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Discriminative Stimulus Properties of the Endocannabinoid Catabolic Enzyme Inhibitor SA-57 in Mice

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ABSTRACT

Whereas the inhibition of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL), the respective major hydrolytic enzymes of *N*-arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), elicits no or partial substitution for Δ^9-tetrahydrocannabinol (THC) in drug-discrimination procedures, combined inhibition of both enzymes fully substitutes for THC, as well as produces a constellation of cannabimimetic effects. The present study tested whether C57BL/6J mice would learn to discriminate the dual FAAH-MAGL inhibitor SA-57 (4-[2-(4-chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester) from vehicle in the drug-discrimination paradigm. In initial experiments, 10 mg/kg SA-57 fully substituted for CP55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol), a high-efficacy CB_1 receptor agonist in C57BL/6J mice and for AEA in FAAH (−/−) mice. Most (i.e., 23 of 24) subjects achieved criteria for discriminating SA-57 (10 mg/kg) from vehicle within 40 sessions, with full generalization occurring 1 to 2 hours postinjection. CP55,940, the dual FAAH-MAGL inhibitor JZL195 (4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate), and the MAGL inhibitors MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate) and JZL184 (4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester) fully substituted for SA-57. Although the FAAH inhibitors PF-3845 ((N-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide) and URB597 (cyclohexylcarbamic acid 3′-(aminocarbonyl)-[1,1′-biphenyl]-3-yl ester) did not substitute for SA-57, PF-3845 produced a 2-fold leftward shift in the MJN110 substitution dose-response curve. In addition, the CB_1 receptor antagonist rimonabant blocked the generalization of SA-57, as well as substitution of CP55,940, JZL195, MJN110, and JZL184. These findings suggest that MAGL inhibition plays a major role in the CB_1 receptor-mediated SA-57 training dose, which is further augmented by FAAH inhibition.

Introduction

Cannabinoid CB_1 (Devane et al., 1988; Matsuda et al., 1990) and CB_2 receptors (Munro et al., 1993) and their endogenous ligands N-arachidonoyl ethanolamine (anandamide; AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) represent primary elements of the endocannabinoid system. This system modulates many physiologic processes, including pain (Hohmann et al., 2005; Kinsey et al., 2010; Woodhams et al., 2012; Ignatowska-Jankowska et al., 2014), memory (Hampson and Deadwyler, 1999), appetite (Kirkham and Tucci, 2006), and reward (Tsou et al., 1998; Marsicano and Lutz, 1999). The primary psychoactive constituent of *Cannabis*, Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964) produces its psychotomimetic effects through CB_1 receptors (Huestis et al., 2001) and induces dopamine release in the nucleus accumbens (Chen et al., 1991), although to a substantially lower magnitude than other abused drugs. Curiously, THC produces reinforcing effects in some (Gardner et al., 1988; Lepore et al., 1996; Justinova et al., 2003, 2005), but not all (Vlachou et al., 2007; Wiebelhaus et al., 2015), preclinical

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ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; ABHD6, α/β-hydrolase domain 6; AEA, *N*-arachidonoyl ethanolamine (anandamide); ANOVA, analysis of variance; CB_1, cannabinoid-1 receptor; CB_2, cannabinoid-2 receptor; CP55,940, ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol); FAAH, fatty acid amide hydrolase; JZL184, 4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester; JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate; KT182, [4-[3′-(hydroxymethyl)[1,1′-biphenyl]-4-yl]-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone; KT195, [4-(4′-methoxy[1,1′-biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone; MAGL, monoacylglycerol lipase; MJN110, 2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate; 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; SR144,528, N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide; URB597, cyclohexylcarbamic acid 3′-aminocarbonyl)-[1,1′-biphenyl]-3-yl ester.

laboratory animal models. In contrast, THC serves as a reliable discriminative stimulus in the drug-discrimination paradigm (Henriksson et al., 1975; Järbe, 1989; Wiley et al., 1997; Vann et al., 2009), an assay that is highly predictive of drug psychoactivity in humans (Chait et al., 1988; Kamien et al., 1993; Lile et al., 2012).

Whereas THC elicits relatively long-lasting pharmacologic effects, AEA and 2-AG produce short-lived effects because of rapid hydrolysis by their respective primary catabolic enzymes fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996, 2001) and monoacylglycerol lipase (MAGL) (Di Marzo et al., 1999; Dinh et al., 2002). Accordingly, inhibitors of these enzymes elevate endocannabinoid brain levels and represent useful investigative tools. Although the selective FAAH inhibitors URB597 (Fu et al., 2005) and PF-3845 (Ahn et al., 2009) elevate AEA brain levels and produce antinociceptive effects, neither compound substitutes for THC (Gobbi et al., 2005; Wiley et al., 2014). Similarly, the MAGL inhibitor JZL184 elevates endogenous 2-AG brain levels and produces antinociception, but it only partially substitutes for THC (Long et al., 2009a,b; Wiley et al., 2014; Walentiny et al., 2015). Conversely, the dual FAAH-MAGL inhibitor JZL195 fully substitutes for THC, elicits a constellation of cannabimimetic effects (Long et al., 2009b; Wise et al., 2012; Hruba et al., 2015), and produces an increased magnitude of antinociceptive effects compared with single enzyme inhibition (Long et al., 2009b; Ghosh et al., 2015). Similarly, the dual FAAH-MAGL inhibitor SA-57 fully substitutes for THC in wild-type mice (Hruba et al., 2015).

As it has yet to be established whether an inhibitor of endocannabinoid hydrolysis can serve as the training drug in drug-discrimination procedures, the present study investigated whether mice will learn to discriminate SA-57 from vehicle. SA-57 inhibits FAAH much more potently than it inhibits MAGL or ABHD6, another serine hydrolase that degrades 2-AG, but to a much less extent than MAGL (Blankman et al., 2007). Thus, SA-57 possesses utility to investigate the consequences of maximally elevating brain AEA levels while dose-dependently increasing brain 2-AG levels (Niphakis et al., 2012). To select the SA-57 training dose, initial experiments examined its dose-effect relationship to substitute for the potent CB_1 receptor agonist CP55,940 in C57BL/6J mice and AEA in FAAH (−/−) mice (to prevent rapid hydrolysis). Having established that mice learn to discriminate SA-57 from vehicle, we then assessed its dose-response relationship and time course. Because various substrates of FAAH (e.g., AEA, palmitoylethanolamide, and oleoylethanolamide and MAGL; e.g., 2-AG) bind CB_1, CB_2, TRPV1 (Smart et al., 2000), and peroxisome proliferator-activated receptor-α (PPARα) receptors (Lo Verme et al., 2005), we tested whether antagonists for these receptors would block the discriminative stimulus effects of SA-57. Additionally, we conducted an extensive series of drug substitution tests to gain further insight into the training dose of the SA-57 discriminative stimulus. Specifically, we tested whether CP55,940, as well as the noncannabinoid psychoactive drugs nicotine and diazepam, would substitute for the SA-57. As MAGL also plays a rate-limiting role in the production of arachidonic acid and prostanoids in brain (Nomura et al., 2011), we examined whether the COX-2 inhibitor valdecoxib, which reduces prostanoid synthesis but does not affect brain endocannabinoid levels, would substitute for SA-57. The final goal of the present study was to elucidate the degree to which relevant endocannabinoid hydrolytic enzyme inhibitors contribute to the SA-57 training dose. Accordingly, we investigated whether individual FAAH, MAGL, and ABHD6 inhibitors, as well as simultaneous inhibition of FAAH and MAGL, would substitute for SA-57.

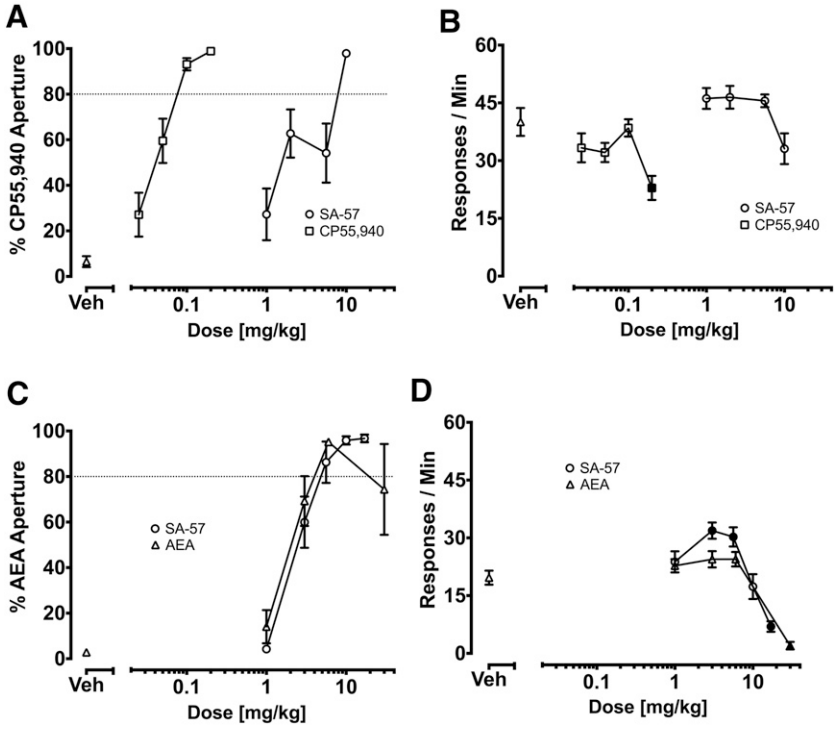

Fig. 1. Effects of CP55,940, AEA, and SA-57 on the percentage of responses in training drug-paired apertures and response rates in C57BL/6J mice trained to discriminate CP55,940 (0.1 mg/kg; (A, B) or FAAH (−/−) mice trained to discriminate AEA (6 mg/kg; C, D). (A) Dose-dependent generalization of CP55,940 and dose-dependent substitution of SA-57 for the CP55,940-discriminative stimulus. The respective ED_{50} (95% confidence interval [CI]) values for CP55,940 generalization and SA-57 substitution in C57BL/6J mice were 0.04 (0.03–0.05) mg/kg and 2.4 (1.6–3.6) mg/kg. (B) CP55,940 (0.2 mg/kg), but not SA-57, significantly decreased rates of responding compared with vehicle. (C) Dose-dependent generalization of AEA and dose-dependent substitution of SA-57. The respective ED_{50} (95% CI) values for AEA and SA-57 in FAAH (−/−) mice were 2.7 (2.3-3.1) mg/kg and 3.1 (2.8–3.4) mg/kg. (D) SA-57 (17 mg/kg) and AEA (30 mg/kg) decreased rates of responding. Values represent mean ± S.E.M. Filled symbols indicate significant difference ($P < 0.001$) versus vehicle; n = 7-10 mice/group.

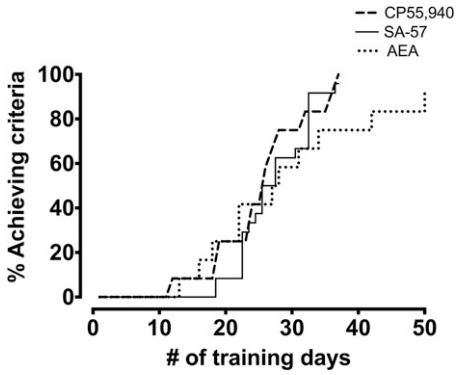

Fig. 2. Acquisition rates of SA-57 (10 mg/kg) in C57BL/6J mice, AEA (10 mg/kg) in FAAH (−/−) mice, and CP55,940 (0.1 mg/kg) in C57BL/6J mice trained in drug discrimination. Values represent the percentage of mice that achieved criteria (see text) across days. n = 24 mice for SA57, 12 for AEA, and 12 for CP55,940.

Materials and Methods

Subjects

Male C57BL6/J mice (Jackson Laboratory; Bar Harbor, ME) and male FAAH (−/−) mice served as subjects. The FAAH (−/−) mice were backcrossed >14 generations on to a C57BL6/J background. The mice were 9–11 weeks of age at the beginning of training and were individually housed in a temperature-controlled (20–22°C) vivarium in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee guidelines. Mice were given water ad libitum and were food-restricted to 85%–90% of free-feed body weight, which was established during a 2-week period of ad libitum food every 6 months.

Drugs

SA-57, MJN110, KT182, KT195, and JZL195 were synthesized in the Cravatt Laboratory, as previously described (Long et al., 2009b; Niphakis et al., 2012, 2013; Hsu et al., 2013). N-arachidonoyl ethanolamine (AEA) was provided by Organix Inc. (Woburn, MA), and valdecoxib was provided by Sigma-Aldrich (Saint Louis, MO). CP55,940, JZL184, PF-3845, rimonabant, and SR144528 were generously supplied by the National Institute on Drug Abuse (NIDA) (Rockville, Maryland). Capsazepine was purchased from Cayman Chemical, and GW6471 was purchased from Tocris Bioscience (Ellisville, MO). Each compound was dissolved in a vehicle consisting of ethanol, emulphor-620 (Rhodia, Cranbury, NJ), and saline in a ratio of 1:1:18. All injections were given via the i.p. route of administration in a volume of 10 μl per 1 g of body weight.

Apparatus

Drug-discrimination was conducted in eight sound-attenuating operant conditioning boxes (18 × 18 × 18 cm) (MED Associates, St. Albans, VT). Each operant box contained two nose-poke apertures and a food dispenser delivering 14-mg food pellets to a receptacle chamber located between apertures. Computer software (MED-PC IV, MED Associates) was used to record nose pokes and to control stimulus presentations and food deliveries.

Drug-Discrimination Paradigm

Training. Separate groups of mice were trained to discriminate each of the following three training drugs from vehicle. Groups 1 and 2 consisted of C57BL6/J mice (n = 8) trained to discriminate CP55,940 and FAAH (−/−) mice (n = 11) trained to discriminate AEA, respectively. The third group of mice consisted of three cohorts of C57BL6/J mice (n = 8/cohort) trained to discriminate SA-57 from vehicle. The treatment conditions for each cohort are described below in the *Testing* section as follows. The pretreatment times for the training drugs were 120 minutes for SA-57 and 30 minutes for CP55,940 and AEA. During each 15-minute training session, both nose-poke apertures were available, but only responses into the correct aperture associated with the appropriate training drug or vehicle resulted in food reinforcement. Each incorrect response reset the response requirement. Injections before training sessions were conducted (Monday–Friday) in a double-alternation sequence of drug (SA-57, CP55,940, or AEA) and vehicle (e.g., vehicle, vehicle, drug, drug).

Testing. Test sessions were scheduled twice per week, with a minimum of 72 hours between test days. To be eligible for testing, subjects were required to meet the following three criteria on nine of the previous 19 consecutive training sessions: 1) correct completion of the first FR10 (i.e., first 10 consecutive responses into the appropriate aperture), 2) ≥80% correct responding, and 3) maintain response rates ≥10 responses/min. During the 15-minute test sessions, responses in either aperture resulted in the delivery of food reinforcement according to an FR10 schedule of reinforcement, without a limitation on the number of reinforcers earned within a session. Before conducting substitution tests, dose-response tests with SA-57, CP55,940, or AEA were conducted to characterize their generalization gradients to their respective discriminative stimulus. For time-course studies, animals were injected with SA-57 (10 mg/kg) and tested 0.25, 1, 2, 4, or 8 hours after injection. To assess whether CB_1 receptors mediated the discriminative effects of SA-57 and the substitution of CP55,940, MJN110, JZL184, and JZL195, we used rimonabant (3 mg/kg; Rinaldi-Carmona et al., 1994). We also examined whether the CB_2 receptor antagonist SR144528 (3 mg/kg; Rinaldi-Carmona et al., 1998), the TRPV1 receptor antagonist capsazepine (5 mg/kg; Kinsey et al., 2009), and the PPARα receptor antagonist GW6471 (2 mg/kg; Lo Verme et al., 2005) would block the discriminative stimulus effects of SA-57. Each antagonist was administered 15 minutes before injections of 10 mg/kg SA-57. The three cohorts of mice trained to discriminate SA-57 were used in the following experiments. All cohorts were included in the SA-57 acquisition curve. Cohort 1 was used in the time-course study, the MJN110 (0.25–5 mg/kg), KT182 (1 and 2 mg/kg), KT195 (40 mg/kg), valdecoxib (10 mg/kg), and

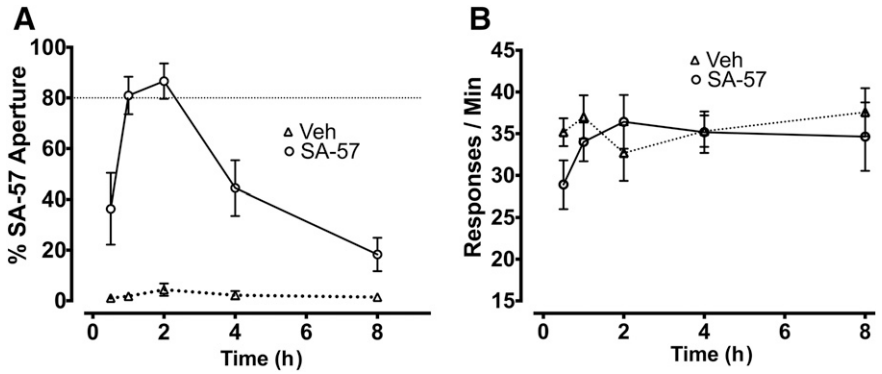

Fig. 3. Time-course effects for occasioning the 10 mg/kg SA-57 training dose. (A) Percentage of responses in the SA-57-associated aperture 0.25, 1, 2, 4, or 8 hours after an injection of vehicle or SA-57 (10 mg/kg). (B) SA-57 did not affect response rates at any time point after administration. Values represent mean ± S.E.M.; n = 7 mice/group.

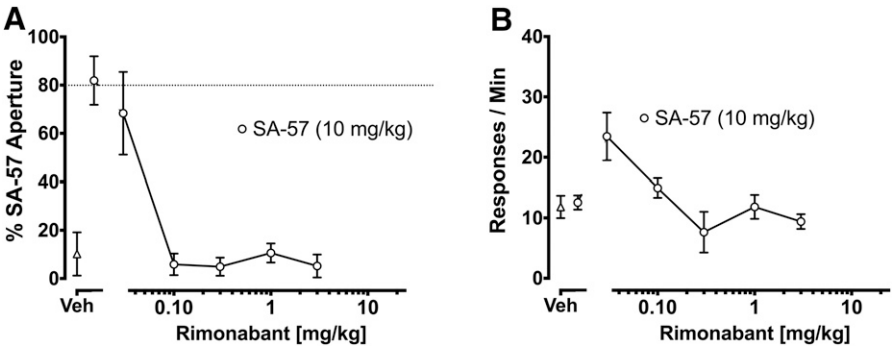

Fig. 4. CB_1 receptors play a necessary role in the SA-57 discriminative stimulus. (A) Rimonabant (0.3–3 mg/kg) significantly attenuated the SA-57 training dose. (B) Rimonabant doses (i.e., 0.1, 0.3, 1, 3 mg/kg) that blocked the SA-57 training dose did not reduce response rates. Values represent mean ± S.E.M.; n = 3–6 mice/group.

MJN110 (2.5 mg/kg) + PF3845 (10 mg/kg) substitution studies; cohort 2 was used to test the psychoactive noncannabinoid drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg) and in substitution tests with JZL195 (2–20 mg/kg), JZL184 (4–100 mg/kg), PF3845 (10 and 30 mg/kg), and URB597 (10 mg/kg); and cohort 3 was used in the receptor antagonist experiments (rimonabant, SR144528, capsazepine, GW6471).

[^{3}H]SR141716A Binding Assay. Cerebella were dissected from adult male ICR mice, stored at −80°C, and membranes were prepared as described previously (Selley et al., 2004). Membrane protein (15 μg) was incubated with 0.94 nM [^{3}H]SR141716A in assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$ and 0.2 mM EGTA) with 0.5% (wt/vol) bovine serum albumin (BSA) in the presence and absence of 5 μM unlabeled SR141716A to determine nonspecific and specific binding, respectively. The assay was incubated for 90 minutes at 30°C and terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that were presoaked in Tris buffer containing 0.5% (wt/vol) BSA (Tris-BSA), followed by five washes with cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency in ScintiSafe Econo 1 scintillation fluid after a 12-hour delay.

Data Analysis. The percentage of drug appropriate responses and response rates (responses/min) were recorded for each experiment. Full substitution was defined as 80% or more nose pokes that occurred into aperture associated with the training drug. Partial substitution was defined as 20% or greater and less than 80% nose pokes in the training drug-paired aperture. Less than 20% nose pokes on the drug-paired aperture was defined as no substitution (Solinas et al., 2006). ED_{50} values (and 95% confidence intervals) for generalization or substitution were calculated using least-squares linear regression analysis. Behavioral data are depicted as mean ± S.E.M. The data were analyzed using one-way or two-way analysis of variance (ANOVA). Dunnett's tests or Bonferroni's post hoc analyses were used after a significant ANOVA for the response rate data. GraphPad Prism 6.0 statistical software (Graph Pad Software, Inc., La Jolla, CA) was used for data analysis.

Binding data were determined in triplicate and are reported as specific binding. Each competition data set was analyzed by one-way ANOVA to determine concentration dependence. Rimonabant competition curves were analyzed by nonlinear regression to determine IC_{50} and Hill coefficients using a four-parameter fit with GraphPad Prism 6.0. The IC_{50} values were then converted to K_i values using the Cheng-Prusoff equation.

Results

SA-57 Substitutes for CP55,940 in C57BL/6J Mice and AEA in FAAH (−/−) Mice. Figure 1 shows that SA-57 fully substituted for CP55,940 and AEA in mice trained to discriminate each of these drugs. C57BL/6J mice administered either CP55,940 or SA-57 completely occasioned the discriminative stimulus effects of CP55,940 (Fig. 1A). SA-57 did not affect response rates; however, CP55,940 significantly reduced response rates [$F(4,55) = 4.7$; $P < 0.01$], with 0.2 mg/kg yielding significant reductions in response rates compared with vehicle (Fig. 1B). In FAAH (−/−) mice trained to discriminate AEA

TABLE 1

Cannaboid 1 receptor (CB_1) receptors mediate the discriminative stimulus effects of the SA-57 (10 mg/kg) training dose

The CB_1 receptor antagonist rimonabant (3 mg/kg) significantly blocked the discriminative stimulus effects of SA-57 (10 mg/kg) as well as substitution of CP55,940 (0.1 mg/kg). The CB_2 receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPARα receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 (10 mg/kg) discriminative stimulus. The vehicle-vehicle and rimonabant-vehicle conditions are the same as those used in Fig. 9. Values represent mean ± S.E.M. n = 6–8 mice/group.

Drug	Antagonist	% SA-57 Substitution ± S.E.M.	Nose Pokes/min ± S.E.M.
Vehicle	Vehicle	12.8 ± 9.4	38.9 ± 3
	Rimonabant	4.0 ± 1.2	24.9 ± 3
	SR144528	0.7 ± 0.3	36.6 ± 3.8
	Capsazepine	1.3 ± 0.4	20.1 ± 2.6
	GW6471	0.3 ± 2.6	24.6 ± 3.1
SA-57	Vehicle	95.7 ± 1.7	27.3 ± 1.9
	Rimonabant	3.4 ± 1.2	20.1 ± 2.5
	SR144528	98 ± 1.5	30.5 ± 5.4
	Capsazepine	86 ± 12.2	15.7 ± 2.9
	GW6471	96.5 ± 1.3	19.0 ± 2
CP55,940	Vehicle	82.5 ± 11	33.1 ± 3.3
	Rimonabant	10.4 ± 5.7	20.7 ± 4.9

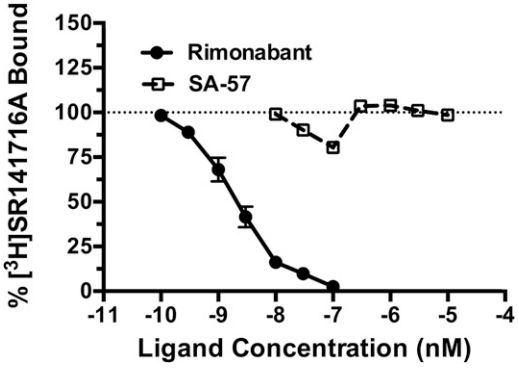

Fig. 5. SA-57 does not compete with [^{3}H]SR141716A binding to CB_1 receptors in mouse cerebellum. Data represent mean [^{3}H]SR141716A bound (pmol/mg) ± S.E.M. in the presence of varying concentrations of rimonabant or SA-57 (n = 3). Specific binding of [^{3}H]SR141716A in the absence of competing ligand was 1.65 ± 0.26 pmol/mg. Similar results were obtained with [^{3}H]CP55,940 binding in membranes prepared from Chinese hamster ovary cells stably expressing the mouse CB_1 receptor, in which concentrations of up to 10 μM SA-57 did not affect binding (data not shown).

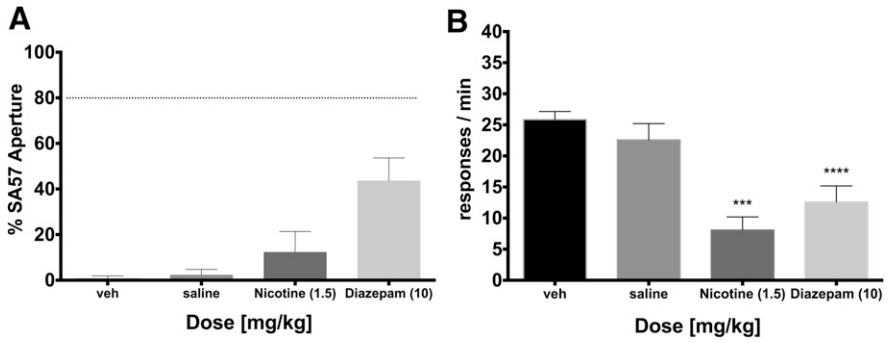

Fig. 6. Substitution experiments of noncannabinoid psychoactive drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg) for the SA-57 training dose. (A) Nicotine did not substitute, whereas diazepam partially substituted for the SA-57 training dose. (B) Nicotine (1.5 mg/kg) and diazepam (10 mg/kg) significantly reduced the rates of responding. Values represent mean ± S.E.M. Asterisks indicate significant difference ($P < 0.05$) versus vehicle; n = 7 to 8 mice/group.

(6 mg/kg) from vehicle, SA-57 also fully substituted for AEA (Fig. 1C). FAAH (−/−) mice administered AEA (1-30 mg/kg) or SA-57 (1–10 mg/kg) dose dependently selected the aperture associated with AEA (Fig. 1C). Both AEA [F (4, 50) = 27.5; $P <$ 0.001] and SA-57 [F (5, 46) = 15.27; $P < 0.001$] significantly reduced response rates (Fig. 1D). The highest doses tested of AEA (i.e., 30 mg/kg) and SA-57 (i.e., 17 mg/kg) significantly depressed response rates compared with vehicle in FAAH (−/−) mice.

SA-57 Discriminative Stimulus. Because 10 mg/kg SA-57 fully substituted for CP55,940 in C57BL/6J mice and for FAAH (−/−) mice, this dose of SA-57 was selected as the training dose in three naïve cohorts of mice (n = 8 mice/group). As shown in Fig. 2, 50% of mice achieved the criteria to discriminate SA-57 from vehicle by the 27th training session, and 23 of 24 mice acquired the discrimination by day 40. The final mouse achieved criteria on day 74 of training but was excluded from subsequent experiments because of its substantial delay in acquisition. Similar rates of acquisition were found for CP55,940 in C57BL/6J mice and AEA in FAAH (−/−) mice.

Figure 3 shows the time-course effects of 10 mg/kg SA-57 versus vehicle for selecting the aperture associated with SA-57 (Fig. 3A) and response rates (Fig. 3B). Whereas mice that received vehicle responded consistently on the vehicle-associated aperture at each of the time points, mice administered 10 mg/kg SA-57 selected the SA-57 aperture ≥80% at 1 and 2 hours postinjection, showed partial substitution at 0.25 and 4 hours and responded predominantly on the vehicle aperture 8 hours after injection. No differences were found in the rates of responding between mice injected with vehicle or SA-57 at any time point (Fig. 3B; $P = 0.48$).

As shown in Fig. 4, the CB_1 receptor antagonist rimonabant (0.03–3 mg/kg) significantly blocked the SA-57 training dose. In contrast, the CB_2 receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPARα receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 training dose (Table 1).

SA-57 Does Not Bind CB_1 Receptors. As the SA-57 discriminative stimulus required CB_1 receptor activation, we next examined whether this compound interacts directly with CB_1 receptors. Accordingly, we tested whether SA-57 would displace [^{3}H]SR141716A binding in mouse cerebellar membranes. As shown in Fig. 5, rimonabant (i.e., unlabeled SR141716A) inhibited [^{3}H]SR141716A binding in a concentration-dependent manner ($P < 0.001$, F = 17.36, df = 7), with a K_i value of 0.75 ± 0.16 nM and a Hill coefficient of 0.97 ± 0.08. In contrast, SA-57 (0.01–10 μM) did not inhibit [^{3}H]SR141716A binding ($P = 0.96$; Fig. 5), indicating that this compound does not directly interact with CB_1 receptors.

Substitution Tests in SA-57 Discriminating Mice. We next tested whether the noncannabinoid, psychoactive compounds nicotine and diazepam would substitute for SA-57. As shown in Fig. 6A, nicotine did not substitute for SA-57, but diazepam produced partial substitution. Both drugs significantly reduced response rates [Fig. 6B; F (3,28) = 14.01; $P <$ 0.001], demonstrating that behaviorally active doses were reached.

Figure 7 shows the dose-effect curves of the mixed CB_1/CB_2 receptor agonist CP55,940, the dual FAAH-MAGL inhibitor JZL195, and SA-57 in mice trained to discriminate SA-57 (10 mg/kg) from vehicle. CP55,940, JZL195, and SA-57 produced dose-related responding into the aperture associated with SA-57 (Fig. 7A). CP55,940 [F (3,28) = 2.99, $P < 0.05$] and SA-57 [F (4,42) = 2.78, $P < 0.05$], but not JZL195, reduced response rates (Fig. 7B).

SA-57 generalized to itself in a dose-dependent fashion, and the MAGL inhibitors MJN110 and JZL184 dose dependently

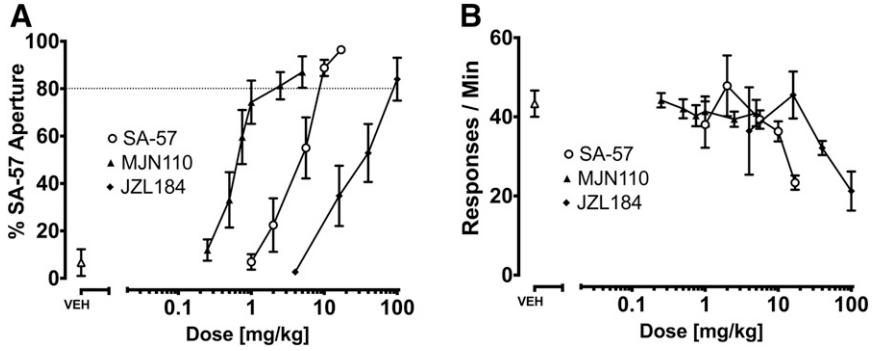

Fig. 7. Evaluation of the dose-response relationships of SA-57, CP55,940, and JZL195 to occasion the SA-57 (10 mg/kg) discriminative stimulus. (A) SA-57 produced dose-dependent generalization, and CP55,940 and JZL195 dose dependently substituted for SA-57. The respective ED_{50} (95% CI) values for CP55,940 substitution, JZL195, and SA-57 generalization were 0.096 (0.076–0.121) mg/kg, 6.2 (3.5–10.9) mg/kg, and 4.4 (3.5–5.4) mg/kg. (B) High doses of CP55,940 (0.2 mg/kg) or SA-57 (17 mg/kg) significantly reduced response rates. Values represent mean ± S.E.M. Filled symbols indicate significant difference ($P < 0.05$) versus vehicle; n = 7 or 8 mice/group.

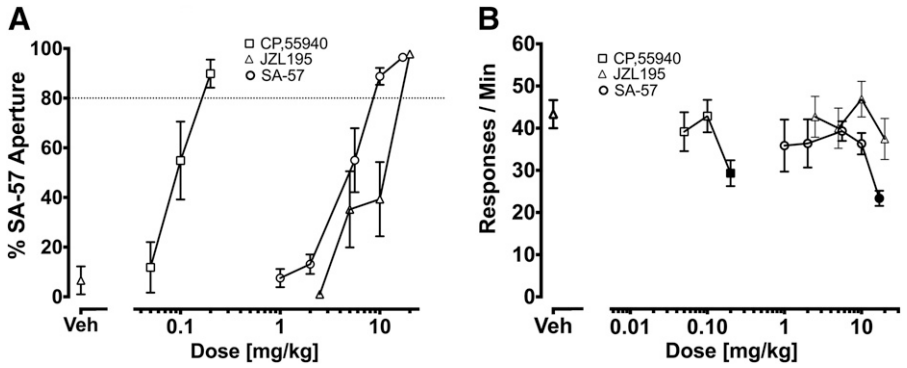

Fig. 8. Evaluation of the dose-response relationships of SA-57, MJN110, and JZL184 to occasion the SA-57 (10 mg/kg) stimulus. (A) Dose-dependent generalization of SA-57 and dose-dependent substitution of MJN110 and JZL184. The respective ED_{50} (95% CI) values for MJN110 and JZL184 generalization and SA-57 substitution in C57BL/6J mice were 0.77 (0.53–1.1) mg/kg and 20.44 (11–37.97) mg/kg, and 4.39 (3.53–5.45) mg/kg. (B) SA-57 (17 mg/kg) and JZL184 (100 mg/kg) significantly decreased the rates of responding. Values represent mean ± S.E.M. **Significant difference ($P < 0.001$) versus vehicle; $n = 7$ or 8 mice/group.

substituted for SA-57 (Fig. 8A). Although MJN110 did not affect response rates [F (6, 48) = 0.33, $P = 0.92$], the highest doses of SA-57 (17 mg/kg) [F (5, 42) = 3.391, $P < 0.05$] and JZL184 (100 mg/kg) [F (4, 18) = 3.985, $P < 0.05$] significantly reduced response rates (Fig. 8B). As shown in Fig. 9A, rimonabant (3 mg/kg) completely blocked substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg), and JZL195 (20 mg/kg) for the SA-57 training dose. Also, rimonabant significantly reduced rates of responding [Fig. 9B; F (1, 29) = 11.91, $P < 0.01$].

In contrast, mice administered high doses of the FAAH inhibitors PF-3845 (10 and 30 mg/kg) and URB597 (10 mg/kg) selected the vehicle aperture (Table 2). Likewise, mice given high doses of the ABHD6 inhibitors KT182 (1 and 2 mg/kg) or KT195 (40 mg/kg), as well as mice given high dose of the selective cyclooxygenase-2 inhibitor valdecoxib (10 mg/kg), selected the vehicle aperture.

Because MAGL inhibitors, but not FAAH inhibitors, substituted for SA-57, we next examined whether full FAAH inhibition would elicit a leftward shift in the MAGL substitution dose-response curve. Accordingly, we tested the dose-response relationship of MJN110 with PF3845 (10 mg/kg) or vehicle for substitution in mice trained to discriminate SA-57 from vehicle. As shown in Fig. 10A, PF-3845 elicited a significant leftward shift in the MJN110 substitution dose-response curve [potency ratio (95% CL) = 1.84 (1.3–2.8)]. No significant changes were found for response rates (Fig. 10B).

Discussion

The present study demonstrates that mice readily learn to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle. Specifically, most (i.e., 23 of 24) subjects learned to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle within 40 training sessions. The 10 mg/kg SA-57 training dose was previously demonstrated to produce significant increases in the brain levels of AEA and 2-AG (Wiebelhaus et al., 2015). As SA-57 fully blocks FAAH activity at lower doses (0.05–1 mg/kg) than those required to inhibit MAGL (1.25–12.5 mg/kg) (Niphakis et al., 2012) it provided a useful tool to examine the consequences of full FAAH inhibition while incrementally elevating brain 2-AG. The observation that 1 mg/kg SA-57, which produces maximal increases in endogenous AEA without detectable increases in 2-AG (Niphakis et al., 2012), did not generalize to the training dose (10 mg/kg SA-57) indicates that FAAH inhibition alone is not sufficient to occasion to the SA-57 training dose. Similarly, neither FAAH inhibitor (i.e., PF-3845 or URB597) substituted for SA-57. In contrast, the dual FAAH-MAGL inhibitor JZL195 and two MAGL inhibitors, MJN110 and JZL184, fully substituted for the SA-57 training dose, suggesting that MAGL inhibition alone may be sufficient for generalization to the 10 mg/kg SA-57 training dose. Interestingly, PF-3845 produced an approximately 2-fold leftward shift in the MJN110 substitution dose-response curve. The observation that rimonabant completely blocked the discriminative stimulus effects of SA-57 indicates that CB_1 receptors play a necessary role in the subjective effects of SA-57. Similarly, rimonabant completely blocked the substitution of both MAGL inhibitors (MJN110 and JZL184) and the dual FAAH-MAGL inhibitor JZL195. These findings suggest that elevating endocannabinoid brain levels through the simultaneous blockade of FAAH and MAGL produces a CB_1 receptor mediated interoceptive stimulus.

Consistent with previous studies reporting that SA-57 or the dual FAAH-MAGL inhibitor JZL195 substitute for the THC discriminative stimulus (Long et al., 2009b; Hruba et al., 2015; Walentiny et al., 2015), we found that SA-57 (10 mg/kg) fully substituted for the discriminative stimulus effects of the potent cannabinoid receptor agonist CP55,940 in C57BL/6J mice and the endogenous cannabinoid AEA (6 mg/kg) in FAAH (−/−) mice. The potency of SA-57 in producing a discriminative

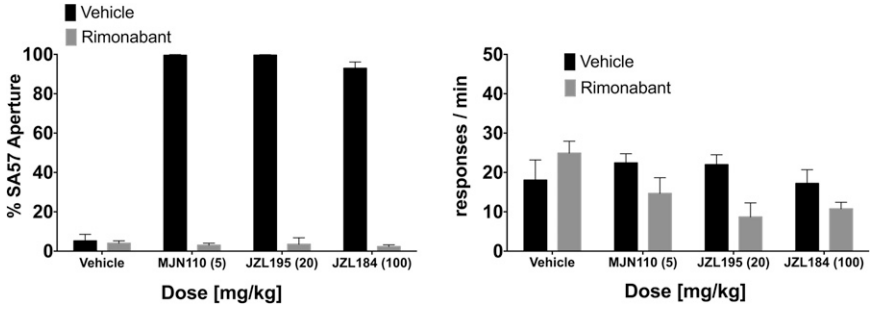

Fig. 9. Substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg), and JZL195 (20 mg/kg) for SA-57 (10 mg/kg) requires CB_1 receptors. (A) Rimonabant (3 mg/kg) completely blocked MJN110, JZL184, and JZL195 substitution. (B) Rimonabant did affect response rates. Values represent mean ± S.E.M.; $n = 7$ or 8 mice/group.

TABLE 2

Fatty acid amide hydrolase (FAAH) inhibitors (PF-3845 and URB597), ABHD6 inhibitors (KT182 and KT195), and the cyclooxygenase-2 (COX2) selective inhibitor valdecoxib do not substitute for the discriminative stimulus effects of SA-57 (10 mg/kg) in C57BL/6J mice and do not affect response rates[a]

Enzyme	Drug (mg/kg)	% SA-57 Substitution	Nose Pokes/Min
	mg/kg	±*S.E.M.*	±*S.E.M.*
FAAH	Vehicle	0.6 ± 0.5	47.5 ± 4.9
	PF-3845 (10)	1.5 ± 0.5	34.4 ± 3.8
	PF-3845 (30)	0.7 ± 0.2	38.9 ± 3.5
	URB597 (10)	2.1 ± 1.0	36.9 ± 5.6
ABHD6	Vehicle	1.1 ± 0.6	46.5 ± 2.6
	KT182 (1)	1.4 ± 0.7	41.1 ± 3.5
	KT182 (2)	1.4 ± 0.8	43.5 ± 2.4
	KT195 (40)	0.8 ± 0.3	38.1 ± 3.1
COX2	Vehicle	1.1 ± 0.6	46.5 ± 2.6
	Valdecoxib (10)	1.1 ± 0.7	31.9 ± 3.9

[a]Values represent mean ± S.E.M.; n = 7 or 8 mice/group.

stimulus was similar to its potency in substituting for either CP55,940 or AEA. Furthermore, SA-57's discriminative stimulus effects occurred at a training dose known to produce maximal increases in AEA and 2-AG (Niphakis et al., 2012). In addition, CP55,940 fully substituted for SA-57, an effect that was completely blocked by rimonabant, further implicating a pivotal role of CB_1 receptors in these effects. Similarly, the dual FAAH-MAGL inhibitor JZL195 dose dependently substituted for SA-57. Time-course investigation revealed that SA-57 partially generalized at 0.5 hour, fully generalized at 1 and 2 hours, partially generalized at 4 hours, and by 8 hours, mice responded mostly on the aperture paired with vehicle.

It is noteworthy that MJN110 and JZL184 fully substituted for the discriminative stimulus effects of SA-57, whereas mice treated with a low dose of SA-57 (which inhibits FAAH and not MAGL), URB597, or PF-3845 selected the vehicle aperture. These findings suggest that MAGL inhibition represents a driving force underlying the SA-57 training dose; however, the observation that PF-3845 increased the potency of MJN110 to substitute for SA-57 suggests that FAAH inhibition increases the effectiveness of the discriminative stimulus produced by MAGL inhibition alone. The fact that 2-AG levels are approximately three orders of magnitude higher than AEA levels in wild-type mouse brain (Ahn et al., 2009; Long et al., 2009b) is consistent with the notion that MAGL inhibition elicits more prominent pharmacologic effects than those produced by FAAH inhibition. Moreover, as FAAH is expressed on the postsynaptic terminal (Gulyas et al., 2004) and MAGL (Dinh et al., 2002) is expressed on the pre-synaptic terminal, it is plausible that AEA and 2-AG activate distinct CB_1 receptor–mediated neuronal circuits.

Because AEA and 2-AG bind CB_1 and CB_2 receptors, AEA also binds TRPV1 receptors, and other FAAH substrates (i.e., palmitoylethanolamide and oleoylethanolamide) bind PPARα receptors (Lo Verme et al., 2005), we examined whether selective antagonists for each of these receptors would block the discriminative stimulus effects of SA-57. Rimonabant, but not the other receptor antagonists, completely blocked the discriminative stimulus effects of SA-57. These findings indicate that CB_1 receptor activation is required for the subjective effects of SA-57, whereas CB_2, TRPV1, and PPARα receptors are dispensable. Moreover, the fact that SA-57 did not affect ligand binding to CB_1 receptors in either a competitive or noncompetitive manner is consistent with the hypothesis that it increases brain endocannabinoid levels that then elicit a CB_1 receptor-mediated discriminative stimulus.

The present study also assessed whether a variety of psychoactive noncannabinoid drugs would substitute for SA-57. Specifically, nicotine did not substitute for the SA-57 training dose, although it significantly reduced response rates. In contrast, diazepam partially substituted for SA-57, but it did so at a dose that reduced response rates. Similarly, diazepam partially substitutes for THC at high doses that produce motor impairment in the rat drug-discrimination paradigm (Wiley and Martin, 1999). Taken together, these studies suggest the possibility of a potential GABAergic component for CB_1 receptor–mediated discriminative stimuli. In addition, because MAGL inhibition reduces brain levels of arachidonic acid as well as various prostanoids (Nomura et al., 2011), we tested whether the cyclooyygenase-2 inhibitor valdecoxib would substitute for SA-57; however, valdecoxib was devoid of action in this assay, suggesting that prostaglandins do not play a necessary role in the discriminative effects of SA-57.

It is noteworthy that the combined inhibition of FAAH and MAGL attenuates somatic signs of opioid withdrawal (Ramesh et al., 2011); however, simultaneous blockade of these enzymes also elicits other cannabimimetic effects, as assessed in the tetrad assay, including hypomotility, antinociception, catalepsy, and hypothermia (Long et al., 2009a; Anderson et al., 2014; Ghosh et al., 2015), as well as impaired performance in a Morris water maze spatial memory task (Wise et al., 2012). These effects of dual FAAH and MAGL inhibition are similar to those of THC, whereas single inhibition of either enzyme produces a decreased spectrum and

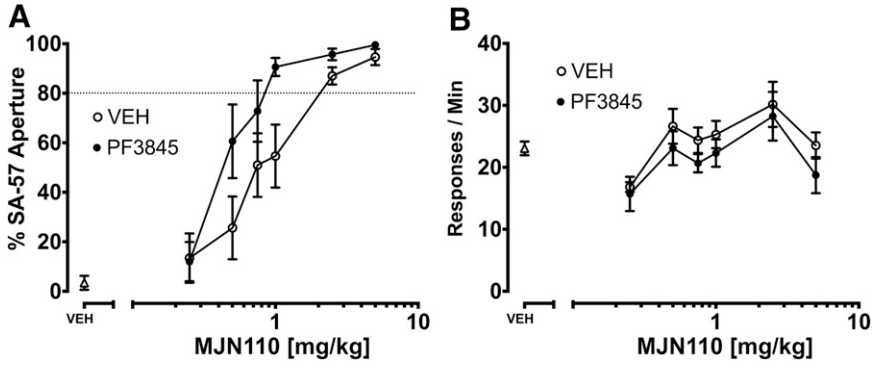

Fig. 10. The FAAH inhibitor PF-3845 augments the MJN110 substitution dose-response curve for SA-57 (10 mg/kg). (A) PF-3845 (10 mg/kg) produced a leftward shift of the MJN110 substitution dose-response curve. (B) None of the drug combinations significantly decreased the rates of responding. Values represent mean ± S.E.M.; n = 7 or 8 mice/group.

magnitude of cannabimimetic effects. Specifically, the MAGL inhibitor JZL184 produces antinociception, hypomotility, and dysregulation of thermoregulation when challenged with manipulations that elicit hypothermia (Nass et al., 2015), whereas FAAH inhibition produces antinociception but not other cannabimimetic effects (Long et al., 2009b). However, drug-discrimination is more sensitive in detecting cannabimimetic effects compared with the tetrad assay. For example, THC is more potent in producing its discriminative stimulus effects than in eliciting the full set of tetrad effects (Long et al., 2009b; Marshell et al., 2014). Given that dual blockade of FAAH and MAGL significantly reduces locomotor activity (Long et al., 2009b) and SA-57 (10 mg/kg) completely inhibits FAAH and MAGL activity (Niphakis et al., 2012), the lack of any rate of suppressive effects of the training dose of SA-57 is interesting. Similarly, THC (5.6 mg/kg) reduces locomotor activity, but it does not reduce response rates in a drug-discrimination procedure (Wiley et al., 2005). This lack of apparent motor depression is consistent with the idea that rate-suppressive effects of drugs undergo tolerance throughout the course of drug-discrimination training (Solinas et al., 2006).

The results of the present study suggest that SA-57 serves as a discriminative stimulus at doses that produce increased levels of both AEA and 2-AG through a CB_1 receptor mechanism of action, although elevated levels of 2-AG may be the main driving force for the SA-57 training dose. Although the brain regions mediating the discriminative stimulus effects of SA-57 and cannabinoid receptor agonists are unknown, it is noteworthy that endogenous cannabinoids and their receptors are located in neural pathways mediating the reinforcing effects of drugs of abuse (i.e., mesolimbic dopamine pathway) (Oleson and Cheer, 2012).

In conclusion, the present study demonstrates that the dual FAAH-MAGL inhibitor SA-57 serves as a reliable discriminative stimulus. The observations that rimonabant completely blocks the SA-57 training dose and that mice trained to discriminate SA-57, CP55,940, and AEA show symmetrical substitution strongly implicate the importance of the CB_1 receptor in this novel interoceptive stimulus. Collectively, these findings raise the provocative possibility that FAAH and MAGL serve as dual brakes to prevent the psychoactive consequences of CB_1 receptor overstimulation caused by elevated levels of AEA and 2-AG.

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Authorship Contributions

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Conducted experiments: Owens, Mustafa, Jali.

Contributed new reagents or analytic tools: Niphakis, Cravatt.

Performed data analysis: Owens, Mustafa, Selley, Lichtman.

Wrote or contributed to the writing of the manuscript: Owens, Ignatowska-Jankowska, Mustafa, Wiley, Beardsley, Selley, Lichtman.

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