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European Journal of Pharmacology 416 (2001) 75–81

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Spontaneous and precipitated withdrawal with a synthetic cannabinoid, WIN 55212-2

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Received in revised form 21 February 2001; accepted 26 February 2001

Abstract

Physical dependence on the synthetic cannabinoid-receptor agonist *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN 55212-2) was demonstrated in rats by the use of a chronic continuous infusion. Spontaneous withdrawal, of moderate intensity, was shown for the first time with this class of drugs of abuse. Behavioral withdrawal signs were also elicited after challenge with (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · HCl (SR141716A), a specific CB_1 cannabinoid-receptor antagonist. In both instances, the high-dose regimen (4, 8, 16 and 16 mg/kg/day, i.p. on days 1–4, respectively) was sufficient to evoke a typical withdrawal syndrome quantified by the signs wet-dog shakes and facial rubs. These results are discussed relative to those obtained with Δ^9-tetrahydrocannabinol and anandamide. With Δ^9-tetrahydrocannabinol, precipitated but not spontaneous or abrupt withdrawal was observed, and this was ascribed to pharmacokinetic properties. Anandamide, which showed little, if any, physical dependence potential, behaved atypically. Possible implications regarding pharmacotherapeutic and human abuse issues are discussed. © 2001 Published by Elsevier Science B.V.

Keywords: WIN 55212–2; SR141716A; Cannabinoid; Withdrawal, spontaneous; Withdrawal, precipitated; (Rat)

1. Introduction

A number of investigators demonstrated that *N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · HCl, (SR141716A), a cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994), precipitated withdrawal in rats receiving chronic dose regimens of delta-9-tetrahydrocannabinol (Aceto et al., 1995, 1996; Tsou et al., 1995; De Fonseca et al., 1997; Beardsley and Martin, 2000). However, withdrawal signs were not observed following spontaneous withdrawal. Precipitated withdrawal was also observed in mice (Cook et al., 1998) and dogs (Lichtman et al., 1998). In subsequent studies, we explored the potential physical dependence liability of anandamide, an endogenous cannabinoid CB_1 receptor agonist (Aceto et al., 1998), as well as arachidonic acid, its precursor and metabolite (Devane et al., 1992), and 2-methylarachidonyl-2-(2′-fluoroethyl)-amide, a metabolically stable analog. Even with the use of a continuous-infusion model, a procedure which increases the likelihood of the development of physical dependence (Aceto, 1990), all three eicosanoids were inactive (Aceto et al., 1998).

Nevertheless, spontaneous withdrawal was noted in human cannabis and delta-9-tetrahydrocannabinol abusers (Jones et al., 1976; Crowley et al., 1998; Kouri et al., 1999; Haney et al., 1999). Needless to say, the experimental parameters and animal model we used do not mimic human practices. Furthermore, the withdrawal signs observed in humans [aggression, irritability and anxiety (Kouri et al., 1999; Haney et al., 1999; Crowley et al., 1998) are much more subtle than those we noted in rats (wet-dog shakes, facial rubs and paw shakes]. Regarding anandamide, although it has many important pharmacological properties in common with delta-9-tetrahydrocannabinol and other cannabinoids, it also manifests significant atypical effects (Aceto et al., 1998).

The lack of correspondence between the results obtained with delta-9-tetrahydrocannabinol and the anandamide-related compounds also raised the possibility that physical dependence was peculiar only to delta-9-tetrahydrocannabinol. To help resolve this question *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,

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0014-2999/01/$ - see front matter © 2001 Published by Elsevier Science B.V.
PII: S0014-2999(01)00873-1

3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN 55212-2), a synthetic, high-affinity cannabinoid CB_1 receptor agonist, was selected for study. As much as possible, these experiments were conducted under the same conditions as were employed in the studies involving delta-9-tetrahydrocannabinol and anandamide.

2. Materials and methods

2.1. Subjects

All rats received care as prescribed in the "Guide for the Care and Use of Laboratory Animals," National Academy Press, Washington, DC, revised, 1996. The American Association for the Accreditation of Laboratory Animal Care certifies these facilities. The Institutional Animal Care and Use Committee approved these studies at Virginia Commonwealth University.

2.2. Continuous-infusion studies in rats

The experimental procedure was described earlier (Aceto et al., 1996, 1998) in similar studies involving delta-9-tetrahydrocannabinol and anandamide. Briefly, adult male Sprague–Dawley rats were purchased from Dominion Laboratories (Dublin, VA). Upon arrival, the rats were examined by a licensed veterinarian and placed in quarantine. They were in the weight range of 250–280 g when assigned to a study. Once selected, they were housed individually in stainless steel cages. The vivarium was temperature- and humidity-controlled, with alternating light–dark cycles (lights on at 06:00 and off at 24:00).

The method described by Teiger (1974) was used, with the modifications indicated below. The rats were acclimated to their new surroundings for at least 3 days before cannulas were implanted. After they were anesthetized with pentobarbital (45 mg/kg i.p.), the lateral side of the lower left abdomen and the back of the neck were shaved, and the exposed skin was cleansed with povidone-iodine solution (Redi-Product, WV). Then, each rat was fitted with a cannula (PE90 tubing, Clay Adams, NJ) that passed s.c. from the nape of the neck to the lateral side of the lower abdomen. The peritoneal end of the cannula was enclosed in silastic tubing to prevent foreign body reaction. It was introduced into the peritoneal cavity through a stab-wound entry site. The cannula was secured with sutures at both sites. Next, each rat was fitted with a harness that consisted of a flat stainless steel plate fitted with a shoulder collar, a narrow strip of Velcro and a spring coil. The collar was passed over the head of the rat, and the harness was secured by means of a strip of Velcro, which girdled the chest. The cannula passed through the harness and spring coil, which protected it. The other end of the enclosed cannula was then attached to a flow-through swivel (Instech Lab., Horsham, PA). The swivel allowed the rat to move about in the cage and to eat and drink normally. An infusion pump (Harvard Apparatus, South Natick, MA, Model-945) delivered the solutions to the swivel in a volume of 8 ml every 24 h. Fresh solutions containing the proper drug dose were prepared daily after the rats were weighed.

2.3. Behavioral observations

During the infusion of WIN 55212, the rats were observed daily for 1 h for overt behavioral signs. In addition, they were observed 1 h before the injection of SR141716A or vehicle (pre-challenge) and 1 h after (post-challenge). The behavioral signs observed were designated: scratching, wet-dog shakes, head shakes, paw shakes, facial rubbing with front paws, chewing, tongue rolling, retropulsion or walking backward, immobility and ptosis (at least 50% closure of both eyelids). The signs wet-dog shake and facial rubbing were quantified. All other signs were simply scored once during an observation period. A trained observer recorded the behavioral signs. The observer was "blind" regarding treatment regimens commencing with the pre-challenge observation period to the termination of the experiment.

2.4. Statistical analysis

A total of three experiments were conducted. Then, the data were collated appropriately by treatment regimen. Three dose regimens expressed as mg/kg/day were used for days 1–4, respectively: low—1, 2, 4 and 8; medium—2, 4, 8 and 16; and high—4, 8, 16 and 16. A synopsis of the treatment regimens and experimental design is provided in Table 1. Analysis of variance (ANOVA) was used to test for overall significance. The conservative Bonferroni/Dunn test was used for planned comparisons. In all cases, significance was $P < 0.05$. The StatView statistical package (Brainpower, Agoura Hills, CA) was utilized for these analyses.

2.5. Chemical supplies

SR141716A was obtained gratis from Pfizer Central Research (Groton, CT). WIN 55212-2 (Research Biochemical International (RBI), Natick, MA), Alkamuls EL-620, formerly Emulphor EL-620 or polyoxyethylated castor oil (Rhône-Poulenc, Cranbury, NJ), and sterile saline and sterile water (Baxter Healthcare, Deerfield, IL) were purchased. SR 141716A was dissolved in Alkamul, ethanol and sterile saline in a ratio of 1:1:18, respectively. WIN 55212-2 was dissolved in a vehicle with the same compo-

Table 1
Synopsis of the WIN 55212-2 pre-treatment-dose regimens infused i.p. for 4 days and the acute challenges with SR141716A or its vehicle

Pre-treatment (8 ml/day)	Dose (mg/kg/day, i.p.)				Challenge	Number[a] of subjects
	Day 1	Day 2	Day 3	Day 4		
Vehicle					Vehicle	13
Vehicle					SR 141716A	16
WIN-Low	1	2	4	8	Vehicle	6
WIN-Low	1	2	4	8	SR 141716A	10
WIN-Medium	2	4	8	16	Vehicle	11
WIN-Medium	2	4	8	16	SR 141716A	12
WIN-High	4	8	16	16	Vehicle	7
WIN-High	4	8	16	16	SR 141716A	7

WIN 55212-2 was dissolved in emulphor–ethanol–sterile saline (1:1:98); SR141716A was dissolved in emulphor–ethanol–sterile saline (1:1:18).
[a]Combined number of subjects from three experiments.

nents as the SR141716A vehicle; however, the ratio of constituents in the order listed above was 1:1:98.

3. Results

3.1. Non quantified behavioral signs

As in the delta-9-tetrahydrocannabinol study (Aceto et al., 1996) many overt behavioral signs were noted and were scored once. Chewing, tongue rolling, paw shakes, and head shakes occurred mostly in WIN 55212-2-treated rats and were associated with withdrawal. Subjects scratching and/or displaying eyelid ptosis were more numerous after SR141716A challenge. Retropulsion was observed in one rat at the high dose prior to and after SR141716A challenge. In addition, myoclonic jerks and immobility were noted only in WIN 55212-2-treated rats, with one exception. The latter two signs were not considered to be withdrawal signs.

3.2. Quantified behavioral signs and body weight

The incidence of wet-dog shakes is depicted in Fig. 1. ANOVA revealed no significant difference among treatment regimens for the pre-challenge observation period ($F = 0.634$, $P = 0.727$). Significant differences were apparent for the 1-h post-challenge and 24-h post-challenge times ($F = 7.314$, $P < 0.0001$ and $F = 4.684$, $P = 0.0002$, respectively). Finally, ANOVA revealed no significant differences among treatments for the intervals designated 48, 72 and 96 h post-challenge ($F = 1.376$, $P = 0.2284$; $F = 0.880$, $P = 0.5282$; and $F = 1.331$, $P = 0.2478$, respectively. Data not shown). The Bonferroni/Dunn test indicated that all rats challenged with SR141716A had significantly increased scores when compared with the vehicle–vehicle group. Importantly, the group that received the high dose of WIN 55212-2 and challenged with SR141716A produced significantly more wet-dog shakes than all other groups. None of the rats that received vehicle, instead of SR141716A, showed significantly elevated scores until 24 h had elapsed. During this time period, the rats administered the medium dose regimen showed significantly increased scores compared to the vehicle controls, indicating the occurrence of spontaneous withdrawal (Fig. 1, panel 24 h post-challenge).

The sign facial rubs exhibited results that were similar to those noted for wet-dog shakes. ANOVA of the pre-challenge data was not remarkable ($F = 0.936$, $P = 0.4845$). Importantly, the F value for the 1-h post-challenge period was 20.437, and it was highly significant ($P < 0.0001$). All rats that received SR141716A showed significantly increased scores for this sign compared to the vehicle–vehicle group; the group that received the high dose regimen had a significantly greater score than all other groups. Evidence for spontaneous withdrawal ($F = 2.871$, $P = 0.0104$) was provided 24 h later: the group that received the medium dose regimen has scores significantly elevated over those of the vehicle/vehicle group. In addition, the F value for the 72-h interval was also significant ($F = 2.150$, $P = 0.0485$). Moreover, P values between WIN 55212-2 high-vehicle and vehicle–vehicle regimens, and between WIN 55212-2 high-SR141716A and vehicle–SR141716A groups approached significance. Finally, F values for comparisons for the 48- and 96-h data indicated no significant differences among treatments ($F = 0.881$, $P = 0.5255$ and $F = 1.698$, $P = 0.1225$, respectively); 48-, 72- and 96-h results are not illustrated.

3.3. Body weights in spontaneously withdrawn and SR141716A challenged rats

The body weights of all animals throughout the duration of infusion and following challenge with either vehicle or SR 141716A are depicted in Fig. 2. For illustrative purposes only, the data were grouped into two categories, designated spontaneously withdrawn (top panel) and SR141716A challenged (bottom panel). However, all statistical treatments were performed on the results of all treatment groups. ANOVA for the body weights before the infusion began (day 0) revealed no significant differences in body weights among the treatment regimens ($F = 1.680$,

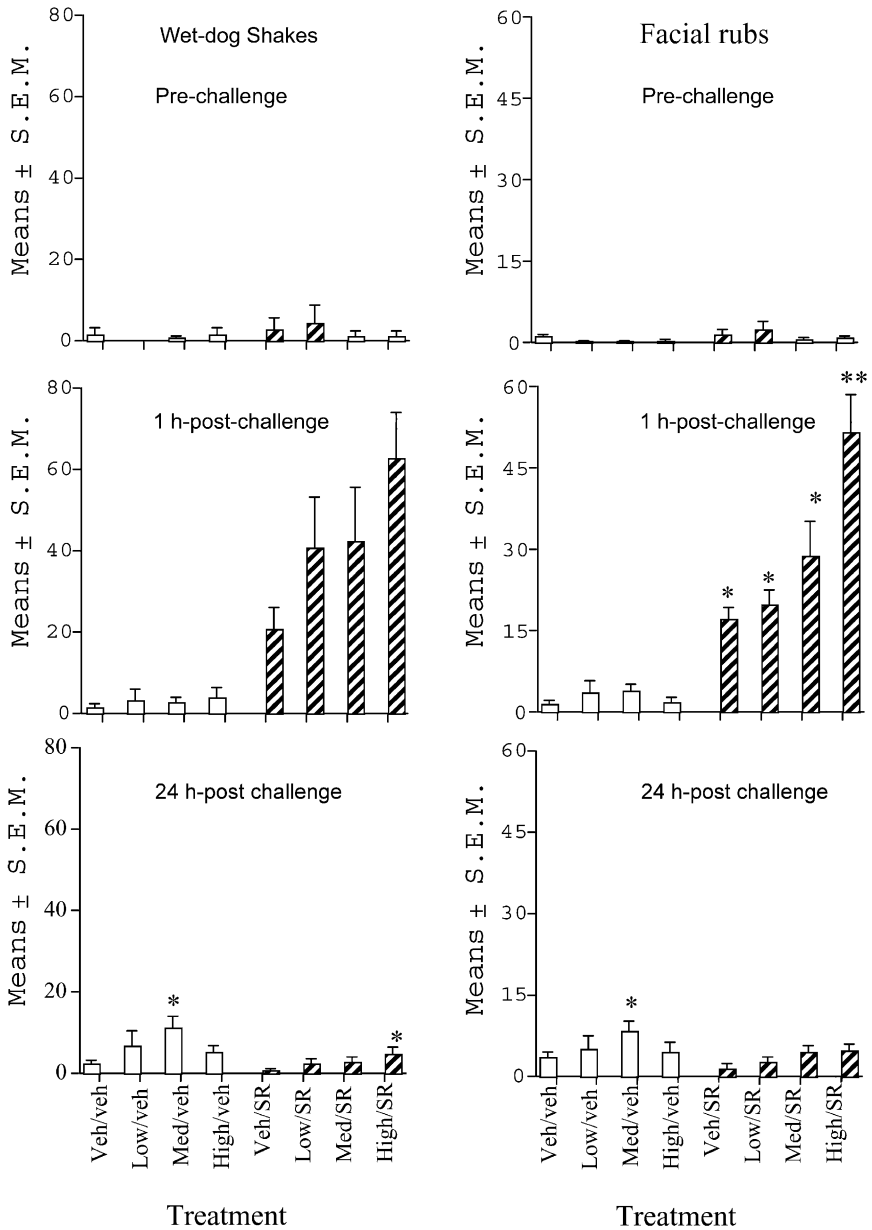

Fig. 1. Wet-dog shakes (left panels) and facial rubs (right panels) in WIN 55212-2- or vehicle-infused rats, 1 h before and after challenge with SR141716A or vehicle. Rats were infused with designated low-, medium- or high-treatment regimens of WIN 55212-2 for 4 days. At the end of this infusion period, the rats were observed before (1 h pre-challenge) and after (1 and 24 h post-challenge) challenge with either vehicle or 10 mg/kg of SR 141716A. The group names on the abscissa signify infusion regime/challenges. Additional details of the experimental design, including the number of subjects per treatment regimen are presented in Table 1. The data are expressed as means ± S.E.M. * Significantly different from vehicle–vehicle group. ** Significantly different from all other groups.

$P = 0.1271$). Significant differences were suggested on day 1 ($F = 2.108$, $P = 0.0530$); however, because P was greater than 0.05, a priori comparisons were not appropriate. Days 2 and 3 did not indicate remarkable differences in body weight among treatment groups ($F = 1.179$, $P = 0.3254$; $F = 1.442$, $P = 0.2017$, respectively). By day 4, significant differences emerged ($F = 3.833$, $P = 0.0013$). When compared to vehicle–vehicle controls, the rats receiving the high dose of WIN 55212-2 lost significant body weight. By day 5 or 24 h after challenge, significant overall changes were evident ($F = 4.947$, $P = 0.0001$). Weight loss now occurred in the group receiving the medium WIN 55212-2 dose but not the high dose (top panel). Compared with the vehicle–vehicle group (top panel), the three groups receiving WIN 55212-2 and challenged with SR141716A (bottom panel) lost a significant amount of body weight. It is tempting to attribute the additional weight loss to precipitated withdrawal; however, we hesitate to draw this conclusion because there were no significant differences among all the treatment groups

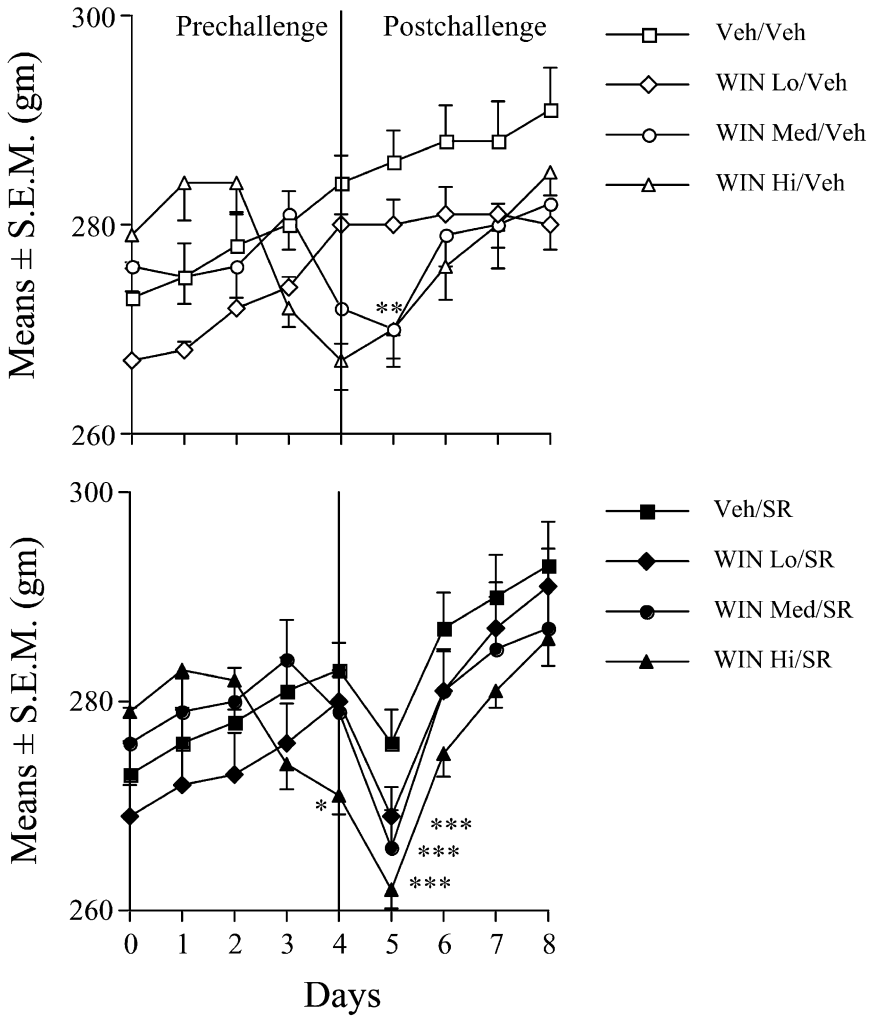

Fig. 2. Body weights of vehicle- or WIN 55212-2-treated rats challenged with vehicle (top panel), and vehicle- or WIN 55212-2 treated rats challenged with SR 141716A (bottom panel). The results are expressed as means ± S.E.M. * The WIN 55212-2 high-treatment group showed a statistically significant weight loss compared to vehicle group on day 4 before challenge. ** The WIN 55212-2 medium-treatment group showed a statistically significant weight loss compared to vehicle–vehicle group 24 h after vehicle challenge. *** The WIN 55212-2 low-, medium- and high-treatment groups showed significantly greater weight losses than the vehicle–vehicle group 24 h after SR 141716A challenge. Body weights for the vehicle-SR141716A group are not significantly different from those of the vehicle–vehicle group at any time.

receiving SR141716A, and the three groups receiving WIN 55212-2 started losing weight before challenge with SR141716A. Afterwards, all rats rapidly regained lost body weight, and by day 8, all body weights appeared greater than those observed at the start of the experiment. This rapid increase suggested that the WIN 55212-2's dose regimens were not in the toxic range but rather suppressed appetite. No significant differences among treatment regimens were calculated on days 6 ($F = 1.599$, $P = 0.1490$), 7 ($F = 0.976$, $P = 0.4550$) and 8 ($F = 1.286$, $P = 0.2645$).

4. Discussion

In this study, we showed that physical dependence on Win 55212-2 could be expressed as precipitated withdrawal, and to a modest degree, as spontaneous withdrawal. This is in contrast with our previous study involving delta-9-tetrahydrocannabinol, in which precipitated, but not spontaneous, withdrawal was expressed. Differences between WIN 55212-2 and delta-9-tetrahydrocannabinol can be reconciled. A basic premise regarding the expression of physical dependence is that the sooner a drug is displaced or eliminated, the faster the onset and the greater the intensity of withdrawal (Aceto, 1990). There is little question that SR141716A displaced both of these cannabinoids from the CB_1 cannabinoid receptor and precipitated withdrawal. On the other hand, during spontaneous withdrawal, delta-9-tetrahydrocannabinol was probably eliminated more slowly than WIN 55212-2. Thus, for delta-9-tetrahydrocannabinol, homeostasis was preserved and spontaneous withdrawal was barely, if at all, perceptible. A direct pharmacokinetic comparison of delta-9-tetrahydrocannabinol and WIN 55212-2 would be needed to confirm

this notion. Another significant difference between delta-9-tetrahydrocannabinol and WIN 55212-2 is that the latter is more efficacious than delta-9-tetrahydrocannabinol in a xenopus oocyte expression system (McAllister et al., 1999).

While anandamide is generally regarded as having many biological properties in common with delta-9-tetrahydrocannabinol and WIN 55212-2, many differences were enumerated (Aceto et al., 1998; Martin et al., 1999), and some are discussed below. It is unlikely that pharmacokinetic factors accounted for anandamide's failure to produce physical dependence. Rapid metabolism is frequently invoked to account for its apparently anomalous effects vis-a-vis delta-9-tetrahydrocannabinol and other exogenous cannabinoids. However, this is probably not involved, because anandamide was given continuously even up to toxic levels. Moreover, neither arachidonic acid, a precursor and metabolite, nor 2-methylarachidonyl-2-(2′-fuoroethyl)-amide, a stable analog, produced physical dependence. The differences may be due to activation of signaling pathways, interactions with non-cannabinoid receptors or other molecular targets. For example, Breivogel (1999) showed that chronic Δ^9-tetrahydrocannabinol treatment produced down-regulation and desensitization of brain cannabinoid receptors. Other investigators were unable to elicit a down-regulation of cannabinoid receptors after chronic exposure to anandamide (Romero et al., 1999). Interestingly, chronic exposure to *R*-methanandamide, another stable analog was able to induce such changes in basal ganglia, cerebellum and hippocampus. However, areas such as cerebral cortex and limbic nuclei were not affected, suggesting regional differences in brain areas (Romero et al., 1999). Furthermore, neither 2-arachidonylglycerol, palmitylethanolamide, another endogenous cannabinoid, nor WIN 55212-2 mimicked anandamide-induced activation of vanilloid receptors (Zygmunt et al., 1999).

It should be noted that SR141716A itself activates behavior in rodents (Compton et al., 1996; De Fonseca et al., 1997; Aceto et al., 1998). In the present study, we also demonstrated variable and limited increases in the number of facial rubbings and wet-dog shakes in all rats receiving SR141716A; however, the effect was much more pronounced in the WIN 55212-2-pre-treated rats. One plausible explanation is that SR141716A blockade of the endogenous cannabinoid system disrupts its tonic inhibitory action. On the other hand, it has been suggested that SR141716A may also act as an inverse agonist (Compton et al., 1996; Richardson et al., 1997). However, it is important to note that SR141716A-induced behavioral activation was not evident during the 24-h post-challenge period when spontaneous withdrawal was occurring.

Physical dependence on smoked marijuana was recently demonstrated in humans. In two carefully controlled clinical trials, abstinence expressed as increased aggression, irritability, anxiety and stomach pain was reported (Kouri et al., 1999; Haney et al., 1999). In a study of cannabis use among adolescents with conduct problems and a high incidence of polysubstance abuse, Crowley et al. (1998) noted similar withdrawal symptoms, including a high incidence of symptoms, designated: tired, sleepy, weak, trouble concentrating, yawn, affect appetite, depressed, trouble sleeping. Importantly, these authors also found evidence that cannabis was as reinforcing as tobacco or alcohol in this population. Needless to say, physical dependence on delta-9-tetrahydrocannabinol was demonstrated in humans many years ago (Jones et al., 1976); however, it was given orally and in high doses.

There is a growing movement to legalize the use of marijuana for the treatment of a wide variety of ailments. Indeed, the voters of eight US states have approved initiatives for its medical use. Cannabinoids have been used clinically to suppress nausea and vomiting and to stimulate appetite in AIDS patients. Other potential uses include the treatment of weight loss associated with serious illnesses, certain sleep disorders, spasticity, chronic pain, asthma, glaucoma, Parkinson's disease and migraine. Clinical trials to fully explore these medical uses have been proposed (Pertwee, 1999; Hollister, 2000; Piomelli et al., 2000).

Our results are in agreement with clinical observations that physical dependence on cannabinoids can develop rapidly and suggest that physical dependence may be more widespread than believed. Some investigators have suggested that marijuana use may be maintained, in part, to alleviate abstinence symptoms (Crowley et al., 1998; Haney et al., 1999). These observations could unduly delay their development and use as therapeutic agents. Since preclinical studies suggest that anandamide's propensity to produce physical dependence is very low, there may be some advantage to targeting the endocannabinoid system regarding therapeutic uses (Pertwee, 1999; Piomelli et al., 2000)

Acknowledgements

Supported by NIDA contract 5.8059 and grant DA 09789.

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