Prolonged Monoacylglycerol Lipase Blockade Causes Equivalent Cannabinoid Receptor Type 1 Receptor–Mediated Adaptations in Fatty Acid Amide Hydrolase Wild-Type and Knockout Mice

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ABSTRACT

Complementary genetic and pharmacological approaches to inhibit monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), the primary hydrolytic enzymes of the respective endogenous cannabinoids 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamine, enable the exploration of potential therapeutic applications and physiologic roles of these enzymes. Complete and simultaneous inhibition of both FAAH and MAGL produces greatly enhanced cannabinimetic responses, including increased antinociception, and other cannabinimetic effects, far beyond those seen with inhibition of either enzyme alone. While cannabinoid receptor type 1 (CB₁) function is maintained following chronic FAAH inactivation, prolonged excessive elevation of brain 2-AG levels, via MAGL inhibition, elicits both behavioral and molecular signs of cannabinoid tolerance and dependence. Here, we evaluated the consequences of a high

dose of the MAGL inhibitor JZL184 [4-nitrophenyl 4-(dibenzo[*d*] [1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; 40 mg/kg] given acutely or for 6 days in FAAH(–/–) and (+/+) mice. While acute administration of JZL184 to FAAH(–/–) mice enhanced the magnitude of a subset of cannabinimetic responses, repeated JZL184 treatment led to tolerance to its antinociceptive effects, cross-tolerance to the pharmacological effects of Δ^9 -tetrahydrocannabinol, decreases in CB₁ receptor agonist-stimulated guanosine 5'-O-(3-[³⁵S]thio)triphosphate binding, and dependence as indicated by rimonabant-precipitated withdrawal behaviors, regardless of genotype. Together, these data suggest that simultaneous elevation of both endocannabinoids elicits enhanced cannabinimetic activity but MAGL inhibition drives CB₁ receptor functional tolerance and cannabinoid dependence.

Introduction

THC (Δ^9 -Tetrahydrocannabinol) and other cannabinoid receptor types 1 and 2 (CB₁/CB₂) agonists produce many beneficial effects in conditions related to pain, inflammation, anxiety, muscle spasticity, feeding, and nausea (Di Marzo, 2008; Blankman and Cravatt, 2013; Mechoulam and Parker, 2013; Pacher and Kunos, 2013). However, psychomimetic effects, memory-impairing actions, and dependence liabilities dampen enthusiasm for therapeutic development of CB₁ receptor

agonists. Conversely, inhibitors of enzymes that hydrolyze the endogenous cannabinoids (endocannabinoids), e.g., AEA (*N*-arachidonylethanolamine; anandamide) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), may offer advantages over direct-acting agonists. In particular, inhibitors of fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996, 2001) and monoacylglycerol lipase (MAGL) (Dinh et al., 2002), the respective primary hydrolytic enzymes of these endocannabinoids, produce antinociceptive (Ahn et al., 2009; Long et al., 2009a), anxiolytic-like (Patel and Hillard, 2006; Sciolino et al., 2011), and antiemetic (Parker et al., 2009; Sticht et al., 2012) actions.

Given that endocannabinoids are produced selectively at their intended sites of action, signaling should occur through cannabinoid receptors via synthesis in a regionally specific

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ABBREVIATIONS: AEA/anandamide, *N*-arachidonylethanolamine; 2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variance; CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; CP55,940, 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl) phenol; FAAH, fatty acid amide hydrolase; [³⁵S]GTP γ S, guanosine 5'-O-(3-[³⁵S]thio)triphosphate; JZL184, 4-nitrophenyl 4-(dibenzo[*d*] [1,3]dioxol-5-yl (hydroxy)methyl)piperidine-1-carboxylate; JZL195, (4-nitrophenyl) 4-[(3-phenoxypheyl)methyl]piperazine-1-carboxylate; MAGL, monoacylglycerol lipase; KML29, 1,1,1,3,3,3-hexafluoropropan-2-yl-4-(bis(benzo[*d*] [1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate; PF-3845, *N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide; THC, Δ^9 -tetrahydrocannabinol; WIN55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-*n*-naphthalenylmethanone mesylate.

manner, which presumably would yield beneficial outcomes with minimal consequences of excessive activation elsewhere. Consistent with this premise, FAAH (Solinas et al., 2007) and MAGL (Long et al., 2009b,c) inhibitors produce a reduced cannabimimetic profile compared with direct-acting CB₁ receptor agonists. However, FAAH and MAGL inhibitors lack full efficacy in preclinical models of acute (Kathuria et al., 2003; Long et al., 2009a,c), neuropathic (Kinsey et al., 2009, 2010), and inflammatory (Alhouayek et al., 2011; Ghosh et al., 2013; Anderson et al., 2014) pain, though they appear fully efficacious in a rat cisplatin-induced peripheral neuropathy assay (Guindon et al., 2011). Conversely, simultaneous blockade of these enzymes produces enhanced antinociceptive efficacy (Long et al., 2009c; Anderson et al., 2014), though at the expense of evoking other CB₁ receptor-mediated effects, including catalepsy, generalization to the discriminative cue of THC in the drug discrimination paradigm, and impaired performance in a repeated-acquisition Morris water maze task (Long et al., 2009c; Wise et al., 2012). Thus, prevention of both AEA and 2-AG hydrolysis within the brain allows for enhanced CB₁ receptor signaling that impacts a variety of behavioral processes.

Prolonged inactivation of FAAH and MAGL yields profoundly distinct consequences for CB₁ receptor function. FAAH(−/−) mice or wild-type mice treated repeatedly with FAAH inhibitors (Schlosburg et al., 2010; Ghosh et al., 2013), such as PF-3845 (*N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxylphenyl]methyl]-1-piperidinecarboxamide), generally display normal CB₁ receptor function, with no signs of cross-tolerance to cannabinoid receptor agonists. Moreover, repeated dosing of high-dose AEA to FAAH(−/−) mice does not reduce CB₁ expression or function (Falenski et al., 2010). In marked contrast, repeated administration of high doses of the MAGL inhibitor JZL184 [4-nitrophenyl 4-(dibenzo[*d*][1,3]dioxol-5-yl (hydroxy)methyl)piperidine-1-carboxylate] leads to tolerance to its antinociceptive effects, cross-tolerance to the pharmacological effects of THC, and reductions in both CB₁ receptor expression and function (Schlosburg et al., 2010; Ghosh et al., 2013). Likewise, MAGL(−/−) mice exhibit a phenotypic loss of CB₁ receptor expression and function (Chanda et al., 2010; Schlosburg et al., 2010; Taschler et al., 2011). Furthermore, repeated high-dose JZL184, but not repeated administration of high-dose PF-3845, leads to physical dependence, evidenced by precipitated somatic withdrawal (Schlosburg et al., 2010). However, it should be noted that repeated low-dose administration of JZL184 continues to elicit anxiolytic-like activity in rats (Busquets-Garcia et al., 2011; Sciolino et al., 2011), as well as sustained antinociceptive effects and normal CB₁ receptor expression in mice (Kinsey et al., 2013).

The objective of the present study was to investigate the consequences of acute and prolonged inhibition of FAAH and MAGL. The observations that AEA behaves as a partial CB₁ receptor agonist (Mackie et al., 1993) with limited capacity to induce CB₁ receptor adaptations *in vivo* (Falenski et al., 2010) and that 2-AG acts as a full agonist (Sugiura et al., 1999) capable of downregulating CB₁ receptors *in vivo* raise the intriguing possibility that their combined blockade over time would result in less CB₁ receptor functional tolerance than prolonged blockade of MAGL alone. At least one previous study suggests that synaptic AEA can inhibit the actions and synthesis of local 2-AG (Maccarrone et al., 2008). Accordingly, the behavioral effects caused by single or dual FAAH-MAGL

inhibition were assessed. Additionally, brain levels of AEA and 2-AG were quantified to compare the impact of single-enzyme inhibition to combined FAAH and MAGL inhibition. Finally, cross-tolerance to THC, physical dependence, and CP55,940 (2-[[1*R*,2*R*,5*R*]-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol)-stimulated guanosine 5'-*O*-(3-[³⁵S]thio)triphosphate ([³⁵S]GTPγS) binding were also evaluated to assess CB₁ receptor function.

Materials and Methods

Animals. Subjects consisted of male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) that were approximately 8 weeks of age at the beginning of the study. In addition, male FAAH(−/−) and age-matched FAAH(+ / +) mice from the Center Transgenic Colony at Virginia Commonwealth University (Richmond, VA), backcrossed onto a C57BL/6J background (13 generations), were used. Mice were housed in a temperature- (20–22°C) and humidity-controlled, Association for Assessment and Accreditation of Laboratory Animal Care–approved facility, with *ad libitum* access to food and water. Subjects weighed approximately 25 g, were housed four to six mice per cage, and were maintained on a 12-hour light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Mice were temporarily individually housed 2 days prior to repeated injections and through all behavioral testing.

Drugs. JZL184 and JZL195 [(4-nitrophenyl) 4-[(3-phenoxyphenyl) methyl]piperazine-1-carboxylate] (Long et al., 2009a,c) as well as the selective FAAH inhibitor PF-3845 (Ahn et al., 2009) were synthesized as previously described. Rimobabant and THC were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). AEA was provided by Organix, Inc. (Woburn, MA). Deuterated standards for AEA and 2-AG were purchased from Cayman Chemical (Ann Arbor, MI). CP55,940, GDP, and unlabeled GTPγS were purchased from Sigma Chemical Co. (St. Louis, MO). [³⁵S]GTPγS was purchased from PerkinElmer (Waltham, MA).

Drug Administration. Drugs were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline in a ratio of 1:1:18, sonicated as necessary, and injected intraperitoneally in a volume of 10 μl/g body mass. Mice were assigned to the following groups: vehicle control (vehicle daily for 6 days), acute JZL184 [vehicle for 5 days and JZL184 (40 mg/kg *i.p.*) on day 6], repeated JZL184 (40 mg/kg *i.p.* for 6 days), repeated JZL195 (20 mg/kg twice-daily injections for 6 days), and repeated PF-3845 (10 mg/kg for 6 days). In an additional experiment, FAAH(−/−) mice were given JZL184 (40 mg/kg *i.p.*) or vehicle once a day and AEA (50 mg/kg *s.c.*) or vehicle twice daily for 6 consecutive days.

Behavioral Assessment. Initial studies examined the pharmacological effects of acute and repeated JZL184 in FAAH(−/−) and (+ / +) mice. Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75-cm diameter) that was elevated 4.5 cm above the surface of the bench top (Kinsey et al., 2013). Mice were scored as cataleptic if they remained motionless (with the exception of respiratory movements) with their forepaws on the bar during the 60-second test. Mice that showed a sudden pushing off the bar or biting/gnawing the bar during placement were recorded as hyper-reflexive.

Antinociceptive effects were assessed in the tail immersion test, in which each mouse was placed head first into a small bag fabricated from absorbent underpads (4-cm diameter, 11-cm length) (VWR Scientific Products, Radnor, PA) with the tail out of the bag (Kinsey et al., 2013). The experimenter gently held the mouse and immersed approximately 1 cm of the tip of the tail into a water bath maintained

at 52.0°C. The latency for the animal to withdraw its tail from the water within a 10-second cutoff time was scored.

Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum, and temperature was obtained from a BAT-10 telethermometer (Physitemp Instruments, Clifton, NJ). Mice previously given repeated injections of vehicle or JZL184 were then tested for cross-tolerance to THC the day following the final injection. A cumulative dosing regimen was used to investigate the dose-response relationship of THC in producing catalepsy, antinociception, and hypothermia (Falenski et al., 2010), which served to reduce the number of animals used and individual animal variability. Thirty minutes after each injection, mice were assessed for catalepsy, antinociception, and hypothermia and then immediately given the next injection. The entire procedure took less than 4 hours to complete.

To evaluate physical dependence, male C57BL/6J FAAH(+/+) or FAAH(-/-) mice were given two daily intraperitoneal injections (8:00 AM and 4:00 PM) of various endocannabinoid catabolic enzyme inhibitors for 5.5 days. At 1.5 hours following the final injection, mice were placed in observation chambers to habituate for 30 minutes. All animals were placed into white (for contrast) acrylic chambers (20 × 20 × 20 cm), with a clear acrylic front panel and a mirrored back panel, enclosed in sound-attenuating cabinets. At the 30-minute time point, the animals were briefly removed from the chambers, given an intraperitoneal injection of the CB₁ receptor antagonist rimonabant, and immediately returned to water-cleaned chambers for a 1-hour observation period. Behavior was recorded through the clear front panel using a series of Fire-i digital cameras (Unibrain, San Ramon, CA), and the videos were processed and saved using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL). The videos were subsequently placed in randomized order in a separate ANY-maze protocol for a trained observer to score somatic behaviors using a keyboard-based behavioral tracking system blinded to treatment group. Videos were scored using time sampling, examining every other 5-minute interval postrimonabant injection (i.e., 5–10 minutes, 15–20 minutes, etc.) and totaled for the 30 minutes of observation over the 1-hour recording. The primary behavior observed was front paw tremors that included a range of behavior from single-paw twitches to full fluttering or shaking of both paws simultaneously. Also recorded were head twitches, which generally manifested as rotational shakes of the head, similar to what is described as “wet dog shakes” in rats.

Extraction and Quantification of Endocannabinoids by Liquid Chromatography–Tandem Mass Spectrometry. Two experiments were conducted in which brain levels of endocannabinoids were quantified. In the first experiment, whole-brain levels of AEA and 2-AG were quantified in FAAH(-/-) and (+/+) mice following acute or repeated JZL184 administration. Two hours after acute or the final JZL184 injection, mice were decapitated, and brains were removed and snap-frozen on dry ice and then stored at -80°C until assay preparation. In the second experiment, male FAAH(-/-) mice were given two daily intraperitoneal injections (10:00 AM and 8:00 PM) of vehicle or AEA (50 mg/kg) for 5.5 days and one daily intraperitoneal injection (9:00 AM) of vehicle or JZL184 (40 mg/kg) for 6 consecutive days. Twenty-four hours following the final injection, the subjects were decapitated, and brains were removed, dissected in half along the midsagittal plane, snap-frozen on dry ice, and stored at -80°C. Half of each brain was used to quantify endocannabinoid levels, and the other half was used for CP55,940-stimulated [³⁵S]GTPγS binding (see below).

On the day of processing, tissues were weighed and homogenized with 1.4 ml of chloroform/methanol [2:1 (v/v), containing 0.0348 g of phenylmethylsulfonyl fluoride/ml] after the addition of internal standards to each sample (2 pmol of AEA-d8 and 1 nmol of 2-AG-d8) (Cayman Chemical). Homogenates were then mixed with 0.3 ml of 0.73% (w/v) NaCl, vortexed, and then centrifuged for 10 minutes at 3220g (4°C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled, and the organic solvents were

evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml of chloroform and mixed with 1 ml of ice-cold acetone. The mixtures were then centrifuged for 5 minutes at 1811g (4°C) to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml of methanol and placed in autosample vials for analysis.

Liquid chromatography–tandem mass spectrometry was used to quantify AEA and 2-AG. The mobile phase consisted of water/methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 2.1 × 150 mm, 4.6 × 15 cm, 3 μm (Supelco, Bellefonte, PA). The mass spectrometer was run in electrospray ionization in positive mode. Ions were analyzed in a multiple-reaction-monitoring mode, and the following transitions were monitored: (348 → 62) and (348 → 91) for AEA; (356 → 62) for AEA-d8; (379 → 287) and (279 → 269) for 2-AG; and (387 → 96) for 2-AG-d8. A calibration curve was constructed for each assay based on linear regression with use of the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.039 to 64 nmol for 2-AG for whole brain.

CP55,940-Stimulated [³⁵S]GTPγS Binding. Brains stored at -80°C were homogenized with an ULTRA-TURRAX T25 (IKA Works Inc., Wilmington, NC) in cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA; pH 7.4) and then centrifuged at 48,000g for 10 minutes at 4°C. Pellets were resuspended in membrane buffer and centrifuged again at 48,000g for another 10 minutes at 4°C. Pellets from the second centrifugation were homogenized in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl; pH 7.7) to measure protein concentration and then incubated for 10 minutes at 30°C in 0.004 U/ml adenosine deaminase (240 U/mg protein) (Sigma Chemical Co). The assay was conducted at 30°C for 2 hours in assay buffer including 10 μg of membrane protein with 0.1% bovine serum albumin, 30 μM GDP, various concentrations of CP55,940 (10, 3, 1, 0.3, 0.1, and 0.03 μM), and 0.10 nM [³⁵S]GTPγS in a final volume of 0.5 ml. Nonspecific binding was determined in the absence of agonist and the presence of 20 μM unlabeled GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters (Brandel, Gaithersburg, MD), followed by three washes with cold Tris-HCl buffer (pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 50% efficiency for ³⁵S after 1-hour shaking of the filters in 4 ml of Budget Solve scintillation fluid (RPI Corp., Mount Prospect, IL).

Data Analysis and Statistics. All results are expressed as mean ± S.E.M. Results were considered to be significant at $P < 0.05$. All endocannabinoid brain concentrations and behavioral endpoints were evaluated by two-way analysis of variance (ANOVA) (genotype and drug treatment) or three-way ANOVA (genotype, drug treatment, and time course). When appropriate, dose-response and JZL184 time course data were further assessed using Dunnett's test, and the Tukey-Kramer post hoc test was used in other experiments. CP55,940 concentration-effect curves for stimulation of [³⁵S]GTPγS binding were performed in triplicate and analyzed by nonlinear regression using GraphPad Prism software (GraphPad Software, La Jolla, CA). The resulting $E_{\max}$ and EC_{50} values were compared by one-way ANOVA with post hoc Dunnett's or Newman-Keuls test to determine significant differences between groups.

Results

The pharmacological effects of acute and repeated administration of JZL184 in FAAH(+/+) and (-/-) mice are shown in Fig. 1. Three-way ANOVA revealed a significant time by treatment by genotype interaction [$F(8,156) = 4.0, P < 0.001$]. Acute administration of JZL184 (40 mg/kg) produced significant increases in tail withdrawal latency in wild-type mice (Fig. 1, top left) and further enhanced the antinociceptive effects in FAAH(-/-) mice (Fig. 1, bottom left). The antinociceptive

Fig. 1. Timelines of behaviors elicited by high-dose JZL184 (40 mg/kg) in wild-type mice (top row) and FAAH(−/−) mice (bottom row). Simultaneous FAAH and MAGL blockade produces enhanced effects that undergo tolerance following repeated JZL184 administration. JZL184 increases tail withdrawal latency (left column) compared with vehicle, an effect whose magnitude is enhanced and prolonged in FAAH(−/−) mice. Similar enhanced effects are seen in FAAH(−/−) mice in hyper-reflexive responses (middle column) and biting responses (right column) when undergoing catalepsy testing in the bar test. All behavioral responses undergo significant tolerance following repeated administration of JZL184 for 6 days. ** $P < 0.01$; *** $P < 0.001$ versus all other treatment groups; $n = 8$ per group.

effects of JZL184 underwent complete tolerance following repeated dosing, regardless of FAAH inactivation genotype.

During the bar test, the occurrence of hyper-reflexive behavior and biting or gnawing of the bar interfered with the assessment of catalepsy. Acute administration of JZL184 elicited hyper-reflexia that followed the antinociceptive time course of this drug. A greater percentage of FAAH(−/−) mice presented with hyper-reflexive behavior than FAAH(+/+) mice [genotype: $F(1,39) = 6.9$, $P < 0.01$; Fig. 1, middle column]. However, this response underwent tolerance following repeated dosing of JZL184 in both genotypes. The hyper-reflexia waned by 4 hours [time by treatment interaction: $F(3,117) = 10.2$, $P < 0.001$]. JZL184 also elicited biting behavior exclusively in FAAH(−/−) mice, with a similar time course as antinociception and hyper-reflexia [time by treatment by genotype interaction: $F(6,117) = 2.5$, $P < 0.05$]. As with the other behavioral measures, repeated JZL184 treatment resulted in nearly complete tolerance of biting responses, regardless of genotype (Fig. 1, right column). In contrast, JZL184 did not produce relevant hypothermic responses (data not shown).

FAAH(+/+) and (−/−) mice were treated repeatedly with vehicle or JZL184 and at 24 hours after the final injection were assessed for cross-tolerance to the antinociceptive, hypothermic, and cataleptic effects of THC. FAAH(−/−) and (+/+) mice treated with vehicle showed typical THC dose-response relationships for each measure. In the tail immersion test (Fig. 2, left column), both genotypes treated repeatedly with JZL184 displayed tolerance to the antinociceptive effects of THC [THC dose by treatment interaction: $F(6,168) = 47.7$, $P < 0.001$]. Similarly, cross-tolerance was observed for THC-induced hypothermia (Fig. 2, middle column), with repeated

JZL184 groups showing minimal loss in body temperature following THC, regardless of genotype [THC dose by treatment interaction: $F(6,168) = 62.3$, $P < 0.001$]. However, neither genotype displayed cross-tolerance to the cataleptic effects of THC (Fig. 2, right column). All groups showed similar dose-responsive immobility in the bar test [main effect of THC dose: $F(6,168) = 26.6$, $P < 0.001$].

The data set depicted in Fig. 3A shows the effects of rimonabant challenge in C57BL/6J mice treated repeatedly with vehicle, PF-3845 (10 mg/kg), JZL184 (40 mg/kg), the combination of PF-3845 and JZL184, or the dual FAAH-MAGL inhibitor JZL195 (20 mg/kg). Rimobant did not elicit cannabinoid withdrawal behavior in mice treated repeatedly with vehicle or PF-3845. However, rimobant did precipitate significant increases in paw tremors in mice treated repeatedly with JZL184, with or without PF-3845, and mice treated repeatedly with the dual FAAH-MAGL inhibitor JZL195 [treatment main effect: $F(4,41) = 9.8$, $P < 0.001$; Fig. 3A]. Rimobant elicited a significant increase in head-twitching behavior in the JZL195 treatment group only [treatment main effect: $F(4,41) = 7.9$, $P < 0.001$; Fig. 3B]. Rimobant precipitated significant increases in paw-fluttering behavior in FAAH(+/+) and (−/−) mice treated repeatedly with JZL184, irrespective of genotype [treatment main effect: $F(1,69) = 45.0$, $P < 0.001$; Fig. 3C]. No significant difference in the magnitude of withdrawal was observed based on FAAH genotype (treatment by genotype interaction: $P = 0.36$). In addition, no significant effect of head twitching was elicited based on either treatment or genotype (Fig. 3D).

Brain levels of AEA and 2-AG were measured following acute and repeated treatment of JZL184 in FAAH(+/+) and (−/−) mice. AEA was significantly elevated in bulk brain

Fig. 2. Cross-tolerance to the antinociceptive and hypothermic effects of THC following repeated JZL184 administration is independent of FAAH activity. Mice treated with JZL184 (40 mg/kg) for 6 days no longer show antinociceptive effects in the tail withdrawal test (left column) or decreases in body temperature (middle column) following increasing doses of THC. No cross-tolerance was seen to THC-induced catalepsy (right column). For each behavioral endpoint, FAAH(+/+) mice (top row) and FAAH(-/-) mice (bottom row) displayed a similar pattern of responses to THC. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus vehicle controls; $n = 8$ per group.

tissue from FAAH(-/-) mice, regardless of JZL184 treatment [genotype: $F(1,29) = 102.6$, $P < 0.001$], with roughly 20-fold elevations above that of respective FAAH(+/+) groups (Fig. 4A). In the FAAH(+/+) mice, repeated JZL184 produced approximately a doubling of AEA content, significantly elevated from vehicle or single JZL184 treatment [treatment: $F(2,14) = 28.7$,

$P < 0.001$]. Acute JZL184 produced roughly 10-fold increases in 2-AG levels in brain, while repeated JZL184 treatment resulted in 20-fold accumulation of 2-AG [treatment: $F(2,29) = 132.1$, $P < 0.001$; Fig. 4B]. FAAH genotype had no significant influence on the levels of 2-AG following vehicle or JZL184 administration [genotype: $P = 0.25$].

Fig. 3. Rimonabant precipitated cannabinoid withdrawal responses following prolonged MAGL inhibition, regardless of FAAH blockade. (A) Rimonabant precipitated paw-fluttering behavior in mice injected repeatedly with high-dose JZL184 (40 mg/kg), with or without repeated treatment with high doses of the FAAH inhibitor PF-3845 (10 mg/kg) or the dual inhibitor JZL195 (20 mg/kg). (B) Rimonabant precipitated head shakes in mice treated repeatedly with JZL195 only. (C and D) Repeated JZL184 but not repeated vehicle produced similar precipitated paw-fluttering response in FAAH(+/+) and (-/-) mice, but did not significantly affect head-shaking behavior in either genotype. ** $P < 0.01$; *** $P < 0.001$ versus vehicle controls; $n = 8-12$ per group.

Fig. 4. Effects of acute and repeated administration of JZL184 in FAAH(+/+) and (-/-) mice on whole-brain AEA (A) and 2-AG (B) levels. FAAH(-/-) mice had AEA levels that were approximately 10-fold higher than those of wild-type mice, while repeated JZL184 also elevated AEA 2-fold in FAAH(+/+) mice. JZL184 enhanced the brain content of 2-AG in bulk tissue samples about 8- to 10-fold above vehicle-treated mice, and the magnitude of this accumulation was further enhanced by repeated JZL184 administration for 6 days. *** $P < 0.001$ versus vehicle controls; ††† $P < 0.001$ versus respective FAAH(+/+) group; # $P < 0.05$ versus respective single JZL184 group; $n = 8$ per group.

Because AEA is a partial CB₁ receptor agonist and 2-AG is a full CB₁ receptor agonist (Hillard, 2000), the final experiment tested whether exogenously administered AEA would prevent CB₁ receptor desensitization caused by repeated administration of high-dose JZL184 (40 mg/kg). Repeated JZL184 caused a significant decrease in the $E_{\max}$ value of CP55,940-stimulated [³⁵S]GTPγS binding [treatment: $F(4,31) = 12.6$, $P < 0.001$; Fig. 5A]. While AEA-treated FAAH(-/-) mice exhibited similar CB₁ receptor activation as FAAH(-/-) mice treated with vehicle or FAAH(+/+) control mice, FAAH(-/-) mice coadministered JZL184 and AEA displayed a further decrease in the CP55,940 $E_{\max}$ value compared with JZL184 treatment alone. No significant differences in CP55,940 EC_{50} values were obtained between groups (not shown). Thus, repeated JZL184 administration decreased maximal CB₁ receptor-mediated G protein activation, and repeated injections of both JZL184 and exogenous AEA further reduced CB₁ receptor activity in FAAH(-/-) mice relative to repeated injections of JZL184 alone.

Repeated administration of JZL184 significantly elevated 2-AG [$F(1,24) = 1088$, $P < 0.001$; Fig. 5B], but did not alter AEA levels [$P = 0.54$; Fig. 5C], in whole brain of FAAH(-/-) mice. Repeated administration of AEA [$F(1,24) = 12.8$, $P = 0.002$], but not JZL184 [$P = 1$], significantly elevated AEA levels in FAAH(-/-) mice [Fig. 5C]. There was no interaction between AEA and JZL184 on 2-AG [$P = 0.35$] or AEA [$P = 0.74$] levels in the brain. FAAH(-/-) mice treated with vehicle showed elevated AEA [$T(13) = 7.9$, $P < 0.001$] but not 2-AG [$P = 0.11$] levels as compared with wild-type mice administered vehicle. However, exogenous AEA administration resulted in an approximately 20-fold increase in brain AEA levels 24 hours after the last administration.

Discussion

As previously reported (Long et al., 2009c; Anderson et al., 2014), complete blockade of both endocannabinoid catabolic enzymes FAAH and MAGL produced enhanced antinociceptive effects that were accompanied with increased cannabinimetic activity. The pharmacological effects of high doses of JZL184 in wild-type mice, as well as the augmented effects of this compound in FAAH(-/-) mice, underwent tolerance following 6 days of daily dosing. Repeated high-dose JZL184 administration also led to cross-tolerance to the antinociceptive and

hypothermic effects of THC that occurred irrespective of FAAH genotype. Likewise, prolonged JZL184 administration produced mild dependence, as indicated by a significant increase in rimonabant-precipitated paw fluttering, previously demonstrated to be roughly equivalent to a mild daily dosing (10 mg/kg) regimen of THC (Schlosburg et al., 2010). This effect was neither abated nor enhanced based on FAAH genotype. Increased AEA content in FAAH(-/-) mice, including substantial exogenously administered AEA, only increases CB₁ receptor desensitization produced by repeated JZL184.

These data continue the line of findings that enhancing both endogenous cannabinoids simultaneously produces a full array of cannabinimetic effects. Simultaneous blockade of FAAH and MAGL produces enhanced antinociceptive effects, but also THC-like subjective effects in the drug discrimination paradigm (Long et al., 2009c; Ignatowska-Jankowska et al., 2014), as well as impaired performance in a repeated-acquisition Morris water maze task (Wise et al., 2012). Here, we show that the pharmacological effects of JZL184 in wild-type mice and its augmented effects in FAAH(-/-) mice undergo a similar magnitude of tolerance and dependence following repeated drug administration. Similarly, FAAH(-/-) or (+/+) mice treated with repeated JZL184 display cross-tolerance to the antinociceptive and hypothermic but not the cataleptic effects of THC. This is consistent with the heterogeneous desensitization of CB₁ receptors in repeatedly JZL184-treated brains, in which reductions in agonist-stimulated [³⁵S]GTPγS binding were found in brain regions associated with pain (i.e., periaqueductal gray and cingulate cortex) but not motor function (i.e., caudate putamen, globus pallidus, substantia nigra, and cerebellum) (Schlosburg et al., 2010). Also in agreement with previous reports, FAAH(-/-) mice showed identical pharmacological effects to THC as their wild-type counterparts (Cravatt et al., 2001; Falenski et al., 2010). Also of interest is the seemingly complete independent regulation of bulk endocannabinoid content within the brain. 2-AG levels were not impacted by FAAH inactivation or repeated injections of AEA to FAAH(-/-) mice, suggesting independent regulation of these two major brain endocannabinoids.

Taken together, the results of the present study indicate that prolonged elevations in 2-AG are capable of CB₁ receptor functional tolerance, regardless of FAAH genotype, though a role for other substrates of JZL184 cannot be ruled out. Off-target effects of JZL184 (e.g., FAAH and ABHD6) are observed

Fig. 5. Effects of repeated JZL184 (40 mg/kg i.p.) and AEA (50 mg/kg s.c. twice daily) on CB₁ receptor function (A) and endogenous cannabinoid levels (B and C) in whole-brain of FAAH(-/-) mice. JZL184 elicited a significant decrease in E_{max} , and coadministration of AEA and JZL184 further reduced CP55,940-stimulated [³⁵S]GTPγS binding. Repeated JZL184 (40 mg/kg i.p.) and AEA (50 mg/kg s.c. twice daily) administration elevated 2-AG and AEA levels in the whole brain of FAAH(-/-) mice. FAAH(-/-) mice showed significantly increased AEA levels compared with wild-type mice treated with vehicle. Data presented as mean ± S.E.M.; $n = 7$ mice per group (each sample in triplicate). (A) * $P < 0.05$; ** $P < 0.01$ versus vehicle; # $P < 0.05$ versus JZL184 alone. (B and C) ** $P < 0.01$; *** $P < 0.001$ versus vehicle; ## $P < 0.01$; ### $P < 0.001$ versus AEA or JZL184 alone; \$\$\$ $P < 0.001$ versus wild-type treated with vehicle.

using a similar repeated-dosing paradigm (Chang et al., 2012), which might contribute to the effects observed here. While JZL184's effect on FAAH is apparent following repeated treatment, especially in the form of moderately enhanced AEA in brain (Fig. 4A), there is evidence that ultimately MAGL inhibition and 2-AG elevations are the common underlying means for CB₁ receptor adaptations. Prolonged enhancement of AEA brain content in FAAH-disrupted mice by itself does not lead to CB₁ receptor tolerance (current paper; Falenski et al., 2010). Also, repeated administration of the far more MAGL-selective

inhibitor KML29 [1,1,1,3,3,3-hexafluoropropan-2-yl-4-(bis(benzo[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate] shows tolerance in numerous pain models, while not showing any increases in AEA (Ignatowska-Jankowska et al., 2014). Finally, profound CB₁ receptor adaptations are observed in MAGL(-/-) mice, with no compensatory alterations in FAAH or ABHD6 activity observed (Chanda et al., 2010; Schlosburg et al., 2010; Pan et al., 2011). In both previous cases, MAGL was selectively inactivated and AEA levels were unaffected. This evidence suggests that MAGL inhibition is sufficient to produce the examined CB₁ receptor adaptations, in which the current studies suggest that concurrent elevation of AEA provides no protection from, nor diminishment of, MAGL adaptations. Whether MAGL is necessary for endocannabinoid dependence and downregulation remains to be seen as studies of knockout models and drugs selective for the other major 2-AG-hydrolyzing enzymes become available (Marrs et al., 2010; Blankman et al., 2013; Hsu et al., 2013).

While the mechanism underlying differential CB₁ receptor desensitization by repeated elevation of 2-AG or AEA concentration is not entirely clear, the fact that 2-AG and AEA interact at the receptor in distinct fashions is likely important. Specifically, AEA behaves as a partial CB₁ receptor agonist (Mackie et al., 1993), and prolonged high levels of AEA provoke no or minimal CB₁ receptor downregulation/desensitization (Falenski et al., 2010). In contrast, 2-AG behaves as a full CB₁ receptor agonist (Sugiura et al., 1999). Differences in efficacy likely account for AEA's attenuation of 2-AG functional activity in human CB₂-transfected Chinese hamster ovary cells (Gonsiorek et al., 2000). Accordingly, we tested whether elevated brain AEA levels caused by FAAH deletion or exogenous AEA administration to FAAH(-/-) mice might protect the CB₁ receptor from functional tolerance following prolonged MAGL inhibition. However, elevating AEA levels did not prevent functional tolerance caused by repeated high-dose JZL184 administration. In fact, the addition of high-dose exogenous AEA in FAAH(-/-) mice enhanced CB₁ receptor desensitization caused by repeated JZL184. These results parallel in vitro findings in isolated cerebellar membranes, in which AEA did not antagonize 2-AG-stimulated G protein activity (Savinainen et al., 2001).

Though the current studies focused specifically on CB₁ receptor function, both AEA and 2-AG elevations may interact through alternative signaling pathways. For example, MAGL(-/-) mice display no apparent decreases in CB₂ receptor binding (Chanda et al., 2010), and numerous examples demonstrate CB₂-mediated antinociceptive effects, particularly in peripheral inflammatory models (Khasabova et al., 2011; Anderson et al., 2014; Ignatowska-Jankowska et al., 2014). Moreover, not only is AEA known to affect nociceptive behavior via transient receptor potential vanilloid-1-mediated mechanisms (Dinis et al., 2004; Maione et al., 2006; Horvath et al., 2008; Guindon et al., 2013), but this endocannabinoid also has been suggested to alter local 2-AG production via transient receptor potential vanilloid-1 receptors (Maccarrone et al., 2008). Though alternative signaling pathways may contribute to overall therapeutic efficacy, for behaviors attributed to CB₁ receptor activation, MAGL inhibition adaptations are reversible by cotreatment with the CB₁ receptor antagonist rimonabant (Schlosburg et al., 2010; Alhouayek et al., 2011; Wise et al., 2012).

Two distinctions emerge from the receptor adaptations observed following functional tolerance produced via either

2-AG or AEA: localization and downregulation. Repeated exogenous AEA given to FAAH(−/−) mice produces cannabinoid tolerance comparable to that of THC, yet with only minimal physical signs of withdrawal and desensitization of receptors in only a few distinct regions (e.g., caudate, cerebellum). Furthermore, exogenous AEA failed to elicit significant loss of cannabinoid receptor-binding pools in these same regions (Rubino et al., 2000; Falenski et al., 2010). Conversely, the magnitude of CB₁ receptor desensitization following repeated administration of JZL184 closely parallels the degree of CB₁ receptor downregulation (Schlosburg et al., 2010), with regions such as caudate and cerebellum notably not altered following repeated JZL184. This bias toward downregulation of receptors is shared with the partial agonist THC and full agonist WIN55,212-2 (*R*-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-*n*-naphthalenylmethanone mesylate) (Sim-Selley and Martin, 2002), suggesting that receptor efficacy alone does not account for the different consequences between prolonged FAAH and MAGL inhibition on CB₁ receptor functional tolerance. One suggested intrinsic property that could be a predictor of ligand-directed adaptations is the rate of CB₁ receptor internalization and recycling; however, in these tests AEA and THC showed equivalently slow desensitization (Luk et al., 2004; Wu et al., 2008). Membrane receptor interactions with intracellular regulators responsible for trafficking, along with new understanding of how ligands can produce biased intracellular signaling, will become important factors to study. The ultimate goal is to determine the crucial differences in why chronic elevation of the two major endocannabinoids produces partial cannabimimetic effects but leads to highly divergent long-term consequences in the overall function of the endocannabinoid system.

Our study indicates that partial rather than full blockade of MAGL may be a preferred strategy for achieving beneficial CB₁ receptor activation while avoiding functional antagonism. While CB₁ receptor function is preserved following repeated low doses of JZL184, generating tempered elevations in brain 2-AG (Kinsey et al., 2013), it remains to be determined whether CB₁ receptor function would also be retained following partial inhibition of MAGL in combination with a FAAH inhibitor for several days. However, the strategy of using drugs that produce complete FAAH inhibition with partial MAGL inhibition alleviated withdrawal signs in morphine-dependent mice, without untoward cannabimimetic effects of loss of CB₁ receptor function following repeated administration (Ramesh et al., 2013).

Author Contributions

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Conducted experiments: Schlosburg, Kinsey, Ignatowska-Jankowska, Ramesh, Abdullah, Tao, Booker, Long.

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Wrote or contributed to the writing of the manuscript: Schlosburg, Kinsey, Ignatowska-Jankowska, Selley, Cravatt, Lichtman.

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