Title: Simultaneous inhibition of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) shares discriminative stimulus effects with Δ⁹-THC in mice

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Running title: FAAH and MAGL inhibitors in mice discriminating Δ⁹-THC

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Abbreviations: AEA, N-arachidonoyl ethanolamine; 2-AG, 2-arachidonoylglycerol; CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; Δ⁹-THC, delta-9-tetrahydrocannabinol; FAAH, fatty acid amide hydrolase; 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; OAE, oleoylethanolamide; PF-3845, N-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidin carboxamide; SA-57, 4-[2-(4-chlorophenyl)ethyl]-1-piperidin carboxylic acid 2-(methylamino)-2-oxoethyl ester; URB597, (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate

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Abstract

Monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) inhibitors exert pre-clinical 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 Δ⁹-tetrahydrocannabinol (Δ⁹-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. Δ⁹-THC dose-dependently increased Δ⁹-THC appropriate responses (ED₅₀ value = 2.8 mg/kg), whereas the FAAH inhibitors PF-3845 and URB597 or a MAGL inhibitor JZL184 alone did not substitute for the Δ⁹-THC discriminative stimulus. The non-selective FAAH/MAGL inhibitors SA-57 and JZL195 fully substituted for Δ⁹-THC with ED₅₀ values equal to 2.4 and 17 mg/kg, respectively. Full substitution for Δ⁹-THC also was produced by a combination of JZL184 and PF-3845, but not by a combination of JZL184 and URB597 (i.e., 52% maximum). The CB₁ receptor antagonist rimonabant attenuated the discriminative stimulus effects of Δ⁹-THC, SA-57, JZL195, and the combined effects of JZL184 and PF-3845. Full substitution for the Δ⁹-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

Δ⁹-tetrahydrocannabinol (Δ⁹-THC) is a cannabinoid CB₁/CB₂ receptor agonist that produces therapeutic effects such as analgesia and anti-emetic effects, but also 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) also are CB₁/CB₂ 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 fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (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 CB₁/CB₂ receptors via increases in AEA and 2-AG, which can only occur in the CB₁ receptor-expressing brain areas that contain these endogenous cannabinoids. In contrast, direct-acting CB₁ receptor agonists such as Δ⁹-THC are expected to produce widespread stimulation of CB₁ receptors in 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 Δ⁹-THC. When assessed with a tetrad of in vivo effects sensitive to CB₁ receptor agonism (Martin et al., 1991), the MAGL inhibitor JZL184 produced some (i.e., analgesic, locomotor-decreasing, and hypothermic effects) 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 Δ⁹-THC in a pre-clinical assay predictive of cannabis-like subjective effects in mice (i.e., drug discrimination; Long et al., 2009a). Moreover, the FAAH inhibitors PF-3845 and URB597
produced antinociceptive and anti-inflammatory effects, but failed to produce locomotor-decreasing, hypothermic, cataleptic, and Δ⁹-THC like discriminative stimulus effects (Booker et al., 2012; Lichtman et al., 2004; Solinas et al., 2007; Long et al., 2009a; Stewart and McMahon, 2011; 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 Δ⁹-THC including discriminative stimulus effects. However, the extent to which simultaneous inhibition of FAAH and MAGL mimics the discriminative stimulus and subjective effects of Δ⁹-THC remains unclear. Whereas a dose of the non-selective FAAH and MAGL inhibitor JZL195 substituted for a Δ⁹-THC discriminative stimulus in mice (Long et al., 2009), a combination of the FAAH inhibitor URB597 with the MAGL inhibitor JZL184 appeared to only partially substitute for Δ⁹-THC (Wiley et al., 2014).

In the current study, the effects of several different FAAH and MAGL inhibitors were studied in C57BL/6J mice discriminating 5.6 mg/kg of Δ⁹-THC i.p. (McMahon et al., 2008), and discriminative stimulus effects were related to changes in brain endocannabinoid content as assessed via gas chromatography/chemical ionization mass spectrometry (Hardison et al., 2006). The drugs included the selective FAAH inhibitors URB597 and PF-3845, the selective MAGL inhibitor JZL184, and the non-selective FAAH/MAGL inhibitors JZL195 and SA-57. JZL195 was chosen because it has been shown to substitute for Δ⁹-THC in mice (Long et al., 2009a), and hence served as a positive control, whereas SA-57 is a relatively new compound that has been shown to share effects with Δ⁹-THC in modifying intracranial self-stimulation and morphine withdrawal in mice (Ramesh et al., 2013; Wiebelhaus et al., 2015). Because non-selective FAAH or MAGL inhibitors appear to substitute for the discriminative stimulus effects of Δ⁹-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 described previously (see Wiley et al., 2014), the current study examined a second drug combination (i.e., PF-3845 with JZL184). The involvement of CB₁ receptors was examined with the CB₁ 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 CB₁ receptor, FAAH, and MAGL (Di Marzo et al., 1988). 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 14/10 h light/dark cycle. For the drug discrimination experiment, sixteen mice received condensed milk during experimental sessions and 2.5 g of food (Dustless Precision Pellets 500 mg, Rodent Grain-Based Diet, Bio-Serv, Frenchtown, NJ) per day after sessions. Forty-five mice used for brain dissection had continuous access to food (Harlan, Teklad 7912, 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 Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio and the Guide for the Care and Use of Laboratory Animals (2011).

Behavioral apparatus

Drug discrimination experiments were conducted in mouse operant conditioning chambers (MedAssociates, 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 cc 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
Discrimination training

Experiments were run once per day 7 days per week at the same time each day during the light period. Vehicle (a mixture of 1 part propylene glycol, 1 part Tween 80, and 18 parts of physiologic saline) was administered in the home cage, and 30 min later, mice were placed inside the operant conditioning chambers. During 30-min 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-s access to 0.01 cc of condensed milk diluted with tap water to 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-s 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 or 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 $\Delta^9$-THC (5.6 mg/kg) i.p. and were placed back in their cages for 30 min. A training dose of 5.6 mg/kg was chosen because 10 mg/kg of $\Delta^9$-THC produced marked decreases in response rate (i.e., less than 20% of control). Thirty min post-injection, mice were placed in the operant chambers during a 30-min 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 $\Delta^9$-THC was administered before the session. Determination of correct holes (e.g., left, vehicle; right, $\Delta^9$-THC) varied among mice and remained the same for that mouse for the duration of the study. Vehicle training sessions alternated non-systematically with $\Delta^9$-THC training sessions; training with vehicle or $\Delta^9$-THC was not repeated for more than two consecutive days. The first test was conducted when, for 5 consecutive or for 6 out 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 criteria specified above during three consecutive sessions, including at least one vehicle training session and one $\Delta^9$-THC training session.

JZL184, JZL195, PF-3845, SA-57, URB597 and a combination of JZL184 + PF-3845 and JZL184 + URB597 were studied by administering dose(s) of drug(s) 2 h (instead of 30 min) before the 30-min test session, in accordance with previous studies showing in vivo effects after 2 h (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 min before the test session. The order of testing among drugs was nonsystematic except that tests with different doses of $\Delta^9$-
THC were conducted at the beginning of the study and again with the same doses of Δ⁹-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 i.p. 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, their brains rapidly collected, frozen in 2-methylbutane (-45 °C), and stored at -80 °C. Frozen brains were placed on a stainless steel mould (Roboz; Rockville, USA) 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 of [²H4]AEA, [²H4]OEA, and [²H5]-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; Varian, USA). Endocannabinoid-containing fractions were analyzed by gas chromatography/chemical ionization mass spectrometry (GC/MS), using an isotope dilution assay.

**Drugs**

The levo enantiomer of Δ⁹-THC (500 mg/ml in absolute ethanol; The Research Technology Branch, National Institute on Drug Abuse, Rockville, MD) was prepared by
evaporating the ethanol under a gentle stream of nitrogen. Δ⁹-THC was re-dissolved in the vehicle consisting of a 1:1:18 mixture of propylene glycol (Sigma), Tween 80 (Sigma), and physiologic saline (i.e., PGT vehicle). SA-57 was synthesized in the laboratory of Dr. Cravatt as previously described (Niphakis et al, 2012). Rimonabant base and URB597 were obtained from The Research Technology Branch, National Institute on Drug Abuse. JZL195, JZL184 and PF-3845 were purchased from Cayman chemicals (Ann Arbor, MI). SA-57, JZL195, PF-3845, URB597, JZL184 and rimonabant were dissolved in the same vehicle used to dissolve Δ⁹-THC. Drugs were administered i.p. 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 Δ⁹-THC appropriate responding (i.e., number of Δ⁹-THC-appropriate responses divided by the total responses made during the duration of the test) and were plotted as a function of dose. Δ⁹-THC was studied in every mouse (n=16). For each drug or drug combination, all doses were studied in the same cohort of eight to ten mice selected from among the sixteen 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% Δ⁹-THC appropriate responding and larger doses including at least one dose producing greater than 80% Δ⁹-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 (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% Δ⁹-THC appropriate responding, including not more than one dose producing less than 20% Δ⁹-THC appropriate responding and not more than one dose producing greater than 80% Δ⁹-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. 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 (ED₅₀ value), potency ratios, and their 95% confidence limits (CL) 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% confidence limits 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 ANOVA 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 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 ANOVA 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 Δ⁹-THC (5.6 mg/kg i.p.), increasing doses of Δ⁹-THC resulted in corresponding increases in Δ⁹-THC appropriate responding (Fig. 1A). A dose of 1.78 mg/kg of Δ⁹-THC produced 9% of responses in the hole associated with the training dose of Δ⁹-THC, whereas 3.2 and 5.6 mg/kg produced 49% and 95% drug-appropriate responding, respectively. Vehicle produced only 3% of Δ⁹-THC appropriate responses. Up to 5.6 mg/kg, Δ⁹-THC did not significantly modify response rate (F₃,₄₅=0.70; p>0.05) (Fig. 1C).

Both non-selective FAAH/MAGL inhibitors SA-57 and JZL195 dose-dependently increased Δ⁹-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 (F₂,₇₇=11.12; p<0.001), i.e., the slope of the Δ⁹-THC dose-response function was greater (i.e., steeper) than the slopes of the SA-57 and JZL195 dose-response functions, the individual slopes were used to estimate the ED₅₀ values. The ED₅₀ values (95% CL) were 2.8 (2.4-3.2) mg/kg for Δ⁹-THC, 2.4 (1.2-4.5) mg/kg for SA-57, and 17 (9.0-32) mg/kg 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% Δ⁹-THC appropriate responding (Fig. 1B). PF-3845 significantly decreased response rate as a function of dose (F₂,₂₀=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 of URB597 and 120 mg/kg JZL184 were not studied.

**Effects of combining JZL184 with either PF-3845 or URB597 in mice discriminating Δ⁹-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 Δ⁹-THC appropriate responding (Fig. 2A, triangles). Drug appropriate responding was 90% at a dose of 120 mg/kg of JZL184 in combination with PF-3845 (10 mg/kg). In the presence of 10 mg/kg of PF-3845, the ED₅₀ value (95% CL) of JZL184 to increase drug-appropriate responding was 25 (16-40) mg/kg. 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 the training dose. Maximum Δ⁹-THC appropriate responding at after 100 mg/kg of URB597 in combination with 120 mg/kg of 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 Δ⁹-THC, JZL195, SA-57, and a combination of JZL184 and PF-3845: antagonism by rimonabrant**
When tested in combination with the dose or dose combination resulting in the highest percentage of Δ⁹-THC appropriate responding (Fig. 3, left, symbols above VEH), discriminative stimulus effects were dose-dependently antagonized by rimonabant. For Δ⁹-THC (5.6 mg/kg) and SA-57 (10 mg/kg), drug-appropriate responding was fully attenuated by a dose of 1 mg/kg of rimonabant. A dose of 3.2 mg/kg of 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 (± S.E.M.) in nmol/g tissue was 8.4 (± 1.4) in the prefrontal cortex, 12 (± 2.1) in the hippocampus, and 12 (± 1.1) in the caudate putamen (Fig 4A-C, black bars). Mean AEA (± S.E.M.) in pmol/g tissue in the respective brain areas was 20 (± 2.0), 29 (± 4.5), and 19 (± 1.3) (Fig. 4D-F, black bars). Mean OEA in pmol/g tissue in the respective brain areas was 70 (± 8.0), 131 (± 16), and 89 (± 4.7) (Fig. 4G-I, black bars).

SA-57, at a dose (10 mg/kg) that produced 93% Δ⁹-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. 4A-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. 4D-F, white bars), and significantly increased OEA by 3.7-, 5.0-, and 3.9-fold (Fig. 4G-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 mg/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. 4A-C, light gray bars), but did not significantly alter AEA or OAE content (Fig. 4D-I, light gray bars). In 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. 4D-I, diagonal hatched light gray bars), but did not significantly alter 2-AG content (Fig. 4A-C, diagonal hatched light gray bars). URB597 (100 mg/kg) produced similar increases in AEA and OEA, but did not alter 2-AG.

Following 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 following administration of each drug individually, i.e., 2-AG was increased similarly after JZL184 alone or in combination with PF-3845 (Fig. 4A-C, compare dark and light gray unhatched bars) and AEA/OEA was increased similarly after PF-3845 alone or in combination with JZL184 (Fig. 4D-I, compare dark gray unhatched bar to 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 as compared with vehicle. The combination of JZL184 (120 mg/kg) and URB597 (100 mg/kg) also increased 2-AG, AEA, and OEA (Fig. 4D-I, rightmost bars). However, the magnitude of increase in 2-AG after the combination of JZL184 and URB597 (2.5-, 1.9-, 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-, 2.8-fold in each respective brain area) (Fig. 4A-C, rightmost bars).
Discussion

The current study used non-selective 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 Δ⁹-THC, whereas inhibition of only one or the other of the enzymes does not. Involvement of CB₁ receptors was confirmed by antagonism of discriminative stimulus effects by rimonabant. FAAH and 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., 2009a; 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 Δ⁹-THC. Collectively, these results suggest that FAAH and MAGL inhibitors are likely to exert Δ⁹-THC like discriminative stimulus effects when they non-selectively inhibit FAAH and MAGL.

The non-selective (i.e., dual) FAAH and MAGL inhibitors JZL195 and SA-57 substituted for the discriminative stimulus effects of Δ⁹-THC, with SA-57 being 7-fold more potent than JZL195. The current results are consistent with a previous study showing that JZL195 (40 mg/kg, i.p) substituted for a Δ⁹-THC discriminative stimulus in mice (Long et al. 2009a). The involvement of CB₁ receptors in mediating the discriminative stimulus effects of SA-57 and JZL195 was evidenced by antagonism by the CB₁ 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 (Figure 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 $\Delta^9$-THC than the dose of SA-57, i.e., the next smaller dose of JZL195 produced
more $\Delta^9$-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 (Ramesh et
al., 2013; Long et al., 2009a), 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 $\Delta^9$-THC resulted in 8- 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 as compared with JZL195.

AEA and OEA are both substrates of FAAH (Piomelli, 2013). Pharmacological
inhibition of FAAH increased both AEA and OEA in the present study. While 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 Δ⁹-THC.

The current and previous studies (Wiley et al., 2014; Long et al., 2009a) strongly suggest that inhibiting either FAAH or MAGL does not mimic the discriminative stimulus effects of Δ⁹-THC. When administered separately, the FAAH inhibitors PF-3845 and URB597 and the MAGL inhibitor JZL184 did not substitute for Δ⁹-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 (present results; see also Ahn et al., 2009; Long et al., 2009abc; Ramesh et al., 2013; Seillier et al., 2014; Wiley et al., 2014). In the current 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 Δ⁹-THC. The combined effects of PF-3845 and JZL184 were antagonized by rimonabant, confirming the involvement of CB₁ 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 CB₁ receptor stimulation from an exogenous agonist. First, although 2-AG and AEA could stimulate the same population of CB₁ 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 CB₁ receptors and stimulation of both populations might be required to mimic the more widespread CB₁ 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 CB₁ receptors stimulated by AEA and 2-AG exists at a level below general brain regions.

Co-administration of the MAGL inhibitor JZL184 and the FAAH inhibitor URB 597 produced less Δ⁹-THC appropriate responding than co-administration 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 Δ⁹-THC discriminative stimulus in rats. Differential effects of the drug combinations on AEA and 2-AG content, as assessed in the current 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 non-selective FAAH and MAGL inhibitors JZL195 and SA-57. In 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 co-administration of these two drugs was lower as 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 the current 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, the current results show that pharmacological increases in endogenous AEA and 2-AG simultaneously through inhibition of FAAH and MAGL, respectively, mimics the discriminative stimulus effects of Δ⁹-THC. On the other hand, selective increases in either AEA or 2-AG do not mimic the discriminative stimulus effects of Δ⁹-THC. Moreover, the FAAH
inhibitor URB597, unlike PF-3845, produces opposing effects on AEA and 2-AG content. Like
Δ⁹-THC, drug inhibitors of FAAH and MAGL exert preclinical effects (i.e., antinociception)
predictive of therapeutic effects. Unlike Δ⁹-THC, however, stimulation of CB₁ receptors
following 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
Δ⁹-THC. To the extent that the discriminative stimulus effects of Δ⁹-THC are predictive of the
abuse liability associated with Δ⁹-THC and cannabis, the present findings suggest that non-
selective inhibitors of FAAH and MAGL are more likely to be abused than selective inhibitors
for one enzyme or the other.
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Authorship Contributions

*Participated in research design:* Hruba, Seillier, Giuffrida, Lichtman, and McMahon

*Conducted experiments:* Hruba, Seillier, and Zaki

*Contributed new reagents or analytical tools:* Lichtman, Cravatt

*Performed data analysis:* Hruba, Zaki, and McMahon

*Wrote or contributed to writing of the manuscript:* Hruba, Seillier, and McMahon
References


produces enhanced anti-withdrawal effects in morphine-dependent mice.

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Footnotes

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Figure legends

Fig. 1
Effects of the non-selective FAAH and MAGL inhibitors JZL195 and SA-57 (A,C), the FAAH inhibitors PF-3845 and URB597 (B,D), and the MAGL inhibitor JZL184 (B,D) in mice discriminating Δ⁹-THC (5.6 mg/kg i.p.). Abscissae: vehicle (VEH) or dose in milligram per kilogram of body weight. Ordinates: mean (± S.E.M.) percentage of responding on the Δ⁹-THC lever (A,B) and mean (± S.E.M) response rate expressed as a percentage of the control rate (C,D).

Fig. 2
Effects of combining the FAAH and MAGL inhibitors PF-3845 and JZL184, respectively (A,C) or URB597 and JZL184, respectively (B,D) in mice discriminating Δ⁹-THC (5.6 mg/kg i.p.). Abscissae: vehicle (VEH) or dose in milligram per kilogram of body weight. Ordinates: mean (± S.E.M.) percentage of responding on the Δ⁹-THC lever (A,B) and mean (± S.E.M) response rate expressed as a percentage of the control rate (C,D).

Fig. 3
Effects of JZL195, SA-57, JZL184 + PF-3845 and Δ⁹-THC in mice discriminating Δ⁹-THC: antagonism by rimonabant. Abscissae: vehicle (VEH) or dose in milligram per kilogram of body weight. Ordinates: mean (±S.E.M.) percentage of responding on the Δ⁹-THC lever (left) and 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 VEH (i.e., JZL195, SA-57, JZL184 + PF-3845, and Δ⁹-THC without rimonabant) are re-plotted 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 brain 2-AG, AEA, and OEA content in prefrontal cortex (A), hippocampus (B), and caudate putamen (C). Values represent mean ± SEM. *p<0.05 vs. VEH, &p<0.05 vs. JZL195, #p<0.05 vs. JZL184, $p<0.05 vs. URB597, +p<0.05 vs. PF-3845, @p<0.05 vs. combined administration of JZL184 and URB597.
Figure 2

A. %Δ9-THC Hole Responding

PF-3845 Dose (mg/kg)

B. URB-597 Dose (mg/kg)

C. Rate (% Control)

JZL-184 Dose (mg/kg)
Figure 3

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Figure 4

Prefrontal Cortex

Hippocampus

Caudate Putamen

2-AG (nmol/g)

AEA (pmol/g)

OEA (pmol/g)

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