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SR 141716 (Rimonabant) precipitates withdrawal in marijuana-dependent mice

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Abstract

Repeated marijuana use is known to lead to physical dependence in humans; however, its dependence liability has yet to be adequately assessed in laboratory animals. The goals of the present study were to: assess whether the CB_1 antagonist SR 141716 (rimonabant) precipitates withdrawal in mice that had been repeatedly exposed to marijuana smoke, and to compare these precipitated withdrawal effects to those elicited following intravenous administration of its chief psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC). SR 141716 elicited a significant increase in paw tremors in mice that were repeatedly dosed with either marijuana or Δ^9-THC. Unexpectedly, the blood and brain concentrations of Δ^9-THC following marijuana exposure were considerably lower than those found following Δ^9-THC injection when comparing an equivalent magnitude of paw tremors in both conditions. Finally, Δ^9-THC dose-dependently alleviated SR 141716-induced paw tremors in marijuana-dependent mice, but marijuana itself failed to reverse the precipitated withdrawal effect. It is likely that marijuana exposure generated insufficient Δ^9-THC brain levels (i.e., 203±19 ng/g) to reverse the withdrawal signs compared with the brain levels following intravenous injection (i.e., 1862±82 ng/g). These findings taken together indicate that mice exposed repeatedly to marijuana smoke exhibit similar precipitated withdrawal effects as Δ^9-THC-injected mice.

Keywords: Marijuana; Δ^9-tetrahydrocannabinol (THC); Cannabis; Cannabinoid; Physical dependence; Physical withdrawal; SR 141716 (rimonabant)

1. Introduction

In addition to being the most commonly used illicit drug in the United States (SAMHSA, 1998), marijuana is believed by its proponents to have a niche in medicine (Grinspoon, 1999) and has been used as an antiemetic/antinauseant in cancer chemotherapy, appetite stimulant in AIDS-related wasting, antispasmodic, antiepileptic, ocular hypotensive agent, and analgesic (Joy et al., 1999). Whether marijuana is used for recreational or therapeutic purposes, it is often used repeatedly, thereby increasing the likelihood of leading to the development of physical dependence. Indeed, smoked marijuana (Haney et al., 1999b, Budney et al., 2001) as well as oral administration of its primary psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC) (Jones and Benowitz, 1976, Haney et al., 1999a) have been shown to lead to physical dependence in humans. Thus, determining the dependence liability of marijuana is of great concern.

While it might be expected that dependence will develop to inhalation exposure to marijuana smoke as it does to repeated i.p. or s.c. injection of Δ^9-THC, there are considerable differences between inhalation of smoked plant material and injection of a pure, synthetic chemical. In addition to Δ^9-THC, marijuana contains over 400 chemical constituents, 66 of which are considered cannabinoids (Turner et al., 1980). Thus, these other components of marijuana may be capable of altering the effects of Δ^9-THC and/or possessing its own pharmacological effects. In addition, route of administration is known to affect the potency of Δ^9-THC (Martin, 1985) and may influence the development of physical dependence.

The first goal of the present study was to assess whether mice would become physically dependent following repeated

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inhalation exposure to marijuana smoke by precipitating withdrawal with the CB_1 receptor antagonist SR 141716 (rimonabant). The second goal of this work was to determine whether the withdrawal effects from marijuana were similar or different to those from pure Δ^9-THC. Because intravenously administered Δ^9-THC and inhaled marijuana smoke have been suggested to have similar pharmacokinetic properties (Rosenkrantz et al., 1974), we compared both routes of administration. Although we have previously demonstrated that SR 141716 precipitates withdrawal in dogs repeatedly given intravenous injections of Δ^9-THC (Lichtman et al., 1998), this was the first experiment examining precipitated withdrawal using this route of administration in mice. We assessed the dependence liability of Δ^9-THC delivered via these routes of administration by evaluating the time course of physical dependence development and the dose–response profile. In addition, we evaluated whether an additional injection of Δ^9-THC or exposure to marijuana following SR 141716 challenge in cannabinoid-dependent mice would reverse the withdrawal effects. Finally, we sought to compare Δ^9-THC dose in marijuana-exposed mice to Δ^9-THC-injected mice at various amounts of each substance that elicited an equivalent magnitude of withdrawal effects. To this end, Δ^9-THC levels in blood and brain were determined through liquid chromatography/mass spectrometry (LC/MS) following administration of each substance.

2. Methods and materials

2.1. Animal

ICR male mice (Harlan Laboratories, Indianapolis, IN) weighing between 20 and 25 g served as subjects. The subjects were housed in the animal care quarters maintained at 22±2 °C on a 12-h light/dark cycle. Food (Harlan Teklab, Madison, WI) and water were available ad libitum. The experimental protocol was approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (IACUC) and is in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs

Marijuana (containing 3.46% Δ^9-THC, 0.18% cannabinol, 0.17% cannabidiol, 0.14% cannabigerol, and 0.05% tetrahydrocannabivarin), ethanol-extracted marijuana (placebo), Δ^9-THC, and SR 141716 were obtained from the National Institute on Drug Abuse (Rockville, MD). SR 141716 (rimonabant) and Δ^9-THC were dissolved in a standard vehicle consisting of ethanol:alkamuls EL-620 (Aventis, Princeton, NJ):saline vehicle in a ratio of 1:1:18, with the exception of one experiment in which both drugs were dissolved in a vehicle comprised of DMSO:alkamuls EL-620:saline in a ratio of 1:1:18. All drug solutions were delivered in a volume of 0.1 ml per 10 g animal weight. Repeated intravenous injections were made into alternating tail veins on alternating days, beginning distally and moving rostrally.

2.3. Marijuana inhalation apparatus

The smoking inhalation apparatus was similar to that described elsewhere (Lichtman et al., 2001b). Marijuana was burned in a corncob pipe that was connected to fresh air intake tubing (Tygon®) and attached to an animal exposure manifold. The manifold was manufactured from Plexiglas® in our facilities and allowed nose-only exposure to six mice at a time. There were glass wool and charcoal air filters beyond the manifold to trap particulate matter. A vacuum pump drew air through the system and an airflow regulator maintained the airflow at approximately 360 ml/min. The entire apparatus was housed in a fume hood. Plant material was burned until it was entirely consumed and required approximately 5 min to burn completely 200 mg of plant material.

2.4. Behavioral evaluations

The dose–response relationship of cannabinoid dependence via exposure to marijuana smoke (50, 100, or 200 mg) or intravenously injected Δ^9-THC (1, 3, or 10 mg/kg) was assessed by giving subjects a single daily dose for five days. An additional study was conducted to assess the time course of physical dependence development, in which subjects were given a daily dose of drug or vehicle for 1,3, 5 or 10 days, before evaluation of withdrawal. In the time course experiments, mice were either exposed daily to smoke from 200 mg of marijuana or given daily injections of 5 mg/kg Δ^9-THC. In a previous experiment performed in our laboratory each of these doses yielded approximately equivalent Δ^9-THC blood levels (Lichtman et al., 2001b).

Precipitated withdrawal was elicited using a procedure previously employed by our group (Cook et al., 1998; Lichtman et al., 2001a) in which subjects were challenged with SR 141716 (10 mg/kg, i.p.) 4 h after the final injection or exposure. Fifteen minutes later, animals were placed in separate cages and withdrawal signs were recorded for 30 min. The dependent measures of interest included bouts of paw tremors (a lateral forepaw clapping behavior), head shakes, and scratching (Cook et al., 1998). An observer who was blind to drug condition tallied the incidence of each occurrence. However, as previously reported (Lichtman et al., 2001a), SR 141716 elicited scratching and head shaking behavior by itself; thus paw tremors is the only reported withdrawal sign.

In the reversal experiments, marijuana-dependent mice, which received daily exposures of drug for five days were challenged with an acute injection of 10 mg/kg SR 141716, i.p. four hours after the final dose. Five minutes after SR 141716, each mouse received an intravenous injection of vehicle or Δ^9-THC (2.5, 5, or 10 mg/kg). In another experiment, each mouse received an exposure to marijuana or placebo smoke (200 mg burned material), five minutes after SR 141716 administration. The timing of Δ^9-THC administration and exposure to marijuana smoke was such that peak Δ^9-THC levels would occur during the thirty minute period observation period. Paw tremors were then tallied for 30 min in both experiments.

In order to determine whether decreases in withdrawal activity following a reversal injection of Δ^9-THC were due to gross motor sedation, locomotor behavior was assessed. A Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH) was

used to count photocell-light beam interruptions. Thirty minutes following reversal drug injection, mice were placed in separate chambers (16.5 cm × 25.5 cm × 11.5 cm high) and activity was monitored for 10 min.

2.5. Determination of Δ^9-THC blood and brain levels

Acute Δ^9-THC levels in blood and brain were determined following inhalation exposure from 50, 100, or 200 mg marijuana and 0.3 or 5 mg/kg intravenous Δ^9-THC. Δ^9-THC concentrations for an intravenous injection of 1, 3, or 10 mg/kg Δ^9-THC were taken from a previous study (Wilson et al., 2002). The mice were decapitated 20 min after drug administration and the blood was collected in heparinized (Elkins-Sinn, Inc., Cherry Hill, NJ) test tubes while the head chilled on ice until the brain was removed. An acute time course of Δ^9-THC blood and brain levels was also determined following intravenously administered 3 mg/kg Δ^9-THC or exposure to smoke from 200 mg marijuana. Additionally, blood and brain samples were also sampled 20 min after the reversal exposure to smoke from 200 mg marijuana or an intravenous injection of 10 mg/kg Δ^9-THC. Finally, Δ^9-THC levels from both matrices were evaluated following 1, 3, 5, or 10 days of either daily intravenous injections of Δ^9-THC (5 mg/kg) or daily inhalation exposures to marijuana (200 mg) smoke. Blood and brain levels were determined 4 h after the last drug exposure.

The Δ^9-THC extraction procedure and quantification procedure were conducted as previously described (Wilson et al., 2002). Specifically, calibration standards were prepared from blank mouse whole blood and homogenized brain (2:1, water: brain, v/w). Fifty nanograms of deuterated Δ^9-THC (Radian Corporation, Austin, TX) was added to the blood sample, brain homogenate, and calibrators as an internal standard. Following an equilibration period, 2.5 ml of cold acetonitrile (HPLC grade, Fisher Scientific, Raleigh NC) was added drop-wise while vortexing. The samples were then centrifuged (Precision Vari-Hi-Speed Centricone, Precision Scientific Co., Chicago, IL) at 2500 rpm for 15 min in order to pelletize solids and then stored in a freezer (−20 °C) overnight, allowing the acetonitrile layer to separate from aqueous layers. The acetonitrile layer was then removed and evaporated to dryness under nitrogen. The Δ^9-THC/deuterated Δ^9-THC was then resolubilized in 0.1 ml methanol (HPLC grade, Fisher Scientific).

LC-MS was used to quantify Δ^9-THC and deuterated Δ^9-THC in blood and brain matrices. The mobile phase consisted of an 85:15 methanol:1% glacial acetic acid (0.1% formic acid). A guard column was used inline with the standard reverse phase C18 column. The mass spectrometer was run in APCI+ mode. Ions analyzed in single ion monitoring mode were 315 for Δ^9-THC and 318 for deuterated Δ^9-THC. A calibration curve was constructed for each assay based on linear regression using the peak-area ratios of Δ^9-THC to deuterated Δ^9-THC of the extracted calibration samples. No peaks were detected above background in the blank control samples, blank blood samples, or blank brain samples. The extracted standard curves ranged from 25 to 5000 ng. Experimental samples that were not linear ($r \geq 0.99$) were excluded from analysis.

2.6. Statistical analyses

Student *t*-tests and analysis of variance (ANOVA) were performed to determine significance at the level of 5% ($p < 0.05$). The Bonferroni test was used for planned comparisons in the time course experiments. Dunnett's post-hoc comparison was used in the dose–response and withdrawal reversal studies in which each experimental group was compared with the control for each respective experiment. In the behavioral experiment a mean of six subjects was used for each group and in the dosimetry studies at least three subjects were used at each time point. In all experiments, subjects were used once.

In order to determine the dose or time required to produce the half-maximal effect, paw tremors at each dose or time point were expressed as a percent of maximum possible effect. The Emax for paw tremors was determined by reciprocal plot. The half-maximal effective dose was then determined using least squares linear regression analysis and calculation of 95% confidence limits.

3. Results

One group of mice was given daily intravenous injections of vehicle or varying doses of Δ^9-THC and a second group was exposed daily to smoke from 200 mg of placebo marijuana or varying quantities of marijuana. As shown in Fig. 1A, five days of daily injections of intravenously administered Δ^9-THC, led to a significant dose-related increase in the number of paw tremors observed during the 30 min observation period, $F(3,20) = 7.9$, $p < 0.05$. The ED_{50} (95% C.L.) value for the repeated intravenous injections of Δ^9-THC per day was 4.1 (2.2–7.7) mg/kg. An additional experiment was conducted in which we compared withdrawal effects elicited by repeated injections of Δ^9-THC (10 mg/kg) and SR 141716 (10 mg/kg) challenge in mice that received both drugs but dissolved in different vehicles; either the standard vehicle (ethanol:alkamuls EL-620:saline) or ethanol-free vehicle (DMSO:alkamuls EL-620:saline). The mean (±s.e.) number of paw tremors for mice receiving the drugs in the standard vehicle and ethanol-free vehicle were 34.0 ± 8.6 and 27.8 ± 9.0, respectively. This magnitude of effect did not differ between the two groups ($p = 0.62$) and is remarkably similar to the number of paw tremors of mice treated with the same dosing regimen of Δ^9-THC in the standard vehicle shown in Fig. 1A. Although these findings suggest that the presence of ethanol in the vehicle had no functional impact on withdrawal under the present conditions, it is possible that ethanol may alter withdrawal resulting from other Δ^9-THC dosing regimens.

The precipitated withdrawal data of mice given five days of daily exposures to marijuana is depicted in Fig. 1B. A significant dose-related increase in paw tremors was found, $F(3,28) = 6.6$, $p < 0.05$. The ED_{50} (95% C.L.) value for the repeated exposure to smoke was 105 (77–143) mg marijuana/day. Based on the percentage of Δ^9-THC present in the marijuana, the ED_{50} (95% C.L.) dose was calculated to be 3.6 (2.7–5.0) mg of Δ^9-THC in marijuana smoke. However, side-stream smoke, as well as other factors related to lung physiology and experimental methodological issues, undoubtedly resulted in each mouse receiving a substantially smaller amount than this estimated value.

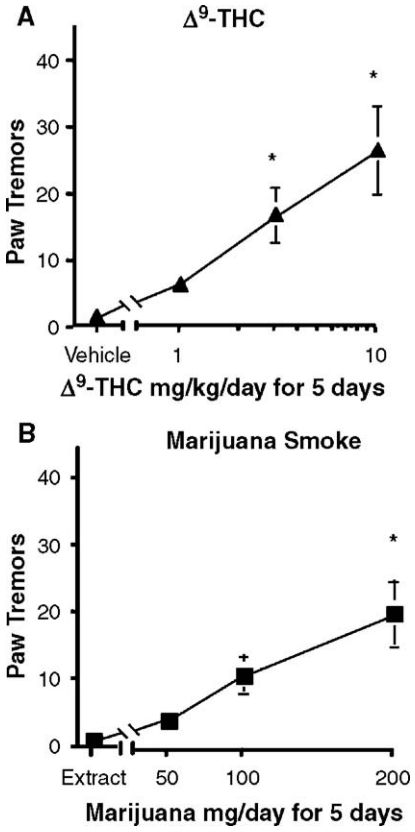

Fig. 1. Effects of SR 141716 on paw tremors following five days of either intravenous Δ^9-THC injections or exposure to marijuana smoke: Panel A. intravenous injection of Δ^9-THC compared to vehicle alone, and Panel B. marijuana smoke compared to placebo smoke (200 mg of ethanol extracted marijuana). Data shown as means±s.e. An asterisk denotes a significant difference ($p<0.05$) from control.

Consequently, we evaluated the concentration of Δ^9-THC in blood and brain following both routes of administration, as a more accurate measurement of dose (see Table 1). Interestingly, a disparity with respect to the proportion of Δ^9-THC in the two matrices was found between mice that were given intravenous injections of Δ^9-THC and mice that were given inhalation exposures to marijuana smoke. Whereas the Δ^9-THC blood levels following exposure to smoke from 100 or 200 mg marijuana were similar to those following intravenous administration of 3 mg/kg Δ^9-THC, the Δ^9-THC brain concentrations following both of these exposures were similar to those following 1 mg/kg Δ^9-THC. We have previously observed a similar pattern of results between inhalation of Δ^9-THC aerosol and intravenous injection (Wilson et al., 2002).

An additional study was performed in which the time course of Δ^9-THC clearance from blood and brain was compared between intravenous injection of 3 mg/kg Δ^9-THC and exposure to 200 mg marijuana (Table 2). Calculating each time point as the percentage of the 20-min value following treatment normalized the data. As shown in Fig. 2, Δ^9-THC blood levels decreased more rapidly after injection than after inhalation of marijuana smoke. In contrast, the Δ^9-THC brain levels decreased at a similar rate in mice given intravenous injections of Δ^9-THC and mice given an inhalation exposure to marijuana smoke.

The effects of SR 141716 in mice given 1, 3, 5, or 10 days of exposures to marijuana smoke or i.v. Δ^9-THC are depicted in Fig. 3. In no case did the groups treated with drug differ from their respective vehicle control group following one exposure to drug. Planned comparisons made between each 200 mg marijuana group (i.e., 6.9 mg Δ^9-THC prior to burning) and its respective 200 mg placebo control revealed significant differences ($p<0.0125$) at day 3 ($t(17)=4.6$), day 5 ($t(16)=3.5$), and day 10 ($t(10)=3.2$). The time required to produce the half-maximal effect in this protocol (95% confidence limits) was 2.8 (1.6 to 4.8) days. Paw tremors were significantly elevated in animals receiving 5 mg/kg Δ^9-THC ($p<0.0125$) for day 3 ($t(10)=6.2$), day 5 ($t(10)=3.3$), and day 10 ($t(10)=3.1$). The number of 5 mg/kg injections necessary to produce a half-maximal effect was 6.7 (3.9–11) injections (days). The blood and brain levels of Δ^9-THC following 1, 3, 5, or 10 daily intravenous injections of Δ^9-THC (5 mg/kg) or 1, 3, 5, or 10 daily inhalation exposures to marijuana (200 mg) smoke are shown in Table 3. The levels of drug present in blood and brain 4 h following exposure were not altered by repeated daily dosing.

Previous research has demonstrated that re-administration of Δ^9-THC can reverse abstinence withdrawal (Jones et al., 1981, Beardsley et al., 1986), as well as SR 141716-precipitated Δ^9-THC withdrawal (Lichtman et al., 2001a). Thus, we evaluated

Table 1
Δ^9-THC blood and brain levels (means±s.e.) 20 min after intravenous Δ^9-THC injection or marijuana smoke exposure

Drug	Dose	Blood	n	Brain	n
		ng/ml Δ^9-THC		ng/g Δ^9-THC	
Intravenous Δ^9-THC	0.3 mg/kg	33±2	6	64±4	6
	1 mg/kg[a]	102±7	5	307±28	5
	3 mg/kg[a]	323±36	8	900±37	8
	5 mg/kg	522±56	3	1170±131	3
	10 mg/kg[a]	1325±190	6	3307±190	6
Marijuana Smoke	50 mg	98±13	9	136±8	8
	100 mg	241±36	11	256±30	12
	200 mg	385±110	10	400±32	12

ED_{50} drug amounts for intravenous Δ^9-THC and marijuana were 4.1 mg/kg and 105 mg of plant material, respectively.
[a] Results previously reported (Wilson et al., 2002).

Table 2
Time course of Δ^9-THC blood and brain concentrations (means±s.e.) following intravenous injection (3 mg/kg) or inhalation exposure to marijuana (200 mg) smoke

Condition	Time point (min)	Blood	Brain
		ng/ml Δ^9-THC (n)	ng/g Δ^9-THC (n)
Intravenous Δ^9-THC	20	365±39 (6)	854±42 (8)
	40	255±29 (3)	614±87 (3)
	60	186±7 (3)	630±10 (3)
	120	56±6 (3)	294±15 (3)
	180	26±3 (3)	141±21 (3)
	240	14±1 (3)	78±5 (3)
Inhalation marijuana	20	365±31 (3)	484±81 (3)
	40	255±43 (3)	529±25 (3)
	60	281±52 (3)	433±66 (3)
	120	221±64 (3)	304±91 (3)
	180	78±11 (6)	52±5 (6)
	240	96±30 (9)	29±4 (4)

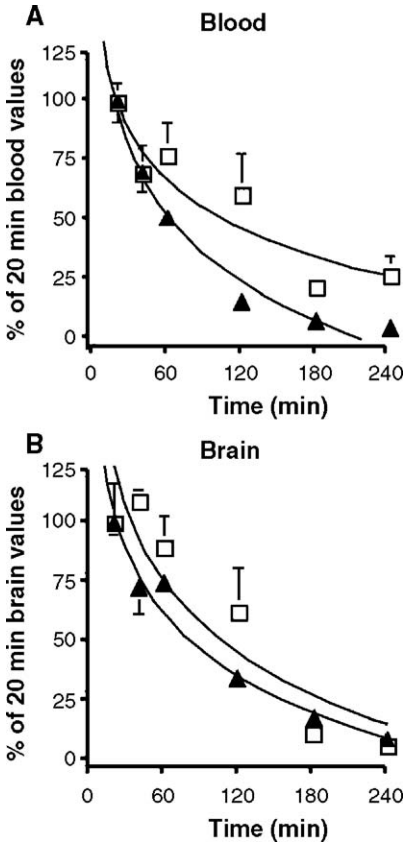

Fig. 2. Time course of Δ^9-THC in blood and brain matrices following injection or smoke exposure. Animals were given an intravenous injection of 3 mg/kg Δ^9-THC (▲) or received a single exposure to smoke from 200 mg of marijuana (□). Data are represented as percentages (means±s.e.) of the 20 min blood (Panel A) and brain (Panel B) concentrations. The concentrations of Δ^9-THC in blood at 20-min were 546 and 356 ng/ml following intravenous administration and marijuana smoke exposure, respectively. The concentrations of Δ^9-THC in brain at 20 min following intravenous administration and marijuana smoke exposure were 854 and 485 ng/g, respectively. Data shown as means±s.e.

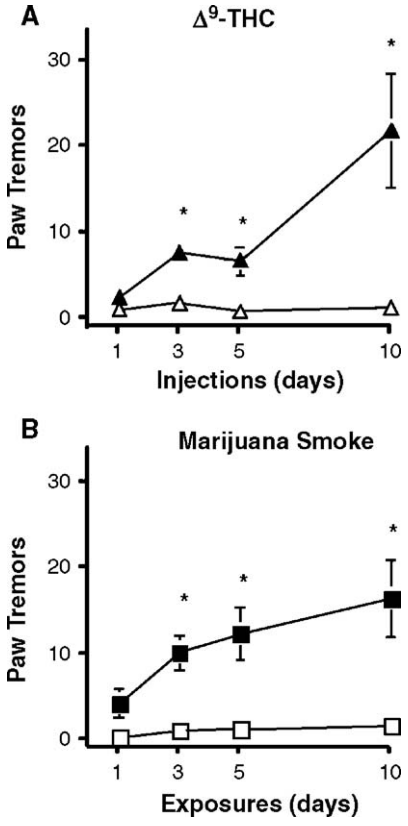

Fig. 3. The occurrence of SR 141716 precipitated paw tremors is dependent on the number of exposures. Paw tremors were observed for 30 min after SR 141716 precipitated withdrawal following injection of 5 mg/kg/day Δ^9-THC (Panel A) or exposure to marijuana smoke from 200 mg/day (Panel B) for 1, 3, 5, or 10 days. Data shown as means±s.e. An asterisk denotes a significant difference ($p<0.0125$) between each drug group (closed symbols) and its respective control group (open symbols).

whether Δ^9-THC or marijuana smoke would alleviate SR 141716-precipitated paw tremors in marijuana-dependent mice. Administration of a reversal dose of intravenously administered Δ^9-THC (0, 2.5, 5.0, or 10 mg/kg) given five minutes after SR 141716 challenge in marijuana-dependent mice led to a significant dose-dependent decrease in precipitated paw tremors, $F(3,21)=7.5$, $p<0.05$, with an ED_{50} dose of 5.0 (3.8–6.7) mg/kg Δ^9-THC (Fig. 4). In order to assess whether the sedative effects of Δ^9-THC interfered with the paw tremor response, locomotor activity was evaluated during the time period that the animals would be observed for withdrawal signs. As depicted in Table 4, there were no significant differences in locomotor activity between the Δ^9-THC and vehicle reversal groups following SR 141716-challenge in marijuana-dependent mice, $t(10)=0.7$, $p=0.49$.

In contrast, exposure to smoke from 200 mg marijuana failed to reverse SR 141716 precipitated paw tremors as compared to the placebo reversal group, $t(14)=1.1$, $p=0.30$, (Table 4). Blood and brain levels generated by a reversal exposure to marijuana were significantly lower than those from an intravenous dose of 10 mg/kg Δ^9-THC, $t(9)=4.0$, $p<0.05$ and $t(9)=20.2$, $p<0.05$, respectively (Table 4). However, higher doses of marijuana were not evaluated because death occurred in about 10% of the mice exposed to smoke from 200 mg of burned material.

4. Discussion

The results from the present study indicate that SR 141716 precipitated a similar withdrawal syndrome in mice after repeated

Table 3
Δ^9-THC blood and brain concentrations (means±s.e.) following 1, 3, 5, or 10 days of either daily intravenous injections of Δ^9-THC (5 mg/kg) or daily exposures to marijuana (200 mg) smoke

Condition	Number of exposures	Blood	Brain
		ng/ml Δ^9-THC (*n*)	ng/g Δ^9-THC (*n*)
Intravenous Δ^9-THC	1	14±1 (11)	78±6 (6)
	3	16±1 (8)	67±8 (3)
	5	16±2 (7)	60±6 (3)
	10	26±4 (9)	66±1 (3)
Inhalation marijuana	1	133±23 (12)	29±4 (4)[a]
	3	134±16 (16)	51±9 (5)
	5	116±12 (8)	31±7 (6)
	10	91±8 (11)	34±1 (3)

Levels were assessed 4 h after drug administration, corresponding to the behavioral assessment.
[a] Value taken from the 4 h time point in Table 2.

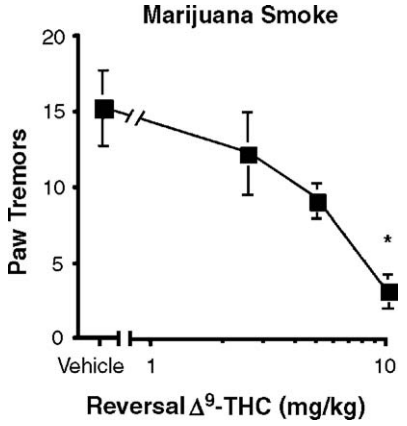

Fig. 4. Δ^9-THC re-administration dose-dependently reverses SR 141716-precipitated increases in paw tremors. Mice were given five days of exposure to smoke from 200 mg marijuana/day. On the fifth day, subjects were treated with SR 141716 (10 mg/kg, i.p.) and then given an intravenous injection of either vehicle or Δ^9-THC (2.5, 5, or 10 mg/kg) to reverse withdrawal signs. Δ^9-THC blocked SR 141716-precipitated paw tremors at an ED_{50} (95% C.L.) dose of 5.0 (3.8–6.7) mg/kg. Data are expressed as means±s.e. An asterisk denotes a significant difference ($p<0.05$) from the vehicle condition.

exposure to marijuana smoke or repeated intravenous injections of Δ^9-THC. These findings are consistent with those from human studies that described similar withdrawal effects following cessation from repeated dosing of smoked marijuana or oral Δ^9-THC (Haney et al., 1999a,b). Induction of physical dependence, as characterized by SR 141716-precipitated increases in paw tremors, was related to the amount of marijuana smoke or intravenous Δ^9-THC to which the mice were exposed. SR 141716 precipitated a similar magnitude of paw tremors following five days of daily exposure to smoke from 200 mg marijuana or intravenous injection of 5 mg/kg Δ^9-THC. Daily exposures to doses of marijuana or injected Δ^9-THC that produced similar behavioral effects resulted in similar temporal patterns for the development of dependence. Dependence occurred with as few as three daily exposures to drug. Interestingly, this time point coincided with published reports of decreased CB_1 receptor binding in the cerebellum (Breivogel et al., 1999), a neuroanatomical site that has been associated with cannabinoid withdrawal (Tzavara et al., 2000). Increasing the number of drug exposures resulted in an increased magnitude of paw tremors following SR 141716 treatment.

Intravenous Δ^9-THC completely alleviated the precipitated increase in paw tremors in a dose-dependent fashion, with an ED_{50} reversal dose of 5.0 mg/kg Δ^9-THC. On the other hand, re-administration of marijuana failed to reverse significantly SR 141716-precipitated paw tremors in marijuana-dependent mice. The blood and brain levels of Δ^9-THC generated following a single reversal exposure to marijuana smoke were likely to have been insufficient to displace SR 141716 from the CB_1 receptor. Consistent with this explanation is that the brain concentration of Δ^9-THC following smoke exposure from 200 mg marijuana was equal to an intravenous injection of 1 mg/kg Δ^9-THC, a dose that was insufficient to reverse SR 141716-precipitated paw tremors (i.e., 2.5 or 5.0 mg/kg Δ^9-THC failed significantly to attenuate this withdrawal sign).

Intravenous administered Δ^9-THC and inhaled marijuana smoke have been shown to result in similar initial Δ^9-THC plasma distribution curves in human subjects (Ohlsson et al., 1981). Consequently, it has been suggested that intravenously administered Δ^9-THC can be used in lieu of marijuana for pharmacological and toxicological studies in rodents (Rosenkrantz et al., 1974). However, it is important to note that in addition to containing Δ^9-THC, marijuana smoke contains more than 60 compounds that are considered cannabinoid in structure as well as consists hundreds of noncannabinoid chemicals (Turner et al., 1980). Additionally, several notable differences were found in the present study between animals given nose-only exposures to marijuana smoke and animals given intravenous injections of Δ^9-THC. Using doses that led to similar degree of dependence, the brain concentrations of Δ^9-THC following intravenous administration were higher than those obtained from the inhaled marijuana smoke. Specifically, the EC_{50} brain levels of Δ^9-THC at 20 min of the half-maximal doses/day for marijuana (i.e., 260 ng/g) were significantly lower than for intravenous injection (i.e., 1000 ng/g). Another difference between inhalation and intravenous routes of administration is that the magnitude of SR 141716-precipitated paw tremors following three and five days of agonist treatment was less for intravenous injection than for marijuana inhalation. Consequently, intravenous administration required a longer number of treatment days to achieve maximal dependence than inhalation administration. It is likely that a pharmacokinetic difference between intravenous and inhalation routes of administration was a contributing factor for this disparity.

Whereas injected Δ^9-THC and marijuana smoke exposure possessed similar Δ^9-THC clearance rates from brain, blood drug levels dropped more slowly following marijuana smoke exposure than after intravenous injection. At 4 h, blood Δ^9-THC concentrations fell 96% from the 20-min intravenous levels and fell 74% from the 20-min smoke exposure levels. In contrast, the respective brain concentrations for injected Δ^9-THC and marijuana smoke exposure fell 91% and 94% from the 20-min values. One explanation for this apparent pharmacokinetic difference is related to absorption issues due to the mouse inhalation procedure. Mice are obligate nose-breathers with an extensive nasal infra-architecture that results in a large portion of the aerosol not reaching the alveoli. It has been reported that up

Table 4
Reversal of SR 141716 precipitated paw tremors following five daily exposures to smoke from 200 mg marijuana

Reversal Drug	Paw tremors	Locomotor activity counts	Blood	Brain
			ng/ml Δ^9-THC	ng/g Δ^9-THC
Placebo marijuana (200 mg)	18±3	ND	ND	ND
Marijuana (200 mg)	13±4	ND	366±97	203±19
Intravenous vehicle	18±4	1624±158[a]	ND	ND
Intravenous Δ^9-THC (10 mg/kg)	3±1[b]	1938±409[a]	839±52[c]	1862±88[c]

Data are expressed as means±s.e.
ND = not determined.
[a] Separate groups of mice were used for locomotor activity experiments.
[b] Significantly different from vehicle reversal group ($p<0.05$).
[c] Significantly different from marijuana reversal group ($p<0.05$).

to 40% of particles ranging in size from 2 to 3 μm deposited in the upper respiratory tract of rats (Schlesinger, 1985) and through empirical modeling it was estimated that less than 15% of particles with a mass median aerodynamic diameter of 2.3 μm would actually reach the alveolar regions of rats compared with 40% in a human (Asgharian et al., 1995). This would result in upper respiratory tract deposition ranging from 60 to 85% of the exposed dose in rats and this percentage would be expected to be even higher in mice. Consequently, prolonged blood levels following marijuana exposure could result from the absorption of Δ^9-THC from the upper respiratory tract as well as from the gastrointestinal tract following ciliary movement of particles to the esophagus where they are swallowed. Despite that humans actively inhale marijuana smoke, while the mice were passively exposed to the smoke through the nose-only exposure system, two observations are consistent with the notion that mice absorbed a relevant amount of drug through the lungs. Specifically, the pharmacological effects occurred within 5-min following inhalation exposure to either marijuana (Lichtman et al., 2001b) or aerosolized Δ^9-THC (Wilson et al., 2002) and a fairly rapid increase in brain levels occurred in the present study (see Table 2).

Alternatively, the prolonged elevation of Δ^9-THC blood levels following inhalation exposure might result from other constituents present in marijuana smoke interfering with metabolism. Previous work demonstrated that pretreatment with large doses of cannabidiol, a non-psychoactive marijuana constituent, led to increases in Δ^9-THC brain levels in mice (Bornheim et al., 1995) and rats (Reid and Bornheim, 2001). On the other hand, cannabidiol failed to alter the pharmacokinetics of Δ^9-THC following oral administration in a 2:1 ratio (Agurell et al., 1981). Recently, we have demonstrated that at equivalent doses cannabidiol does not modify the acute pharmacological effects (e.g., antinociception, catalepsy, and hypothermia) of Δ^9-THC in mice (Varvel et al., 2006). Regardless, the low percentage of cannabidiol present in the marijuana used in the present study indicates that it did not play a relevant role in the clearance of Δ^9-THC. In any event, the lack of alterations of Δ^9-THC in blood and brain following daily exposures to intravenous Δ^9-THC or marijuana (see Table 3) suggest that changes in pharmacokinetic factors are unlikely to account for the differences observed between injected Δ^9-THC and inhaled marijuana smoke.

As in the case of opioids, cocaine, and other drugs of abuse, there is not a monotonic relationship of cannabinoid dose and effect between mice and humans. In the present study, the low dose marijuana exposure (i.e., 50 mg marijuana containing 3.46% Δ^9-THC), which failed to elicit any evidence of physical dependence in mice (see Fig. 1), led to Δ^9-THC plasma levels of 98±13 ng/ml (mean+SE) at 20 min (see Table 1). This blood level is comparable to the 77±19 ng/ml Δ^9-THC plasma level found in humans after smoking a large amount of marijuana (i.e., 900 mg of marijuana containing 3.55%) (Huestis et al., 1992). Although mice generally require substantially higher doses of cannabinoids than humans to achieve comparable effects, the fact that cannabinoids elicit similar pharmacological effects in both species supports the utility of the mouse model. Likewise, it has been estimated that on a body weight basis, humans are generally more vulnerable to chemicals than are experimental animals by a factor of 10 (Eaton and Klaassen, 1996).

The degree of physical dependence observed in the present study, as measured by precipitated increases in paw tremors, was less than previous reports of precipitated withdrawal in mice. In one study, SR 141716 precipitated approximately 40–55 paw tremors during a 45-min observation period in mice given 10 to 20 mg/kg Δ^9-THC twice a day for 6 days (Hutcheson et al., 1998). In another study, SR 141716 given to mice injected with 10 mg/kg Δ^9-THC twice a day for 6.5 day led to approximately 100 paw tremors during a 30-min period (Cook et al., 1998). In yet another study, mice dosed with Δ^9-THC 10 mg/kg twice a day for only 2.5 days and challenged with SR 141716 exhibited between 30 and 45 paw tremors (Lichtman et al., 2001a). Thus, dose of Δ^9-THC and dosing schedule affects the degree of physical dependence as measured by precipitated increases in paw tremors.

Unlike some previous studies, head shaking did not represent a precipitated withdrawal sign as SR 141716 elicited this effect regardless of agonist treatment. Others have also reported that SR 141716 given alone elicits head shakes. Although Cook et al. (1998) reported that Δ^9-THC-dependent mice exhibited a significant increase in head shaking upon challenge with SR 141716, head shakes were also elevated in control mice receiving an injection of SR 141716. Similarly, Hutcheson et al. (1998) found that SR 141716 elicited increases in shaking as well as grooming behavior in control mice. Finally, in another study that employed a shortened Δ^9-THC dosing regimen, SR 141716 elicited a dose-dependent increase in head shakes regardless of Δ^9-THC treatment (Lichtman et al., 2001a). In general, studies in which head shakes were indicative of withdrawal used higher dosing regimens (Cook et al., 1998, Hutcheson et al., 1998) than that used in the present study and others (Lichtman et al., 2001a) in which SR 141716 failed to precipitate head shakes above the controls levels. Thus, higher doses of Δ^9-THC than those used in the present study might be required to induce other withdrawal behaviors, such as head shakes.

In the present study, SR 141716 alone also produced an elevation in scratching behavior regardless of repeated treatment group. Similarly, SR 141716 has been reported to elicit scratching in rats (Aceto et al., 1996) and mice (Cook et al., 1998) and has also been reported to have other behavioral actions such as anxiety-like responses in the defensive withdrawal test and elevated plus-maze (Navarro et al., 1997), increased locomotor activity (Compton et al., 1996), hyperalgesia (Richardson et al., 1998), anorexic effects (Di Marzo et al., 2001), and memory enhancing effects (Terranova et al., 1996). Although these pharmacological effects might reflect the blockade of endogenous cannabinoid tone, they may also be due to intrinsic effects of SR 141716. For example, SR 141716 has been shown to have inverse agonist activity in $G_{i/o}$-protein binding studies (Landsman et al., 1997, Bouaboula et al., 1997), though its inverse agonist activity is approximately 7000 fold less potent than its activity as a CB_1 receptor antagonist (Sim-Selley et al., 2001). Nonetheless, whether SR 141716 elicits head shaking and scratching through inverse agonism, inhibition of endogenous cannabinoid activity, or at a site other than the CB_1 receptor remains to be determined.

It was noted that the inhalation experiments were not without lethality. Approximately 10% of mice died after the first or second exposure to smoke generated from 200 mg of plant material.

Rarely did an animal die after surviving the first two exposures. It is unlikely that the restraint alone contributed to the lethality as indicated by the lack of deaths in our previously reported Δ^9-THC aerosol exposure study (Wilson et al., 2002) in which mice were restrained twice as long as the mice in the smoke exposure study reported here. It is more likely that hypoxia contributed to the deaths. In more recent experiments, we have found that virtually no deaths occur during exposure to marijuana smoke when the flow rate is increased from 360 ml/min (i.e., flow rate in the present study) to at least 500 ml/min. Consequently, when rodents are restrained, as is the case of our nose-only exposure system, they are more apt to experience toxicity during periods of hypoxia when compared to non-restrained animals. In support of this notion, the LD_{50} values following inhalation exposure to carbon monoxide were lower in restrained rats than in unrestrained rats (Lapin and Burgess, 1981). Thus in the present study, the mice might have habituated to the restraint system following the repeated exposure sessions, which resulted in a decrease in lethality over sessions.

In conclusion, we found that repeated exposure to marijuana, as well as repeated intravenous injections of Δ^9-THC, led to physical dependence in mice as reflected by an increase in SR 141716-precipitated paw tremors. Similar to human (Budney et al., 2001) and nonhuman primate (Beardsley et al., 1986) cannabinoid abstinence withdrawal studies, re-administration of Δ^9-THC reversed SR 141716 precipitated withdrawal signs in marijuana-dependent mice. Thus similar to its chief psychoactive constituent Δ^9-THC, as well as synthetic cannabinoids, marijuana possesses dependence liability in laboratory animals. The observation that SR 141716 could precipitate withdrawal effects following as few as three exposures to marijuana or Δ^9-THC in mice raises concern that even a few consecutive days of recreational or therapeutic cannabinoid use could lead to the development of physical dependence in humans. Nonetheless, it will be important in future studies to examine the utility of the SR 141716-precipitated cannabinoid withdrawal rodent model in predicting cannabinoid abstinence withdrawal that occurs in humans.

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