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Central mediation and differential blockade by cannabinergics of the discriminative stimulus effects of the cannabinoid CB₁ receptor antagonist rimonabant in rats

Torbjörn U. C. Järbe, Brian J. LeMay, V. Kiran Vemuri, Subramanian K. Vadivel, Alexander Zvonok, and Alexandros Makriyannis

Center for Drug Discovery and Department of Pharmaceutical Sciences, Northeastern University, 116 Mugar Hall, 360 Huntington Ave., Boston, MA 02115, USA

Torbjörn U. C. Järbe: t.jarbe@neu.edu

Abstract

Rationale—Discovery of an endocannabinoid signaling system launched the development of the blocker rimonabant, a cannabinoid CB₁ receptor (CB₁R) antagonist/inverse agonist. Due to untoward effects, this medication was withdrawn and efforts have been directed towards discovering chemicals with more benign profiles.

Objective—This study aims to comparatively evaluate new ligands using a rimonabant discriminated drinking aversion procedure.

Methods—Rats discriminated between rimonabant (5.6 mg/kg) and vehicle. The 30 min saccharin (0.1%) drinking after rimonabant pretreatment was followed by injection of lithium chloride (120 mg/kg) in the experimental animals. After vehicle pretreatment, experimental animals were given i.p. NaCl (10 ml/kg). Postdrinking treatment for controls was NaCl, irrespective of pretreatment condition (rimonabant or vehicle).

Results—The centrally acting neutral CB₁R antagonist AM4113, but not the limited brain penetrating CB₁R neutral antagonist AM6545, substituted for rimonabant. The CB₁R agonists THC (1–10 mg/kg), AM1346 (1–10 mg/kg) did not substitute. The rimonabant-induced conditioned suppression of saccharin drinking was attenuated when CB₁R agonists AM5983 (0.01–1 mg/kg) and THC (10 mg/kg), but not the CB₁R agonist AM1346 (0.1–18 mg/kg), were combined with rimonabant (5.6 mg/kg). By varying the injection-to-test interval, we gauged the relative duration of the cueing effects of rimonabant, and the in vivo functional half-life was estimated to be approximately 1.5 h.

Conclusion—A neutral CB₁R antagonist (AM4113) produced cueing effects similar to those of rimonabant and generalization likely was centrally mediated. The functional cueing effects of rimonabant are relatively short-acting, pharmacologically selective, and differentially blocked by cannabinergics.

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Correspondence to: Torbjörn U. C. Järbe, t.jarbe@neu.edu.

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Keywords

Drug discrimination; Cannabinoid antagonists; Rimonabant–AM4113–AM6545; Cannabinoid agonists; AM5983–AM1346– Δ^9 -THC; Time-course; Rats

Introduction

The surge of information regarding endocannabinoid signaling in recent decades attests to the significance the scientific community is placing on this system and its implications for normal physiology and pathology. The cannabinoid 1 receptor (CB₁R) is the most abundant member of the 7-transmembrane G-protein coupled receptor (GPCR) family in the central nervous system (CNS). This receptor is particularly abundantly expressed in brain areas involved with motor function (basal ganglia, cerebellum), emotion/memory (limbic system), and cognition (prefrontal cortex). CB₁R is also expressed in the periphery, notably in the gut, liver, and the reproductive system. The other cloned cannabinoid receptor is named CB₂R and linked to immune function. Although viewed in the past primarily as a peripheral target, recent observations suggest a functional role for CB₂R also in the CNS, not only in pathophysiology/inflammation but also for normal physiological processing. Endogenous ligands for above receptors include anandamide and 2-arachidonoyl glycerol and possibly also other fatty acid derivatives. Enzymatic cleavage terminates the activity of the two major endocannabinoids anandamide and 2-arachidonoyl glycerol (Hanus 2009).

The role of CB₁R in energy balance is being extensively studied and launched the first discovered CB₁R antagonist rimonabant as an “anti-obesity” medication. Although efficacious in reducing body weight, psychiatric side effects prompted the withdrawal of this CB₁R selective antagonist/inverse agonist from the market. Neutral CB₁R antagonists such as the rimonabant analogs AM4113 and AM6527 were synthesized as alternatives (Janero et al. 2009; Vemuri et al. 2008) and in vivo preclinical comparisons suggested that neutral CB₁R antagonists might be less prone to emesis and anxiogenic-like reactions, including fear retention, than inverse agonists such as rimonabant and AM251 (Chambers et al. 2007; Sink et al. 2009a, 2010). The “anti-obesity” profile is retained by these neutral CB₁R antagonists (Chambers et al. 2007; Sink et al. 2008, 2009b) as are other characteristic CB₁R antagonism related effects such as e.g., increased frequency of scratching in rodents (Järbe et al. 2008a). AM6545, a more polar analog of AM4113, was recently synthesized (Tam et al. 2010) and its preclinical profile described. As expected, CNS access of AM6545 was limited in wild-type mice, but in blood–brain barrier compromised mice, pharmacological effects of a centrally acting CB₁R agonist were blocked by AM6545 and this CNS dependent activity was accompanied by increased tissue levels of the ligand in brain (Tam et al. 2010). Although not affecting basal colonic propulsion rate in mice, AM6545 significantly blocked CB₁R agonist (WIN55,212-2) induced delaying of gastrointestinal transit. Thus, the AM6545-produced blockade of a peripherally CB₁R mediated response demonstrates functional activity (Cluny et al. 2010). Neutral CB₁R antagonists do not reduce GTP γ S binding (Tam et al. 2010) or affect cAMP levels (Cluny et al. 2010), nor do they elicit conditioned gaping, a disgust reaction in rats, whereas the inverse CB₁R agonists rimonabant and AM251 do (Limebeer et al. 2010).

In spite of rimonabant’s slow pharmacokinetic clearance after oral dosing in humans (Huestis et al. 2007), and its relatively long functional in vivo half-life ($\approx$ 15 h) in suppressing food intake in rats (McLaughlin et al. 2003), we found that antagonism by rimonabant of both conditioned and unconditioned Δ^9 -tetrahydrocannabinol (THC) produced behavioral effects in rats was relatively short lasting, with functional in vivo half-life estimates being around 2 h (Järbe et al. 2010).

The very short half-life of anandamide has precluded extensive pharmacological or behavioral investigation of the ligand. Anandamide analogs with better enzymatic resistance have been developed (Thakur et al. 2005). One such analog, methanandamide, has been fairly extensively studied and although the compound exhibits many *in vivo* similarities with, e.g., THC, notable differences in the pharmacological spectrum between these two CB₁R agonists have surfaced (Baskfield et al. 2004; Järbe et al. 2001; McMahon et al. 2008). Although less studied, the anandamide derivative AM1346 appears functionally more similar to THC compared to methanandamide, in addition to displaying a considerable duration of effect *in vivo* (Järbe et al. 2009). Thus far, blockade of the cueing effects of rimonabant has only been shown with the phytocannabinoid THC (but not methanandamide). Therefore, inclusion of AM1346 and the cannabinergic aminoalkylindole AM5983 would provide new insights regarding types of ligands (anandamide-derived or not?) able to block the cueing effects of rimonabant.

Drug discrimination may be considered a pharmacologically selective animal model of “perceived” effects resulting from drug administration. Thus, the discriminative stimulus effect complex of the training drug defines the end-point by which new compounds are compared. In this study, rimonabant was established as a discriminative stimulus using a discriminated drinking aversion (DDA) baseline (Järbe et al. 2004, 2008b) as operant approaches tried thus far have been unsuccessful (Mansbach et al. 1996; McMahon 2006; Pério et al. 1996). When reliably discriminating the presence/absence of rimonabant effects (see “Results” section), other drugs (and doses) were examined for their capacity to substitute for, or to antagonize the effects of rimonabant. Thus, AM4113 testing addressed the issue of possible divergence in the perceptual effects between a neutral CB₁R antagonist and rimonabant, an antagonist exhibiting inverse biochemical properties. We tested AM6545 to shed light on the issue of whether the discrimination was mediated centrally or peripherally. Examining potential blockade of the cueing effects of rimonabant involved separate co-administrations of the CB₁R agonists THC, AM5983, and AM1346. By varying the injection-to-test interval, we gauged the relative duration of the discriminative stimulus effects of rimonabant. Nicotine was primarily included to assess if the discrimination was based on general malaise/emesis rather than pharmacological selectivity.

Materials and methods

Subjects

Sixteen male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA), weighing between 275 and 300 g upon arrival to the laboratory, were used. Rats were individually housed in polycarbonate cages in a colony room with an average temperature of 20°C and a non-reversed 12-h light/dark cycle (lights on at 7 AM) at the University’s vivarium. The animals had unlimited access to pellet food, but after a week of acclimation to the vivarium access to fluid was limited (see below). All procedures were approved by the Animal Care and Use Committee of Northeastern University, Boston, MA, USA. The “Principles of animal laboratory care” (NIH publication NO. 85–23, revised 1996) were followed.

General procedure for DDA acquisition and testing

During the week before the start of the experiment proper, access to tap water was freely available and thereafter gradually restricted to two drinking sessions per weekday (11–11:30 AM and 4:30 to 5 PM). For conditioning, access to saccharin (0.1%) flavored fluid was limited to the 30 min morning session and presented in Nalgene® 250 ml plastic bottles with #7 rubber stoppers. In the afternoon, the animals had access to tap water for 30 min in reduced height 266 ml plastic bottles (model PC9RH; AncareCorp®, Bellmore, NY, USA),

equipped with 2.5 cm nozzle open-ended screw tops (consumption not recorded). All experimental procedures took place during the morning sessions.

For discrimination training, i.p. administrations of the training drug rimonabant (5.6 mg/kg), a dose for which most previous comparison data are available (Järbe 2011), or vehicle (2 ml/kg) occurred 20 min prior to the 30 min morning saccharin presentation (Järbe and Lamb 1995). Following rimonabant pretreatment, the experimental animals (EXP; $n=8$) were injected i.p. with 10 ml/kg lithium chloride (LiCl, 120 mg/kg) immediately after the 30 min morning saccharin presentation, whereas vehicle controls (CONT; $n=8$) received 10 ml/kg of 0.9% saline (NaCl) postsession. The CONT group is used to differentiate potential unconditioned effects from conditioned drug effects relative to the EXP group. Drug sessions alternated with sessions during which administration of the drug vehicle was followed by 30 min saccharin presentation and subsequent i.p. injection of 10 ml/kg NaCl for all animals. There was an equal number of drug and non-drug discrimination training sessions.

Animals were trained and tested Monday through Friday. Once saccharin consumption was reliably reduced for the EXP rats during rimonabant discrimination days (individual EXP rats consuming 5 g of fluid), tests with different drugs and doses occurred on average once a week. Except when evaluating rimonabant's time-course, test drugs were administered i.p. 20 min prior to session. In antagonism testing, the two drugs were given in rapid succession on opposite sides of the peritoneal midline. Animals were habituated to receiving two injections prior to antagonism tests. Drugs and doses were examined in a mixed order. After test sessions, neither LiCl nor NaCl were given. To ensure maintenance of the discrimination, conditioning continued on interim days. Thus, at least two training sessions under each of the two training conditions followed one test day before testing resumed provided above EXP criterion was met; if not met, additional conditioning ensued before further testing. Training, testing, and afternoon drinking took place in the rats' home cages in the vivarium. The animals had free access to tap water from Friday afternoon until Sunday in the vivarium (consumption not recorded).

Drugs

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 provided by the National Institute on Drug Abuse (NIDA; Bethesda, MD, USA) and stored at -20°C until used. For preparing suspensions, appropriate amounts of ethanol/THC was withdrawn, the ethanol evaporated under a stream of nitrogen, the residue dissolved (*v/v*) in a solution of dimethyl sulfoxide (DMSO), propylene glycol (PG), and Tween-80 (T-80) before the solute was diluted with normal (0.9%) saline after the solute had been sonicated for 20 min. Above procedure was also used for suspending AM1346 [alkoxyacid amide of *N*-eicosa-(5*Z*, 8*Z*, 11*Z*, 14*Z*)-tetraenylamide; K_i (CB₁)=1.5 nM; K_i (CB₂)=152 nM]. 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 DMSO/PG/T-80 (by volume) mixture before being diluted with saline. For the most part, the final suspension consisted of 2% DMSO, 4% PG, 4% T-80 and 90% saline, exceptions being 10 mg/kg THC, 5.6 mg/kg AM4113 and 18 mg/kg AM1346 where the amounts of organic solvents were doubled, i.e., 4, 8 and 8%, respectively, at the expense of saline (80%). The doses of 10 mg/kg AM6545 and AM4113 were delivered in 100% DMSO. Racemic AM5983 [(1-((1-methylpiperidin-2-yl)methyl)-1*H*-indol-3-yl)(naphthalen-1-yl) methanone; K_i (CB₁)=0.75 nM; K_i (CB₂)=3.5 nM] was handled the same way as rimonabant as were also AM6545 [5-(4-[4-cyanobut-1-ynyl]phenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1,1-dioxo-thiomorpholino)-1*H*-pyrazole-3-carboxamide; k_i (CB₁)=3.3 nM; k_i (CB₂)=624 nM], and AM4113 (N-piperidin-1-yl-2,4-dichlorophenyl-1*H*-

pyrazole-3-carboxamide analog); K_i (CB₁)=1.73; k_i (CB₂)=463. AM4113, AM6545, AM5983 and AM1346 were synthesized at the Center for Drug Discovery, Northeastern University, Boston, MA, USA. (–)-Nicotine di-d-tartrate (Research Biochemicals International, RTI, Natick, MA, USA) dose was calculated as the base and dissolved in saline (1 ml/kg). The cannabinoid ligands generally were administered in a volume of 2 ml/kg, the exceptions being 10 mg/kg THC and 18 mg/kg AM1346 where 3 ml/kg were used. DMSO (100%) was given in volumes of 1 ml/kg. Suspensions were prepared fresh daily just prior to the i.p. administrations. Saccharin and LiCl were obtained from Sigma (St. Louis, MO, USA). LiCl was dissolved in normal saline.

Analysis

Overall analyses were performed with two-way repeated measures (RM) analysis of variance (ANOVA), using Sigma-Stat (v. 3.1, Systat Software). When ANOVA was significant ($p < 0.05$), *post hoc* analyses were carried out with the Holm–Sidak all pair-wise comparison test procedure with alpha, two-tailed, set at 0.05 experiment-wise, i.e., for the collection of comparisons. To better meet the assumptions of homogeneity of error variances and normality of treatment-level distributions, all saccharin intake data were square root transformed for statistical analysis (Kirk 1968). Non-linear regression analyses of dose-generalization and antagonism data after log- X transformation were performed using Prism (v. 5, GraphPad Software) to provide estimates of the independent variables when the coordinates of X intersected with $Y=50$ (ED₅₀) and the 95% confidence limits [(±95% C.L.; regression model: log dose or log time vs. response–variable slope with the top and bottom of the curves constrained to the highest and lowest value (mean fluid consumed) for each individual regression curve generated by the experimental group)].

Results

The left portion of all figure panels below illustrates the average (±SEM) fluid intake during maintenance conditioning sessions immediately preceding the test results depicted in the right portion of the panels for the EXP (squares) and CONT (circles) groups. Throughout the study, the EXP group consumed significantly less fluid after 5.6 mg/kg rimonabant (D) compared to vehicle (V), indicated by positive sign, and all EXP-D and CONT-D fluid intake levels were significantly different, indicated by asterisks. There were two instances of a significant difference between CONT-D and CONT-V, indicated by section signs (see Fig. 1d and c below), and two instances of a significant difference between EXP-V and CONT-V, indicated by asterisks (see Fig. 1a and b below).

Figure 1 shows the average (±SEM) fluid intake in the EXP (squares) and CONT (circles) groups when tested with different doses of rimonabant, AM4113, AM6545 and nicotine.

In tests with rimonabant (graph 1a), RM ANOVA was significant for dose [$F(5, 64)=20.88$; $p<0.001$], and the group×dose interaction [$F(5, 64)=17.44$; $p<0.001$]. Select pair-wise comparisons are indicated in graph 1a. *Within-group comparisons*: fluid intake after rimonabant doses 0.3, 1, and 1.8 mg/kg was significantly different compared to that of EXP-D (+). Intake was significantly different from vehicle in the EXP group after 1 mg/kg and higher doses of rimonabant (#). *Between-group comparisons*: compared to the corresponding data points for CONT, fluid consumption was significantly less reduced following rimonabant doses of 0.3 and 1 mg/kg, but more reduced at 3 mg/kg (*). The median dose effect estimate (±95% C.L.) and goodness of fit, r^2 , were ED₅₀=1.71 (±1.31–2.24) mg/kg rimonabant; $r^2=0.98$. This analysis included the mean EXP-D saccharin intake (graph 1a), defining the bottom constraint entered for the non-linear regression model. Thus, rimonabant discriminative control was dose-dependent.

In tests with AM4113 (graph 1b), RM ANOVA indicated significance for dose [$F(6, 75)=29.45$; $p<0.001$], and the group $\times$ dose interaction [$F(6, 75)=25.90$; $p<0.001$]. Select pair-wise comparisons are indicated in graph 1b. *Within-group comparisons*: significantly different intakes compared to EXP-D occurred after AM4113 doses 0.3, 1, 3, and 5.6 mg/kg (+), and fluid consumption after doses of 1 mg/kg AM4113 and above were significantly different from EXP-V (#). *Between-group comparisons*: compared to corresponding data points for CONT, fluid consumption was significantly less reduced with AM4113 doses of 0.3 and 1 mg/kg, but significantly lower after 5.6 and 10 mg/kg (*). The median dose effect estimate ($\pm 95\%$ C.L.) and goodness of fit, r^2 , were $ED_{50}=3.72 (\pm 2.67-5.18)$ mg/kg AM4113; $r^2=0.96$. Thus, tests with AM4113 indicated a dose-dependent substitution for rimonabant.

In tests with AM6545 (graph 1c), RM ANOVA indicated significance for dose [$F(4, 51)=30.32$; $p<0.001$], and the group $\times$ dose interaction [$F(4, 51)=16.85$; $p<0.001$]. *Within-group comparisons*: all AM6545 doses resulted in more fluid intake compared to EXP-D (+). *Between-group comparisons*: there were no significant differences regarding dose. A separate test (not shown) with 1 ml/kg DMSO (100%) alone resulted in average fluid intakes of 14.6 and 10.4 g for the EXP and CONT groups, respectively. Thus, AM6545 did not substitute for rimonabant.

In tests with nicotine (graph 1d), RM ANOVA indicated significance for dose [$F(5, 64)=86.07$; $p<0.001$], and the group $\times$ dose interaction [$F(5, 64)=10.67$; $p<0.001$]. Select pair-wise comparisons are indicated in graph 1d. *Within-group comparisons*: intake levels with nicotine doses 0.1, 0.3, and 0.56 mg/kg were significantly different from EXP-D, indicated by positive sign, and consumption was significantly different from EXP-V (#), as well as CONT-V (§), after all doses of nicotine. The 1 mg/kg nicotine dose suppressed drinking significantly below the level observed for CONT-D (x). *Between-group comparisons*: there were no significant differences in intakes regarding nicotine dose. It appears that nicotine did not substitute, suggesting pharmacological selectivity rather than general emesis/malaise as the basis for the rimonabant discrimination.

Figure 2 shows the average ($\pm$ SEM) fluid intake in the EXP (squares) and CONT (circles) groups when examined with AM5983 in combination with 5.6 mg/kg rimonabant, THC alone and the combination of 10 mg/kg THC with 5.6 mg/kg rimonabant, AM1346 alone, and AM1346 in combination with 5.6 mg/kg rimonabant.

In tests with 5.6 mg/kg rimonabant and different doses of AM5983 (graph 2a), RM ANOVA was significant for group [$F(1, 63)=17.09$; $p<0.001$], dose [$F(5, 63)=16.10$; $p<0.001$], as well as for the group $\times$ dose interaction [$F(5, 63)=5.69$; $p<0.001$]. Select pair-wise comparisons are indicated in graph 2a. *Within-group comparisons*: a less reduced intake compared to EXP-D occurred after 0.3 and 1 mg/kg AM5983 combined with rimonabant (+), and fluid consumption after doses of 0.01, 0.1, and 0.3 mg/kg AM5983 in combination with 5.6 mg/kg rimonabant were significantly different from EXP-V (#). Fluid consumption was significantly lower after AM5983 (plus rimonabant) doses of 0.01 and 0.1 mg/kg compared to CONT-V (§). *Between-group comparisons*: compared to corresponding data points for CONT, fluid consumption was significantly lower in the EXP group after AM5983 (plus rimonabant) doses of 0.1 and 0.3 mg/kg (*). The median dose effect estimate ($\pm 95\%$ C.L.) and goodness of fit, r^2 , were $ED_{50}=0.38 (\pm 0.18-0.80)$ mg/kg AM5983; $r^2=0.98$. Thus, AM5983 attenuated the cueing effects of rimonabant.

In tests with THC alone and in combination with rimonabant (graph 2b), RM ANOVA was significant for dose [THC alone: $F(4, 52)=66.16$; $p<0.001$] [THC plus rimonabant: $F(2, 26)=38.98$; $p<0.001$], and the group $\times$ dose interaction [THC alone: $F(4, 52)=10.63$;

$p < 0.001$] [THC plus rimonabant: $F(4, 52) = 10.63$; $p < 0.001$]. Select pair-wise comparisons are indicated in graph 2b. *Within-group comparisons*: (1) THC alone—increased consumption in the EXP group occurred following THC doses 1 and 3 mg/kg (+), and fluid consumption after doses of 3 and 10 mg/kg THC were significantly different from EXP-V (#), as was the intake after 10 mg/kg THC in the CONT group (§); (2) THC plus rimonabant—for the EXP group, fluid consumption was significantly higher with the drug combination compared to EXP-D (+), but lower than EXP-V (#). *Between-group comparisons*: (1) THC alone—compared to corresponding data points for CONT, fluid consumption for the EXP group was significantly higher with 1 mg/kg THC (*). (2) THC plus rimonabant—fluid intakes between the EXP and the CONT groups were not significant. Thus, THC did not substitute for rimonabant when examined alone. The combination of 10 mg/kg THC and 5.6 mg/kg rimonabant resulted in a fluid intake level significantly higher than that observed when testing each of these ligands alone in the EXP group, indicative of mutual antagonism.

In tests with AM1346 alone (graph 2c), RM ANOVA indicated significance for dose [$F(5, 55) = 61.94$; $p < 0.001$], and the group $\times$ dose interaction [$F(5, 55) = 15.52$; $p < 0.001$]. Select pair-wise comparisons are shown in graph 2c. *Within-group comparisons*: in EXP, intake after all doses of AM1346 was significantly above EXP-D (+), and fluid consumption after 10 mg/kg AM1346 was significantly different from that of vehicle in the EXP group (#). Intake in CONT after 10 mg/kg AM1346 was significantly lower compared to CONT-V (§), and also significantly below the level for CONT-D (x). *Between-group comparisons*: the EXP and CONT groups differed significantly after 10 mg/kg AM1346, indicated by (*). Thus, AM1346 did not substitute for rimonabant.

In tests with 5.6 mg/kg rimonabant and different doses of AM1346 (graph 2d), RM ANOVA was significant for group [$F(1, 67) = 13.40$; $p = 0.003$], dose [$F(6, 67) = 22.71$; $p < 0.001$], and for the group $\times$ dose interaction [$F(6, 67) = 3.56$; $p = 0.004$]. Select pair-wise comparisons are indicated in graph 2d. *Within-group comparisons*: fluid consumption after all doses of AM1346 in combination with 5.6 mg/kg rimonabant was significantly different from EXP-V (#), but not EXP-D. With the exception of 1 mg/kg AM1346 (plus rimonabant), fluid consumption was significantly lower after all AM1346 (plus rimonabant) doses compared to CONT-V (§). The combination of 10 mg/kg AM1346 and rimonabant resulted in a significantly lower intake compared to CONT-V (x). *Between-group comparisons*: the EXP and CONT groups differed significantly regarding doses 1, 10, and 18 mg/kg AM1346 (plus rimonabant), indicated by asterisks. Thus, AM1346 did not significantly attenuate the cueing effects of rimonabant.

Figure 3 shows average ($\pm$ SEM) fluid consumption in the EXP (squares) and CONT (circles) groups as a function of elapsed time since administration of 5.6 mg/kg rimonabant.

RM ANOVA was significant for dose [$F(5, 62) = 25.74$; $p < 0.001$], and the group $\times$ dose interaction [$F(5, 62) = 8.63$; $p < 0.001$]. Select pair-wise comparisons are indicated in graph 3. *Within-group comparisons*: there was no significant difference between intake at 20 (EXP-D) and 40 min post-administration, but from 90 min post-injection and onwards, intake for the experimental animals was significantly different compared to EXP-D (+). In the EXP group, fluid consumption at all time intervals after 5.6 mg/kg rimonabant was significantly different from EXP-V (#). In the controls, fluid consumption was significantly lower at 90 and 180 min post-administration compared to CONT-V (§). *Between-group comparisons*: the EXP and CONT groups differed significantly at 20, 40, and 90 min post-administration (*). The in vivo functional half-life estimate was 95.2 (± 53.7 –168.5) min post-administration and $r^2 = 0.93$. Thus, the discrimination was sensitive to manipulation of time since injection, and data suggest a relatively short functional in vivo half-life for the cueing effects of rimonabant.

Discussion

Saccharin intake after treatment with 5.6 mg/kg rimonabant was consistently significantly lower in the EXP group compared to the corresponding vehicle condition, as well as in comparison to the intake levels noted for the CONT group, in spite of unconditioned effects. Thus, rimonabant served a cue function for the EXP group in the current DDA conditioning procedure, which is consistent with previous data (Järbe et al. 2004, 2008b). Given that these studies were carried out in different facilities provides some generality for the phenomenon across different contexts. A caveat of drug DDA studies may be the absence of LiCl exposure in the controls making the EXP and CONT groups not equal regarding drug history, yet generating data seemingly consistent with those obtained in the more commonly employed two lever choice procedure. Conversely, an advantage of DDA is its sensitivity to the cueing effects of certain drugs (e.g., naloxone, rimonabant), not readily established as cues in the conventional two lever task (<http://www.drugrefs.org/>). The window for gustatory aversive conditioning is long (Welzl et al. 2001) and requiring two daily drinking episodes as used here makes it logistically difficult to accommodate a long enough delay between the drinking bout and LiCl administration where the likelihood for the formation of an association between the drinking episode and the LiCl effects is minimized for CONT. Since taste per se is not necessary (Järbe et al. 2004; Järbe and Lamb 1999), other aspects related to ingestion sustain conditioning.

The discriminative stimulus control was dose-dependent with a median dose–effect estimate of 1.71 mg/kg rimonabant. In tests with the neutral, centrally acting CB₁R antagonist AM4113 generalization occurred (ED₅₀ = 3.72 mg/kg), albeit with a potency significantly different from that of rimonabant as there were no overlap in the 95% confidence limits between the two ED₅₀ estimates. Previous data indicated generalization to 5.6 mg/kg rimonabant also with the CB₁R antagonists/inverse agonists AM251 and AM281, although in the latter case the fluid intake by the controls was also affected. Those data recalculated yielded the following ED₅₀ estimates: ($\pm$ 95% C.L.): 1.47 ($\pm$ 0.59–3.68) mg/kg and 2.72 ($\pm$ 1.70–4.37) mg/kg for suspensions of rimonabant and AM251, respectively (Järbe et al. 2004), and 1.41 ($\pm$ 1.04–1.92) mg/kg and 0.68 ($\pm$ 0.18–2.61) mg/kg for rimonabant and AM281 (both drugs dissolved in pure DMSO), respectively (Järbe et al. 2008b). Of note is that above rank order potencies parallel those observed when these inverse agonists were used as antagonists for THC based operant drug discriminations in rats (Järbe 2011; Järbe et al. 2006b); comparable antagonism data are not yet available for AM4113. The CB₁R binding affinity and CB₁R/CB₂R selectivity (Sink et al. 2008) does not seem to explain the potency difference between AM4113 and rimonabant. Notwithstanding, it is tempting to speculate that the various differences in end-point results noted in the “Introduction” section for the two ligands may make AM4113 a less than perfectly matching substitute for rimonabant leading to the conclusion that although similar, the discriminative stimulus effects of the compounds may not necessarily be identical (perhaps a reflection of neutral antagonism vs. inverse agonism). Thus, since DDA presumably involves aversive conditioning related to ingestion, nauseating aspects of the procedure may be particularly salient (McLaughlin et al. 2005).

The lack of generalization in tests with the neutral CB₁R AM6545, a ligand with limited brain permeability, would be consistent with a central mediation for the cueing effects of rimonabant and its generalization to AM4113, and presumably also other centrally acting CB₁R antagonists. Central mediation is likely for the vast majority of drug discrimination studies published thus far (<http://www.drugrefs.org/>), including the cannabinergic aminoalkylindole WIN55,212-2, the cueing effects of which were blocked by rimonabant but not by a rimonabant analog (structure undisclosed) claimed to have limited brain permeability (Pério et al. 1996). Discriminative control by isopropamide, a muscarinic

cholinergic antagonist with limited blood–brain barrier permeability, and its generalization to methylscopolamine, suggests that peripheral effects induced by drugs can exert differential control over choice behaviors (Colpaert et al. 1975), and such control may even differ qualitatively from that of centrally mediated drug-produced discriminative control (Colpaert et al. 1976). It thus appears that central action is not a prerequisite for drug discrimination, but if the reference (i.e., drug training dose) is primarily centrally acting, the contribution of peripheral effects in controlling the discrimination generally appear negligible, as reviewed elsewhere (Järbe 1989). No apparent effect of AM6545 was demonstrated in our study, thus questioning the inclusion of a behaviorally effective dose range of this ligand. However, previous data (see Introduction) have shown that at least the highest dose (10 mg/kg) examined here is sufficient to block food intake in rats and mice as well as blocking cannabinergic-agonist delaying of colonic transit in mice, a peripherally mediated response. It remains to be determined if a peripherally restricted CB₁R ligand exhibiting more inverse agonism would challenge currently obtained support for a central mediation of the cueing effects of rimonabant and AM4113. Limited generalization with naltrexone methobromide, an opioid antagonist with reduced brain permeability, in rats trained to discriminate between naloxone and vehicle suggests that also opioid-antagonist based DDA may be centrally mediated (Davis et al. 2009).

The inclusion of the non-cannabinoid nicotine was based primarily on its well-documented emetic/nauseating effects across a variety of species, including man, and the presumed neuromodulatory role ascribed to the endocannabinoid system in the expression of such reactions (Solinas et al. 2008). For example, anandamide, particularly when combined with the fatty acid amide hydrolase enzyme blocker URB597, significantly attenuated nicotine induced vomiting in *Suncus murinus* (shrews), and the effect blocked by rimonabant (Parker et al. 2009). The strong unconditioned effect (reflected primarily by the suppressed fluid intake of the controls) in the current study complicates interpretation and does not allow any firm conclusions. Note though that fluid intake generally was higher in the EXP group relative to the controls. Previously, pharmacological selectivity in this DDA procedure was based primarily on tests with naloxone and flumazenil (Järbe et al. 2004). Receptor sub-type selectivity was based on tests with the CB₂R antagonists AM630 and SR144528 (Järbe et al. 2008b). Additionally, rimonabant neither substituted for, nor antagonized the cue effects of nicotine in rats trained a two-lever choice discrimination based on the presence/absence of the cholinergic agonist (Cohen et al. 2002; Le Foll and Goldberg 2004), implying distinct pharmacological profiles for the drugs.

The lack of generalization in current tests with the CB₁R agonists THC and AM1346 combined with previous tests with methanandamide (Järbe et al. 2008b) also supports pharmacological specificity for this rimonabant-based DDA. Co-administration of THC and rimonabant attenuated both the conditioned and unconditioned effects of the drugs, replicating previous findings (Järbe et al. 2004). Somewhat surprising, and in contrast to the outcome for the THC/rimonabant combination, there was only limited attenuation when AM1346 was combined with rimonabant. This outcome also contrasts with the dose-related attenuation of the cueing effects of rimonabant observed when combined with the CB₁R agonist AM5983. Due to a paucity of material, 1 mg/kg AM5983 was the highest dose that could be assessed. This was also the reason why AM5983 was not examined singly. Previous operant THC discrimination data suggested that AM5983 was about eight times more potent than THC. When combined with 1 mg/kg rimonabant, the dose effect curve for AM5983 was shifted tenfold to the right in a parallel fashion (Tai et al. 2009). Intriguingly, we also noted right-ward parallel shifts of the generalization curves when AM1346 was combined with 0.3 and 1 mg/kg rimonabant in animals discriminating between vehicle and THC (1.8 mg/kg), or vehicle and AM1346 (3 mg/kg), respectively (Järbe et al. 2006a, 2009). This was in contrast to the outcomes for similar combinations involving the

anandamide analog methanandamide in operant studies (Järbe et al. 2001, 2009). Likewise, attenuation of rimonabant's (or AM251) conditioned effects in DDA was limited when these CB₁R antagonists were combined with methanandamide (Järbe et al. 2008b). Additionally, whereas rimonabant attenuated THC induced open-field effects including circling, a motor disturbance produced by higher doses of various CB₁R agonists including, cannabinal, AM411, HU210, WIN55,212-2, and to some extent also AM1346 (Järbe et al. 2007), behaviors such as ambulation and rearing were dose dependently augmented by the CB₁R antagonist in methanandamide treated rats (Järbe et al. 2002, 2003). Circling did not occur after methanandamide administration (Järbe et al. 1998). Particularly regarding anandamide analogs, we suspect that key factors are the relative agonist/antagonist dose combinations. Indeed, for rats discriminating between the relatively high dose of 5.6 mg/kg AM1346 and vehicle, co-administration of this agonist with 1 mg/kg rimonabant resulted in a right-ward shift of the dose–effect curve that was not parallel whereas the right-ward shift for the THC generalization curve in the same animals was parallel as was also the right-ward shift for the curve of the CB₁R agonist WIN55,212-2 (unpublished).

By varying the injection-to-test interval when examining the effect of the maintenance dose of rimonabant, the *in vivo* functional half-life for rimonabant was estimated to be approximately 1.5 h. This estimate is in agreement with a previous study where the focus was on rimonabant's (1 mg/kg) time–course to block behavioral effects of THC, using both conditioned (drug discrimination) and unconditioned (response rate) operant measures (Järbe et al. 2010). Thus, the time–course for the perceptual effects of rimonabant alone as a cue and as a blocker of THC-induced CB₁R agonism correlates closely. Our estimates, however, contrast with that derived from a similar study examining blockade of the cueing effects of THC by *i.p.* administered 1 mg/kg rimonabant (Solinas et al. 2003). We suspect that the vehicle (0.3% Tween-80 in saline) in which rimonabant was suspended in the latter report may be key in explaining the difference in the onset and duration of action of rimonabant between the two studies (Järbe et al. 2010; Solinas et al. 2003). Thus, a vehicle with lower compound solubility would be expected to result in delayed absorption and therefore a seemingly more protracted time–course.

In conclusion, this study provided evidence that a neutral CB₁R antagonist (AM4113) produced a discriminative stimulus complex sufficiently similar to that of the inverse agonist rimonabant, and that this generalization likely was centrally mediated as there was no substitution with a more polar CB₁R antagonist (AM6545) with limited capacity to pass the blood–brain barrier. Further work will be needed to determine if these neutral antagonists are viable alternative medications to replace inverse agonists such as rimonabant. The cannabinergics AM5983 and THC, but not AM1346, significantly attenuated the rimonabant induced conditioned suppression of drinking. The limited blockade by AM1346 of rimonabant conditioned suppression of drinking in DDA is similar to that of another anandamide analog, methanandamide. Whether this divergence among cannabinergics reflects differential interactions with other GPCRs such as the vanilloid type 1 receptor, for which anandamide may be an endogenous ligand (Di Marzo 2010), or other non-CB₁R/CB₂R targets (Pertwee 2010), remains to be determined. The functional *in vivo* half-life of 1.5 h post-rimonabant administration as a discriminative stimulus is in accord with our previous data examining rimonabant's time–course as an antagonist of THC produced operant behavioral effects in rats. Regardless of the previously noted caveat, current and previous DDA studies using rimonabant as a cue continue to suggest the usefulness of this aversion-based conditioning model for discerning perceptual aspects of cannabinoid signaling not readily amenable by other *in vivo* means.

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Fig. 1.

Average ($\pm$ SEM) fluid intake (g) in experimental (EXP; *squares*) and control (CONT; *circles*) rats after treatment with different doses of rimonabant (graph 1a), AM4113 (graph 1b), AM6545 (graph 1c), and nicotine (graph 1d). Each data point is based on the performance of seven to eight individual rats in each group. Data points above D and V represent the average ($\pm$ SEM) fluid intake during the two (drug and vehicle) training, maintenance sessions immediately preceding testing. (*asterisks*) Significant pair-wise difference between EXP and the corresponding data point in the CONT group. (*positive sign*) Significantly different from EXP drug base-line (D) in the EXP group. (*multiplication symbol*) Significantly different from CONT drug base-line (D) in the CONT group. (*number*)

sign) Significantly different from EXP vehicle base-line (V) in the EXP group. (*section*
sign) Significantly different from CONT vehicle base-line (V) in the CONT group; ($p \leq 0.05$)

Fig. 2.

Average ($\pm$ SEM) fluid intake (g) in experimental (EXP; *squares*) and control (CONT; *circles*) rats when examined with AM5983 in combination with 5.6 mg/kg rimonabant (graph 2a), THC alone and the combination of 10 mg/kg THC with 5.6 mg/kg rimonabant (graph 2b), AM1346 alone (graph 2c), and AM1346 in combination with 5.6 mg/kg rimonabant (graph 2d). Each data point is based on the performance of seven to eight (graphs a and b) and six to seven (graphs c and d) individual rats in each group. Data points above D and V represent the average ($\pm$ SEM) fluid intake during two (drug and vehicle) training, maintenance sessions immediately preceding testing. (*asterisks*) Significant pair-wise difference between EXP and the corresponding data point in the CONT group. (*positive sign*) Significantly different from EXP drug base-line (D) in the EXP group. (*multiplication symbol*) Significantly different from CONT drug base-line (D) in the CONT group. (*number sign*)

Significantly different from EXP vehicle base-line (V) in the EXP group. (*section sign*)
Significantly different from CONT vehicle baseline (V) in the CONT group; (*p* 0.05)

Fig. 3.

Average ($\pm$ SEM) fluid consumption (g) in experimental (EXP; *squares*) and control (CONT; *circles*) rats as a function of elapsed time since i.p. administration of 5.6 mg/kg rimonabant. Data points were determined on separate test days and reflect the performance of seven to eight individual rats in each group except at the 360 min. interval where $n=6-7$. The in vivo functional half-life estimate was $95.2 (\pm 53.7-168.5)$ min post-administration. (*asterisks*) Significant pairwise difference between EXP and the corresponding data point in the CONT group. (*positive sign*) Significantly different from EXP drug base-line (D) in the EXP group. (*number sign*) Significantly different from EXP vehicle base-line (V) in the EXP group. (*section sign*) Significantly different from CONT vehicle base-line (V) in the CONT group; ($p < 0.05$)