1521-0103/353/2/261–268$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.115.222836
J Pharmacol Exp Ther 353:261–268, May 2015

Simultaneous Inhibition of Fatty Acid Amide Hydrolase and Monoacylglycerol Lipase Shares Discriminative Stimulus Effects with Δ^9-Tetrahydrocannabinol in Mice

Lenka Hruba, Alexandre Seillier, Armia Zaki, Benjamin F. Cravatt, Aron H. Lichtman, Andrea Giuffrida, and Lance R. McMahon

Department of Pharmacology, University of Texas Health Science Center, San Antonio, Texas (L.H., A.S., A.Z., A.G., L.R.M.); Department of Chemical Physiology, Skaggs Institute for Chemical Biology, Scripps Research Institute, La Jolla, California (B.F.C.); and Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia (A.H.L.)

Received January 9, 2015; accepted February 23, 2015

ABSTRACT

Monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) inhibitors exert preclinical effects indicative of therapeutic potential (i.e., analgesia). However, the extent to which MAGL and FAAH inhibitors produce unwanted effects remains unclear. Here, FAAH and MAGL inhibition was examined separately and together in a Δ^9-tetrahydrocannabinol (Δ^9-THC; 5.6 mg/kg i.p.) discrimination assay predictive of subjective effects associated with cannabis use, and the relative contribution of *N*-arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) in the prefrontal cortex, hippocampus, and caudate putamen to those effects was examined. Δ^9-THC dose-dependently increased Δ^9-THC appropriate responses (ED_{50} value = 2.8 mg/kg), whereas the FAAH inhibitors PF-3845 [*N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide] and URB597 [(3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate] or a MAGL inhibitor JZL184 [4-nitrophenyl-4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate] alone did not substitute for the Δ^9-THC discriminative stimulus. The nonselective FAAH/MAGL inhibitors SA-57 [4-[2-(4-chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester] and JZL195 [4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate] fully substituted for Δ^9-THC with ED_{50} values equal to 2.4 and 17 mg/kg, respectively. Full substitution for Δ^9-THC was also produced by a combination of JZL184 and PF-3845, but not by a combination of JZL184 and URB597 (i.e., 52% maximum). Cannabinoid receptor type 1 antagonist rimonabant attenuated the discriminative stimulus effects of Δ^9-THC, SA-57, JZL195, and the combined effects of JZL184 and PF-3845. Full substitution for the Δ^9-THC discriminative stimulus occurred only when both 2-AG and AEA were significantly elevated, and the patterns of increased endocannabinoid content were similar among brain regions. Overall, these results suggest that increasing both endogenous 2-AG and AEA produces qualitatively unique effects (i.e., the subjective effects of cannabis) that are not obtained from increasing either 2-AG or AEA separately.

Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is a cannabinoid receptor type 1 (CB_1)/cannabinoid receptor type 2 (CB_2) agonist that produces therapeutic effects such as analgesia and antiemersis, as well as unwanted effects, such as reinforcing effects that drive repeated use (Hollister, 1986; Pertwee et al., 2010). The endogenous lipid neurotransmitters *N*-arachidonoyl ethanolamine (AEA; also known as anandamide; Devane et al., 1992) and 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995) are also CB_1/CB_2 receptor agonists. AEA levels are regulated in vivo by fatty acid amide hydrolase (FAAH; Cravatt et al., 1996), whereas 2-AG levels are regulated by monoacylglycerol lipase (MAGL; Dinh et al., 2002). Drug inhibitors of FAAH and MAGL that inhibit degradation of AEA and 2-AG, respectively, have made it possible to examine the contribution of endogenous cannabinoids to physiology and behavior. Moreover, there is great interest in the therapeutic potential of FAAH and MAGL inhibitors, in part, due to indirect stimulation of CB_1/CB_2 receptors via increases in AEA and 2-AG, which can only occur in the CB_1 receptor–expressing brain areas that contain these endogenous cannabinoids. By contrast, direct-acting CB_1 receptor agonists such as Δ^9-THC are expected to produce

This research was supported by the National Institutes of Health National Institute on Drug Abuse [Grants R01-DA19222, R01-DA26449, and R01-DA32933]; and a Toni Rosenberg Fellowship (to B.F.C.).
dx.doi.org/10.1124/jpet.115.222836.

ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; 95% CL, 95% confidence limit; AEA, *N*-arachidonoyl ethanolamine; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; Δ^9-THC, Δ-9-tetrahydrocannabinol; FAAH, fatty acid amide hydrolase; FR, fixed ratio; GC, gas chromatography; JZL184, 4-nitrophenyl-4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate; JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate; MAGL, monoacylglycerol lipase; MS, chemical ionization mass spectrometry; OEA, oleoylethanolamide; PF-3845, *N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide; SA-57, 4-[2-(4-chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester; URB597, (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate.

widespread stimulation of CB_1 receptors in the brain regardless of the presence of AEA and 2-AG.

FAAH and MAGL inhibitors produce in vivo effects that are similar though not always identical to those of Δ^9-THC. When assessed with a tetrad of in vivo effects sensitive to CB_1 receptor agonism (Martin et al., 1991), the MAGL inhibitor JZL184 [4-nitrophenyl-4-(dibenzo[*d*][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate] produced some (i.e., analgesic and locomotor-decreasing) but not all (i.e., catalepsy) of the tetrad (Kinsey et al., 2009, 2011; Sciolino et al., 2011). JZL184 also did not share effects with Δ^9-THC in a preclinical assay predictive of cannabis-like subjective effects in mice (i.e., drug discrimination; Long et al., 2009c). Moreover, the FAAH inhibitors PF-3845 [*N*-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide] and URB597 [(3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate] produced antinociceptive and anti-inflammatory effects, but failed to produce locomotor-decreasing, hypothermic, cataleptic, and Δ^9-THC–like discriminative stimulus effects (Lichtman et al., 2004; Solinas et al., 2007; Long et al., 2009c; Stewart and McMahon, 2011; Booker et al., 2012; Wiley et al., 2014). Collectively, these studies suggest that selective inhibition of either FAAH or MAGL is not sufficient to mimic all of the in vivo effects of Δ^9-THC including discriminative stimulus effects. However, the extent to which simultaneous inhibition of FAAH and MAGL mimics the discriminative stimulus and subjective effects of Δ^9-THC remains unclear. Whereas a dose of the nonselective FAAH and MAGL inhibitor JZL195 [4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate] substituted for a Δ^9-THC discriminative stimulus in mice (Long et al., 2009c), a combination of the FAAH inhibitor URB597 with the MAGL inhibitor JZL184 appeared to only partially substitute for Δ^9-THC (Wiley et al., 2014).

In this study, the effects of several different FAAH and MAGL inhibitors were studied in C57BL/6J mice discriminating 5.6 mg/kg Δ^9-THC i.p. (McMahon et al., 2008), and discriminative stimulus effects were related to changes in brain endocannabinoid content as assessed via gas chromatography (GC)/chemical ionization mass spectrometry (MS) (Hardison et al., 2006). The drugs included the selective FAAH inhibitors URB597 and PF-3845, the selective MAGL inhibitor JZL184, and the nonselective FAAH/MAGL inhibitors JZL195 and SA-57 [4-[2-(4-chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester]. JZL195 was chosen because it has been shown to substitute for Δ^9-THC in mice (Long et al., 2009c) and hence served as a positive control, whereas SA-57 is a relatively new compound that has been shown to share effects with Δ^9-THC in modifying intracranial self-stimulation and morphine withdrawal in mice (Ramesh et al., 2013; Wiebelhaus et al., 2015). Because nonselective FAAH or MAGL inhibitors appear to substitute for the discriminative stimulus effects of Δ^9-THC but selective inhibitors of either FAAH or MAGL do not, drug combinations were used to test the hypothesis that dual inhibition of both FAAH and MAGL produces qualitatively unique effects. In addition to combining URB597 and JZL184 as previously described (see Wiley et al., 2014), this study examined a second drug combination (i.e., PF-3845 with JZL184). The involvement of CB_1 receptors was examined with the CB_1 receptor antagonist rimonabant. Endogenous levels of AEA and 2-AG were measured in the medial prefrontal cortex, hippocampus, and caudate putamen, areas containing high levels of CB_1 receptor, FAAH, and MAGL (Di Marzo et al., 1998). Oleoylethanolamide (OEA), an endogenous lipid also degraded by FAAH, was quantified to provide a secondary measure of FAAH inhibition.

Materials and Methods

Subjects. Sixty-one male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) at 6 weeks of age at the beginning of the study were housed individually on a 14-hour/10-hour light/dark cycle. For the drug discrimination experiment, 16 mice received condensed milk during experimental sessions and 2.5 g food (500 mg Dustless Precision Pellets Grain-Based Rodent Diet; Bio-Serv, Frenchtown, NJ) per day after sessions. Forty-five mice used for brain dissection had continuous access to food (Teklad 7912; Harlan, Houston, TX) in the home cage. Water was continuously available in the home cage for all mice. Mice were maintained and experiments were conducted in accordance with the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee and the 2011 Guide for the Care and Use of Laboratory Animals.

Behavioral Apparatus. Drug discrimination experiments were conducted in mouse operant conditioning chambers (Med Associates, Inc., St. Albans, VT) that were housed within ventilated, sound-attenuating enclosures. The center of one wall of the operant conditioning chamber contained a light (i.e., house light) positioned above a hole 2.2 cm in diameter through which milk could be obtained. Condensed milk in a volume of 0.01 ml was available via a dipper that could be raised from a tray positioned outside the hole. On the opposite wall, there were three recessed holes (2.2-cm diameter) spaced 5.5 cm apart and each of these holes contained a photobeam and a light. The center of each hole was positioned 1.6 cm from the floor. An interface connected the operant conditioning chambers to a computer, and experimental events were controlled and recorded with Med-PC software (Med Associates, Inc.).

Discrimination Training. Experiments were run once per day 7 days per week at the same time each day during the light period. Vehicle (a 1:1:18 mixture of propylene glycol, Tween 80, and physiologic saline) was administered in the home cage, and 30 minutes later, mice were placed inside the operant conditioning chambers. During 30-minute sessions, the left and the right recessed holes were illuminated and mice could insert their noses to disrupt a photobeam. A single disruption of the photobeam (FR1) in either hole resulted in 10-second access to 0.01 ml condensed milk diluted with tap water to one-half of the original concentration, during which time the lights in each hole were turned off and the house light was illuminated. Disruptions of a photobeam during the 10-second period of milk availability had no programmed consequence. Training under continuous reinforcement continued until a minimum of 100 reinforcers was obtained in three consecutive sessions. Thereafter, the number of photobeam disruptions required to obtain a reinforcer (i.e., fixed ratio [FR]) was increased in the following increments: FR3, FR5, and FR10. To minimize odor traces between sessions, holes were wiped, and milk dippers and trays were rinsed with tap water.

Once responding was maintained at an FR10, mice received vehicle or Δ^9-THC (5.6 mg/kg i.p.) and were placed back in their cages for 30 minutes. A training dose of 5.6 mg/kg was chosen because 10 mg/kg Δ^9-THC produced marked decreases in response rate (i.e., less than 20% of control). Thirty minutes after injection, mice were placed in the operant chambers during a 30-minute response period. Lights in each hole were illuminated and mice could respond under the FR10 schedule of milk presentation described above except that responding in only one of the holes (i.e., correct hole) was reinforced depending on whether vehicle or Δ^9-THC was administered before the session. Determination of correct holes (e.g., left, vehicle; right, Δ^9-THC) varied among mice and remained the same for that mouse for the duration of the study. Vehicle training sessions alternated nonsystematically with Δ^9-THC training sessions; training with vehicle or Δ^9-THC was not repeated for more than 2

consecutive days. The first test was conducted when, for 5 consecutive days or for 6 of 7 days, at least 80% of the total responses occurred on the correct hole and fewer than 10 responses occurred in the incorrect hole before completion of the first FR on the correct hole.

Discrimination Testing. Test sessions were identical to training sessions except that 10 responses in either hole resulted in milk presentation and mice received vehicle or a dose of drug. After the first test, subsequent tests were conducted only after mice satisfied the above-specified criteria during three consecutive sessions, including at least one vehicle training session and one Δ^9-THC training session.

JZL184, JZL195, PF-3845, SA-57, URB597, and a combination of JZL184 plus PF-3845 and JZL184 plus URB597 were studied by administering dose(s) of drug(s) 2 hours (instead of 30 minutes) before the 30-minute test session, in accordance with previous studies showing in vivo effects after 2 hours (Fegley et al., 2005; Ahn et al., 2009; Kinsey et al., 2009; Long et al., 2009) and in accordance with the current analysis of AEA and 2-AG content (see *Results*). Rimonabant was studied in combination with other drugs by administering a dose 30 minutes before the test session. The order of testing among drugs was nonsystematic except that tests with different doses of Δ^9-THC were conducted at the beginning of the study and again with the same doses of Δ^9-THC at the end of the study. Tests with rimonabant in combination with other test drugs were conducted after establishing the individual dose-response functions for those other test drugs.

Biochemical Studies. Two hours after the intraperitoneal administration of vehicle, SA-57 (10 mg/kg), JZL195 (120 mg/kg), JZL184 (120 mg/kg), PF-3845 (10 mg/kg), URB597 (100 mg/kg), a combination of JZL184 (120 mg/kg) and PF-3845 (10 mg/kg), and a combination of JZL184 (120 mg/kg) and URB597 (100 mg/kg), animals were anesthetized with halothane and their brains rapidly collected, frozen in 2-methylbutane (−45°C), and stored at −80°C. Frozen brains were placed on a stainless steel mold (Roboz, Rockville, MD) kept at 17°C and sliced into 1-mm coronal sections using razor blades to dissect out the following brain areas: medial prefrontal cortex (prelimbic and infralimbic cortices), hippocampus, and caudate putamen. Tissue samples were spiked with 50 pmol [^{2}H4]AEA, [^{2}H4]OEA, and [^{2}H5]2-AG (internal standards) and processed as previously described (Hardison et al., 2006). Briefly, lipids were extracted by adding methanol/chloroform/water (1:2:1 [v/v/v]), and the chloroform layer was further purified by solid phase extraction using C18 Bond Elut cartridges (100 mg; Agilent Technologies, Inc., Santa Clara, CA). Endocannabinoid-containing fractions were analyzed by GC/MS, using an isotope dilution assay.

Drugs. The levo enantiomer of Δ^9-THC (500 mg/ml in absolute ethanol; National Institute on Drug Abuse Research Technology Branch, Rockville, MD) was prepared by evaporating the ethanol under a gentle stream of nitrogen. Δ^9-THC was redissolved in the vehicle consisting of a 1:1:18 mixture of propylene glycol (Sigma-Aldrich, St. Louis, MO), Tween 80 (Sigma-Aldrich), and physiologic saline (i.e., vehicle). SA-57 was synthesized in the laboratory of B.F.C. as previously described (Niphakis et al., 2012). Rimonabant base and URB597 were obtained from the National Institute on Drug Abuse Research Technology Branch. JZL195, JZL184, and PF-3845 were purchased from Cayman Chemical Company (Ann Arbor, MI). SA-57, JZL195, PF-3845, URB597, JZL184, and rimonabant were dissolved in the same vehicle used to dissolve Δ^9-THC. Drugs were administered intraperitoneally in a volume of 0.1 ml/kg at doses (milligrams per kilogram) expressed as the weight of the forms listed above.

Data Analysis. Discrimination data were expressed as an average ± S.E.M. percentage of Δ^9-THC appropriate responding (i.e., number of Δ^9-THC appropriate responses divided by the total responses made during the duration of the test) and were plotted as a function of dose. Δ^9-THC was studied in every mouse (n = 16). For each drug or drug combination, all doses were studied in the same cohort of 8–10 mice selected from among the 16 mice. For each mouse contributing to a particular drug treatment, the dose-effect function for that mouse was defined by one dose producing less than 20% Δ^9-THC appropriate responding and larger doses including at least one dose producing greater than 80% Δ^9-THC appropriate responding or decreasing rate of responding to less than 20% of the control response rate.

The dose-response functions for producing discriminative stimulus effects were analyzed with linear regression by simultaneously fitting straight lines to the individual dose-response data by means of GraphPad Prism software (GraphPad Software, Inc., San Diego, CA), using the following equation: effect = slope × log(dose) + intercept. Straight lines were fitted to the linear portion of dose-effect curves, defined by doses producing 20%–80% Δ^9-THC appropriate responding, including not more than one dose producing less than 20% Δ^9-THC appropriate responding and not more than one dose producing greater than 80% Δ^9-THC appropriate responding. Other doses were excluded from the analyses. The slopes of dose-effect curves were compared with an F-ratio test using GraphPad software. If the slopes were not significantly different, then a common, best-fitting slope was used for further analyses (for detailed examples of this approach, see Kenakin, 1997). Doses corresponding to the 50% level of the effect (ED_{50} values), potency ratios, and their 95% confidence limits (95% CLs) were calculated by parallel line analyses of data from individual subjects (Tallarida, 2000). The potencies of different inhibitors were considered significantly different when the 95% CLs of their potency ratio did not include 1.

Response rate was averaged (± S.E.M.) among mice and plotted as a function of dose. Response rate for an individual mouse was calculated by expressing the response rate during a test as a percentage of control; the control response rate was defined as the average response rate for the five preceding vehicle training sessions during which the criteria for testing were satisfied. The effects of a drug on response rate were analyzed with one-way repeated-measures analysis of variance including only one ineffective dose in the analysis; Dunnett's post hoc test was used to examine significant differences from control. Discrimination data were not included for analysis when the response rate for an individual mouse was less than 20% of the vehicle control rate for that mouse; however, all response rate data were included in the group average.

Data from the GC/MS analyses were analyzed by one-way analysis of variance separately for each brain area. The Newman–Keuls test was used for post hoc comparisons. The level of significance was set at $P < 0.05$.

Results

Effects of SA-57, JZL195, JZL184, PF-3845, and URB597 in Mice Discriminating Δ9-THC. Sixteen mice satisfied the criteria for testing after a median of 34 training sessions (range, 9–55). In mice discriminating Δ^9-THC (5.6 mg/kg i.p.), increasing doses of Δ^9-THC resulted in corresponding increases in Δ^9-THC appropriate responding (Fig. 1A). A dose of 1.78 mg/kg Δ^9-THC produced 9% of responses in the hole associated with the training dose of Δ^9-THC, whereas 3.2 and 5.6 mg/kg produced 49% and 95% drug-appropriate responding, respectively. Vehicle produced only 3% of Δ^9-THC appropriate responses. Up to 5.6 mg/kg, Δ^9-THC did not significantly modify response rate ($F_{3,45} = 0.70$; $P > 0.05$) (Fig. 1C).

Both nonselective FAAH/MAGL inhibitors SA-57 and JZL195 dose-dependently increased Δ^9-THC responding to a mean of 93% at a dose of 10 mg/kg and 92% at a dose of 120 mg/kg, respectively (Fig. 1A, diamonds and squares). Because the slopes of the three dose-response functions were significantly different from each other ($F_{2,77} = 11.12$; $P < 0.001$); that is, the slope of the Δ^9-THC dose-response function was greater (i.e., steeper) than the slopes of the SA-57 and JZL 195 dose-response functions, the individual slopes were used to estimate the ED_{50} values. The ED_{50} values were 2.8 mg/kg (95% CL, 2.4–3.2) for Δ^9-THC, 2.4 mg/kg (95% CL, 1.2–4.5) for SA-57, and

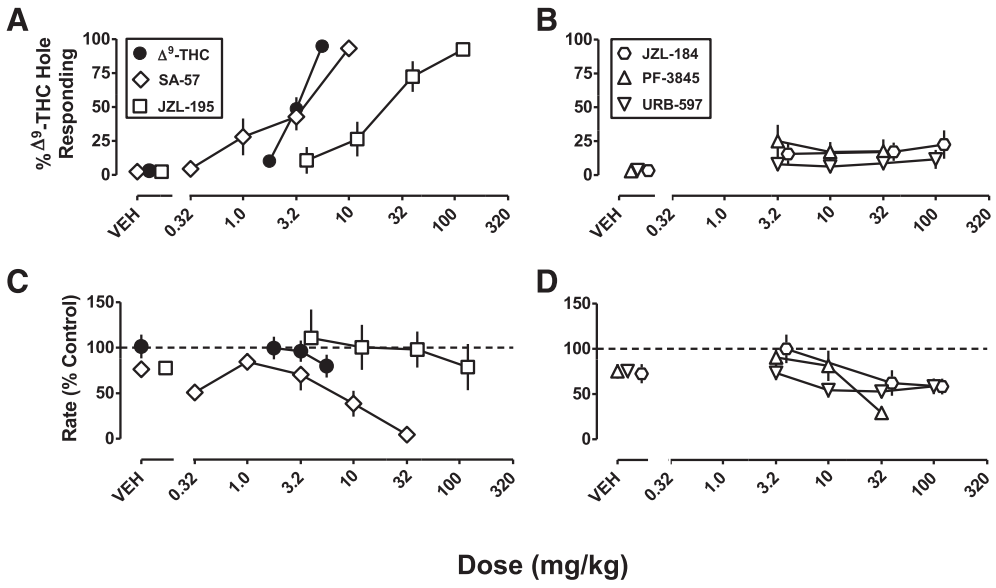

Fig. 1. Effects of the nonselective FAAH and MAGL inhibitors JZL195 and SA-57 (A and C), the FAAH inhibitors PF-3845 and URB597 (B and D), and the MAGL inhibitor JZL184 (B and D) in mice discriminating Δ^9-THC (5.6 mg/kg i.p.). Abscissae show vehicle (VEH) or dose in milligrams per kilogram of body weight. Ordinates show the mean ± S.E.M. percentage of responding on the Δ^9-THC lever (A and B) and the mean ± S.E.M response rate expressed as a percentage of the control rate (C and D).

17 mg/kg (95% CL, 9.0–32) for JZL195. SA-57 was studied up to a dose (32 mg/kg) that significantly decreased response rate to 5% of control (Fig. 1C), whereas JZL195 did not significantly alter response rate up to a dose of 120 mg/kg. Larger doses of JZL195 were not studied due to poor solubility.

When studied up to doses that significantly decreased response rate or that reached the limits of solubility, the MAGL inhibitor JZL184 and the FAAH inhibitors PF-3845 and URB597 produced no greater than 25% Δ^9-THC appropriate responding (Fig. 1B). PF-3845 significantly decreased response rate as a function of dose ($F_{2,20} = 3.61$; $P < 0.05$); response rate at 32 mg/kg (29% of control) was significantly different from the vehicle control (Fig. 1D, triangles). Doses larger than 100 mg/kg URB597 and 120 mg/kg JZL184 were not studied.

Effects of Combining JZL184 with Either PF-3845 or URB597 in Mice Discriminating Δ^9-THC. When an ineffective dose (3.2 mg/kg) of PF-3845 was studied in combination with ineffective doses of JZL184 (4–120 mg/kg), drug-appropriate responding did not exceed 40% (Fig. 2A, circles). However, when combined with a larger, still ineffective dose of PF-3845 (10 mg/kg), JZL184 dose-dependently increased Δ^9-THC appropriate responding (Fig. 2A, triangles). Drug-appropriate responding was 90% at a dose of 120 mg/kg JZL184 in combination with PF-3845 (10 mg/kg). In the presence of 10 mg/kg PF-3845, the ED_{50} value of JZL184 to increase drug-appropriate responding was 25 mg/kg (95% CL, 16–40). When various doses (10–100 mg/kg) of URB597 were combined with JZL184 (Fig. 2B), drug-appropriate responding was not increased to the same percentage as that obtained with

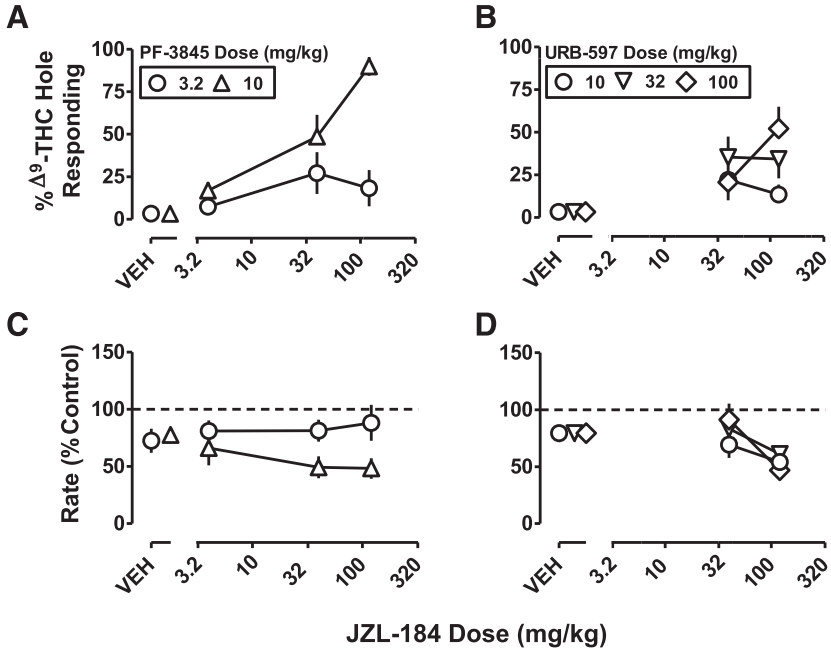

Fig. 2. Effects of combining the FAAH and MAGL inhibitors PF-3845 and JZL184, respectively (A and C), or URB597 and JZL184, respectively (B and D), in mice discriminating Δ^9-THC (5.6 mg/kg i.p.). Abscissae show vehicle (VEH) or dose in milligrams per kilogram of body weight. Ordinates show the mean ± S.E.M. percentage of responding on the Δ^9-THC lever (A and B) and the mean ± S.E.M response rate expressed as a percentage of the control rate (C and D).

the training dose. Maximum Δ^9-THC appropriate responding after 100 mg/kg URB597 in combination with 120 mg/kg JZL184 was a mean of 52% and was significantly less than drug-appropriate responding produced by JZL184 in combination with PF-3845. Response rate was not significantly modified relative to vehicle controls at any dose of JZL184 in combination with either PF-3845 or URB597 (Fig. 2, C and D).

Effects of Δ^9-THC, JZL195, SA-57, and a Combination of JZL184 and PF-3845: Antagonism by Rimonabant. When tested in combination with the dose or dose combination resulting in the highest percentage of Δ^9-THC appropriate responding (Fig. 3, left, symbols above vehicle), discriminative stimulus effects were dose-dependently antagonized by rimonabant. For Δ^9-THC (5.6 mg/kg) and SA-57 (10 mg/kg), drug-appropriate responding was fully attenuated by a dose of 1 mg/kg rimonabant. A dose of 3.2 mg/kg rimonabant was required to produce near full antagonism of drug-appropriate responding produced by JZL195 (120 mg/kg) and by JZL184 (120 mg/kg) in combination with PF-3845 (10 mg/kg). The effects of drugs to decrease response rate did not vary in the absence versus the presence of various doses of rimonabant (Fig. 3, right).

AEA, 2-AG, and OEA Brain Content. After vehicle treatment, mean 2-AG was 8.4 ± 1.4 nmol/g tissue in the prefrontal cortex, 12 ± 2.1 nmol/g tissue in the hippocampus, and 12 ± 1.1 nmol/g tissue in the caudate putamen (Fig. 4, A–C, black bars). Mean AEA in the respective brain areas was 20 ± 2.0, 29 ± 4.5, and 19 ± 1.3 pmol/g tissue (Fig. 4, D–F, black bars). Mean OEA in the respective brain areas was 70 ± 8.0, 131 ± 16, and 89 ± 4.7 pmol/g tissue (Fig. 4, G–I, black bars).

SA-57, at a dose (10 mg/kg) that produced 93% Δ^9-THC appropriate responding, significantly increased 2-AG in all three brain regions (i.e., 12-fold in prefrontal cortex, 7.8-fold in the hippocampus, and 7.9-fold in the caudate putamen) (Fig. 4, A–C, white bars). In each respective brain area, SA-57 (10 mg/kg) significantly increased AEA by 4.0-, 3.0-, and 3.7-fold (Fig. 4, D–F, white bars), and significantly increased OEA by 3.7-, 5.0-, and 3.9-fold (Fig. 4, G–I, white bars). JZL195 (120 mg/kg) also significantly increased 2-AG, AEA, and OEA as much as 5.3-, 3.1-, and 4.0-fold, respectively, among the various brain regions. However, the increase in 2-AG produced by JZL195 (120 mg/kg) was significantly less than the increase in 2-AG produced by SA-57 (10 m/kg).

The three other inhibitors increased either 2-AG or both AEA and OEA. JZL184 (120 mg/kg) significantly increased 2-AG between 2.8- and 4.4-fold among brain regions (Fig. 4, A–C, light gray bars), but did not significantly alter AEA or OEA content (Fig. 4, D–I, light gray bars). By contrast, PF-3845 significantly increased AEA between 2.7- and 3.5-fold as well as OEA 3.3- and 6.3-fold among brain regions (Fig. 4, D–I, diagonal hatched light gray bars), but did not significantly alter 2-AG content (Fig. 4, A–C, diagonal hatched light gray bars). URB597 (100 mg/kg) produced similar increases in AEA and OEA, but did not alter 2-AG.

After the combination of JZL184 (120 mg/kg) and PF-3845 (10 mg/kg) that produced 90% drug-appropriate responding, there was a significant increase in 2-AG, AEA, and OEA. For the combination, the magnitude of increase was similar to that obtained after administration of each drug individually (i.e., 2-AG was increased similarly after JZL184 alone or in combination with PF-3845) (Fig. 4, A–C, compare dark and light gray unhatched bars). AEA and OEA were increased similarly after PF-3845 alone or in combination with JZL184 (Fig. 4, D–I, compare dark gray unhatched bar with diagonal hatched light gray bar). The only exception was for 2-AG in the prefrontal cortex after the combination of JZL184 and PF-3845, which was not significantly increased compared with vehicle. The combination of JZL184 (120 mg/kg) and URB597 (100 mg/kg) also increased 2-AG, AEA, and OEA (Fig. 4, D–I, rightmost bars). However, the magnitude of increase in 2-AG after the combination of JZL184 and URB597 (2.5-, 1.9-, and 1.4-fold in prefrontal cortex, hippocampus, and caudate putamen, respectively) was less than the magnitude of increase after JZL184 alone (4.4-, 3.2-, and 2.8-fold in each respective brain area) (Fig. 4, A–C, rightmost bars).

Discussion

This study used nonselective FAAH and MAGL inhibitors as well as a combination of inhibitors selective for either FAAH or MAGL to demonstrate that inhibition of both enzymes mimics the discriminative stimulus effects of Δ^9-THC, whereas inhibition of only one or the other of the enzymes does not. Involvement of CB_1 receptors was confirmed by antagonism of discriminative stimulus effects by rimonabant. FAAH and

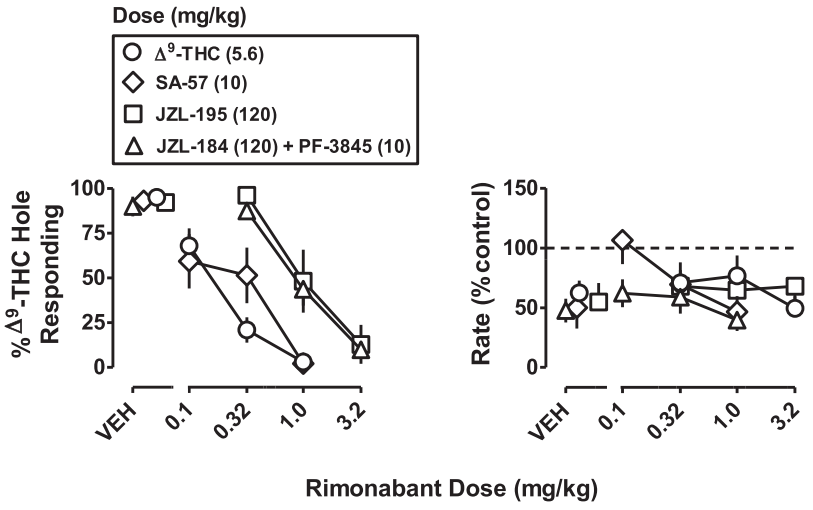

Fig. 3. Effects of JZL195, SA-57, JZL184 plus PF-3845, and Δ^9-THC in mice discriminating Δ^9-THC: antagonism by rimonabant. Abscissae show vehicle (VEH) or dose in milligrams per kilogram of body weight. Ordinates show the mean ± S.E.M. percentage of responding on the Δ^9-THC lever (left) and the mean ± S.E.M response rate expressed as a percentage of the control rate (right). The number in parentheses next to a drug is the dose for that particular drug. Control data above vehicle (i.e., JZL195, SA-57, JZL184 + PF-3845, and Δ^9-THC without rimonabant) are replotted from Figs. 1 and 2.

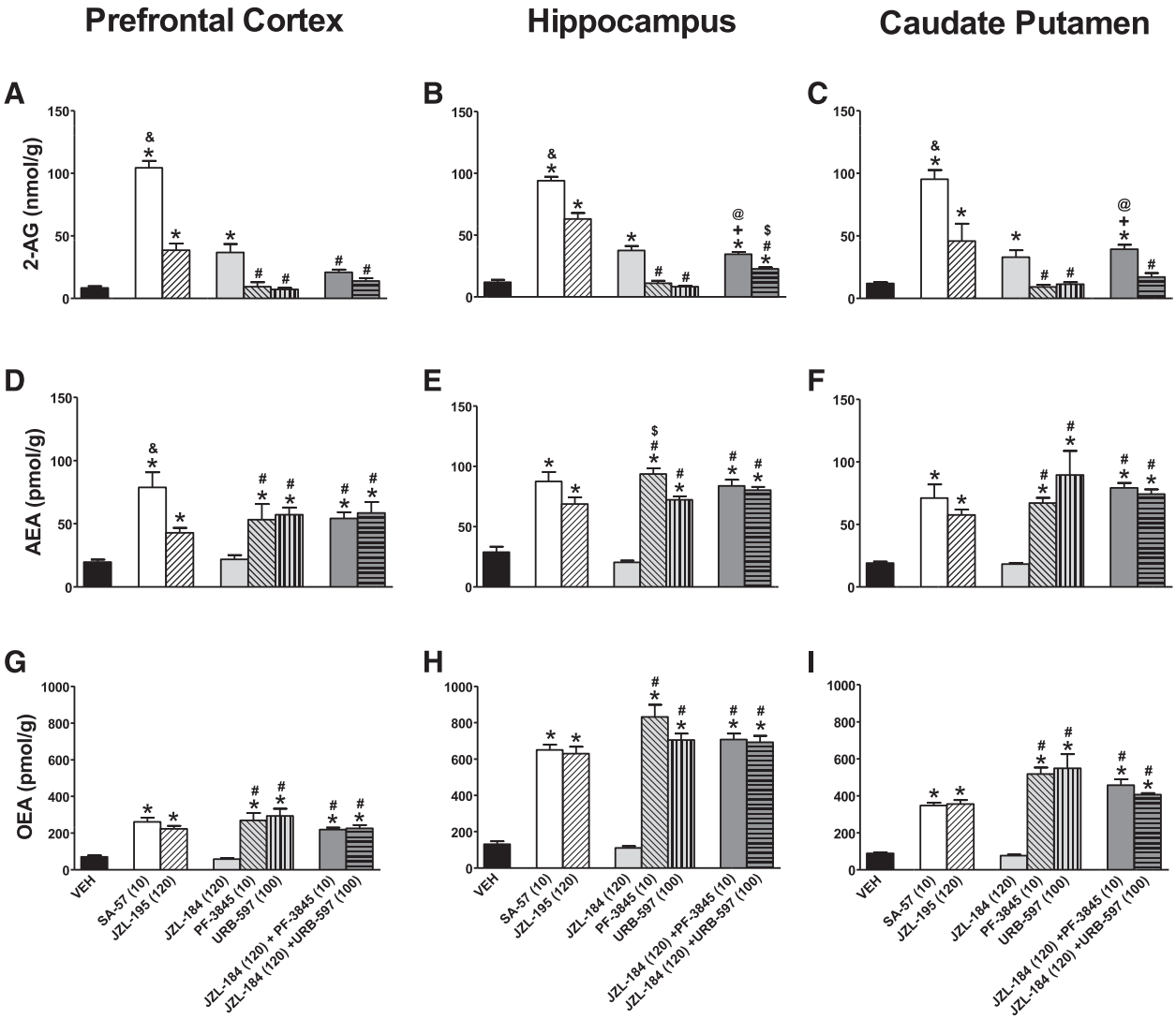

Fig. 4. The separate and combined effects of vehicle (VEH), JZL184, PF-3845, and URB597, as well as the effects of SA-57 and JZL195 on 2-AG, AEA, and OEA content in prefrontal cortex (A, D, and G), hippocampus (B, E, and H), and caudate putamen (C, F, and I). Values represent the mean ± S.E.M. $^{*}P < 0.05$ versus vehicle; $^{\&}P < 0.05$ versus JZL195; $^{\#}P < 0.05$ versus JZL184; $^{\$}P < 0.05$ versus URB597; $^{+}P < 0.05$ versus PF-3845; $^{@}P < 0.05$ versus combined administration of JZL184 and URB597.

MAGL inhibition was evidenced by quantification of increases in AEA/OEA and 2-AG, respectively, in the medial prefrontal cortex, hippocampus, and caudate putamen of mouse brain. In general, the degree of selectivity of each drug for FAAH and MAGL, as reported previously (Fegley et al., 2005; Ahn et al., 2009; Long et al., 2009c; Niphakis et al., 2012), was confirmed under the current experimental conditions. Because changes in endocannabinoid content were not region dependent, these particular brain areas did not appear to differ in their potential contribution to the discriminative stimulus effects of Δ^9-THC. Collectively, these results suggest that FAAH and MAGL inhibitors are likely to exert Δ^9-THC–like discriminative stimulus effects when they nonselectively inhibit FAAH and MAGL.

The nonselective (i.e., dual) FAAH and MAGL inhibitors JZL195 and SA-57 substituted for the discriminative stimulus effects of Δ^9-THC, with SA-57 being 7-fold more potent than JZL195. Our results are consistent with a previous study showing that JZL195 (40 mg/kg i.p.) substituted for a Δ^9-THC discriminative stimulus in mice (Long et al., 2009c). The involvement of CB_1 receptors in mediating the discriminative stimulus effects of SA-57 and JZL195 was evidenced by antagonism by the CB_1 receptor antagonist rimonabant. There was a difference in the position of the dose-effect function for rimonabant to antagonize the effects JZL195 and SA-57 (Fig. 3, compare squares to diamonds), which could be due to a difference in the effectiveness of the single doses of SA-57 and JZL195 that were studied. The doses chosen were the smallest doses of each inhibitor producing near maximal discriminative stimulus effects, as reflected by the group mean. However, the dose of JZL195 was more effective in substituting for Δ^9-THC than the dose of SA-57 (i.e., the next smaller dose of JZL195 produced more Δ^9-THC appropriate responding than the next smaller dose of SA-57). That JZL195 was studied at a functionally larger dose than SA-57 was likely responsible for the difference in position of the rimonabant dose-effect function.

In agreement with previous measurements of brain endocannabinoid content (Long et al., 2009c; Ramesh et al., 2013), SA-57 and JZL195 significantly increased both AEA and 2-AG. SA-57 and JZL195 produced the same magnitude of

increase in AEA (i.e., 3- to 4-fold). However, the smallest dose of SA-57 producing full substitution for Δ^9-THC resulted in 8-fold to 12-fold increases in 2-AG (depending on the brain region), as opposed to only 4- to 5-fold increases in 2-AG at doses of JZL195 that were equally or more effective than SA-57 in producing discriminative stimulus effects. The overall greater increase in 2-AG produced by SA-57 might have contributed to its greater potency compared with JZL195.

AEA and OEA are both substrates of FAAH (Piomelli, 2013). Pharmacological inhibition of FAAH increased both AEA and OEA in our study. Although OEA is not a CB_1 receptor agonist (Piomelli, 2013) and is not expected to have contributed to the discriminative stimulus effects of the inhibitors, the high concordance between AEA and OEA data reported here provides strong evidence that AEA/OEA levels are highly correlative with FAAH inhibition. In general, the effects of the FAAH and MAGL inhibitors to modify 2-AG, AEA, and OEA content did not vary as a function of brain region (i.e., prefrontal cortex, hippocampus, and caudate putamen) so there is no evidence from these data to suggest differential involvement of these brain regions in mediating the discriminative stimulus effects of Δ^9-THC.

This and previous studies (Long et al., 2009c; Wiley et al., 2014) strongly suggest that inhibiting either FAAH or MAGL does not mimic the discriminative stimulus effects of Δ^9-THC. When administered separately, the FAAH inhibitors PF-3845 and URB597 and the MAGL inhibitor JZL184 did not substitute for Δ^9-THC. Selectivity of the drugs for the respective enzymes was evidenced here by measurement of AEA and 2-AG. PF-3845 and URB597 increased AEA but not 2-AG, whereas JZL184 increased 2-AG but not AEA (our results; see also Ahn et al., 2009; Long et al., 2009a,b,c; Ramesh et al., 2013; Seillier et al., 2013, 2014; Wiley et al., 2014). In this study, administration of a selective FAAH inhibitor (PF-3845) in combination with a selective MAGL inhibitor (JZL184) fully substituted for the discriminative stimulus effects of Δ^9-THC. The combined effects of PF-3845 and JZL184 were antagonized by rimonabant, confirming the involvement of CB_1 receptors. Two potential explanations that are not mutually exclusive could account for the inability of endogenous 2-AG alone or AEA alone to mimic the effects induced by CB_1 receptor stimulation from an exogenous agonist. First, although 2-AG and AEA could stimulate the same population of CB_1 receptors, the overall magnitude of receptor stimulation from either is less than that produced by an exogenous agonist. That is, AEA and 2-AG must both be increased to achieve the magnitude of stimulation from exogenous agonist. Second, AEA and 2-AG could stimulate different subsets or populations of CB_1 receptors and stimulation of both populations might be required to mimic the more widespread CB_1 receptor stimulation produced by exogenous agonist. Here, increases in AEA and 2-AG did not vary among prefrontal cortex, hippocampus, and caudate putamen, suggesting that any difference in the population of CB_1 receptors stimulated by AEA and 2-AG exists at a level below general brain regions.

Coadministration of the MAGL inhibitor JZL184 and the FAAH inhibitor URB597 produced less Δ^9-THC appropriate responding than coadministration of JZL184 and the FAAH inhibitor PF-3845. In a previous study (Wiley et al., 2014), a combination of a dose of JZL184 and a dose of URB597 also was reported to not fully substitute for a Δ^9-THC discriminative stimulus in rats. Differential effects of the drug combinations on AEA and 2-AG content, as assessed in this study, provide a plausible explanation. The combination of JZL184 (120 mg/kg) and PF-3845 (10 mg/kg) significantly increased both 2-AG and AEA relative to control in a manner similar to the nonselective FAAH and MAGL inhibitors JZL195 and SA-57. By contrast, the combination of JZL184 (120 mg/kg) and URB597 (100 mg/kg) increased AEA but not 2-AG. Whereas AEA content was similar regardless of whether URB597 was administered alone or in combination with JZL184, 2-AG content after coadministration of these two drugs was lower compared with administration of JZL184 alone. These results are consistent with previous results showing that URB597 decreases 2-AG content in squirrel monkey brain (Justinova et al., 2008), and specifically 2-AG content in the caudate putamen of rodents (Maccarrone et al., 2008), the area of greatest disparity in this study. The mechanism responsible for the effects of URB597 on 2-AG is not clear, although FAAH-independent mechanisms could be involved (Bosier et al., 2013).

Collectively, our results show that pharmacological increases in endogenous AEA and 2-AG simultaneously through inhibition of FAAH and MAGL, respectively, mimic the discriminative stimulus effects of Δ^9-THC. On the other hand, selective increases in either AEA or 2-AG do not mimic the discriminative stimulus effects of Δ^9-THC. Moreover, the FAAH inhibitor URB597, unlike PF-3845, produces opposing effects on AEA and 2-AG content. Like Δ^9-THC, drug inhibitors of FAAH and MAGL exert preclinical effects (i.e., antinociception) predictive of therapeutic effects. Unlike Δ^9-THC, however, stimulation of CB_1 receptors after administration of a FAAH and/or MAGL inhibitor is indirect and depends on endogenous cannabinoid content. Accordingly, inhibitors of FAAH and MAGL have been suggested as a novel therapeutic strategy that could lack many of the side effects associated with Δ^9-THC. To the extent that the discriminative stimulus effects of Δ^9-THC are predictive of the abuse liability associated with Δ^9-THC and cannabis, these findings suggest that nonselective inhibitors of FAAH and MAGL are more likely to be abused than selective inhibitors for one enzyme or the other.

Acknowledgments

The authors thank G. Weiss and D. Schulze for technical assistance.

Authorship Contributions

Participated in research design: Hruba, Seillier, Lichtman, Giuffrida, McMahon.

Conducted experiments: Hruba, Seillier, Zaki.

Contributed new reagents or analytic tools: Cravatt, Lichtman.

Performed data analysis: Hruba, Zaki, McMahon.

Wrote or contributed to the writing of the manuscript: Hruba, Seillier, McMahon.

References

Ahn K, Johnson DS, Mileni M, Beidler D, Long JZ, McKinney MK, Weerapana E, Sadagopan N, Liimatta M, Smith SE, et al. (2009) Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol* **16**:411–420.

Booker L, Kinsey SG, Abdullah RA, Blankman JL, Long JZ, Ezzili C, Boger DL, Cravatt BF, and Lichtman AH (2012) The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice. *Br J Pharmacol* **165**:2485–2496.

Bosier B, Muccioli GG, and Lambert DM (2013) The FAAH inhibitor URB597 efficiently reduces tyrosine hydroxylase expression through CB_1- and FAAH-independent mechanisms. *Br J Pharmacol* **169**:794–807.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, and Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**:83–87.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946–1949.

Di Marzo V, Melck D, Bisogno T, and De Petrocellis L (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci* **21**:521–528.

Dinh TP, Freund TF, and Piomelli D (2002) A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem Phys Lipids* **121**:149–158.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2005) Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. *J Pharmacol Exp Ther* **313**:352–358.

Hardison S, Weintraub ST, and Giuffrida A (2006) Quantification of endocannabinoids in rat biological samples by GC/MS: technical and theoretical considerations. *Prostaglandins Other Lipid Mediat* **81**:106–112.

Hollister LE (1986) Health aspects of cannabis. *Pharmacol Rev* **38**:1–20.

Justinova Z, Mangieri RA, Bortolato M, Chefer SI, Mukhin AG, Clapper JR, King AR, Redhi GH, Yasar S, Piomelli D, et al. (2008) Fatty acid amide hydrolase inhibition heightens anandamide signaling without producing reinforcing effects in primates. *Biol Psychiatry* **64**:930–937.

Kenakin TP (1997) *Pharmacologic Analysis of Drug-Receptor Interaction*, Raven Press, New York.

Kinsey SG, Long JZ, O'Neal ST, Abdullah RA, Poklis JL, Boger DL, Cravatt BF, and Lichtman AH (2009) Blockade of endocannabinoid-degrading enzymes attenuates neuropathic pain. *J Pharmacol Exp Ther* **330**:902–910.

Kinsey SG, O'Neal ST, Long JZ, Cravatt BF, and Lichtman AH (2011) Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay. *Pharmacol Biochem Behav* **98**:21–27.

Lichtman AH, Shelton CC, Advani T, and Cravatt BF (2004) Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* **109**:319–327.

Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavón FJ, Serrano AM, Selley DE, Parsons LH, et al. (2009a) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5**:37–44.

Long JZ, Nomura DK, and Cravatt BF (2009b) Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chem Biol* **16**:744–753.

Long JZ, Nomura DK, Vann RE, Walentiny DM, Booker L, Jin X, Burston JJ, Sim-Selley LJ, Lichtman AH, Wiley JL, et al. (2009c) Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. *Proc Natl Acad Sci USA* **106**:20270–20275.

Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agrò A, et al. (2008) Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. *Nat Neurosci* **11**:152–159.

Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R, and Ward SJ (1991) Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacol Biochem Behav* **40**:471–478.

McMahon LR, Ginsburg BC, and Lamb RJ (2008) Cannabinoid agonists differentially substitute for the discriminative stimulus effects of Delta(9)-tetrahydrocannabinol in C57BL/6J mice. *Psychopharmacology (Berl)* **198**:487–495.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.

Niphakis MJ, Johnson DS, Ballard TE, Stiff C, and Cravatt BF (2012) O-hydroxyacetamide carbamates as a highly potent and selective class of endocannabinoid hydrolase inhibitors. *ACS Chem Neurosci* **3**:418–426.

Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, et al. (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. *Pharmacol Rev* **62**:588–631.

Piomelli D (2013) A fatty gut feeling. *Trends Endocrinol Metab* **24**:332–341.

Ramesh D, Gamage TF, Vanuytsel T, Owens RA, Abdullah RA, Niphakis MJ, Shea-Donohue T, Cravatt BF, and Lichtman AH (2013) Dual inhibition of endocannabinoid catabolic enzymes produces enhanced antiwithdrawal effects in morphine-dependent mice. *Neuropsychopharmacology* **38**:1039–1049.

Sciolino NR, Zhou W, and Hohmann AG (2011) Enhancement of endocannabinoid signaling with JZL184, an inhibitor of the 2-arachidonoylglycerol hydrolyzing enzyme monoacylglycerol lipase, produces anxiolytic effects under conditions of high environmental aversiveness in rats. *Pharmacol Res* **64**:226–234.

Seillier A, Dominguez Aguilar D, and Giuffrida A (2014) The dual FAAH/MAGL inhibitor JZL195 has enhanced effects on endocannabinoid transmission and motor behavior in rats as compared to those of the MAGL inhibitor JZL184. *Pharmacol Biochem Behav* **124**:153–159.

Seillier A, Martinez AA, and Giuffrida A (2013) Phencyclidine-induced social withdrawal results from deficient stimulation of cannabinoid CB_1 receptors: implications for schizophrenia. *Neuropsychopharmacology* **38**:1816–1824.

Solinas M, Tanda G, Justinova Z, Wertheim CE, Yasar S, Piomelli D, Vadivel SK, Makriyannis A, and Goldberg SR (2007) The endogenous cannabinoid anandamide produces delta-9-tetrahydrocannabinol-like discriminative and neurochemical effects that are enhanced by inhibition of fatty acid amide hydrolase but not by inhibition of anandamide transport. *J Pharmacol Exp Ther* **321**:370–380.

Stewart JL and McMahon LR (2011) The fatty acid amide hydrolase inhibitor URB 597: interactions with anandamide in rhesus monkeys. *Br J Pharmacol* **164** (2b): 655–666.

Tallarida RJ (2000) *Drug Synergism and Dose-Effect Data Analysis*, Chapman and Hall/CRC, Boca Raton, FL.

Wiebelhaus JM, Grim TW, Owens RA, Lazenka MF, Sim-Selley LJ, Abdullah RA, Niphakis MJ, Vann RE, Cravatt BF, Wiley JL, et al. (2015) Δ^9-Tetrahydrocannabinol and endocannabinoid degradative enzyme inhibitors attenuate intracranial self-stimulation in mice. *J Pharmacol Exp Ther* **352**:195–207.

Wiley JL, Walentiny DM, Wright MJ, Jr, Beardsley PM, Burston JJ, Poklis JL, Lichtman AH, and Vann RE (2014) Endocannabinoid contribution to Δ9-tetrahydrocannabinol discrimination in rodents. *Eur J Pharmacol* **737**:97–105.

Address correspondence to: Dr. Lance R. McMahon, Department of Pharmacology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. E-mail: mcmahonl@uthscsa.edu