Cannabinoid CB₁ Discrimination:
Effects of Endocannabinoids and Catabolic Enzyme Inhibitors

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ABSTRACT

An improved understanding of the endocannabinoid system has provided new avenues of drug discovery and development toward the management of pain and other behavioral maladies. Exogenous cannabinoid type-1 (CB₁) receptor agonists such as Δ⁹-tetrahydrocannabinol are increasingly utilized for their medicinal actions; however, their utility is constrained by concern regarding abuse-related subjective effects. This has led to growing interest in the clinical benefit of indirectly enhancing the activity of the highly labile endocannabinoids N-arachidonylethanolamine (anandamide; AEA) and/or 2-arachidonoylglycerol (2-AG) via catabolic enzyme inhibition. The present studies were conducted to determine whether such actions can lead to CB₁ agonist-like subjective effects, as reflected in the presence or absence of CB₁-related discriminative-stimulus effects in laboratory subjects. Squirrel monkeys (n=8) that discriminated the CB₁ full agonist AM4054 (0.01 mg/kg) from vehicle were used to study, first, inhibitors of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MGL) alone or in combination [FAAH (URB597, AM4303); MGL (AM4301); FAAH/MGL (JZL195, AM4302)] and, second, the ability of the endocannabinoids AEA and 2-AG to produce CB₁ agonist-like effects when administered alone or after enzyme inhibition. Results indicate that CB₁-related discriminative-stimulus effects were produced by combined, but not selective, inhibition of FAAH and MGL, and that these effects were non-surmountably antagonized by low doses of rimonabant. Additionally, FAAH- or MGL-inhibition revealed CB₁-like subjective effects produced by AEA, but not 2-AG. Taken together, the present data suggest that therapeutic effects of combined, but not selective, enhancement of AEA or
2-AG activity via enzyme inhibition may be accompanied by CB$_1$ receptor-mediated subjective effects.
INTRODUCTION

Accumulating evidence confirming medicinal effects of the cannabinoid type-1 (CB₁) partial agonist Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the principal psychoactive constituent of marijuana, has led to the current availability of medicinal marijuana in the majority of states in the US. Notwithstanding such growing popularity, concerns regarding its safety, especially during adolescence (Gruber et al., 2014), cloud the further development of Δ⁹-THC and other exogenous CB₁ agonists as pharmacotherapies. An alternative approach to the use of exogenous CB₁ agonists for medicinal purposes may lie in increasing the activity of endogenous CB₁ receptor ligands, e.g., the endocannabinoids N-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG). Although AEA and 2-AG are highly labile, complicating their in vivo evaluation, each has been reported to produce CB₁-mediated behavioral effects. For example, infusion of 2-AG into the dorsolateral periaqueductal grey has been reported to produce CB₁-mediated increases in anxiolytic-like behavior (Almeida-Santos et al., 2013; Gobira et al., 2016). Furthermore, both anandamide and 2-AG, like Δ⁹-THC, maintain i.v. self-administration behavior in, respectively, monkeys and rats, presumably due to rapid delivery to relevant brain regions permitted by intravenous administration (DeLuca et al., 2014; Justinova et al. 2005, 2011). Findings such as these suggest that AEA and 2-AG can independently produce CB₁ receptor-mediated behavioral effects.

In addition to the CB₁-mediated actions of exogenous AEA and 2-AG, their effects also have been examined indirectly by inhibiting their rapid enzymatic degradation in vivo. Compounds that inhibit the activity of fatty acid amide hydrolase
(FAAH) and monoacylglycerol lipase (MGL) can increase the concentrations of, respectively, AEA and 2-AG at CB₁ receptors and, following single or combined administration, have been shown to produce varying levels of effect in the CB₁ tetrad test (Little et al., 1988; Ghosh et al., 2015; Schlosburg et al., 2014; Wilkerson et al., 2017). Thus, dual FAAH/MGL inhibition was found to produce full effects on all measures (antinociception, hypomotility, hypothermia, catalepsy) whereas selective inhibition of either FAAH or MGL has been reported to produce only antinociception in rodent models of acute and/or chronic pain (Ahn et al., 2011; Ignatowska-Jankowska et al., 2014). FAAH inhibitors additionally have been reported to modulate addiction-related behavior (Justinova et al., 2015; Wilkerson et al., 2017) and, in assays of emotional control, mood (Rossi et al., 2010; Varvel et al., 2007) and post-traumatic stress (Zer-Aviv and Akirav, 2016), have produced results supporting their further development as anxiolytic and antidepressant drugs (Gaetani et al., 2009).

Although the potential medicinal benefits of modulating endocannabinoid activity have received considerable attention, there is relatively little information on possibly adverse effects, including CB₁-related abuse liability. Along these lines, drug discrimination procedures have been used to study CB₁ receptor-mediated discriminative-stimulus effects, which can serve as a valid behavioral marker of subjective effects of CB₁ agonists (e.g., Δ⁹-THC) that are linked to abuse liability. The discriminative-stimulus effects of directly-acting CB₁ receptor agonists have been extensively evaluated in both rodent and nonhuman primate species (e.g., Järbe et al., 2014; Kangas et al., 2013; McMahon, 2006; Vann et al., 2009). However, there have been few comparable studies of endocannabinoids to date. Solinas et al. (2007) initially
reported that the metabolically stable anandamide analog, methanandamide, or co-administration of the FAAH inhibitor URB597 and anandamide fully substituted for Δ⁹-THC in Δ⁹-THC-trained rats—findings that have been supported in subsequent studies in mice and nonhuman primates (Long et al., 2009; Wiley et al., 2014; Stewart and McMahon, 2011). The discriminative-stimulus effects of 2-AG, on the other hand, are even less understood. Wiley et al. (2014) found that 2-AG, with or without a MGL inhibitor, failed to substitute for Δ⁹-THC whereas partial substitution was produced by an MGL inhibitor (JZL184) in mice and by co-administration of a FAAH (URB597) and MGL (JZL184) inhibitor in rats. Owens et al. (2016) reported that the dual FAAH-MGL inhibitor, SA-57, can be readily trained as a discriminative stimulus in mice and substitutes for synthetic cannabinoid agonists in an antagonist-sensitive manner. Of interest, selective MGL, but not FAAH, inhibitors fully substituted for the training stimulus, suggesting that the CB₁ receptor-mediated effects of anandamide and 2-AG differ in a fundamental, though as yet unclear, manner.

The present research was conducted to further examine the ability of endocannabinoid action to produce CB₁ receptor-mediated discriminative-stimulus effects. Monkeys trained to discriminate the CB₁ full agonist AM4054 from vehicle were used to evaluate the agonist-like effects of nonselective enzyme inhibitors, selective FAAH and MGL inhibitors alone and in combination, and to compare the ability of the endocannabinoids anandamide and 2-AG to produce CB₁ agonist-like effects alone or after enzyme inhibition.

METHODS

Subjects
Eight adult male squirrel monkeys (*Saimiri sciureus*) served in the present studies. Subjects were housed individually in a temperature- and humidity-controlled vivarium with a 12-h light/dark cycle (lights on at 7am). This facility is licensed by the U.S. Department of Agriculture and complies with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2011). All procedures in the present studies were approved by the McLean Hospital Institutional Animal Care and Use Committee. Throughout the present studies, all subjects were maintained at approximate free-feeding weights by post-session access to a nutritionally balanced diet of high protein banana-flavored biscuits (Purina Monkey Chow, St. Louis, MO) and had unlimited access to water in their home cage. In addition, they were provided with fresh fruit and environmental enrichment daily. Four subjects (31, 101, 103, and 115) had served in cannabinoid drug discrimination studies prior to the present experiments; the remaining subjects (2, 88, 113, and 134) had previously served in experiments examining other psychoactive drugs (e.g., opioids, monoaminergic stimulants); however, prior to the present studies, had not received drug treatments for at least two months. Additionally, 3 subjects (88, 113, 134) were prepared with intravenous (i.v.) catheters (Herd et al., 1969) for studies of the endocannabinoids anandamide (AEA) and 2-arachidonoyl ethanolamide (2-AG).

**Apparatus**

Subjects were seated in a Plexiglas chair (Spealman et al., 1977; Kangas et al., 2013) within a ventilated light- and sound-attenuating enclosure facing an intelligence panel equipped with two response levers, 6 cm left and right of center. Each lever-press
with a force of at least 0.25 N closed a microswitch, produced an audible relay click, and was recorded as a response. Red stimulus lights were positioned 10 cm above each lever. Prior to each session, a shaved portion of each subject’s tail was coated with electrode paste and placed under brass electrodes for the delivery of brief, low-intensity shock stimuli (see below). All experimental events and data collection were controlled via Med Associates (St. Albans, VT) operating software and interfacing hardware.

**Experimental Procedures**

**CB₁ Discrimination Training Procedure.** Experimental sessions were conducted daily (Mon-Fri). Initially, subjects were trained to respond on the lever to terminate visual stimuli associated with the delivery of a brief, low intensity shock stimulus (200 ms; 3 mA; see above). Next, subjects were trained to discriminate between the intramuscular (i.m.) injection of the CB₁ agonist AM4054 (0.01 mg/kg; Järbe et al., 2016; Kangas et al., 2013) and vehicle using two-lever drug discrimination procedures. Briefly, one response lever was designated as the drug (AM4054) lever and the other as the vehicle lever. Assignment remained the same for each subject throughout the study, but was counterbalanced across subjects. AM4054 or vehicle was administered i.m. 50 min prior to each training session. Training sessions began with a 10 min timeout period during which all lights were extinguished and responding had no programmed consequences. Following the timeout period, two red stimulus lights above each lever were illuminated and completion of ten consecutive responses (FR10) on the injection-associated (correct) lever extinguished all stimulus lights and initiated a 50 s timeout. Responses on the other (incorrect) lever reset the FR requirement. Shock
delivery was scheduled for every 10 sec until either the FR10 was completed on the correct lever or 30 sec elapsed, whichever came first. Each presentation of the FR schedule constituted a trial, and sessions were comprised of 20 trials. A double-alternation injection schedule (i.e., drug-drug-vehicle-vehicle) was employed throughout training, with a third drug or vehicle training session arranged intermittently to avoid associations based on the periodicity of the double alternation schedule.

**CB$_1$ Discrimination Testing Procedures.** Drug tests for substitution were conducted only when the first FR10 was completed on the injection-appropriate lever and overall discrimination performance was at least 90% accurate for 4 of the last 5 sessions and in the immediately preceding session. Test sessions differed procedurally from training sessions in 3 ways. First, 10 consecutive responses on either the drug or vehicle lever extinguished the stimulus lights and initiated the 50 sec timeout. Second, cumulative dosing procedures were used to study the effects of a range of doses of each drug, permitting the determination of dose-response relationships for the discriminative-stimulus effects of drugs that substituted for the training drug. Test sessions consisted of 4 components of 10 trials, each component beginning with a 30-min timeout period, and generally were completed within 2.5 hrs. This procedure permitted the study of up to 4 incremental doses of a drug delivered at the onset of sequential timeout periods of a single test session (e.g., Kangas et al., 2013; Lamb et al., 2000; Spealman, 1985). The effects of 5 or more doses were determined by administering overlapping ranges of cumulative doses over different test sessions. For each drug except AM4301, doses ranged up to those that fully substituted for the training drug or produced a $\geq$50% decrease in response rate from control values.
(AM4301 did not appreciably decrease response rates at the highest doses studied.) Third, no shock deliveries were scheduled during test sessions so as to preclude their possible influences on performance. All other experimental contingencies were identical.

**Discriminative-Stimulus Effects of Endocannabinoids.** Five sets of experiments were conducted in the present study. First, the effects of i.m. injections of AEA (0.3-32 mg/kg) and 2-AG (0.3-18 mg/kg) were studied in a group of four subjects; the effects of cumulative i.v. doses of 2-AG (0.3-18 mg/kg) also were evaluated in three subjects. Inasmuch as both compounds are rapidly metabolized *in vivo* (Willoughby et al., 1997; Savinainen et al., 2001; Stewart and McMahon, 2011), the drug pretreatment time preceding test components were shortened from 30 to 10 min. Experiments to determine the effects of i.v. AEA also were initiated; however, as described below in Results, these experiments were discontinued due to adverse effects of the highest dose of AEA (32 mg/kg) in the absence of evidence for CB<sub>1</sub>-related discrimination in the first subject studied.

**Discriminative-Stimulus Effects of FAAH and MGL Inhibitors.** Next, studies were conducted to examine the effects of five enzyme inhibitors previously characterized in *in vitro* studies of their CB<sub>1</sub> receptor affinity and their potency in inhibiting both FAAH and MGL (See Table 1 and Kathuria et al., 2003; Long et al., 2009). These included enzyme inhibitors that were designated as: a) FAAH-selective [URB597 (0.3-5.6 mg/kg); AM4303 (0.3-10 mg/kg)], b) MGL-selective [AM4301 (0.3-10 mg/kg)], and c) FAAH/MGL [JZL195 (0.1-5.6 mg/kg); AM4302 (0.1-5.6 mg/kg)].
Discriminative-Stimulus Effects of Endocannabinoids Following Enzyme Inhibition. In a third set of experiments, the effects of the endocannabinoids AEA and 2-AG were re-determined following pretreatment with selective (URB597, AM4303, AM4301) and nonselective enzyme inhibitors (JZL195 and AM4302). Based upon previous studies of the time course of FAAH inhibition in rats and monkeys (Fegley et al., 2005; Justinova et al., 2008, Long et al., 2009), all enzyme inhibitors were administered 60 min prior to treatment with AEA or 2-AG. Cumulative doses of the endocannabinoids were administered 10 min prior to each test component.

Discriminative-Stimulus and Time Course Effects of Selective Enzyme Inhibitor Combinations. Additional studies with URB597, AM4303, and AM4301 were conducted to determine the lowest doses of FAAH- and MGL-selective inhibitors that, when combined, would fully mimic the discriminative-stimulus effects of AM4054. Thus, in the presence of 1.0 mg/kg URB597 administered 60 min prior to the test session, the effects of cumulative doses of AM4301 (0.03-1.0 mg/kg) delivered 30 min prior to each test component were studied to determine the lowest effective dose of AM4301. Next, the lowest effective dose of AM4301 (1.0 mg/kg) was administered 60 min before sessions in which cumulative doses of either AM4303 (0.003-0.1 mg/kg) or URB597 (0.01-0.3 mg/kg) were administered 30 min prior to each test component. Finally, a time course function for the duration of action of the dose combination of 1.0mg/kg AM4301 and 0.3mg/kg URB597 was determined by conducting test sessions 15, 60, 240, and 480 min following their i.m. administration.

Antagonism of CB₁ Discriminative-Stimulus Effects of Selective Enzyme Inhibitor Combinations. A last set of experiments was conducted to examine
antagonism of the CB₁-related discriminative-stimulus effects resulting from combined FAAH (URB597) and MGL (AM4301) enzyme inhibition. In these experiments, doses of the selective CB₁ inverse agonist/antagonist SR141716A (rimonabant; 0.003-0.3 mg/kg) were administered 60 min prior to sessions in which either a fixed dose of AM4301 was given before cumulative dosing with URB597 (0.01-3.2 mg/kg) or, alternatively, a fixed dose of URB597 was given before cumulative dosing with AM4301 (0.03-3.2 mg/kg). Pretreatment times were the same as described above.

Data Analysis

In the present studies, the two primary dependent measures were the allocation of responding to the AM4054-associated lever, expressed as % CB₁-lever responding, and overall response rate. The % CB₁-lever responding was calculated by dividing the number of responses on the AM4054-associated lever by the total number of responses. Response rate was calculated by dividing the total number of responses on both levers by the total session time (excluding all timeout periods). Doses of drugs were considered to substitute fully when the subject responded on the AM4054-associated lever >90% and response rates were >0.2 responses/sec.

Drugs

2-AG, AEA, AM4054, AM4301, AM4302, AM4303, JZL195, and URB597 were synthesized for these studies by the present authors (SOA, LJ, VGS, YL, SPN, AM) in the Center for Drug Discovery at Northeastern University (Boston, MA). Rimonabant was provided by the NIDA Drug Supply Program (Rockville, MD). All drugs usually were
prepared for administration in a 20:20:60 mixture of 95% ethanol, Tween-80, and saline. When concentrations >10 mg/ml were necessary, drugs were dissolved in dimethyl sulfoxide. Drug solutions were refrigerated and protected from light. Injections of drug or vehicle were prepared in volumes of 0.3 ml/kg body weight or less and administered i.m. in calf or thigh muscle and i.v. through the venous access ports.

RESULTS

CB₁ Discrimination. All subjects acquired the drug discrimination in approximately 30-60 sessions. Throughout subsequent control sessions, injections of the training dose of 0.01 mg/kg AM4054 produced an average of >99% responding on its associated lever. Injections of vehicle produced <1% responding on the AM4054-associated lever. Response rates following i.m. training doses of AM4054 were somewhat lower than vehicle administration in all subjects, with group averages of 3.1 ±0.6 and 3.6 ±0.7 responses/sec, respectively (mean ±SEM). This small (<20%) difference in response rate was evident at the outset of training and remained constant throughout the present studies.

Discriminative-Stimulus Effects of Endocannabinoids. As shown in the upper panels of Fig. 1, neither cumulative i.m. doses of the endocannabinoids 2-AG or AEA up to 18 and 32 mg/kg, respectively, nor cumulative i.v. doses of 2-AG up to 18 mg/kg engendered responding on the AM4054-associated lever. The effects of cumulative i.v. doses of AEA up to 32 mg/kg were studied in one subject and, similarly, produced no responding on the CB₁-associated lever. However, the subject collapsed immediately following the cumulative i.v. dose of 32 mg/kg AEA, most likely due to non-CB₁
mechanisms—perhaps vanillloid-1 receptor activation (Panlilio et al., 2009)—and further studies were not conducted in other subjects to avoid similar adverse events. As shown in the lower panels of Fig. 1, the effects of i.m. AEA on response rate were dose-dependent: cumulative doses below 32 mg/kg were within the range of control values or produced increases in response rate, whereas the highest cumulative dose of 32 mg/kg produced, on average, an approximately 25% decrease in responding after i.m. administration and abolished responding after i.v. injection. Like AEA, most cumulative doses of i.m. or i.v. 2-AG (0.3-18 mg/kg) were either within the range of control values or only increased response rates; however, the i.v. injection of 18 mg/kg 2-AG completely abolished responding and produced a short-lived episode of syncope in at least one subject.

**Discriminative-Stimulus Effects of FAAH and MGL Inhibitors.** Fig. 2 presents mean data for responding on the CB$_1$-associated lever (upper panels) and response rate (lower panels) following cumulative doses of selective and nonselective enzyme inhibitors. Selective inhibitors including the FAAH inhibitors AM4303 and URB597 (left panels) and the MGL inhibitor AM4301 (middle panel) did not substitute for AM4054 but, excepting AM4301, produced a >50% decrease in response rate following the highest cumulative dose of 10 mg/kg. The MGL inhibitor AM4301 did not alter responding but was available in limited supply, precluding the evaluation of cumulative doses >10 mg/kg. In contrast, both FAAH/MGL inhibitors, AM4302 and JZL195 (right panels), produced dose-dependent AM4054-related discriminative-stimulus effects and fully substituted following the cumulative dose of 5.6 mg/kg. Additionally, both AM4302 and JZL195 produced dose-related decreases in responding, with an approximately 34%
and 64% reduction in response rates, respectively, after the cumulative dose of 5.6 mg/kg.

**Discriminative-Stimulus Effects of Endocannabinoids Following Enzyme Inhibition.** The left panels of Fig. 3 present mean data for responding on the CB$_1$-associated lever following cumulative i.m. doses of AEA alone (open diamonds), and after i.m. treatment with either FAAH-, MGL- or FAAH/MGL enzyme inhibitors. Exogenously administered AEA after treatment with the FAAH-selective inhibitors (URB597 and AM4303; upper panel) produced dose-dependent cannabimimetic discriminative-stimulus effects. Full substitution was observed in all subjects following a cumulative dose of 10 mg/kg AEA when pretreated with a dose of 1.0 mg/kg URB597, and 18 mg/kg AEA when pretreated with a dose of 3.2 mg/kg AM4303. Likewise, both FAAH/MGL inhibitors (AM4302 and JZL195; lower panel) also disclosed dose-related cannabimimetic discriminative-stimulus effects of exogenously administered AEA. Thus, after treatment with 0.1 mg/kg of AM4302 and JZL195, full substitution for AM4054 occurred in all subjects following cumulative doses of, respectively, 10 and 18 mg/kg AEA. In contrast, pretreatment with the MGL-selective inhibitor AM4301 (3.2 mg/kg) failed to alter the effects of AEA up to a cumulative dose of 10 mg/kg which, following pretreatment, produced an approximating 50% decrease in response rate (data not shown).

The right panels of Fig. 3 present mean data for responding on the CB$_1$-associated lever following cumulative i.m. doses of 2-AG alone (open triangles), and after i.m. treatment with the MGL-selective inhibitor AM4301 (middle panel), and the FAAH/MGL inhibitors AM4302 and JZL195 (lower panel). Cumulative doses of 2-AG up
to 18 mg/kg administered i.m. or i.v. failed to produce AM4054-associated responding alone or after treatment with any of the three enzyme inhibitors. As in initial studies of its effects alone, the highest i.v. dose of 2-AG (18 mg/kg) produced >75% decreases in response rate in the two monkeys in which it was studied after treatment with AM4301 (data not shown).

**Discriminative-Stimulus and Time Course Effects of Selective Enzyme Inhibitor Combinations.** Fig. 4 presents mean data for CB₁-associated responding (upper panels) and response rate (lower panels) following administration of the FAAH-selective inhibitors URB597 or AM4303 alone and after treatment with the selective MGL-inhibitor AM4301 (1.0 mg/kg). Although URB597 or AM4303 alone failed to produce AM4054-associated responding up to doses that decreased response rate by >50% (open symbols; see also Fig 2), cumulative doses of both URB597 and AM4303 engendered dose-related increases in AM4054-associated responding following pre-session administration of AM4301. Full substitution for the training dose of AM4054 was observed in the latter experiments after cumulative doses of 0.3 mg/kg and 0.1 mg/kg, respectively, of URB597 and AM4303.

Fig. 5 presents the time course of CB₁-associated discriminative-stimulus effects after treatment with the dose combination of 0.3 mg/kg of URB597 and 1.0 mg/kg AM4301. The full time course of action for the AM4054-related discriminative-stimulus effects of this dose combination was captured within 8-hr, i.e., all subjects responded ≤20% on the AM4054-associated lever 15 min after treatment, ≥90% after 60 minutes, and 0% at 8-hours post-treatment. At 4-hr after treatment, the response distribution varied among individual subjects, yielding a mean value of approximately 50%
responding on both levers. Inspection of individual data shows that two subjects responded exclusively on the AM4054-associated lever at the 4-hr post-treatment time, whereas ≥80% responding occurred on the vehicle-associated lever for the remaining two subjects.

**Antagonism of CB₁ Discriminative-Stimulus Effects of Selective Enzyme Inhibitor Combinations.** Fig. 6 presents the effects of rimonabant on the cannabinomimetic discriminative-stimulus effects produced by the combination of selective enzyme inhibitors. The upper panel of Fig. 6 shows modification by rimonabant of the cannabinomimetic effects of cumulative doses of URB597 administered in the presence of 1.0 mg/kg AM4301. As shown, the doses of 0.003 and 0.01 mg/kg rimonabant slightly attenuated the effects of lower doses of URB597 without altering the effects of the highest cumulative dose of URB597 (0.32 mg/kg) in the presence of AM4301. However, 0.03 mg/kg of rimonabant completely blocked CB₁ discriminative-stimulus effects of URB597/AM4301 up to the highest cumulative dose of URB597 that could be studied (3.2 mg/kg). The lower panel of Fig. 6 shows the effects of rimonabant on the effects of cumulative doses of AM4301 following pretreatment with 0.3 mg/kg URB597 [AM/URB]. Pretreatment with 0.03 mg/kg rimonabant produced a small (<3-fold) rightward movement of the dose-effect function, whereas a dose of 0.1 mg/kg rimonabant completely blocked the CB₁ discriminative-stimulus effects of AM/URB up to the cumulative dose of 3.2 mg/kg AM4301.

**DISCUSSION**

In the present studies, ligands that selectively inhibit the metabolic deactivation of the endocannabinoids AEA by FAAH (URB597, AM4303) and 2-AG by MGL (AM4301)
failed to produce CB₁ discriminative-stimulus effects when administered alone. The present results with FAAH inhibitors agree well with data from previous studies of URB597 in monkeys that were trained to discriminate ∆⁹-THC from vehicle (e.g., Stewart and McMahon, 2011; Long et al., 2009, Solinas et al., 2007), and extend those observations to the novel and selective FAAH inhibitor, AM4303. The highest doses of URB597 in the present studies have been shown previously to produce up to a 10-fold enhancement of brain AEA levels in squirrel monkeys (Justinova et al., 2008), indicating that such high levels of endogenous AEA alone do not sufficiently activate CB₁ receptors to engender CB₁-mediated discriminative-stimulus effects.

Administration of exogenous AEA or 2-AG also failed to produce CB₁-mediated discriminative-stimulus effects in the present experiments. The inability of AEA to produce CB₁-related discriminative-stimulus effects likely reflects its rapid enzymatic degradation in vivo (Savinainen et al., 2001; Stewart and McMahon, 2011; Willoughby et al., 1997). Supporting this view, the relatively stable analog of AEA, methanandamide, has been shown to reproduce CB₁-mediated discriminative-stimulus effects in both rats and monkeys (Järbe et al., 2014; Kangas et al., 2013), despite having lesser efficacy than associated with ∆⁹-THC or CB₁ full agonists like WIN55,212-2 (see also Kangas et al., 2016). Moreover, the behavioral effects of AEA could be revealed in the present studies following inhibition of its metabolism by either FAAH-selective or dual FAAH/MGL ligands. The full and dose-dependent substitution observed with AEA under these conditions is consistent with previous reports that either pharmacological (Solinas et al., 2007; Stewart and McMahon, 2011) or genetic
inactivation of FAAH can potentiate CB₁-mediated discriminative-stimulus effects.

The absence of CB₁-like effects of both URB597 and i.v. AEA in the present studies stands in contrast to the results of previous i.v. self-administration studies. URB597 is not readily self-administered, perhaps reflecting a lack of rewarding effect of URB597-increased levels of AEA or kinetics of FAAH inhibition that are not conducive to the demonstration of reinforcing effects (i.e., a too gradual accumulation of AEA). On the other hand, i.v. AEA, which has fast onset but is rapidly degraded, has been shown to have reinforcing effects in squirrel monkeys (Justinova et al., 2005; 2008). It is important to note, however, that the pretreatment times for AEA and URB597 in the present studies were 10- and 60-min, respectively, whereas, i.v. infusions were delivered immediately in the self-administration studies. Whether this can be attributed to differences in the temporal requirements for discriminative and reinforcing stimuli to achieve behavioral efficacy remains to be determined.

The effectiveness of dual FAAH/MGL inhibition, but not FAAH inhibition alone, to substitute for AM4054 in the present experiments suggests a functional role for 2-AG in CB₁-mediated discriminative-stimulus effects. However, in contrast to results with AEA after FAAH inhibition, 2-AG displayed no capacity for producing such behavioral effects alone, even after selective MGL- or dual FAAH/MGL inhibition. The reasons for this are unclear. Endocannabinoids are thought to modulate brain permeability, and it may be that, unlike AEA, exogenously-administered 2-AG itself does not adequately penetrate the blood-brain barrier to sufficiently activate CB1 receptors in the central nervous system (CNS) (Hind et al., 2015; Pelkover et al., 2013). Notwithstanding this caveat, the
highest i.v. dose of 2-AG in the present studies produced clear behavioral effects in all monkeys, consistent with CNS activity. It is possible that these effects of 2-AG were mediated by its activation of peroxisome proliferator-activated receptors (PPARs) or through the prostanoid system consequent to its oxygenation (Alhouayek et al., 2013; Pertwee et al., 2010; Sticht et al., 2015). However, 2-AG, like AEA, has previously been reported to maintain rimonabant-sensitive i.v. self-administration in squirrel monkeys, which is thought to reflect CB1 receptor-mediated actions in the CNS (Justinova et al. 2005; 2011). Alternatively, and presuming that 2-AG does adequately penetrate the CNS to bind CB1 receptors, it may be that 2-AG alone cannot trigger CB1 discriminative-stimulus effects in the absence of elevated levels of AEA. Mounting evidence suggests a functional interaction between AEA and 2-AG, and they may play differential and, perhaps, cooperative roles in mediating CB1-related discriminative-stimulus effects. For example, dual FAAH/MGL inhibition has been shown to produce a distinctly different behavioral profile in the rodent tetrad test than selective blockade of either enzyme alone (Long et al., 2009). Similarly, the effects of dual FAAH/MGL inhibition in the present study strengthen the idea that complementary, but not individual, actions of the AEA and 2-AG are required to produce CB1-related discriminative-stimulus effects. However, this explanation for the absence of CB1-like effects of 2-AG, while plausible, does not easily comport with the ability of i.v. 2-AG to produce reinforcing and other behavioral effects in squirrel monkeys, and must be viewed with caution. Additional work is necessary to more clearly understand the role of both CB1 and non-CB1 mechanisms that may mediate the behavioral effects of 2-AG.
Both FAAH-selective and dual FAAH/MGL inhibitors, but not the MGL-selective inhibitor, produced dose-related decreases in response rate in squirrel monkeys—differences that also have been observed in both rats and mice (Järbe et al., 2001; Wiley et al., 2014). The rate-decreasing effects of the enzyme inhibitors studied may result from actions of AEA metabolites at off-site targets, e.g., vanilloid (TRPV1) receptors (Wiley et al., 2006; Panlilio et al., 2009). Similar non-CB\textsubscript{1} mechanisms may also explain the profound behavioral effects of i.v. AEA observed at high doses. Importantly, neither AEA nor 2-AG produced CB\textsubscript{1}-related discriminative-stimulus effects when administered i.v. up to doses that substantially decreased response rate, supporting the idea that the rate-decreasing effects of cannabinoids like \(\Delta^9\)-THC, which typically are produced by discriminable doses and have been attributed to CB\textsubscript{1}-mediated actions, can be distinguished from those of the endocannabinoids. Unfortunately, the adverse effects observed with rate-decreasing doses of AEA and 2-AG in the present studies precluded further antagonism experiments to confirm this possibility.

Although neither selective FAAH nor MGL inhibitors produced AM4054-related discriminative-stimulus effects, the dual FAAH/MGL inhibitors JZL195 and AM4302, consistently substituted for the CB\textsubscript{1} agonist. Moreover, the combined administration of selective FAAH and MGL inhibitors similarly produced AM4054-like effects. These findings provide the first example of CB\textsubscript{1}-mediated discriminative-stimulus effects of the combination of FAAH and MGL inhibition in nonhuman primates, and are consistent with previous findings in drug discrimination studies in which neither maximal FAAH nor MGL inhibition alone sufficed to reproduce CB\textsubscript{1} discriminative-stimulus effects in rodents.
or monkeys (e.g., Stewart and McMahon, 2011; Long et al., 2009; Solinas et al., 2007). They also agree with reports of CB₁ discriminative-stimulus effects in mice that result from dual MGL/FAAH inactivation via genetic and/or pharmacological means (Long et al., 2009; see also Ignatowska-Jankowska et al., 2013). Taken together, these previous and present findings indicate that endocannabinergic production of CB₁ discriminative stimulus requires dual FAAH and MGL inhibition, presumably reflecting an integrated signaling mechanism resulting from elevated levels of both AEA and 2-AG in brain.

Administration of the selective CB₁ receptor antagonist rimonabant verified that the discriminative-stimulus effects produced by FAAH/MGL inhibitors were mediated by the CB₁-receptor. Interestingly, unlike direct CB₁ agonists, escalating doses of FAAH/MGL inhibitors failed to surmount a modest CB₁-receptor blockade by rimonabant. For example, the relatively small pretreatment dose of 0.1 mg/kg rimonabant fully reversed the effects of FAAH/MGL inhibition, but only produced a 3-fold shift in the AM4054 dose-response curve (see Kangas et al., 2013). These findings disclose the limited extent to which enzyme inhibition can elevate AEA and 2-AG levels to exert CB₁-receptor-mediated actions. These data also suggest that a near-complete inhibition of the two enzymes may be required to produce discriminative-stimulus effects, as the dose-response curve for co-administered FAAH and MGL inhibitors was not shifted rightward (i.e., the antagonistic effects of rimonabant could not be surmounted). These observations are consistent with previous findings showing that approximately 85% of FAAH must be inactivated by URB597 in order to maintain an elevated AEA tone (Fegley et al., 2005).
In conclusion, the present studies suggest that discriminative stimulus, and perhaps subjective, effects associated with CB₁ agonists also can be provoked through endocannabinoid mechanisms involving both AEA and 2-AG. Although AEA can mediate such cannabimimetic effects directly when administered exogenously in the presence of a FAAH-inhibitor, exogenous 2-AG administered alone or after enzyme inhibition, failed to produce similar effects up to doses that dramatically reduced response rate. The discriminative-stimulus effects elicited by dual FAAH/MGL inhibition, but not by either type of inhibition alone, further suggest a functionally cooperative interaction between the endocannabinoids that remains to be clearly defined. Finally, the insurmountable antagonism by rimonabant of the CB₁-related effects of combined FAAH and MGL inhibition strongly suggests that the magnitude of tolerance and dependence produced by directly-acting CB₁ agonists, e.g., Δ⁹-THC, and cannabinomimetic enzyme inhibitors may differ qualitatively. From the perspective of medication development, such findings encourage the idea that endocannabinoid enzyme inhibitors may display less abuse liability than associated with currently available CB₁-related drugs.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Leonard, Bergman, and Kangas.

Conducted experiments: Leonard.

Contributed new reagents or analytic tools: Alapafuja, Ji, Shukla, Liu, Nikas, Makriyannis.

Performed data analysis: Leonard and Kangas.

Wrote or contributed to the writing of the manuscript: Leonard, Bergman, and Kangas.
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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Left panels: Dose-effect functions for AEA administered either i.m. (open triangle, n=4) or i.v. (closed inverted-triangle, n=1) in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top-left panel), response rate (bottom-left panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. Right panels: Dose-effect functions for 2-AG, administered either i.m. (open diamond, n=4) or i.v. (closed square, n=3) in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top-left panel), response rate (bottom-left panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects.

Figure 2. Left panels: Dose-effect functions for the selective FAAH-inhibitors URB597 (closed diamond, n=3) and AM4303 (closed circle, n=3), in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top-left panel), response rate (bottom-left panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. Middle panels: Dose-effect functions for the selective MGL-inhibitor AM4301 (closed triangle, n=4), in subjects trained to
discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top-middle panel), response rate (bottom-middle panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. **Right panels:** Dose-effect functions for nonselective FAAH/MGL-inhibitors AM4302 (closed inverted-triangle, n=4) and JZL195 (closed square, n=3), in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top-right panel), response rate (bottom-right panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects.

**Figure 3. Top panel:** Dose-effect functions for AEA alone (open diamonds, n=4), and following pretreatment with the selective FAAH-inhibitors URB597 (1.0mg/kg; closed diamond, n=4) and AM4303 (3.2mg/kg; closed circle, n=4), in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever. Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. **Middle panels:** Dose-effect functions for AEA (left-middle panel) and 2-AG (right-middle panel) administered alone i.m (AEA; open diamond, n=4. 2-AG; open triangle; n=4), alone i.v. (2-AG; open inverted-diamond, n=3), or following pretreatment with the selective MGL-inhibitor AM4301 (3.2mg/kg) in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle.
vehicle. After pretreatment with AM4301, AEA was administered i.m. (left-middle panel; closed triangle, n=3), and 2-AG was administered either i.m. (right-middle panel; closed triangle, n=3) or i.v. (right-middle panel; semi-filled triangle, n=2). Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (left- and right-middle panels). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. Bottom panels: Dose-effect functions for AEA (bottom-left panel) and 2-AG (bottom-right panel) following pretreatment with nonselective FAAH/MGL-inhibitors AM4302 (0.1mg/kg) and JZL195 (0.1mg/kg), in subjects trained to discriminate 0.01mg/kg AM4054 from vehicle. AEA was administered i.m. alone (open diamond, n=4), after pretreatment with JZL195 (closed square, n=4), and after pretreatment with AM4302 (closed inverted-triangle, n=4). 2-AG was administered i.m. alone (open triangle, n=4) and after AM4302 (closed inverted triangle, n=3), as well as i.v. alone (open inverted-triangle, n=3) and after AM4302 (semi-filled inverted-triangle, n=2). Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (bottom-left and -right panels). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects.

Figure 4. Dose-effect functions for selective FAAH-inhibitors alone (URB597, open inverted-triangle, n=3; AM4303, open diamond, n=3), and in combination with 1.0mg/kg of the selective MGL-inhibitor AM4301 (URB597, closed circle, n=3; AM4303, closed square, n=3), administered in subjects trained to discriminate 0.01 mg/kg AM4054 from
vehicle. Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever (top panel), response rate (bottom panel). Symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects.

**Figure 5.** Time course of 0.3 mg/kg URB597 in combination with 1.0 mg/kg AM4301 (n=4) in subjects trained to discriminate 0.01 mg/kg AM4054 from vehicle. Abscissae, time interval following injection in which discrimination session occurred; ordinate, percent of responses on the AM4054-associated lever. Points represent averages (±SEM) for the groups of subjects.

**Figure 6.** Top panel: Dose-effect function of URB597 following a pretreatment with 1.0 mg/kg AM4301 in subjects trained to discriminate 0.01 mg/kg AM4054 from vehicle, administered alone (open triangle, n=4) or following various pretreatment doses of SR141716A (closed symbols). Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever. Open symbols left of abscissae break indicate performance during Vehicle (V) and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects. **Bottom panel:** Dose-effect function of AM4301 following pretreatment with 0.3 mg/kg URB597 in subjects trained to discriminate 0.01 mg/kg AM4054 from vehicle, administered alone (open triangle, n=2) or following various pretreatment doses of SR141716A (closed symbols). Abscissae, cumulative dose, log scale; ordinate, percent of responses on the AM4054-associated lever. Open symbols left of abscissae break indicate performance during Vehicle (V)
and AM4054 (AM) control sessions. Points represent averages (±SEM) for the groups of subjects.
<table>
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<tr>
<th>Compound</th>
<th>rFAAH IC$_{50}$ (nM)</th>
<th>hMGL IC$_{50}$ (nM)</th>
<th>FAAH/MGL Selectivity Ratio</th>
<th>CB1 K$_i$</th>
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<tr>
<td>URB597</td>
<td>4.6 ± 1.6</td>
<td>&gt;30,000</td>
<td>&gt;100,000</td>
<td>&gt;300,000</td>
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<tr>
<td>AM4303</td>
<td>1.92 ± 0.24</td>
<td>&gt;10,000</td>
<td>&gt;5208</td>
<td>&gt;1000</td>
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<td>AM4301</td>
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<td>10.65 ± 2.5</td>
<td>&lt;0.01</td>
<td>&gt;1000</td>
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<tr>
<td>JZL195</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>&gt;20,000</td>
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<tr>
<td>AM4302</td>
<td>31.2 ± 3.8</td>
<td>41.9 ± 3.3</td>
<td>0.74</td>
<td>940</td>
</tr>
</tbody>
</table>

**Table 1.** IC$_{50}$ values, selectivity ratio, and CB$_1$-receptor affinity (K$_i$) for each compound studied. Values were derived for URB597 from Kathuria et al. (2003), JZL195 from Long et al. (2009). Values obtained for AM4303, AM4301, and AM4302 from the present authors (SOA, LJ, VGS, YL, SPN, AM) from in vitro data using rat and/or human enzyme assays using rat and/or human enzyme assays and by following previously described procedures (Alapafuja et al., 2012; see also Makriyannis et al., 2016; Parker et al., 2016).
Fig. 1

The graphs show the cumulative dose-response curves for Anandamide and 2-AG following intramuscular (i.m.) and intravenous (i.v.) administration.

**Anandamide**
- Drug Lever Responding (%)
- Rate (Response/Sec)
- Cumulative Dose (mg/kg)

**2-AG**
- Drug Lever Responding (%)
- Rate (Response/Sec)
- Cumulative Dose (mg/kg)
Fig. 2

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Fig. 3

**FAAH-Selective Inhibitors**

- Diamond: alone
- Square: + 1.0 mg/kg URB597
- Circle: + 3.2 mg/kg AM4303

**MGL-Selective Inhibitors**

- Diamond: alone
- Triangle: + 3.2 mg/kg AM4301

**Non-Selective Inhibitors**

- Diamond: alone
- Square: + 0.1 mg/kg JZL195
- Circle: + 0.1 mg/kg AM4302
- Triangle: + 0.1 mg/kg AM4302

**Drug Lever Responding (%)**

**Anandamide Cumulative Dose (mg/kg)**

**2-AG Cumulative Dose (mg/kg)**
Fig. 4

The graph shows the relationship between cumulative dose (mg/kg) and drug lever responding (%). It compares the effects of different doses of URB597, AM4303, AM4303 + AM4301, and URB597 + AM4301 on the rate of response per second.
Fig. 6

+ AM4301 (1.0 mg/kg)

URB597 Cumulative Dose (mg/kg)

+ Rimonabant

- 0.003 mg/kg
- 0.01 mg/kg
- 0.03 mg/kg
- 0.1 mg/kg

Drug Lever Responding (%)

+ URB597 (0.3 mg/kg)

AM4301 Cumulative Dose (mg/kg)

Δ alone

- 0.003 mg/kg
- 0.01 mg/kg
- 0.03 mg/kg
- 0.1 mg/kg