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Increasing the selectivity of the discriminative stimulus effects of Δ^9-tetrahydrocannabinol: complete substitution with methanandamide

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Abstract

In an attempt to increase the selectivity of the discriminative stimulus effects of Δ^9-tetrahydrocannabinol (THC), rats were trained to discriminate 3.2 mg/kg of this compound from a group of "other" drugs consisting of morphine (3.2 mg/kg), PCP (2.5 mg/kg), and vehicle. Acquisition of the Δ^9-THC-other discrimination was rapid (38 days) and did not differ significantly from that of a group of "control" animals trained to discriminate Δ^9-THC (3.2 mg/kg) from its vehicle (33 days). In substitution (generalization) tests, a high dose of anandamide, which also severely decreased response rate, substituted partially in both the control and the Δ^9-THC-other group; (*R*)-methanandamide, an analog of anandamide which is metabolized more slowly, substituted completely for Δ^9-THC in the control, and partially in the Δ^9-THC-other group; neither pentobarbital nor diazepam substituted completely for Δ^9-THC under any experimental condition. Regardless of the level of Δ^9-THC lever responding, all drugs except diazepam substituted less in the Δ^9-THC-other than in the control group. For this reason, the Δ^9-THC-other training procedure might be described as being more selective than the commonly used drug–no drug procedure.

Keywords: Drug discrimination; Drug-other; Δ^9-Tetrahydrocannabinol (THC); Anandamide; (*R*)-methanandamide; Pentobarbital; Phencyclidine (PCP); Morphine

1. Introduction

Delta-9-tetrahydrocannabinol (Δ^9-THC) produces a wide variety of pharmacological effects in rats and other animals that are not related to its marijuana-like properties in humans; these include hypokinesia, hypothermia, and antinociception (Pertwee, 1988). However, the discriminative stimulus effects of Δ^9-THC seem to be relatively specific; that is, they generalize to other, naturally occurring psychoactive cannabinoids with potencies in drug discrimination (DD) procedures that are highly correlated with potencies of subjective effects in humans (Balster and Prescott, 1992; Overton, 1988). In addition, compounds that bind to the cannabinoid receptor substitute completely for Δ^9-THC when this substance is used as the training drug and do so at doses that do not also disrupt rate of responding (responses/min): these include, but are not limited to various 11-hydroxy metabolites of THC (Jarbe and McMillan, 1979), CP 55,940 (Gold et al., 1992), (+)-WIN 55,212 (Compton et al., 1992), cannabinol (Jarbe and Hiltunen, 1988), and Δ^8-tetrahydrocannabinol (Jarbe and McMillan, 1979). Since Δ^9-THC also substitutes for CP 55,490 or WIN 55,212 when animals are trained to discriminate either of these compounds from appropriate vehicles, it is probably safe to conclude that there are no differences in the stimulus effects of various cannabinoids other than their relative potencies (Perio et al., 1996; Wiley et al., 1995).

The discriminative stimulus effects of anandamide, the endogenous cannabinoid receptor ligand (Smith et al., 1994), are less clear. This substance does not substitute reliably for Δ^9-THC when given exogenously, except at

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doses that decrease response rates significantly and are therefore difficult, if not impossible, to interpret; among other things, this suggests that different mechanisms of action are involved in the stimulus effects of anandamide and those of (other) cannabinoids (Wiley et al., 1995, 1997). On the other hand, (*R*)-methanandamide and related, metabolically more stable methylated analogs of anandamide do substitute for Δ^9-THC (Burkey and Nation, 1997; Wiley et al., 1997). Thus, it is possible that the rapid metabolism of exogenously administered anandamide may contribute to its lack of Δ^9-THC like stimulus properties.

Much of the information concerning the pharmacological selectivity of Δ^9-THC comes from two comprehensive studies in which a large number of compounds from a variety of pharmacological classes were compared with respect to their ability to substitute for Δ^9-THC (3.2 mg/kg). These included althesin, amphetamine, atropine, BRL13776, baclofen, buspirone, chlordiazepoxide, cimetidine, clonidine, clozapine, cyclazocine, dextromethorphan, dexamethazone, diazepam, dizocilpine, gamma hydroxy butyrate (GHB), haloperidol, imipramine, iproniazid, ketocyclazocine, loperamide, LSD, MDL 72222, midazolam, morphine, muscimol, oxotremorine, pargyline, pentazocine, pentobarbital, phencyclidine, phenobarbital, phenytoin, L-phenylisopropyl adenosine, pilocarpine, piroxicam, thujone, and yohimbine (Barrett et al., 1995; Browne and Weissman, 1981). While none of these drugs substituted completely (>80%) for Δ^9-THC, both pentobarbital and diazepam substituted partially and thus can be considered to be the only *potential* false positives in the cannabinoid discrimination literature (Browne and Weissman, 1981; Jarbe and Hiltunen, 1988; Mokler et al., 1986; Wiley, 1999).

Both the unreliability of the substitution of the endogenous cannabinoid receptor ligand anandamide and the possibility that two false positives exist suggested a need to increase the pharmacological selectivity of the Δ^9-THC discrimination. This was attempted in the present study by using a "drug-other" (D-O) procedure first suggested by Overton (1988), and recently utilized successfully in our laboratory to eliminate the substitutions of three "false positives" (lisuride, quipazine, and yohimbine) for LSD (D-lysergic acid diethylamide). In this procedure, which is about as efficient as the traditional drug vs. no drug (D–ND) method in terms of the number of sessions required to reach criterion (at least 80% responding on the drug-appropriate lever), animals are trained to discriminate a training drug not from only its vehicle, but also from a group of other drugs. In the present study, the "other" group consisted of phencyclidine hydrochloride (PCP, 2.5 mg/kg), morphine sulfate (3.2 mg/kg), and the vehicle in which Δ^9-THC was dissolved (below). These drugs were chosen because anandamide has been reported to interact with both NMDA glutamate (PCP) and μ-opioid (morphine) as well as cannabinoid receptors (Hampson et al., 1998; Wiley et al., 1995). Since no such interactions purportedly occur with Δ^9-THC, it seemed reasonable to suppose that training animals to discriminate between this cannabinoid and these non-cannabinoid cues might enhance the discriminability of Δ^9-THC from anandamide in particular and, perhaps, from other drugs that act at least in part at non-cannabinoid receptors.

2. Method

2.1. Animals

Twenty experimentally naïve Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing approximately 300 g at the beginning of the experiment were used. They were housed individually in a colony maintained on a 12 h light–dark cycle with lights on from 07:00 to 19:00 h. Temperature and humidity were held constant at 20–22 °C and 45–50%, respectively. Initially, both food and water were freely available. Five days prior to training, access to water was restricted to 1 h per day. Water was then restricted to amounts obtained during training sessions and on weekends (Friday afternoon to Sunday morning).

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of South Carolina and is in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Apparatus

Twelve commercially available experimental chambers (MED Associates ENV 018) housed in light- and sound-attenuating shells (MED Associates ENV 008) were used. Each chamber contained two retractable levers on either side of a dipper programmed to deliver 0.1 ml of water for 3 s whenever a reinforcer was scheduled. An IBM compatible computer using MED State software was used to program and record experimental events.

2.3. Experimental design and procedures

2.3.1. Training procedure

Half of the animals (n=10) were trained to discriminate Δ^9-THC from an equal volume of its vehicle (below); the remaining animals (n=10) were trained to discriminate Δ^9-THC from a group of "other" drugs (other group) consisting of morphine (3.2 mg/kg), PCP (2.5 mg/kg) or the Δ^9-THC vehicle. Doses were chosen on the basis of previous results obtained under similar conditions in our laboratory (Hernandez et al., 1978; Poling et al., 1979). All drugs were given intraperitoneally (IP), 25 min before daily (Monday–Friday) experimental sessions, except anandamide, which was administered 15 min before sessions. Animals in the

other group were given Δ^9-THC on 50% of the sessions and one of the other compounds (morphine [33.33%], PCP [33.33%], or vehicle [33.33%]) was given on the remaining 50% of the training sessions; thus, on any given training session, animals had an equal chance of receiving either Δ^9-THC or any one of the other three substances. Each session lasted 20 min.

During the first stage of experiment, only the Δ^9-THC-appropriate lever was present. The position of the lever (left or right) was assigned randomly within and between groups of animals to control for lever bias. The order of stimulus (drug) presentation in both groups of rats was also assigned randomly with the restriction that no drug was administered for more than three consecutive sessions. Conditioning of lever pressing began under a fixed-ratio schedule of reinforcement (FR 1); as response rates stabilized, the ratio was raised gradually to FR 20.

2.3.2. Discrimination training

After all animals were responding reliably under the FR 20 schedule, both levers were presented simultaneously. Responses on the correct lever (the Δ^9-THC-appropriate lever following an injection of Δ^9-THC or the other lever following an injection of vehicle, PCP or morphine) were reinforced under the FR 20 schedule. Responses on the incorrect lever were recorded, but had no further consequences. Training continued until all animals in each group reached a criterion of 80% of the first 20 responses occurring on the condition-appropriate lever for seven consecutive sessions.

2.3.3. Substitution testing

Substitution tests were given with Δ^9-THC (0.8–3.2 mg/kg), pentobarbital (2.5–10 mg/kg), diazepam (2.5–10 mg/kg), anandamide (5–20 mg/kg), and methanandamide (0.8–3.2 mg/kg). For the control dose (0 mg/kg), the last vehicle session before the first testing dose of each test drug was used. Tests were terminated after the first 20 responses on either lever were completed and were conducted under extinction conditions, one or two times/week. Complete substitution was defined as at least 80% responding on the Δ^9-THC-appropriate lever: partial substitution was defined as >20% but <80% responses on the Δ^9-THC-appropriate lever.

2.4. Drugs

Δ^9-THC and diazepam were dissolved in absolute ethanol and diluted with an equal concentration of Emulphor-620 (Rhone-Poulenc, Paris, France) to make a solution of 100 mg/ml. Saline was added to this solution to form a suspension that consisted of emulphor–ethanol–saline (1:1:18). Morphine sulfate and PCP hydrochloride were dissolved in 0.9% saline and equal concentrations of ethanol–Emulphor-620 were added to these solutions. All drugs were given IP in a volume of 1 ml/kg and were obtained from the National Institute on Drug Abuse (NIDA) Rockville, MD.

2.5. Data analysis

The number of sessions to reach criterion (above) was compared across training conditions with Student's *t*-test. During substitution test sessions, the data of primary interest were the proportions of responses occurring on the Δ^9-THC appropriate lever. These values were used to calculate ED50s and 95% confidence intervals (CIs) in the manner of Tallarida and Murray (1987). To be included in the ED50 analysis of the effects of each test drug, each rat was required to make at least 50% drug state-appropriate responding. The bottom limit of rate of responding was 20 response/min. Animals which did not complete at least 20 responses during the 20 min test session were excluded from the analysis of the effects of each test drug.

The maximum proportion of responses on the Δ^9-THC appropriate lever that occurred following each test drug under each of the two training conditions were compared with Student's *t*-tests. Rates of responding prior to the completion of the first 20 responses were also analyzed

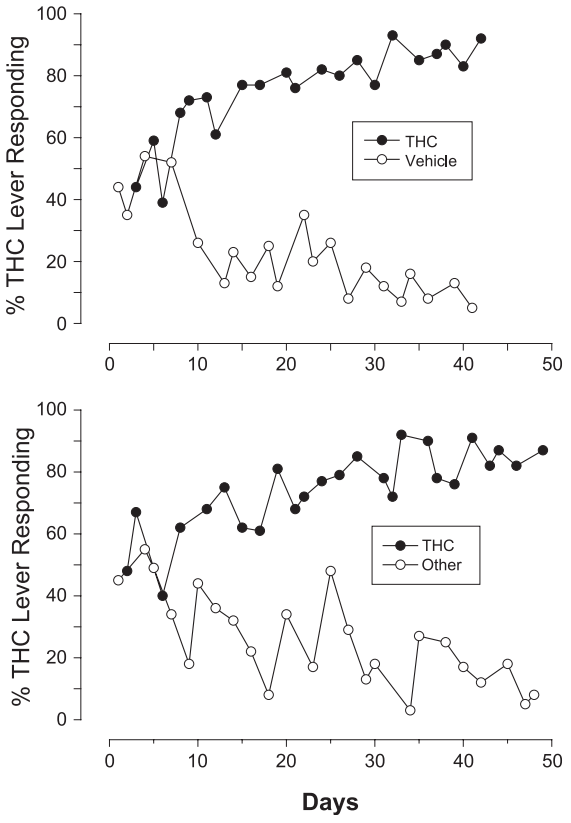

Fig. 1. Acquisition of the Δ^9-THC discrimination. Average percent Δ^9-THC-appropriate responding when subjects are being trained to discriminate Δ^9-THC from either vehicle (top panel) or group of other drugs (bottom panel).

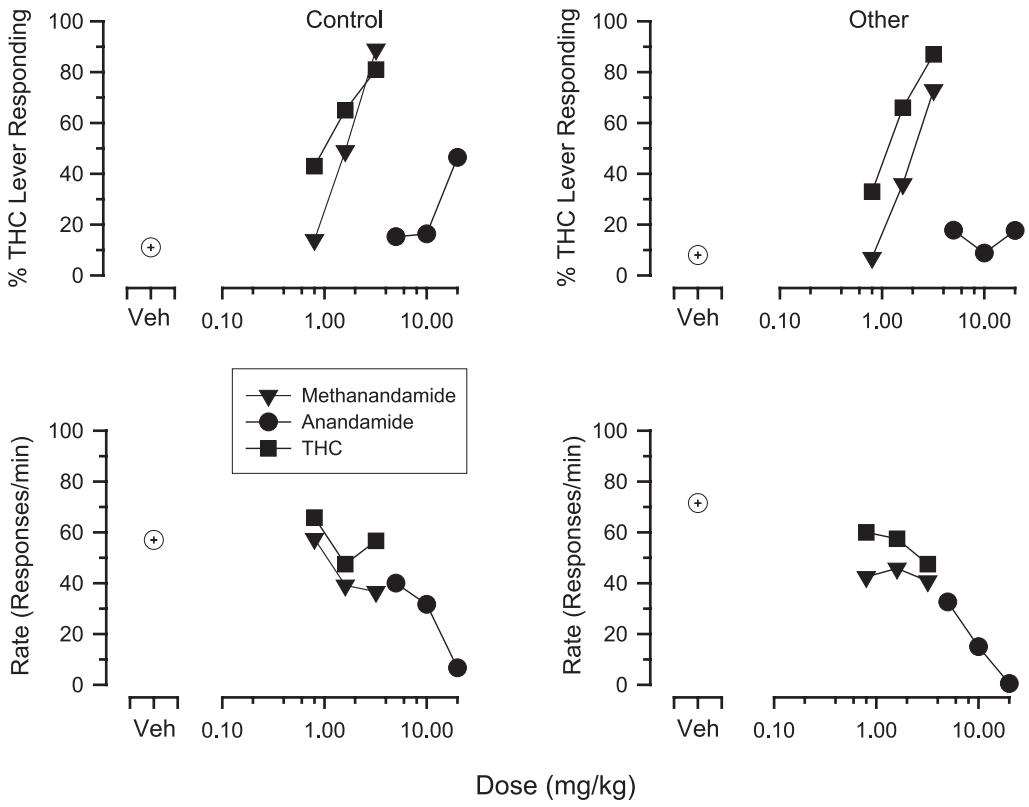

Fig. 2. Results of substitution tests with Δ^9-THC, (*R*)-methanandamide, and anandamide in animals trained to discriminate Δ^9-THC (3.2 mg/kg IP) from either vehicle (left panels) or a group of other drugs (right panels).

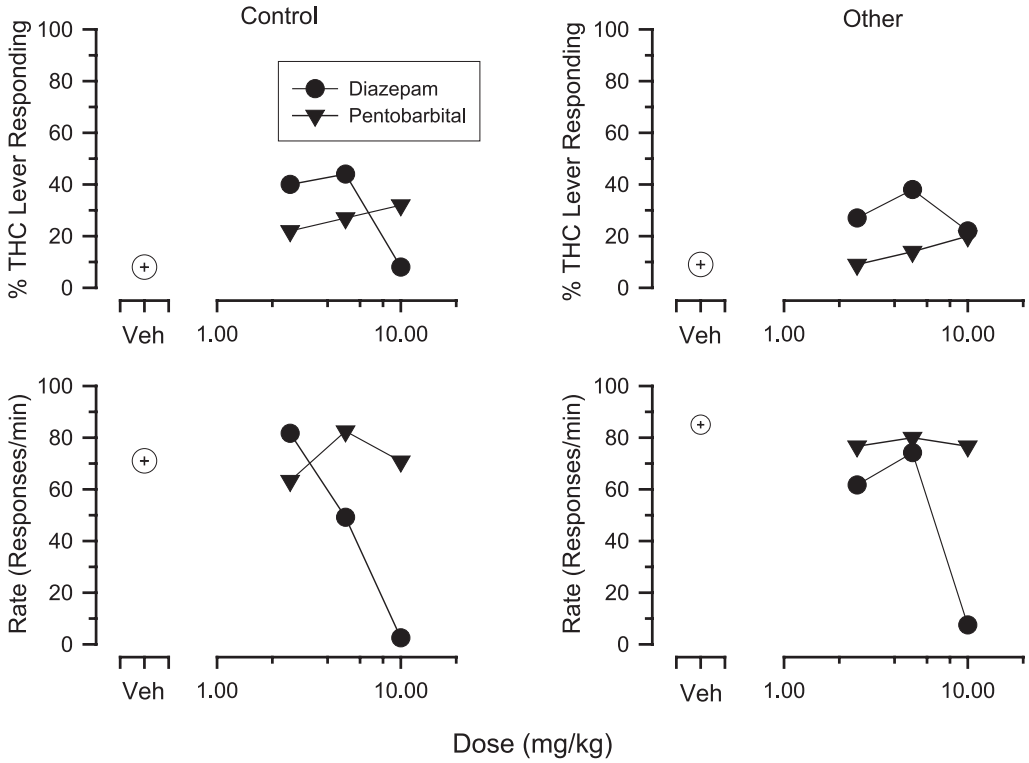

Fig. 3. Results of substitution tests with diazepam and pentobarbital in animals trained to discriminate Δ^9-THC (3.2 mg/kg IP) from either vehicle (left panels) or a group of other drugs (right panels).

with repeated-measures ANOVAS. When F-values were significant ($p<0.05$), post hoc tests were performed using Tukey's Studentized t-test.

3. Results

3.1. Acquisition of drug discriminations

The acquisition of the Δ^9-THC-vehicle (control group) and Δ^9-THC-other (other group) discriminations are shown in Fig. 1. Criterion appeared to be attained more rapidly in the control (33 days) than in Δ^9-THC-other (38 days) group; however, this difference was not significant (t (18)=1.36; $p>0.05$).

3.2. Substitution tests

The results of substitution tests are shown in Figs. 2 and 3; ED50s and 95% confidence intervals (CIs) are summarized in Table 1. Not surprisingly, Δ^9-THC substituted for itself (3.2 mg/kg) in both the control (left panel) and Δ^9-THC-other (right panel) groups in a dose-dependent manner. Anandamide substituted partially (52.62%) for Δ^9-THC in the control group but this effect occurred only at a high dose (20 mg/kg), which also severely disrupted response rate (Fig. 2); significantly less substitution (21.56%) occurred in the Δ^9-THC-other following treatment with this substance than in the control group (t (14)=2.17, $p<0.05$). On the other hand, (R)-methanandamide substituted completely (91.44%) for Δ^9-THC in the control, but only partially (75.50%) in the Δ^9-THC-other group; once again, this difference was significant (t (16)=2.52, $p<0.05$).

Fig. 3 shows the results of substitution tests with pentobarbital and diazepam in both groups. While no dose of pentobarbital substituted completely for Δ^9-THC under either training condition, significantly more Δ^9-THC-lever responding occurred in the control than in the "other" group (t (15)=2.07, $p<0.05$). Thus, training condition had a small, but significant effect.

While diazepam produced slightly more substitution than pentobarbital, the maximal level (44%) occurred following the 5 mg/kg dose in the control group. In addition, the effect of the highest dose of diazepam was accompanied by a significant decrease in response rate (F(3, 15)=7.29, $p<0.05$). In fact, four animals (two in the control, and two in the other group) did not complete 20 responses in 20 min following the administration of 10 mg/kg of diazepam; thus, they were excluded from the calculation of the group average for the highest dose (Fig 3). Training condition had no significant effect on amount of drug-lever responding following diazepam administration (t (14)=0.21, $p>0.05$).

4. Discussion

The results indicate that: (1) rats can be trained to discriminate Δ^9-THC from a group of other drugs consisting of morphine, PCP, and vehicle (no drug) and that such training takes little more time than the commonly used "drug vs. no drug" procedure. (2) The endogenous cannabinoid receptor ligand anandamide substitutes partially for Δ^9-THC in both the control and the Δ^9-THC-other group and, that this partial substitution occurs only at a dose that severely decreases response rate. (3) (R)-methanandamide, an analog of anandamide, which is more resistant to metabolism, substitutes completely for Δ^9-THC in the control, and partially in the drug-other group. (4) Neither pentobarbital nor diazepam substitutes fully for Δ^9-THC under either training condition. (5) For all drugs except diazepam, significantly more substitution occurs following drug-vehicle than drug-other training.

As mentioned previously, the purpose of the present study was to try to increase the selectivity of discriminative stimulus effects of Δ^9-THC by using a differential training procedure, which involved other training drugs. In addition, it was the first study to demonstrate that rats could learn to discriminate Δ^9-THC from an NMDA antagonist, phencyclidine hydrochloride, a μ-opioid receptor agonist morphine, as well as vehicle. Indeed, such training required little more time to reach criterion than the standard "Δ^9-THC vs. vehicle" procedure. This could have been caused by the high rates of responding that were observed under both training conditions, the use of chambers equipped with retractable

Table 1
Results of substitution tests in animals trained to discriminate Δ^9-THC (3.2 mg/kg) from vehicle or from a group of other compounds consisting of vehicle, morphine (3.2 mg/kg) and PCP (2.5 mg/kg)

Drug	Doses tested (mg/kg)	Δ^9-THC-vehicle			Δ^9-THC-other		
		n[a]	ED50	95% CI	n[a]	ED50	95% CI
Δ^9-THC	0.8, 1.6, 3.2	10	0.924[b]	0.49–1.74	10	0.916[b]	0.46–1.79
Methanandamide	0.8, 1.6, 3.2	9	1.316[b]	0.29–4.01	9	1.87[c]	0.74–4.74
Anandamide	5, 10, 20	8	–[c]		8	–	
Diazepam	2.5, 5, 10	8	–[d]		8	–	
Pentobarbital	2.5, 5, 10	9	–[d]		9	–	

[a] Number of animals completing the test.
[b] Complete substitution: ≥80% responding on Δ^9-THC appropriate lever.
[c] Partial substitution: 50% ≥79% responding on Δ^9-THC appropriate lever.
[d] No substitution: ≤49% responding on Δ^9-THC appropriate lever.

levers, or by restricting the availability of reinforcement to a limited duration (3 s; Appel et al., 1999).

Anandamide failed to substitute completely for Δ^9-THC (defined as rats allocating >80% of responses on the drug state-appropriate lever), indicating that, when given exogenously, this substance does not have Δ^9-THC like stimulus effects. This result is reasonably consistent with those of Burkey and Nation (1997) who also obtained partial substitution following a smaller training dose (2 mg/kg). However, the fact that, in both experiments, the highest doses of anandamide tested disrupted response rates severely makes interpretation of such data difficult.

On the other hand, the greatest amount of substitution of anandamide for Δ^9-THC found in the control group (53%) in the present study is much lower than the complete substitution (>80%) of anandamide reported by Wiley et al. (1995). While the training dose of Δ^9-THC used in the earlier experiment was almost the same as the one used herein (3 mg/kg), the doses of anandamide tested ranged from 0.3 to 45 mg/kg; complete substitution was reported only at the highest doses (45 mg/kg), which caused a substantial decrease in response rate.

It would seem that two conclusions can be drawn from the results of these studies: (1) one of the major determinants of the substitution of anandamide for Δ^9-THC seems to be test dose. (2) While anandamide may sometimes substitute for Δ^9-THC, it does so only at doses that significantly decrease response rates, in contrast to Δ^9-THC itself and other structurally diverse cannabinoids, which substitute completely and dose-dependently at doses that do not reduce response rates. Given that partial or incomplete "substitution" accompanied by response rate suppression is induced by high doses of a variety of psychoactive drugs, including pentobarbital (10 mg/kg) and diazepam (10 mg/kg) in the present experiment, there is no reason to argue that such effects are selective to anandamide.

The fact that (*R*)-methanandamide substituted completely (>80%) for Δ^9-THC in the control group, in which animals were trained to discriminate 3.2 mg/kg of Δ^9-THC from vehicle, is consistent with previously reported data indicating that this substance substitutes for Δ^9-THC in rats trained to discriminate a dose of 2 mg/kg of Δ^9-THC (Burkey and Nation, 1997). However, it should be noted that the patterns of substitution found in the two studies differ in at least one important way: in the earlier experiment, the mean percentage of complete substitution was obtained in only 6 of the 14 animals tested whereas complete substitution occurred in the present experiment in all animals and did so at doses that did not significantly alter rates of responding. The results of another study involving structural manipulations including saturation of the arachidonyl constituent, substitution for the ethanolamide constituent or C2 hydroxyl group, and addition of a methyl group, are also consistent with those described in the present report. In addition, they support the hypothesis that methylation of anandamide, which decreases its rate of metabolism, increases its ability to substitute for Δ^9-THC (Wiley et al., 1998).

In the present experiment, the substitutions of both pentobarbital and diazepam for Δ^9-THC were found to be considerably less than those reported previously (Barrett et al., 1995; Mokler et al., 1986). At least some of this apparent "discrepancy" can be explained by the way in which other investigators analyzed their data. For example, Mokler et al. (1986) reported that over 90% Δ^9-THC-lever responding occurred after administration of 3.0 mg/kg of diazepam in 13 of 20 animals, which they called diazepam-responders; the remaining seven animals (diazepam-non-responders) emitted only 14% of their responses on the Δ^9-THC appropriate lever. At least partial substitution of pentobarbital (10 mg/kg) occurred in both groups, with 65% Δ^9-THC appropriate responding in the diazepam-responder group and 84% in the diazepam-non-responder group. While differentiating subjects into "responders" and "non-responders" is not an uncommon practice in drug discrimination research, it is based solely on retrospective analyses of the dependent measure itself and can therefore have no predictive value. If, for example, in the Mokler et al. study (1986), we might assume that the diazepam-responders were somehow more susceptible to GABAergic influences, we would expect a similar pattern for pentobarbital; however, this was not the case. Regardless of how one interprets the data, the fact that neither diazepam nor pentobarbital substituted completely for Δ^9-THC in the present experiment limits the generality of previous findings.

In a more recent study, among three benzodiazepines tested (chlordiazepoxide, diazepam, and midazolam), only diazepam substituted partially for Δ^9-THC; maximum levels of substitution (67%) were obtained with a dose of 3 mg/kg (Barrett et al., 1995). A higher dose (10 mg/kg) produced less Δ^9-THC-lever responding (48%) and decreased response rate significantly. This is consistent with the pattern of diazepam substitution in the control group of the present experiment (an inverted U).

As mentioned previously, one aim of the present research was to eliminate false positives by using the drug-other procedure which did, indeed, significantly reduce responding on the Δ^9-THC related lever following treatment with drugs such as pentobarbital and diazepam that are not considered to be similar to cannabinoids. However, the drug-other procedure did not eliminate responding on the Δ^9-THC lever completely, probably because of the drugs included in the "other" group. PCP and morphine were chosen, as stated previously, because of our interest in the mechanism of action of anandamide, which has been reported to interact with NMDA glutamate and μ-opioid as well as cannabinoid receptors (Hampson et al., 1998; Wiley et al., 1995). It seems likely that if we had used GABAergic agents or at least one other CNS depressant in the other group, false positives would have been eliminated

completely; however, the answer to this question awaits the outcome of future research.

To the extent that both diazepam and pentobarbital engender more responding on the Δ^9-THC related lever than vehicle and may (or may not) substitute for cannabinoids under other experimental conditions, why they might do so requires further explanation. The role of benzodiazepine receptors in the discriminative stimulus properties of Δ^9-THC has been investigated in some of the studies mentioned previously. The reported substitutions of both diazepam and pentobarbital were significantly antagonized by the benzodiazepine antagonists Ro15-1788 and flumazenil (Mokler et al., 1986; Wiley, 1999) but not by the cannabinoid CB1 receptor antagonist SR141716A, which completely and dose-dependently blocked the discriminative stimulus effects of Δ^9-THC (Mansbach et al., 1996; Mokler et al., 1986; Perio et al., 1986; Wiley, 1999; Wiley et al., 1995; Wiley and Martin, 1999). In addition, benzodiazepines and other GABAergic drugs can enhance some of the pharmacological effects of cannabinoids and this enhancement can be blocked by GABA antagonists (Pertwee, 1988). One possible conclusion from all of these data is that the Δ^9-THC like effects of these compounds are mediated by actions at GABA receptors. Among other things, this means that pentobarbital, diazepam and other benzodiazepines do not have any direct effects on cannabinoid CB1 receptors and, hence, should not be expected to substitute for Δ^9-THC in drug discrimination experiments.

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