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Discriminative stimulus functions of methanandamide and Δ^9 -THC in rats: tests with aminoalkylindoles (WIN55,212-2 and AM678) and ethanol

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Abstract

Objective—The aim of the study was to characterize in vivo the aminoalkylindoles WIN55,212-2 (WIN) and AM678 (naphthalen-1-yl(1-pentyl-1*H*-indol-3-yl)methanone) as cannabinoid receptor (CB₁R) ligands using drug discrimination. Tests also involved Δ^9 -tetrahydrocannabinol (THC) and *R*-(+)-methanandamide (mAEA), a metabolically stable analog of the endogenous ligand anandamide, as well as the CB₁R selective antagonist/inverse agonist rimonabant; tests with ethanol assessed pharmacological specificity. We used two different drug discriminations (mAEA and THC) allowing us to explore potential differences in CB₁R activation which could be attributed to variations in their respective CB₁R signaling mechanisms.

Methods—There were two concurrently trained groups of rats. One group discriminated between i.p. injected vehicle and 10 mg/kg mAEA. The other group was trained to discriminate between vehicle and 1.8 mg/kg THC.

Results—Dose generalization curves for AM678, WIN55,212-2, THC, and mAEA suggested the following rank order of potency: AM678>WIN55,212-2 THC>mAEA in both drug discrimination groups. Challenge by 1 mg/kg rimonabant resulted in shifts to the right of the generalization curves for the two aminoalkylindoles (4.4-fold for AM678 and 11.3-fold for WIN in the mAEA group, whereas for the THC group, the corresponding values were 13 and 2.6, respectively), suggesting surmountable antagonism. Ethanol did not generalize in either of the two groups, suggesting pharmacological specificity.

Conclusion—Data are congruent with the general observation that there is substantial overlap in the discriminative stimulus effects of CB₁R ligands across different chemical classes. However,

the quantitative differences in the interactions between the two aminoalkylindoles and rimonabant in the two discrimination groups suggest subtle variations in the ligand–receptor activation(s).

Keywords

Δ^9 -THC; *R*-(+)-Methanandamide; Aminoalkylindoles; AM678; WIN55,212-2; Rimonabant; Drug discrimination; Rats

Introduction

An interesting aspect of cannabinoid research is the considerable structural diversity of the known classes of ligands affecting the endocannabinoid signaling system. These include but are not limited to (1) classical (tricyclic, e.g., Δ^9 -THC (THC)) and nonclassical (bicyclic, e.g., CP55,940) cannabinergics acting as agonists on either cannabinoid receptor 1 (CB₁R) and 2 (CB₂R), (2) aminoalkylindoles acting as CB₁R and CB₂R agonists (e.g., WIN55,212-2 (WIN)) or alternatively as CB₂R antagonists (e.g., AM630), (3) anandamide (arachidonoyl ethanol-amine, AEA) analogs as CB₁R selective agonists (e.g., methanandamide, mAEA), (4) 2-arachidonoylglycerol analogs as CB₁R agonists, (5) pyrazole analogs acting as CB₁R and CB₂R antagonists/inverse agonists (e.g., rimonabant), and (6) diaryl ether sulfonate CB₁R/CB₂R agonists (for overview, see Thakur et al. 2005). Thus, several structural chemotypes have been used as templates for designing novel ligands to probe the endocannabinoid system regarding the (patho)physiological consequences of CB₁R/CB₂R activation/inactivation.

The pharmacological effects produced by ligands belonging to different chemical classes appear to display considerable overlap. However, there are also subtle but clear differences in the pharmacological profiles of these ligands (see Howlett (2004) for overview). Existing evidence indicates that many G-coupled protein receptors (GPCRs) including the cannabinoid receptors may be activated through multiple signaling mechanisms (e.g., G_{i/o}, G_s, G_q) and that different classes of ligands may engage different signaling pathways. Elucidation of these differences will help to further probe the mechanism(s) of cannabinoid activity. Alternatively, CB₁R and CB₂R agonists, especially those that are structurally related to AEA, may also interact with other GPCRs such as the vanilloid type-1 receptor (TRPV1) or the recently deorphanized GPR55 (Brown 2007; Howlett 2004; Pacher et al. 2006; Oz 2006 for reviews). Identifying these differences in receptor activation and subsequent downstream signaling might provide clues for more rational drug (D) design through the development of novel ligands with enhanced pharmacological selectivity and specificity (for further discussion(s) on ligand/receptor interaction(s) and signaling pathways, see, e.g., Wallace et al. 2009 and Glass and Northup (1999) concerning cannabinoids and Saidak et al. 2006 regarding opioids).

The most commonly employed aminoalkylindole ligand in cannabinoid research is WIN. Although to date there are numerous published data, Compton et al. (1992) in an early in vivo study pointed out differences between cannabinergic aminoalkylindoles (including WIN) and THC using drug discrimination (see also Pério et al. (1996) using WIN as the training cue). Drug discrimination is a pharmacologically selective behavioral procedure commonly used as an animal model of “perceived” (or “subjective”) effects of psychoactive agents (Solinas et al. 2006).

This study used drug discrimination to establish 10 mg/kg mAEA (“endogenous” ligand) and 1.8 mg/kg THC (“exogenous” ligand) as cues for two groups of rats. The above two ligands mostly substitute for each other’s discriminative stimulus effects (Järbe et al. 1998, 2000). However, in earlier work, we also pointed out differences in their pharmacological

effects (e.g., Järbe et al. 2001, 2003a, b, 2009). Using the two different discriminations allows us to explore in more detail potential differences in CB₁R activation which could be attributed to variations in ligand–receptor binding interaction(s) that may reflect differences in signaling mechanisms. Once reliably discriminating the training drug condition from vehicle (V), other doses and drugs were examined. Given that in vivo effects of cannabinergic aminoalkylindoles other than WIN is scanty and particularly in view that WIN has been shown to also interact with noncannabinoid binding sites (see Pertwee 2008), the current study included the high affinity CB₁R selective agonist AM678 for direct comparison with WIN, both when administered alone and when combined with the selective CB₁R antagonist/inverse agonist rimonabant. Cannabinergic indole ligands like WIN55,212-2 and AM678 appear to interact with CB₁R differently than, e.g., THC and CP55,940 (e.g., Huffman et al. 2003). Ethanol was included primarily as a positive control in this study. The effects of ethanol ingestion have, however, been linked to the endocannabinoid system. For example, cannabinoid receptor activation appears involved in inducing pharmacodynamic tolerance to ethanol and CB₁R agonists promote ethanol drinking in rodents; conversely, rimonabant reduces ethanol intake (Basavarajappa and Hungund 2005). Thus, the general hypothesis for these studies is that different CB₁R agonists may activate the receptor(s) through different signaling pathways (e.g., different G proteins).

Materials and methods

Animals

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA) were individually housed in a colony room with an average temperature of 20°C and a 12-h light/dark cycle (rats were trained and tested during the light phase). Animals (≈90 days old at the beginning of the study) were experimentally naïve at the time of shaping the lever pressing response (see below). The average ($\pm$ standard error of the mean (SEM)) weights of the rats were 306.5 ($\pm$ 3.3) g (mAEA) and 293.4 ($\pm$ 2.8) g (THC) at the beginning and 461.8 ($\pm$ 8.8) g (mAEA) and 387.5 g (THC) at the end of the study. Postsession supplemental feeding with Harlan Rat Chow® (# 2018) was restricted to approximately 12 to 14 g/day. All procedures were approved by the Animal Care and Use Committee of Temple University, Philadelphia, PA, USA. The *Principles of Animal Laboratory Care* (National Institutes of Health 1996) was followed.

Apparatus

Training and testing occurred in eight chambers (ENV-001, Med. Associates, St Albans, VT, USA) equipped with two nonretractable response levers, house and lever lights, and a grid floor. Each chamber was enclosed within sound- and light-attenuating boxes with an exhaust fan and interfaced with a DOS/Windows compatible computer. Response contingencies were programmed using Med-PC software (v. 1.16; Med. Associates).

Training

Rats were trained to eat food pellets (45 mg, BioServe®) from a food receptacle located midway between the two response levers and shaped to lever press for food until they responded ten times for each reinforcer (fixed ratio 10 schedule of reinforcement; FR-10). Under our conditions, when the house light was off and the stimulus lights above the response levers being lit, completion of ten presses on the state-appropriate lever resulted in the delivery of two 45-mg food pellets, followed by a 10-s timeout period with only the house light on. At the end of the 10-s timeout period, the stimulus lights above the levers were lit, the house light was turned off, and the FR-10 schedule of reinforcement contingency reinstated. Sessions ended by all lights in the box being turned off.

Once daily, beginning 20 min after i.p. injection, the rats were trained in this two-choice task to respond on drug- or vehicle-appropriate levers. The position of drug-appropriate levers was randomly assigned among subjects so that it was to the right of the food cup for half the subjects and left for the other half. Throughout the session, the aforementioned FR-10 schedule of reinforcement was in effect. Presses on the incorrect lever were recorded, but had no programmed consequences. The order of drug or vehicle administrations was nonsystematic, with no more than two consecutive drug or vehicle sessions. Approximately an equal number of drug and vehicle training sessions occurred throughout the study. To avoid the potential influence of odor cues left in a chamber by a preceding subject, the order in which drug and vehicle training sessions were conducted for animals trained in the same chamber was randomized (Extance and Goudie 1981). Training took place Monday through Friday and lasted 20 min. Training continued until and beyond animals reached the acquisition criterion of selecting the lever appropriate for the training condition on at least eight out of ten consecutive training days. Correct selection was defined as total presses before the first reinforcement (FRF) being equal to or less than 14 (i.e., the incorrect lever not pressed more than four times before completing ten responses on the lever appropriate for the prevailing training condition; FRF $\leq$ 14).

Testing

Once stable, drug discriminations were achieved (see “Results” section), and test (T) sessions were conducted on average three times every 2 weeks; on interim days, regular D or V training sessions of 20-min duration took place. Approximately 2 weeks before initial testing, animals began receiving two i.p. injections 20 min before the training sessions (i.e., drug and vehicle, or vehicle and vehicle) to accustom the animals to a double injection procedure such as that used for antagonism testing. Typically, the order of sessions was D, V, T, V, D (week 1); V, T, V, D, T (week 2); V, D, T, D, V (week 3); and D, T, D, V, T (week 4). Tests were conducted only if responding during the preceding training sessions had been correct (FRF $\leq$ 14) during the initial six FR-10 cycles of the session. If incorrect, animals were retrained for at least three sessions where FRF $\leq$ 14 before additional testing took place. In test sessions, food pellets were delivered for ten presses on either lever for six reinforcement cycles or until 20 min had elapsed, whichever occurred first. There was one session per test day. Doses and drugs were examined in a mixed order. For each dose tested, the percentage of responding on the drug-appropriate lever was calculated from the ratio of the number of presses on the drug (mAEA in one group and THC in the other group)-associated lever to the total number of lever presses in a test session (excluding responding during the timeout periods). Only data for animals receiving at least one reinforcer during the test session were considered for this measure, i.e., animals must have made a minimum of ten presses on one of the two levers. Additionally, response rate (responses per second) across all subjects was calculated. This measure was based on the performance of all animals, including nonresponders. Responding during timeout periods was not included in the rate data.

Statistics

Response rate was averaged ($\pm$ SEM) among rats and plotted as a function of dose. The effects of a drug on response rate were considered significant when the mean rate of responding was not within the 95% confidence limits ($\pm$ 95% CL) of the mean control response rate. This was defined in individual rats as the mean response rate pertaining to the initial six reinforcement cycles calculated from vehicle training sessions in which the criteria for testing were met. The dotted horizontal lines in the graphs presented in the “Results” section represent the $\pm$ 95% CL of the vehicle rate immediately preceding the tests in question. Data points outside these dotted lines are considered significant.

Linear regression analyses of dose generalization and antagonism data after log-dose transformation were performed using Prism 5 software (v. 5.0, GraphPad Software, San Diego, CA, USA; www.graphpad.com) to provide ED₅₀ estimates and their $\pm 95\%$ CL; data were entered as the mean, SEM, and the number of observations (*N*). Using the *F*-test, the Prism program estimates if slopes are parallel or not and if parallel evaluates whether the intercepts are equal or not (a measure of potency). All data shown in “Results” section were obtained from test sessions.

Drugs

mAEA ((5*Z*,8*Z*,11*Z*)-*N*-((*R*)-1-hydroxypropan-2-yl)eicosa-5,8,11,14-tetraenamide; K_i (CB₁)=28 nM; K_i (CB₂)= 867 nM), synthesized according to Abadji et al. (1994) and sent to the site of behavioral evaluation in argon capped vials on a monthly basis. This shipment schedule was implemented to minimize the likelihood of drug decomposition over time. Upon arrival, mAEA was dissolved in ethanol, appropriate amounts withdrawn, the ethanol evaporated under a stream of nitrogen, the residue dissolved (*w/v*) in a solution of propylene glycol (PG) and Tween-80 (T-80), and stored at -20°C . Shortly before being used, the solute was diluted with normal (0.9%) saline after the solute had been sonicated for 20–30 min. This procedure was followed for preparing suspensions of THC as well. The levo isomer of Δ^9 -THC (6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6*H*-benzo[*c*]chromen-1-ol), dissolved in ethanol (200 mg/ml), was kindly provided by the National Institute on Drug Abuse (NIDA; Bethesda, MD, USA) and stored at -20°C until used. Rimonabant, as the base (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) was also provided by NIDA and stored refrigerated at 4°C before being dissolved in the PG/T-80 (*v/v*) mixture (final suspension 5/3% for all cannabinergic ligands) before being diluted with saline (92%). AM678 (naphthalene-1-yl(1-pentyl-1*H*-indol-3-yl)methanone; K_i (CB₁)=4.5 nM; K_i (CB₂)=33.6 nM; M_w =341) is a crystalline compound that was handled the same way as rimonabant and WIN. The ligands mAEA and AM678 were synthesized in the Center for Drug Discovery, University of Connecticut at Storrs, and (*R*)-(+)-WIN55,212-2 mesylate ((*R*)-(5-methyl-3-(morpholinomethyl)-2,3-dihydro-[1,4]oxazino[2,3,4-*h*] indol-6-yl)(naphthalene-1-yl)methanone; K_i (CB₁) = 1.9 nM; K_i (CB₂)=0.3 nM; purity 98%) was purchased from Biomol (Plymouth Meeting, PA, USA; for structures, see Fig. 1 (K_i values for THC and WIN were obtained from Thakur et al. 2005)). Cannabinergic ligand doses were administered i.p. in a volume of 2 ml/kg (THC, AM678, WIN, and rimonabant) or 3 ml/kg (mAEA). Suspensions were prepared fresh daily just prior to administration. Ethanol (95%) was purchased from Temple University hospital (Philadelphia, PA, USA) and diluted with physiological saline (*w/v*) and administered i.p. in volumes of 3 (0.3 g), 5.6 (0.56 g), and 10 (1 g) ml/kg. Drugs were administered 20 min (mAEA, THC and ethanol) or 30 min (WIN, AM678 and rimonabant) prior to testing. Doses are expressed as the forms indicated.

Results

Acquisition of the drug discriminations

mAEA Rats required an average ($\pm$ SEM) of 29 ($\pm$ 1.2) training sessions with the presence/absence of 10 mg/kg mAEA to fulfill the eight out of ten acquisition criterion (range 24 to 38 sessions). However, testing did not commence until all the animals had been trained the mAEA drug discrimination for 68 sessions. Although there initially were 12 animals in the group, one rat died of unknown cause(s) early in training and is not included in above data.

THC Rats required an average ($\pm$ SEM) of 20 ($\pm$ 3.0) training sessions with the presence/absence of 1.8 mg/kg THC to fulfill the eight out of ten acquisition criterion (range 11 to 39

sessions). However, testing did not commence until all the animals had been trained the THC drug discrimination for 75 sessions.

Substitution tests with CB₁R agonists (mAEA and THC) and ethanol

mAEA The left panel of Fig. 2 (top) shows the generalization results of mAEA, THC, and ethanol for animals trained to discriminate between vehicle and 10 mg/kg mAEA. The ED₅₀ estimates ($\pm 95\%$ CL) for the mAEA and THC generalization curves are summarized in Table 1 (the dose of 1 mg/kg THC was not included in the regression analysis). Clearly, THC substituted for mAEA (i.e., exceeded 80% drug appropriate responding), but ethanol by occasioning 34% mAEA responding did not (i.e., did not exceed 80% drug appropriate responding). THC was more potent than mAEA. The relatively poor linear fit for the THC curve precluded calculation of its 95% CL. However, the *F* test indicated that the difference in potency between the two CB₁R agonists was significant ($F=27.58$ (DFn=1; DFd=6); $P=0.002$). The ratio between the ED₅₀ estimates for mAEA and THC was 14.61. The lower left panel in Fig. 2 shows the mean ($\pm$ SEM) rate of responding during the above generalization tests. Notably, marked response suppression occurred in tests with 1 g/kg ethanol, indicating the inclusion of a clearly behaviorally active dose in our tests. Some of the tests with mAEA also were associated with response rate decreases.

THC Figure 2 (top right) shows the generalization results of THC and ethanol for the rats discriminating between vehicle and 1.8 mg/kg THC. The ED₅₀ estimate ($\pm 95\%$ CL) for THC is summarized in Table 1 (the dose of 3 mg/kg THC was not included in the regression). Ethanol produced a maximum of 48% THC-like responding and hence did not generalize (i.e., did not exceed 80% drug appropriate responding). Figure 2 (bottom right) shows the mean ($\pm$ SEM) response rate for the THC and ethanol tests. Notably, marked response suppression occurred with 1 g/kg ethanol, indicating the inclusion of a behaviorally effective dose of ethanol also for the THC-trained rats. While mAEA was not examined in the current THC-trained animals, Table 2 summarizes generalization test data for mAEA and THC obtained from two previous separate studies from this laboratory using male Sprague–Dawley rats (Taconic) discriminating between 1.8 mg/kg THC and vehicle. Both studies found THC to be more potent than mAEA, the ratios between the ED₅₀ values being 16.05 ($F=29.06$ (DFn=1; DFd=6); $P=0.002$; Järbe et al. 1998) and 13.51 ($F=65.57$ (DFn=1; DFd=5); $P=0.0005$; Järbe et al. 2000), respectively.

Tests for generalization with WIN and surmountable antagonism by rimonabant

mAEA Figure 3 (top left) shows the generalization gradients (dose–response curves) for WIN alone and when combined with 1 mg/kg rimonabant in the mAEA-trained rats. The ED₅₀ values are listed in Table 1. WIN was 26.9 times more potent than mAEA and appeared 1.8 times more potent than THC. However, the difference in the intercepts between WIN and THC were not significant ($F=1.67$ (DFn=1; DFd=8); $P>0.05$). In the presence of 1 mg/kg rimonabant, there was a parallel 11.3-fold shift to the right of the WIN curve, suggesting surmountable antagonism ($F=32.86$ (DFn=1; DFd=9); $P=0.0003$). The outcomes essentially were the same when performing the regressions where the two highest test doses in each of the two WIN curves were excluded (ED₅₀=0.092 $\pm$ 0.054–0.159 and 1.078 $\pm$ 0.540–2.477 mg/kg, respectively). Response rate was reduced in tests with the highest dose of WIN alone (1.8 mg/kg), as well as in tests involving 5.6 and 10 mg/kg WIN and rimonabant (Fig. 3, bottom left).

THC Figure 3 (top right) shows the generalization gradients for WIN alone and when combined with 1 mg/kg rimonabant in the THC-trained rats. The ED₅₀ values are listed in Table 1. WIN was slightly, though nonsignificantly ($F=1.0$ (DFn=1; DFd=9); $P=0.34$) more potent than THC and the curves were parallel ($F=1.35$ (DFn=1; DFd=8); $P=0.28$). In the

presence of 1 mg/kg rimonabant, there was a significant parallel 2.6-fold shift to the right of the WIN curve ($F=10.95$ (DFn=1; DFd=9); $P=0.009$), suggesting surmountable antagonism. Response rate was reduced when testing 5.6 mg/kg WIN together with 1 mg/kg rimonabant (Fig. 3, bottom right). For all the other tests, the response rate was within the 95% CL of the vehicle control rate (dotted lines in lower right panel of Fig. 3).

Tests for generalization with AM678 and surmountable antagonism by rimonabant

mAEA Figure 4 (top left) shows the gradients for AM678 alone and when combined with 1 mg/kg rimonabant in the mAEA-trained rats. The ED₅₀ values are listed in Table 1. AM678 was 57 times more potent than mAEA, 3.9 times more potent than THC ($F=18.67$ (DFn=1; DFd=6); $P=0.005$), and 2.1 times more potent than WIN; the difference in the intercepts between AM678 and WIN was not determined because the slopes were significantly different ($F=14.48$ (DFn=1; DFd=8); $P=0.005$). In the presence of rimonabant, there was a parallel 4.4-fold shift to the right of the AM678 curve, suggesting surmountable antagonism ($F=340.10$ (DFn=1; DFd=7); $P<0.0001$). Response rate during these tests generally was within the 95% CL of the vehicle control rate (dotted lines in lower left panel of Fig. 4).

THC Figure 4 (top right) shows the gradients for AM678 alone and when combined with 1 mg/kg rimonabant in the THC-trained rats. The ED₅₀ values are listed in Table 1. AM678 was 11.3 times more potent than THC ($F=68.37$ (DFn=1; DFd=7); $P<0.0001$) and 8.4 times more potent than WIN55,212-2 ($F=67.71$ (DFn=1; DFd=9); $P<0.0001$). In the presence of rimonabant, there was a 13-fold shift to the right of the AM678 curve, suggesting surmountable antagonism. Response rate increased during tests with AM678 alone, whereas tests with combinations of AM678 and rimonabant resulted in a comparatively lowered rate of responding that nonetheless was within the 95% CL of the vehicle control rate (dotted lines in lower left panel of Fig. 4).

Discussion

This study was undertaken to further characterize the discriminative stimulus functions of the CB₁R AEA analog mAEA and the “classical” cannabinoid THC in vivo. To that end, rats were trained to discriminate between vehicle and 10 mg/kg mAEA in one group and another group discriminated between vehicle and 1.8 mg/kg THC; the two groups were run concurrently. After acquiring the discriminations, the two groups were tested with CB₁R agonists as well as ethanol. For both groups, the rank order of potencies was AM678>WIN>THC>mAEA. Ethanol did not substitute for the training drug in either of the two groups. Antagonism by 1 mg/kg rimonabant of the discriminative stimulus effects of both AM678 and WIN was surmountable. Thus, there was a parallel shift to the right (4.4-fold for AM678 and 11.3-fold for WIN) of the two CB₁R agonist dose generalization curves in the presence of rimonabant compared to the curves when the two CB₁R agonists were evaluated alone in the mAEA-trained animals. For the THC-trained animals, the corresponding values were 13 and 2.6 for AM678 and WIN, respectively.

The observed differences in ED₅₀ values for THC between the two training conditions is in agreement with previous data suggesting that in vivo THC is more potent than mAEA (e.g., Järbe et al. 2001) notwithstanding that in vitro, mAEA exhibits higher CB₁R affinity and CB₁R/CB₂R selectivity compared to THC (Abadji et al. 1994; Khanolkar et al. 1996). Most published data (e.g., Järbe et al. 1998, 2000, 2001, 2006a; McMahon 2006a, 2009; Solinas et al. 2007b) concur with this observation although in more limited reports, the two drugs were found to be approximately equipotent (Alici and Appel 2004; Burkey and Nation 1997). A number of reasons may account for this discrepancy regarding the relative potencies of these widely used CB₁R agonists, including differences in the vehicles used in different laboratories to prepare drug suspensions of these lipophilic molecules. An added complexity

is that there may also be potential species differences (McMahon et al. 2008; Vann et al. 2009).

However, the observation that discriminative stimulus effects are overlapping does not necessarily mean that the drug produced cues are identical. Information on this issue concerning cannabinergics is very limited but some insight may be gained from studies examining the high-affinity CB₁R selective AEA analog AM1346 (Järbe et al. 2006a, 2009). Using the training dose of 3 mg/kg AM1346, the following ED₅₀ doses (milligrams per kilogram) were calculated: 0.35 (THC), 1.0 (AM1346), and 2.61 (mAEA), and all three dose-effect lines were parallel. After retraining with 5.6 mg/kg AM1346, the ED₅₀ estimates were 0.42, 1.45, and 10.05 mg/kg, respectively (Järbe et al. 2009). Thus, the relative potencies for THC and AM1346 remained the same with parallel slopes irrespective of the AM1346 training dose while the relative potency (and slope) for mAEA changed considerably. This illustrates that the cueing effects of cannabinergics can be sensitive to training dose manipulation (see also Järbe et al. 2006a). In opioid drug discrimination, such an approach has been used to characterize full and partial as well as mixed opioid receptor agonists (Bergman et al. 2000). Given that AEA may activate also TRPV1 (see “Introduction” section), it is noteworthy that vanilloid mechanism(s) do not seem significant for the discriminative stimulus effects of CB₁R agonists because, firstly, the TRPV1 antagonist capsazepine did not block the THC-like stimulus effects of AEA (Solinas et al. 2007b) and, secondly, the TRPV1 agonist O-1839 did not substitute for either the AEA analog O-1812 or THC in rats discriminating between vehicle and either of these two CB₁R agonists (Wiley et al. 2004). Yet, administration of O-1839 produced a behavioral profile similar to that of other cannabinergics in the tetrad assay in mice (Di Marzo et al. 2001), suggesting greater pharmacological specificity with drug discrimination.

WIN's ability to substitute for THC is in agreement with previously described drug discrimination results employing different species although the potency estimates have varied. Some studies found that the discriminative stimulus effects of WIN were more potent than those of THC (e.g., Compton et al. 1992; McMahon et al. 2008; Solinas et al. 2007a). Although our data may be viewed as supporting the above conclusion, the statistical analysis comparing the WIN and THC data when tested alone did not detect a significant difference between the two generalization gradients in either of the two discrimination groups (see also De Vry and Jentsch 2002, 2004; Mauler et al. 2002; McMahon 2006a, b; Wiley et al. 1995a).

When combined with the CB₁R antagonist rimonabant, increasing doses of WIN overcame the receptor blockade in a surmountable fashion in both groups as has been described before using THC-trained animals (e.g., McMahon 2006a; Wiley et al. 1995b). However, the magnitude of the rightward shift appeared different in the two groups. Thus, for the THC group, the difference between the two ED₅₀ values (0.37 mg/kg for WIN alone and 0.96 mg/kg for WIN plus rimonabant) was 2.6, whereas in the mAEA group, there was an 11.3-fold difference (0.11 versus 1.22 mg/kg). Thus, rimonabant appeared more efficacious in blocking the effects of WIN in the mAEA-trained rats compared to the THC-trained rats. Apparent pA₂ analysis (Schild plot) of CB₁R agonists in combination with CB₁R antagonists/inverse agonists (rimonabant and AM251) led McMahon (2006a) to conclude that the same receptor population (i.e., CB₁) was responsible for the discriminative stimulus effects of all three CB₁R agonists (THC, CP55,940, and WIN) included in the study design using rhesus monkeys discriminating between THC and vehicle (see also McMahon 2009 regarding mAEA). However, conventional Schild plot analysis does not necessarily reflect events occurring downstream after G-protein receptor activation. Accumulating evidence suggests that structurally different CB₁R agonists regulate G-protein coupling (see Howlett 2004 for review) resulting in subtle, yet different signaling mechanisms and exhibit different

downstream pharmacological profiles. Perhaps such a mechanism(s) is responsible for the quantitative differences outlined above in our *in vivo* model (see also, e.g., Georgieva et al. (2008); Mukhopadhyay and Howlett 2005). Such *in vitro* functional data were a major impetus for including the two different training drug conditions in our analysis.

AM678 elicited discriminative stimulus effects similar to both mAEA ($ED_{50} = 0.051$ mg/kg) and THC ($ED_{50} = 0.044$ mg/kg). One interpretation of the very similar ED_{50} estimates for both groups would be that there is a closer correspondence in the pharmacological mechanism(s) underlying the discriminative stimulus effects between AM678 and THC compared to mAEA or else one would have expected the ED_{50} values to have been more differentiated in the two groups as was the case with WIN. Like WIN, surmountable antagonism occurred in combination tests with rimonabant in both groups. Thus, there was a 4.4-fold rightward shift in the mAEA-trained group and a 13-fold shift in the THC group. Notably, this pattern of rightward shifts is opposite to that seen with WIN as described above. Whatever the explanation for the differential outcome between these two aminoalkylindoles in their interaction with rimonabant, AM678 clearly is a potent CB_1R agonist. Actually, AM678 is the most potent aminoalkylindole cannabinergic agonist thus far described in the drug discrimination literature (Compton et al. 1992; Wiley et al. 1998; Vann et al. 2009). Apparently, the CB_1R binding affinities (see “Materials and methods” section) for these two aminoalkylindoles would not predict the *in vivo* difference in potency. If CB_2R activation is a determinant for the differential generalization results, it is noteworthy that WIN primarily is CB_2R selective, whereas AM678 is CB_1R selective to a similar extent, i.e., 6- to 7-fold in the binding ratios for the two receptor subtypes. However, previous work does not substantiate a significant role for CB_2R involvement in drug discriminations based on either CB_1R agonism (THC, ajulemic acid, and mAEA) or antagonism/inverse agonism (rimonabant) in monkeys and rats (Järbe et al. 2004, 2006b, 2008; McMahon 2006a; Vann et al. 2007). If WIN activates also non- CB_1R/CB_2R binding sites (see “Introduction” section), AM678 may represent a more specific cannabinergic aminoalkylindole ligand and thus serve as an alternative to WIN in future cannabinoid research.

The lack of substitution by ethanol at behaviorally depressant doses in both groups is consistent with previous findings in gerbils and rats discriminating between ethanol and vehicle (Bueno et al. 1976; Järbe 1977; Lamb and Järbe 2001) as well as in mice and rats discriminating between THC and vehicle (Bueno et al. 1976; Kubena and Barry 1972; McMahon et al. 2008), adding further support for the pharmacological specificity attributed to the *in vivo* cannabinoid drug discrimination model. The current data extend the pharmacological specificity to a lower training dose of THC than that (3–3.2 mg/kg) most commonly used for rats in the past as well as to mAEA. Previous validation of the pharmacological specificity of the mAEA (10 mg/kg) drug discrimination involved morphine and *d*-amphetamine (Järbe et al. 2006a). This is important considering that drug discrimination assays tend to become pharmacologically less selective or, stated otherwise, more sensitive with lower compared to higher training doses (e.g., Bergman et al. 2000).

In conclusion, the present data are congruent with the general observation that there is substantial overlap in the discriminative stimulus effects of CB_1R ligands across different chemical classes. However, differential quantitative interactions between the two aminoalkylindoles and rimonabant in the two groups support the hypothesis that there are differences in the manner with which WIN55,212-2 and AM678 interact with CB_1R as a function of training drug, thus leading to variations in their respective signaling effects. Also, AM678 is considerably more potent than WIN55,212-2.

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Δ⁹-THC
6,6,9-trimethyl-3-pentyl-6a,7,8,10a-
tetrahydro-6H-benzo[c]chromen-1-ol

AM356
(5Z,8Z,11Z,14Z)-N-((R)-1-hydroxypropan-2-yl)icosa-5,8,11,14-tetraenamide

WIN55,212-2
(R)-5-methyl-3-(morpholinomethyl)-2,3-dihydro-
[1,4]oxazino[2,3,4-*h*]indol-6-yl)(naphthalen-1-yl)methanone

AM678
naphthalen-1-yl(1-pentyl-1H-indol-3-yl)methanone

Fig. 1.
 Chemical structures of cannabinergic agonists used in study

Fig. 2.

Generalization test results (*top*) and corresponding response rate data (*bottom*) for mAEA (*R*-(+)-methanandamide; $n=10-11$), Δ^9 -THC ($n=10-20$) as well as for ETOH (ethanol; $n=9-18$) in the mAEA (10 mg/kg)- versus vehicle-trained rats (*left*), and Δ^9 -THC ($n=12$) as well as for ETOH (ethanol; $n=9-19$) in the Δ^9 -THC (1.8 mg/kg)-versus vehicle-trained rats (*right*); sessions began 20 min after i.p. administration. The generalization results represent the mean ($\pm$ SEM) percentage of lever presses on the drug (mAEA, *top left*; Δ^9 -THC, *top right*) appropriate lever out of the total number of lever presses emitted during a test session (*Y*-axis); doses examined in milligrams per kilogram (*X*-axis). Rate refers to the mean ($\pm$ SEM) number of lever presses per second emitted during a test session (*Y*-axis); doses in milligrams per kilogram (*X*-axis). *Dotted lines* represent the $\pm 95\%$ confidence limits of vehicle control response rate determined from the initial six reinforcement cycles of the vehicle training sessions immediately preceding these tests; *symbols* outside the confidence limits are considered significantly different from control. Data points are based on one to two observations for each rat and were obtained on separate test days. *Numbers within brackets* indicate the number of rats responding (i.e., accumulating at least ten responses on either lever and thus obtaining at least one reinforcement) out of the total number used for the test. Test results are based on sessions of a maximum of six reinforcements (12 food pellets) or 20 min, whichever occurred first. *V* vehicle

Fig. 3.

Generalization test results (*top*) and the corresponding response rate data (*bottom*) for WIN55,212-2 alone (*open hexagons*) and when combined with 1 mg/kg rimonabant (*filled hexagons*), 30 min after i.p. administration. Test results for the 10-mg/kg mAEA-versus vehicle-trained rats are shown in the *two left panels* (*open hexagons*; $n=9-19$; *filled hexagons*; $n=8-18$ except for the highest test dose where $n=7$), and the results for the 1.8-mg/kg Δ^9 -THC-versus vehicle-trained rats are depicted in the *two right panels* (*open hexagons*; $n=9-21$; *filled hexagons*; $n=9-19$). Drug lever responding results represent the mean ($\pm$ SEM) percentage of lever presses on the drug (mAEA, *top left*; Δ^9 -THC, *top right*) appropriate lever out of the total number of lever presses emitted during a test session (*Y-axis*); doses examined in milligrams per kilogram (*X-axis*). Rate refers to the mean ($\pm$ SEM) number of lever presses per second emitted during a test session (*Y-axis*); doses in milligrams per kilogram (*X-axis*). Other details are as described in the legend for Fig. 2

Fig. 4. Generalization test results (*top*) for AM678 alone (*open triangles*) and when combined with 1 mg/kg rimonabant (*filled triangles*) and the corresponding response rate data (*bottom*), 30 min after i.p. administration. Test results for the 10-mg/kg mAEA- versus vehicle-trained rats are shown in the *two left panels* (*open triangles*; $n=8-18$; *filled triangles*; $n=8-10$) and the results for the 1.8-mg/kg Δ^9 -THC- versus vehicle-trained rats are depicted in the *two right panels* (*open triangles*; $n=20-21$; *filled triangles*; $n=10-21$). Drug lever responding results represent the mean ($\pm$ SEM) percentage of lever presses on the drug (mAEA, *top left*; Δ^9 -THC, *top right*) appropriate lever out of the total number of lever presses emitted during a test session (*Y-axis*); doses examined in milligrams per kilogram (*X-axis*). Rate refers to the mean ($\pm$ SEM) number of lever presses per second emitted during a test session (*Y-axis*); doses in milligrams per kilogram (*X-axis*). Other details are as described in the legend for Fig. 2

Table 1

Summary of generalization test data

Drug	ED ₅₀ ±95% CL (mg/kg)
mAEA-trained rats	
mAEA	2.907 (±2.551–3.300)
WIN	0.108 (±0.059–0.181)
WIN + R	1.219 (±0.532–2.113)
AM678	0.051 (±0.045–0.056)
AM678 + R	0.223 (±0.177–0.282)
THC	0.198 (ND)
THC-trained rats	
WIN	0.369 (±0.214–0.808)
WIN + R	0.963 (±0.444–2.215)
AM678	0.044 (±0.037–0.050)
AM678 + R	0.570 (±0.233–0.910)
THC	0.497 (±0.213–1.353)

ND not determined (due to insufficient data), R rimonabant (1 mg/kg)

Table 2

Summary of generalization test data for mAEA and THC obtained in two previous studies

THC-trained rats	
Drug	ED₅₀±95% CL (mg/kg)
mAEA	5.409 (±1.451–30.620) ^a
THC	0.337 (±0.164–0.789) ^a
mAEA	6.108 (±1.741–11.402) ^b
THC	0.452 (±0.351–0.556) ^b

^aData from Järbe et al. (1998; log-dose transformed linear regressions based on doses 0.1, 0.18, 0.3, 0.56, and 1 mg/kg (THC); 1, 3, 10, and 18 mg/kg (mAEA), respectively). Rats were initially trained to discriminate between i.p. injected 3 mg/kg THC and vehicle before being retrained with 1.8 mg/kg THC before conducting above tests. Tests began 30 min postinjection

^bData from Järbe et al. (2000; log-dose transformed linear regressions based on doses 0.18, 0.56, 1, and 1.8 mg/kg (THC); 3, 5.6, 10, and 18 mg/kg (mAEA), respectively). Rats were trained to discriminate between i.p. injected 1.8 mg/kg THC and vehicle from the outset of the drug discrimination study. Tests began 30 min postinjection