}S]GTPγS Binding Assay

Mouse brain membranes (5 µg protein) were preincubated for 30 min at 30°C with adenosine deaminase (0.5 U ml^{-1}). The membranes were then incubated with the agonist ± modulator or vehicle for 60 min at 30°C in assay buffer (50 mM Tris; 5 mM $MgCl_2$; 1 mM EDTA; 100 mM NaCl; 1 mM DTT; 0.1% BSA) in the presence of 0.1 nM [^{35}S]GTPγS and 30 µM GDP. Binding was initiated by the addition of [^{35}S]GTPγS. Nonspecific binding was measured in the presence of 30 µM GTPγS. The reaction was terminated by rapid vacuum filtration (50 mM Tris-HCl; 50 mM Tris-Base; 0.1 % BSA) using a 24-well sampling manifold (cell harvester; Brandel, Gaitherburg, MD) and GF/B filters (Whatman, Maidstone, UK).

Data Analysis: Raw data were presented as cpm. Basal level was defined as zero. Results were calculated as a percentage change from basal level of [^{35}S]GTPγS binding (in the presence of vehicle). Data were analysed by nonlinear regression analysis of sigmoidal dose response curves using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis are presented as Emax with 95% confidence limits and pEC_{50} (logEC50) ± SEM.

PathHunter® CB_1 β-Arrestin Assays

PathHunter® hCB_1 β-arrestin cells were plated 48 h before use and incubated at 37°C, 5% CO_2. Compounds were dissolved in DMSO and diluted in OCC media. 5 µl of allosteric modulator or vehicle solution was added to each well and incubated for 60 min. 5 µl of agonist was added to each well followed by a 90 min incubation. 55 µl of detection reagent is then added followed by further 90 min incubation at room temperature. Chemiluminescence, indicated as RLU, was measured on a standard luminescence plate reader.

Data Analysis: Raw data were relative light units (RLU). Basal level was defined as zero. Results were calculated as the percentage of CP55940 maximum effect. Data were analyzed by nonlinear regression analysis of sigmoidal dose response curves using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis are presented as E_{max} with 95% confidence limits and pEC_{50} ($logEC_{50}$) ± SEM.

AlphaScreen® SureFire® ERK 1/2 phosphorylation assay

ERK1/2 MAP-kinase phosphorylation assay. For experimental studies of ERK1/2 MAP-kinase phosphorylation, hCB_1R cells (40,000 cells/well) were plated onto 96 well plates and serum-starved for 24 h. Cells were then washed with DMEM before the addition of agonist ± Org 27569 or vehicle at the desired concentration. After a 6 minute incubation at 37ºC in a humidified atmosphere, ice cold lysis buffer (provided with the AlphaScreen® SureFire® kit) was added to each well and the plate was placed at -80°C for at least 1 hour.

AlphaScreen® SureFire® ERK Assay. The assay was performed in 384 well white Proxiplates according to the manufacturer instructions. Briefly, 4 µl samples were incubated with 7 µl of mixture containing: 1 part donor beads: 1 part acceptor beads: 10 parts activation buffer: 60 parts reaction buffer. Plates were incubated for 3 hours at 25°C in the dark and read with the Envision® system (PerkinElmer) using AlphaScreen® settings.

Data Analysis. Raw data were presented as 'Envision units'. Basal level was defined as zero. Results were presented as means and variability as SEM or 95% confidence limits (CL) of the percent stimulation of phosphorylated ERK1/2 above the basal level (in the presence of vehicle). Data were analyzed by nonlinear analysis of log agonist versus-response curves using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis were presented as E_{max} with 95% confidence limits and pEC_{50} ($logEC_{50}$) ± SEM.

DiscoverX® cAMP Assays

For experimental studies of inhibition of cAMP formation, hCB1R cells (20,000 cells/well) were plated into 96 well plates and serum-starved for 24 h. Cells were then washed with serum and phenol free DMEM before the addition of agonist with vehicle/allosteric modulator in the presence of 10µM rolipram and 10µM forskolin. Cells were stimulated for 30 minutes at 37°C in a humidified atmosphere. The DiscoverX® cAMP kit was then used and a standard curve was included in every assay. Antibody solution was added to each well followed by working solution: 1 part ED solution and 1 part combination- lysis (19), Emerald solution (5) and Gal (1). Plates were incubated at room temperature for 60 minutes. A final addition of EA reagent to each well was followed by incubation at room temperature for no less than 3 but no more than 18 hours and plates were read using a luminescence plate reader.

Data Analysis: Results were calculated as the percentage inhibition of forskolin-stimulated cAMP production. Data were analyzed by nonlinear regression analysis of sigmoidal dose response curves using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis were presented as E_{max} with 95% confidence limits and pEC_{50} ($logEC_{50}$) ± SEM.

Chronic Constriction Nerve Injury (CCI) Model of Neuropathic Pain

CCI nerve injury was induced according to surgical procedures described previously (Kinsey *et al*, 2009), as detailed in supplementary methods. ZCZ011 (0, 10, 20, 40 mg/kg) was injected via the i.p. route of administration and mice were tested for mechanical and cold allodynia 75 min later. ZCZ011 was administered in a counterbalanced Latin square within subject design with at least a five day wash out period between tests. In order to assess the effects of repeated treatment of ZCZ011 (40 mg/kg, i.p.) on mechanical and cold allodynia induced by CCI, mice were divided into the following three experimental groups: 1) Vehicle Control (six days of vehicle injections); 2) Acute ZCZ011 (five days of vehicle injections and injected with 40 mg/kg ZCZ011 on day 6); and 3) Repeated ZCZ011 (six days of injections of 40 mg/kg ZCZ011). In the repeated ZCZ011 group, mechanical and cold allodynia were assessed 1, 2, 4, 12 and 24 h following the first ZCZ011 injection to determine duration of acute anti-allodynic effects of ZCZ011. The subjects in all groups were tested 1 h following ZCZ011 administration on day 6. In experiments assessing cannabinoid receptor mechanism of action, rimonabant (3 mg/kg) or SR144528 (3 mg/kg) was administered 10 min prior to ZCZ011 or vehicle. Mechanical allodynia was assessed using von Frey filaments and the acetone flinching test was used to assess cold allodynia, as described previously (Kinsey *et al*, 2009) and detailed in **Supplementary Information**.

Carrageenan Model of Inflammatory Pain

Edema was induced *via* intraplantar injection of 0.3 % carrageenan (Sigma, St Louis, MO) in a 20 µl volume using a 30 gauge needle into the hind left paw. Paw thickness was measured with an electronic digital micrometer (Traceable Calipers, Friendswood, TX) prior to and 5 h following carrageenan administration, which corresponds to peak edema (Wise *et al*, 2008). Paw edema data are expressed as the difference in paw thickness between the 5 h and pre-injection measures. Mechanical allodynia was assessed using von Frey filaments at the same peak time point (see **Supplementary Information**).

Tetrad Assay

The behavioral testing was conducted in the following order: bar test (catalepsy), tail withdrawal test, and rectal temperature. A separate group of mice was used to assess the effects of ZCZ011 on locomotor activity. Testing was performed according to previously described procedures (Long *et al*, 2009b; Schlosburg *et al*, 2010). For a full description, see **Supplementary Information**.

Drug Discrimination

Male C57BL/6J and FAAH (-/-) mice (20–25 g) trained to discriminate CP55,940 (0.1 mg/kg) or AEA (6 mg/kg) from vehicle, respectively, were tested in a nose-poke operant task according to procedures described previously (Long *et al*, 2009b) with minor modifications. For complete description of these procedures, see **Supplementary Information**.

ZCZ011 Place Conditioning

An unbiased mouse CPP paradigm was utilized, as previously described (Kota *et al*, 2008) in which vehicle, ZCZ011 (40 mg/kg), or cocaine (10 mg/kg; positive control) were randomly paired with one of two distinct chambers. On the test day, mice did not receive an injection and were allowed to roam freely for 15 min while the percentage of time spent in both chambers was scored as the dependent measure. For further details, see **Supplementary Information**.

Extraction and Quantification of Endocannabinoids by Liquid Chromatography-tandem Mass Spectrometry (see Supplementary Information)

C57BL/6J mice were administered ZCZ011 (40 mg/kg) either acutely and sacrificed 45 min later. Brains were harvested and the concentrations of 2-AG, AEA, palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and arachidonic acid (AA) levels were quantified, as previously described (Ignatowska-Jankowska *et al*, 2014) and detailed in **Supplementary Information**.

Data Analyses

All in vivo data are presented as mean ± standard error (SEM) or 95 % confidence limits (CL). *In vitro* data were analysed using log agonist versus response curves in GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis were presented as E_{max} with 95 % confidence limits and pEC_{50} ($logEC_{50}$) ± SEM. *In vivo* data were analyzed using one-way or two-way analysis of variance (ANOVA). Dunnett's test was used for *post hoc* analysis in the dose-response experiments, and the Tukey test was used for *post hoc* analyses comparing different treatment groups. Multiple comparisons following two-way ANOVA were conducted with Bonferroni *post hoc* comparisons. Differences were considered significant at the level of $P < 0.05$. Statistical analysis was performed with GraphPad Prism version 5.00 (GraphPad, San Diego, CA).

Results

ZCZ011 Enhanced CB_1 Receptor Agonist Binding

In equilibrium binding experiments, ZCZ011 (**Figure 1a**) produced a significant and concentration-dependent increase in the specific binding of the CB_1 receptor orthosteric agonist, [^{3}H]CP55,940 to mouse brain membranes with an E_{max} of 207 % (95 % confidence limits, 191-223) and a pEC_{50} of 6.90 ± 0.23 (**Figure 1b**). ZCZ011 also produced a significant and concentration-dependent increase in the specific binding of the CB_1 receptor orthosteric agonist, [^{3}H]WIN55212 to mouse brain membranes with an E_{max} of 225 % (95 % confidence limits, 182-269) and a pEC_{50} of 6.31 ± 0.33 (**Figure 1b**). In contrast, ZCZ011 produced a significant and concentration-dependent decrease in the specific binding of the CB_1 receptor orthosteric inverse agonist, [^{3}H]SR141716A to mouse brain membranes with an E_{max} of 17 % (95 % confidence limits, -2.7 - 37) and a pEC_{50} of 6.21 ± 0.21 (**Figure 1b**).

In saturation binding experiments, [^{3}H]CP55,940 bound in a saturable manner to the CB_1 receptor with a B_{max} of 1.37 pmol/mg (95 % confidence limits, 1.042 – 1.704) and K_d of 3.10 ± 0.9 nM (**Figure 1c**). ZCZ011 (1 µM) significantly increased the B_{max} value of [^{3}H]CP55,940 to 1.88 pmol/mg (95 % confidence limits, 1.728-2.025) without significantly affecting binding affinity (K_d = 1.97 ± 0.2 nM; **Figure 1c**), suggesting an increase in the number of available binding sites for [^{3}H]CP55,940.

ZCZ011 Enhanced CB_1 Receptor Agonist Signalling

[^{35}S]GTPγS binding

AEA stimulated [^{35}S]GTPγS binding in mouse brain membranes with a pEC_{50} value of 6.5 ± 0.2 and E_{max} (efficacy) of 46.5% (95% confidence limits, 40-54; **Figure 1d**). Addition of 100 nM ZCZ011, significantly enhanced AEA-stimulated [^{35}S]GTPγS binding [E_{max} of 115.2% (95% confidence limits, 104 - 127)], but there was no significant change in the pEC_{50} value (potency). A concentration of 10 nM ZCZ011 enhanced AEA-stimulated [^{35}S]GTPγS binding with an E_{max} of 65.4% (95% confidence limits, 51-80); however, this effect was not statistically significant. ZCZ011 alone caused no stimulation of [^{35}S]GTPγS binding.

Similarly, CP55,940 stimulated [^{35}S]GTPγS binding in mouse brain membranes with a pEC_{50} value of 8.34 ± 0.25 and E_{max} of 61% (95% confidence limits, 50-72) (**Supplementary Figure**

1a). Addition of 1µM ZCZ011, significantly enhanced AEA-stimulated [^{35}S]GTPγS binding [E_{max} of 100% (95% confidence limits, 82 – 112)], but there was no significant change in the pEC_{50} value (8.80 ± 0.26).

PathHunter® hCB1 β-arrestin recruitment assay

In the PathHunter® β-arrestin assay in hCB_1 cells, AEA stimulated β-arrestin recruitment with a pEC_{50} of 6.1 ± 0.10 and an E_{max} of 100% (95% confidence limits, 87-113; **Figure 1e**). ZCZ011 caused a concentration dependent enhancement of AEA-stimulated β-arrestin recruitment at 10 nM, 100nM and 1µM with E_{max} values of 99% (95% confidence limits, 90-107) and 157% (95% confidence limits, 138-175) and 195% (95% confidence limits, 185-205), respectively. There was no significant change to the pEC_{50} value of 6.78 ± 0.11 and 7.02 ± 0.12 in the presence of 10 nM and 100 nM ZCZ011, respectively. At 1 nM AEA, the maximum stimulation observed was 0.0% (95% confidence limits, -6.6 – 6.6); however, the addition of 10 nM, 100 nM and 1µM ZCZ011 significantly increased stimulation to 18.1% (95% confidence limits, 10.8 – 25.4), 65.1% (95% confidence limits, 47 – 83) and 161.6% (95% confidence limits, 148 – 175), respectively. These findings indicate positive cooperativity between the endogenous cannabinoid and ZCZ011. When tested alone, ZCZ011 produced an increase in β-arrestin recruitment that was 35.9% (95% confidence limits, 33-39) of maximal stimulation.

AlphaScreen® surefire® ERK 1/2 phosphorylation assay

Using an AlphaScreen® surefire® ERK 1/2 phosphorylation assay kit, we measured the effect of ZCZ011 on activation of ERK 1/2 phosphorylation by CB_1 agonist, AEA in hCB_1R cells (**Figure 1f**). AEA induced ERK 1/2 phosphorylation with an E_{max} of 98.2% (95% confidence limits, 79-118) and pEC_{50} of 6.5 ± 0.3. Neither 10 nM nor 100 nM ZCZ011 significantly affected AEA E_{max} (efficacy), but pEC_{50} value (potency) was significantly increased to 8.3 ± 0.3 and 8.4 ± 0.5, respectively (one-way ANOVA, Dunnett's multiple comparison test, $p < 0.05$). With the exception of the highest concentration (1 µM), ZCZ011 alone did not induced ERK 1/2 phosphorylation.

CP55,940 induced ERK 1/2 phosphorylation with an E_{max} of 101% (95% confidence limits, 86-116) and pEC_{50} of 7.85± 0.25 (**Supplementary Figure 1b**). 1µM ZCZ011 did not affect the E_{max}

(efficacy) of CP55,940 [E_{max} of 119% (95% confidence limits, 107-132)] but pEC_{50} value (potency) was significantly increased to 8.95 ± 0.3 (one-way ANOVA, Dunnett's multiple comparison test, $p < 0.05$).

DiscoverX® hCB_1 cAMP production assay

Using a DiscoverX® cAMP production assay, we measured the effect of ZCZ011 on activation of ERK 1/2 phosphorylation by CB_1 agonist, AEA in hCB_1 cells (**Figure 1g**). AEA inhibited forskolin stimulated cAMP production with an E_{max} of 59% (95% confidence limits, 35-83) and pEC_{50} of 6.97 ± 0.35. Alone, ZCZ011 acted as an agonist; inhibiting forskolin stimulated cAMP production with an E_{max} of 84% (95% confidence limits, 46-122) and pEC_{50} of 5.68 ± 0.33. 100 nM ZCZ011 did not significantly affected AEA. There was evidence positive cooperatively at a concentration of 1μM ZCZ011.

CP55,940 inhibited forskolin stimulated cAMP production with an E_{max} of 83% (95% confidence limits, 75 - 92) and pEC_{50} of 8.27 ± 0.13. 100 nM ZCZ011 did not significantly affected AEA. 1μM ZCZ011 did not significantly affect the Emax or pEC50 of CP55,940 (**Supplementary Figure 1c**).

ZCZ011 Does Not Produce Psychoactive Effects in Mice

ZCZ011 (40 mg/kg) was detected in whole brain, 6.5 ± 0.6 (mean ± SEM) ng/wet (g), as determined by HPLC/MS/MS (Poklis *et al*, in press). Given alone, 40 mg/kg ZCZ011 did not produce catalepsy (0s immobility), hypothermia ($p = 0.4$; **Supplementary Figure 2a**), antinociception in tail withdrawal ($p = 0.9$; **Supplementary Figure 2b**) or hot plate tests ($p = 0.8$; **Supplementary Figure 2c**) , or locomotor depression ($p = 0.9$; **Supplementary Figure 2d**). ZCZ011 did not substitute for either CP55,940 **(Supplementary Figure 2e**) or AEA (**Supplementary Figure 3a**) in the drug discrimination assay and did not affect respective response rates for either training drug ($p = 0.9$; **Supplementary Figure 2f** and $p = 0.9$, $p = 0.1$; **Supplementary Figure 3b**). Also, ZCZ011 (40 mg/kg) did not elicit a conditioned place preference or aversion compared with vehicle ($p = 0.2$; **Supplementary Figure 4**).

ZCZ011 Potentiates the Pharmacological Effects of AEA and CP55,940 in Mice

In contrast to its ineffectiveness to elicit cannabimimetic effects when administered alone, ZCZ011 significantly augmented the antinociceptive [$F(1,14)=8.0$, $p<0.01$], cataleptic [$F(2,28)=3.84$, $p<0.05$], and hypothermic [$F(1,14)=5.5$; $p<0.05$] effects of CP55,940 (**Figure 2a-c)**. It also enhanced AEA-induced hypothermia [$F(4,64)=2.93$; $p<0.05$; **Figure 2d**], but did not affect the antinociceptive ($p=0.8$; **Figure 2e**) or cataleptic ($p=1$; **Figure 2f**) effects of AEA in FAAH (-/-) mice. Also, this compound did not alter the antinociceptive effects of 1 mg/kg nicotine (**Supplementary Figure 5**).

In the drug discrimination assay, ZCZ011 (40 mg/kg) significantly increased the potency of the discriminative stimulus effects of AEA in FAAH (-/-) mice [$F(4,48)=10.47$, $p<0.001$; **Supplementary Figure 3a**]. The respective ED_{50} (95% CI) values of AEA in the vehicle-pretreated mice and ZCZ011-pretreated mice were 4.0 (2.7-5.9) mg/kg and 1.4 (1.2-1.7) mg/kg. ZCZ011 increased AEA potency 2.2-fold compared with the vehicle-pretreated mice. ZCZ011 (20 mg/kg) also significantly enhanced the discriminative cue of AEA. While ZCZ011 (40 mg/kg) given alone or in combination with AEA did not affect response rates of FAAH (-/-) mice in the drug discrimination paradigm (**Supplementary Figure 3b**), it potentiated the depressive effects of AEA (5.6 mg/kg) on operant responding for food in a separate group of mice [$F(1,4)=6.88$, $p<0.05$; **Supplementary Figure 6**].

ZCZ011 Reverses Nociceptive Behavior in Neuropathic and Inflammatory Pain Models

ZCZ011 enhanced the pharmacological effects of AEA and CP55,940 in *in vitro* and in behavioral assays, but did not produce common cannabimimetic effects on its own. Accordingly, we next investigated whether it would reverse nociceptive behavior in well-established models of neuropathic and inflammatory pain. As has been previously shown (Kinsey *et al*, 2010), the FAAH inhibitor, PF-3845 reversed mechanical (**Figure 3a**) and cold allodynia (**Figure 3b**) in the CCI model of neuropathic pain. These findings are consistent with the idea that AEA, which is rapidly hydrolyzed by FAAH, has an autoprotective role in this model. Similarly, ZCZ011 completely reversed mechanical [$F(3,42)=7.6$, $p<0.001$; **Figure 3a**] and cold allodynia [$F(3,42)=3.6$, $p<0.05$; **Figure 3b**] in the CCI model of neuropathic pain. Unlike endocannabinoid catabolic enzyme inhibitors; however, ZCZ011 (40 mg/kg) did not affect whole brain levels of 2-

AG ($p=0.3$), AEA ($p=0.3$), palmitoylethanolamide ($p=0.3$), or oleoylethanolamide ($p=1$) in C57BL/6J mice (**Supplementary Figure 7**).

These anti-allodynic actions of ZCZ011 were prevented by the CB_1 receptor antagonist, rimonabant (3 mg/kg), but not by the CB_2 receptor antagonist, SR144528 (3 mg/kg), [$F(3,28)=8.9$, $p<0.001$; **Figure 3c and d**], indicating a CB_1 receptor-mediated mechanism of action.

ZCZ011 (40 mg/kg) blocked mechanical allodynia [$F(4,56)=6.0$, $p<0.001$] and cold allodynia [$F(4,56)=4.44$, $p<0.01$; **Figure 4a and b**] for durations of 12 h and 4 h, respectively. The anti-allodynic effects of ZCZ011 (40 mg/kg) to mechanical [$F(2,21)=8.9$, $p<0.01$; **Figure 4c**] and cold [$F(2,21)=7.0$, $p<0.01$; **Figure 4d**] stimuli were retained following six days of daily injections and did not differ from the antinociceptive effects produced by acute ZCZ011. Thus, the antinociceptive effects of ZCZ011 were resistant to tolerance.

Likewise, ZCZ011 (40 mg/kg) partially reversed carrageenan-induced mechanical allodynia [$F(2,21)=22.3$, $p<0.001$; **Figure 5a**], which required CB_1, receptors, but not CB_2 receptors [$F(1,16)=14.7$, $p<0.01$; **Figure 5b**]. Consistent with its lack of CB_2 receptor action, ZCZ011 did not reduce carrageenan-induced paw edema (**Figure 5c and d**; $p=0.8$).

DISCUSSION

Here we report the development and pharmacological characterization of the novel synthetic CB_1 receptor PAM, ZCZ011. This compound increased equilibrium binding of the potent CB_1 receptor orthosteric agonist, CP55,940. Additionally, ZCZ011 enhanced the efficacy of AEA in stimulating [^{35}S]GTPγS binding in whole brain as well as β-arrestin recruitment and the potency of AEA in ERK phosphorylation assays in hCB_1 cells. In mice, ZCZ011 potentiated CP55,940-induced catalepsy, hypothermia, and antinociception. It also increased the potency of AEA in several *in vivo* assays employing FAAH (-/-) mice, including the discriminative stimulus effects of AEA, AEA-induced depression of operant responding for food, and AEA-induced hypothermia. Most strikingly, when administered alone, ZCZ011 completely reversed allodynia in the CCI model of neuropathic pain and partially reversed carrageenan-induced allodynia, but did not elicit any apparent cannabimimetic side effects. Its actions in the CCI model required CB_1 receptors, were of long duration (i.e., up to 12 h), and did not undergo tolerance after six days of treatment. Accordingly, we hypothesize that ZCZ011 blocked neuropathic pain, without eliciting general cannabimimetic activity, by augmenting the actions of endocannabinoids at CB_1 receptors in pathways mediating nociceptive responses following sciatic nerve injury. Likewise, ZCZ011 reduced carrageenan-induced allodynia through a CB_1 receptor mechanism of action, and did not reduce the edematous effects of carrageenan. Thus, this study provides compelling parallel *in vitro* and *in vivo* evidence that ZCZ011 acts as a CB_1 receptor PAM. Accordingly, ZCZ011 represents a valuable pharmacological tool for mechanistic studies as well as for exploring potential therapeutic applications of CB_1 receptor allosteric target(s).

Our *in vivo* observations showing that ZCZ011 potentiated the pharmacological effects of either CP55,940 or AEA in behavioral assays confirm *in vitro* observations showing that ZCZ011 acts as a CB_1 PAM and enhances the signalling of the bound agonist. ZCZ011 (1 μM) caused an increase in the B_{max} of [^{3}H]CP55,940 and [^{3}H]WIN55212, which implies an increase in the number of available receptors for CP55,940 to bind. Intriguingly, ZCZ011 elicited an apparent displacement of the CB_1 receptor inverse agonist, SR141716A. With the exception of cAMP activity, ZCZ011 increased CB_1 orthosteric agonist potency and/or efficacy in all the functional assays performed. Specifically, ZCZ011 potentiated AEA and CP55,940 signaling efficacy in the

[^{35}S]GTPγS binding in mouse brain membranes and increased the potency of AEA and CP55,940 in the ERK phosphorylation assay in hCB_1 cells, and enhanced the potency of AEA-mediated β-arrestin recruitment. This evidence strongly suggests that ZCZ011 acts as a CB_1 PAM both *in vitro* and *in vivo*. In the cAMP assay, ZCZ011 acted as an agonist alone, but did not affect the potency or efficacy of AEA or CP55,940 at a concentration of 1μM.

A highly novel finding in the present study was that ZCZ011 produced anti-allodynic effects in CCI model of neuropathic pain. Previous studies have also demonstrated that the anti-allodynic effects of FAAH inhibitors (Kinsey *et al*, 2010; Kinsey *et al*, 2009), as well as MAGL inhibitors JZL184 and KML29 (Ignatowska-Jankowska *et al*, 2014; Kinsey *et al*, 2010; Kinsey *et al*, 2009) elicited anti-allodynic effects in the CCI assay. Repeated administration of high dose ZCZ011 retained its antinociceptive effects, which is similar to the finding that the antinociceptive effects produced by FAAH inhibition also do not undergo tolerance (Schlosburg *et al*, 2010). While repeated high doses of MAGL inhibitors leads to antinociceptive tolerance associated with CB_1 receptor down-regulation and desensitization (Ignatowska-Jankowska *et al*, 2014; Schlosburg *et al*, 2010), CB_1 receptor function is retained following repeated low doses of the MAGL inhibitor JZL184 (Kinsey *et al*, 2013; Sciolino *et al*, 2011). The most notable difference between ZCZ011 and endocannabinoid catabolic enzyme inhibitors is that ZCZ011 did not alter the concentration of endocannabinoids or other N-acylethanoloamines in brain (**Supplementary Figure 7**). In contrast, FAAH and MAGL inhibitors produce increased brain levels of AEA (Kathuria *et al*, 2003) and 2-AG (Long *et al*, 2009a), respectively. The anti-allodynic effects of ZCZ011 in the CCI assay, were CB_1, but not CB_2, receptor dependent. In contrast, FAAH inhibitors require both CB_1 and CB_2 receptors to reverse CCI-induced allodynia (Kinsey *et al*, 2010; Kinsey *et al*, 2009). Additionally, FAAH (Holt *et al*, 2005) and MAGL (Ghosh *et al*, 2013) inhibitors produce anti-edematous actions in the carrageenan assay, which in each case was completely blocked by a CB_2 receptor antagonist and not a CB_1 receptor antagonist. Thus, a CB_1 PAM would not be expected to reduce carrageenan-induced paw edema, as the case in the present study. Collectively, these findings are consistent with the idea that the anti-allodynic effects of ZCZ011 in the CCI and carrageenan assays are mediated through its actions as a CB_1 PAM by enhancing the activity of endocannabinoids at CB_1 receptors.

An important observation from a drug development perspective is that ZCZ011 did not produce cannabimimetic side effects (i.e., catalepsy, hypothermia, thermal antinociception, or hypomotility) and did not substitute for AEA or CP55,940 in the drug discrimination paradigm. However, ZCZ011 enhanced many pharmacological effects produced by these orthosteric agonists, consistent with *in vitro* data showing that it acts as a CB_1 receptor PAM. While it is interesting that ZCZ011 augmented more of the measured actions produced by CP55,940 than those elicited by AEA, it is known that allosteric modulators affect orthosteric agonists in a ligand-dependent manner. The failure of ZCZ011 to affect nicotine-induced antinociception in the tail withdrawal and hot plate tests (**Supplementary Figure 5**) shows that its effects are selective to cannabinergic ligands.

Pamplona et al. (2012) presented the first *in vivo* and *in vitro* evidence demonstrating that the endogenous anti-inflammatory mediator, lipoxin A4, acts as a CB_1 PAM. This naturally occurring lipid enhanced both CB_1 receptor binding of AEA and AEA-induced cAMP inhibition. Moreover, when given via the i.c.v. route of administration lipoxin A4 produced cannabimimetic effects (i.e., catalepsy, hypothermia, hypomotility, and antinociceptive effects in the hotplate test). Notably, systemic administration of an inhibitor of 5-lipoxygenase, the primary biosynthetic enzyme of lipoxin A4, attenuated the cataleptic effects of i.c.v administered AEA, suggesting that this endogenous lipid contributes to the behavioral actions of CB_1 orthosteric agonists. In addition, i.c.v. administration of lipoxin A4 protected mice from impaired spatial memory performance in the Morris water maze task elicited by i.c.v. injection of β-amyloid (1-40) protein. This protective effect was blocked by rimonabant, indicating a CB_1 receptor-mediated mechanism of action. The data presented here with ZCZ011, together with those previously published with lipoxin A4 (Pamplona *et al*, 2012), provide compelling proof of principle that CB_1 PAMs offer promise as therapeutic strategies for neurodegenerative diseases and pain states related to nerve injury. The identification of the putative binding site(s) of ZCZ011 and lipoxin A4 at the CB_1 receptor will be a crucial step for future development of allosteric modulators of the CB_1 receptor as well as better understanding of the physiological function of CB_1 allosteric modulation for the development of novel pharmacotherapies based on this mechanism. Nonetheless, the present study demonstrates that the synthetic CB_1 PAM, ZCZ011, produces CB_1-mediated anti-allodynic effects in murine models of neuropathic and inflammatory pain without the development of tolerance or the occurrence of cannabimimetic side effects.

Funding and Disclosure

Research was supported by: NIH grants DA-009789, DA-026449, DA-003672 and CIHR Proof of Principal Phase 1 grant 288645.

Conflict of Interest: RAR, IRG, and MZ are inventors on patent applications filed by the Universities of Toronto and Aberdeen, which disclose pharmaceutical agents targeting molecular pathways described in the present article. RAR, IRG and MZ have an equity share in Signal Pharma Ltd, a University spin-out company developing CB1 positive allosteric modulators. The remaining authors declare no competing financial interests.

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Figure legends

Figure 1: The CB_1 positive allosteric modulator, ZCZ011 enhances CB_1 receptor binding and signaling (**a**) Chemical structure of ZCZ011. (**b**) ZCZ011 significantly increased [^{3}H]CP55,940 binding in mouse brain membranes. (**c**) ZCZ011 caused a significant increase in the B_{max} for [^{3}H]CP55,940 and [^{3}H]WIN55212 whilst having no effect on the K_d. ZCZ011 caused an apparent displacement of [^{3}H]SR141617A. (**d**) ZCZ011 caused a significant increase in the efficacy of AEA-stimulated [^{35}S]GTPγS binding in mouse brain membranes. (**e**) ZCZ011 caused a significant increase in AEA-stimulated β-arrestin recruitment in hCB_1 cells. (**f**) ZCZ011 caused a significant increase in the potency of AEA to stimulate ERK 1/2 phosphorylation in hCB_1 expressing cells. (**g**) Effect of ZCZ011 on forskolin-stimulated cAMP production in hCB_1 expressing cells; ZCZ011 alone inhibits forskolin-stimulated cAMP production. Symbols represent mean values ± S.E.M from 2-7 independent experiments.

Figure 2: ZCZ011 (40 mg/kg) potentiated the pharmacological effects of orthosteric CB_1 receptor agonists CP55,940 and AEA. ZCZ011 significantly enhanced CP55,940-induced hypothermia (**a**), catalepsy (**b**), and antinociception (**c**) in C57BL/6J mice and AEA-induced hypothermia (**d**), but not AEA-induced catalepsy (**e**) or antinociception (**f**), in FAAH (-/-) mice. Data presented as mean ± SEM; n = 8-9 mice per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle.

Figure 3: ZCZ011 significantly reduced mechanical (a) and cold (b) allodynia induced by chronic constriction nerve injury (CCI). The anti-allodynic effects of ZCZ011 (40 mg/kg, i.p.) were blocked by the CB_1 receptor antagonist rimonabant (SR141716A, SR1) (3 mg/kg; **c**), but not by the CB_2 receptor antagonist SR144528 (SR2) (3 mg/kg; **d**). The FAAH inhibitor, PF-3845 (10 mg/kg, i.p.) was included for comparison. Results presented as mean ± SEM; (n = 9-12 mice per group) *$p<0.05$, **$p<0.01$, ***$p<0.001$, ###$p<0.001$ vs vehicle; $$$p<0.01$, $$$$p<0.001$ vs contralateral paw.

Figure 4. The anti-allodynic effects of ZCZ011 (40 mg/kg) are of long duration and do not undergo tolerance after six days of daily injections in the CCI model of neuropathic pain. ZCZ011 significantly reversed mechanical allodynia for up to 12 h (**a**) and cold allodynia for up to 4 h (**b**). The anti-allodynic effects of ZCZ011 are retained following six days of repeated administration in response to tactile (**c**) and cold (**d**) stimulation. Values represent mean ± SEM, n=8 mice per group, *p<0.05, **p<0.01, ***p<0.001 vs vehicle, $$p<0.01, $$$p<0.001 vs. contralateral paw.

Figure 5: ZCZ011 partially reversed mechanical allodynia in the carrageenan model of inflammatory pain. The CB_1 receptor antagonist rimonabant (SR141716A, SR1; 3 mg/kg, **a**), but not the CB_2 receptor antagonist SR144528 (SR2; 3 mg/kg; **b**) blocked the anti-allodynic effects of ZCZ011. Results presented as mean ± SEM; (n = 9-12 mice per group). ZCZ011 (40 mg/kg, i.p.) did not attenuate carrageenan-induced paw edema (**c and d**). Values represent mean ± SEM, n=8 mice per group.*p<0.05, **p<0.01, ***p<0.001, ###p<0.001 vs vehicle; $$p<0.01, $$$p<0.001 vs. contralateral paw.

Supplementary Figure 1: ZCZ011 (ZCZ) affects the signaling of the orthosteric CB_1 receptor agonist, CP55,940 (CP). (**a**) ZCZ011 caused a significant increases the efficacy of CP55-stimulated [^{35}S]GTPγS binding in mouse brain membranes. (**b**) ZCZ011 caused a significant increase in the potency of CP55 to stimulate ERK 1/2 phosphorylation in hCB_1 CHO expressing cells. (**c**) ZCZ011 (1 μM) did not significantly affect the potency or efficacy of CP55 to inhibit forskolin-stimulated cAMP production in hCB_1 expressing cells. Symbols represent mean values ± S.E.M from 2-4 independent experiments.

Supplementary Figure 2: ZCZ011 does not produce cannabimimetic effects when given alone. ZCZ011 (40 mg/kg, 15 min pretreatment) did not affect thermal antinociception in **(a)** the warm water tail withdrawal and **(b)** hot plate tests, did not affect **(c)** body temperature **(d)** did not elicit locomotor depression and **(e)** did not substitute for CP55,940 or **(f)** depress response rates in the drug discrimination assay. Also, ZCZ011 did not produce catalepsy (0 s immobility time). Values expressed as mean ± SEM, n=8 mice per group, ***p<0.001 vs vehicle, ###p<0.001 vs CP55,940.

Supplementary Figure 3: (a) ZCZ011 significantly increased the AEA potency as a discriminative stimulus in FAAH (-/-) mice, but did not affect response rates (**b**). Data presented as mean ± SEM; n = 8-9 mice per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle.

Supplementary Figure 4: ZCZ011 [40 mg/kg, i.p.] did not produce a conditioned place preference or conditioned place aversion. In contrast, cocaine (10 mg/kg), which served as a positive control, produced a significant place preference [$F(2,23)=8.9$, $p<0.01$. Data are presented as mean ± SEM, n = 7-10 mice per group. *$p < 0.05$ vs. vehicle, ##$p<0.01$ vs. cocaine.

Supplementary Figure 5: ZCZ011 (40 mg/kg, i.p., 45 min pretreatment) did not affect nicotine-induced antinociception in tail withdrawal (**a**) or hot plate **(b**) tests. Data presented as mean ± SEM, n = 8 mice per group.

Supplementary Figure 6: ZCZ011 (40 mg/kg) significantly potentiated the rate-suppressing effects of AEA (5.6 mg/kg) in a nose-poke operant procedure for food reinforcement. When given alone ZCZ011 (40 mg/kg, i.p.) or AEA (5.6 mg/kg, s.c.) did not alter operant responses compared with the vehicle controls. Mean ± SEM, n = 8-10 mice per group, *$p < 0.05$ vs. each other group.

Supplementary Figure 7: ZCZ011 (40 mg/kg, i.p.) did not affect whole brain levels of (**a**) 2-arachidonoylglycerol (2-AG), (**b**) anandamide (AEA), (**c**) palmitoylethanolamide (PEA), or (**d**) oleoylethanolamide (OEA) in C57BL/6J mice. Data presented as mean ± SEM, n = 5 mice per group.