

# Opioid and Cannabinoid Modulation of Precipitated Withdrawal in $\Delta^9$ -Tetrahydrocannabinol and Morphine-Dependent Mice

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## ABSTRACT

The goal of the present study was to elucidate the relationship between cannabinoid and opioid systems in drug dependence. The CB<sub>1</sub> cannabinoid receptor antagonist SR 141716A precipitated both paw tremors and head shakes in four different mouse strains that were treated repeatedly with  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC). SR 141716A-precipitated  $\Delta^9$ -THC withdrawal was ameliorated in  $\mu$ -opioid receptor knockout mice compared with the wild-type control animals and failed to occur in mice devoid of CB<sub>1</sub> cannabinoid receptors. An acute injection of morphine in  $\Delta^9$ -THC-dependent mice undergoing SR 141716A-precipitated withdrawal dose dependently decreased both paw tremors, antagonist dose 50 (AD<sub>50</sub>) (95% CL) = 0.035 (0.03–0.04), and head shakes, AD<sub>50</sub> (95% CL) = 0.07 (0.04–0.12). In morphine-dependent mice, the opioid antagonist naloxone precipitated head shakes, paw tremors, di-

arrhea, and jumping. As previously reported, naloxone-precipitated morphine withdrawal failed to occur in  $\mu$ -opioid knockout mice and was significantly decreased in CB<sub>1</sub> cannabinoid receptor knockout mice. Acute treatment of  $\Delta^9$ -THC in morphine-dependent mice undergoing naloxone-precipitated withdrawal blocked paw tremors, AD<sub>50</sub> (95% CL) = 0.5 (0.3–1.0), and head shakes AD<sub>50</sub> (95% CL) = 0.6 (0.57–0.74) in dose-dependent manners, but failed to diminish the occurrence of diarrhea or jumping. Finally, naloxone and SR 141716A failed to elicit any overt effects in  $\Delta^9$ -THC-dependent and morphine-dependent mice, respectively. These findings taken together indicate that the  $\mu$ -opioid receptor plays a modulatory role in cannabinoid dependence, thus implicating a reciprocal relationship between the cannabinoid and opioid systems in dependence.

*Cannabis sativa* has been the most prevalently used illicit drug in the United States for the last several decades (Johnston et al., 1998). Also contributing to increases in the use of this drug is the growing popular support for its decriminalization for medicinal purposes. The occurrence of a positive correlation between marijuana use and marijuana dependence (Chen et al., 1997) raises concern that physical withdrawal might become an issue when a recreational user or patient abruptly discontinues the drug. Indeed, more than 25 years ago, an abrupt cannabinoid withdrawal syndrome was described in human subjects following discontinuation from chronic oral  $\Delta^9$ -THC (Jones and Benowitz, 1976; Jones et al., 1976). More recently, subjects undergoing abrupt withdrawal from repeated administration of either oral  $\Delta^9$ -THC (Haney et al., 1999a) or marijuana smoke inhalation (Haney et al., 1999b) exhibited abstinence symptoms that included subjective effects of anxiety, irritability, and stomach pain, as well as decreases in food intake.

Before the availability of the CB<sub>1</sub> cannabinoid antagonist

SR 141716A (Rinaldi-Carmona et al., 1994), studies investigating cannabinoid abstinence withdrawal in laboratory animals yielded contradictory findings. Whereas some evidence supported the occurrence of cannabinoid dependence (Kaymakcalan, 1979; Beardsley et al., 1986), other studies failed to observe any abrupt withdrawal signs (McMillan et al., 1970; Leite and Carlini, 1974). In contrast, administration of SR 141716A precipitated reliable withdrawal signs in several species that had been treated repeatedly with cannabinoids, including mice (Cook et al., 1998; Hutcheson et al., 1998), rats (Aceto et al., 1995; Tsou et al., 1995), and dogs (Lichtman et al., 1998). As in the case of opioids, mice will self-administer the cannabinoid aminoalkylindole WIN 55,212-2 (Martellotta et al., 1998). Through the use of these models, the neurochemical mechanisms underlying cannabinoid dependence can now be systematically investigated.

Substantial evidence is mounting that the antinociceptive effects, drug reinforcing actions, and dependence liability of morphine and  $\Delta^9$ -THC share common neuroanatomical sites. In particular, converging data suggest that cannabinoids influence opioid withdrawal. Anatomical studies have found that CB<sub>1</sub> cannabinoid receptor and  $\mu$ -opioid receptor mRNA

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**ABBREVIATIONS:**  $\Delta^9$ -THC,  $\Delta^9$ -tetrahydrocannabinol; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl; AD<sub>50</sub>, antagonist dose 50; CL, confidence limits.

are colocalized in brain limbic areas associated with dependence (Navarro et al., 1998). It has long been known that  $\Delta^9$ -THC produces a moderate amelioration of naloxone-precipitated withdrawal in morphine-dependent mice (Bhargava, 1976a,b, 1978) and rats (Hine et al., 1975). The endogenous cannabinoid anandamide was also shown to decrease naloxone-induced morphine withdrawal (Vela et al., 1995). Other compelling evidence supporting a link between opioid dependence and the cannabinoid system is that CB<sub>1</sub> cannabinoid receptor knockout mice exhibited substantial decreases in both morphine self-administration and naloxone-precipitated morphine withdrawal (Ledent et al., 1999). This amelioration of opioid withdrawal suggests the possibility that endocannabinoids modulate opioid dependence.

Alternatively, epistasis in which the effect of gene disruption is modified by the genetic background on which it is placed can also account for amelioration of naloxone-precipitated morphine withdrawal in CB<sub>1</sub> cannabinoid receptor knockout mice. Whereas a CD-1 background strain was used for the CB<sub>1</sub> cannabinoid receptor knockout mice in the opioid dependence report (Ledent et al., 1999), the present study used a C57BL/6 background strain for the knockouts. A reduction in opioid dependence in both backgrounds would provide further support for the involvement of the cannabinoid system in opioid dependence. In addition, we examined whether  $\Delta^9$ -THC would reduce naloxone-precipitated morphine withdrawal reactions and whether SR 141716A would precipitate withdrawal in morphine-dependent mice.

In contrast to the growing body of research that is establishing a role for cannabinoid receptors on modulating opioid dependence, relatively few studies have focused on the influence of opioid receptors on cannabinoid dependence. The finding that SR 141716A-precipitated  $\Delta^9$ -THC withdrawal was significantly attenuated in preproenkephalin-deficient mice suggests that opioid systems may modulate cannabinoid dependence (Valverde et al., 2000). In the present study, we investigated whether the expression of SR 141716A-precipitated cannabinoid withdrawal would also be altered in  $\mu$ -opioid receptor-deficient mice. In addition, the effects of acute morphine administration on cannabinoid withdrawal were examined. Because previous research has yielded mixed results on whether an opioid antagonist can precipitate withdrawal in  $\Delta^9$ -THC-dependent animals (McMillan et al., 1971; Hirschhorn and Rosecrans, 1974; Kaymakcalan et al., 1977), we also evaluated whether naloxone would precipitate withdrawal effects in  $\Delta^9$ -THC-dependent mice.

In all experiments, mice were evaluated for head shakes and paw tremors, two cannabinoid withdrawal behaviors that can be reliably quantitated (Cook et al., 1998), as well as for diarrhea and jumping, indices indicative of opioid withdrawal (Way et al., 1969).

## Materials and Methods

**Subjects.** Swiss-Webster and ICR mice were purchased from Harlan Laboratories (Dublin, VA). C57BL/6 and DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice deficient of the  $\mu$ -opioid receptor (Loh et al., 1998) and CB<sub>1</sub> cannabinoid receptor (Zimmer et al., 1999) mice were born in the Virginia Commonwealth University vivarium from breeding pairs that were initially provided by Drs. Horace Loh (University of Michigan, Ann Arbor, MI) and Andreas Zimmer (National Institutes of Health, Bethesda, MD), respectively. All subjects were male, weighed 22 to

30 g, and were housed six animals per cage in an American Association for the Accreditation of Laboratory Animal Care-approved facility. The study was approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Mice were given unlimited access to food and water and were maintained on a 12:12-h light/dark cycle.

**Drugs.**  $\Delta^9$ -THC, SR 141716A, morphine sulfate, and morphine sulfate pellets (25 or 75 mg) were provided by the National Institute on Drug Abuse (Bethesda, MD). Naloxone hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO).  $\Delta^9$ -THC and SR 141716A were dissolved in ethanol, followed by addition of Emulphor-620 (Rhone-Poulenc, Princeton, NJ), and diluted with 0.9% saline to form a vehicle mixture of ethanol/Emulphor/saline in a ratio of 1:1:18. Morphine and naloxone were dissolved in 0.9% saline. All drug injection volumes were made based on mouse body weight, with 0.1 ml of dissolved drug volume given for every 10 g of body weight.  $\Delta^9$ -THC, morphine, and naloxone were given s.c. and SR 141716A was given i.p.

**Morphine Sulfate Pellet Implantation.** Mice were made dependent to morphine as previously described (Way et al., 1969). Each subject was anesthetized with diethyl ether and a 2-cm lateral incision was made 1 cm posterior to its ears on the midline of its back. The skin was then separated from the muscle and the pellet was inserted subcutaneously. The incision site was closed using Autoclip 9-mm wound clips (Becton-Dickinson and Company, Sparks, MD).

**Evaluation of Withdrawal Symptoms.** In the cannabinoid dependence studies, mice were administered a daily s.c. injection of either  $\Delta^9$ -THC (10 mg/kg, unless otherwise noted) or vehicle between 9:00 and 10:00 AM on five consecutive days. On the 5th day, each subject was challenged with an i.p. injection of SR 141716A (10 mg/kg) 4 h after its morning injection. In the opioid dependence studies, each mouse was challenged with naloxone (1 mg/kg) on the 5th day following implantation of the placebo or morphine sulfate pellet. In both studies, the mice were placed in clear cages and 15 min following the i.p. injection the number of paw tremor (lateral forepaw clapping behavior), head shake (i.e., turning or twisting of the head from side to side), and scratching incidences were noted during a 30-min observation period, as previously described (Cook et al., 1998). The number of writhing (i.e., a stretching of the abdomen) occurrences and whether the mice exhibited eyelid ptosis, diarrhea, and jumping from an elevated pedestal (1 foot in height  $\times$  4 inches in diameter) was also noted (Way et al., 1969). The observer was blind to the drug condition and challenge drug.

**Statistical Analysis.** Statistical analysis of quantified data was performed by analysis of variance, with significance set at  $p < 0.05$ . Post hoc tests included the Scheffé test for multiple comparisons and Dunnett's test to compare drug-treated mice to the appropriate vehicle group. The Bonferroni  $t$  test was used for planned comparisons. ED<sub>50</sub> values were determined by least-squares linear regression analysis followed by calculation of 95% confidence limits (Bliss, 1967).

## Results

### Influence of Opioid Receptor Manipulations on Cannabinoid Dependence

Because published reports investigating cannabinoid dependence use different mouse strains that often assess different endpoints following different treatment regimens, an initial experiment was conducted to assess the effects of SR 141716A challenge in different strains of mice that received the same regimen of  $\Delta^9$ -THC. Two outbred strains, ICR and Swiss-Webster mice, and two inbred strains, C57BL/6 and DBA/2 mice, were selected. Our laboratory has traditionally used ICR mice (Cook et al., 1998) and Swiss-Webster mice (Bhargava, 1976a,b; Vela et al., 1995) have been used to

evaluate the involvement of cannabinoids on opioid tolerance. The C57BL/6 strain is the background strain for both the CB<sub>1</sub> cannabinoid receptor knockout mice (Zimmer et al., 1999) and  $\mu$ -opioid receptor knockout mice (Loh et al., 1998) used in the present study. Finally, DBA/2 mice have not been previously evaluated for cannabinoid dependence. Mice were given daily injections of either vehicle or  $\Delta^9$ -THC (10 mg/kg) and challenged with vehicle or SR 141716A on the 5th day. To ascertain whether there was an effect between strain and treatment, the paw tremor, head shake, and scratching data were analyzed by three-way analyses of variance in which the factors included strain,  $\Delta^9$ -THC, and SR 141716A.

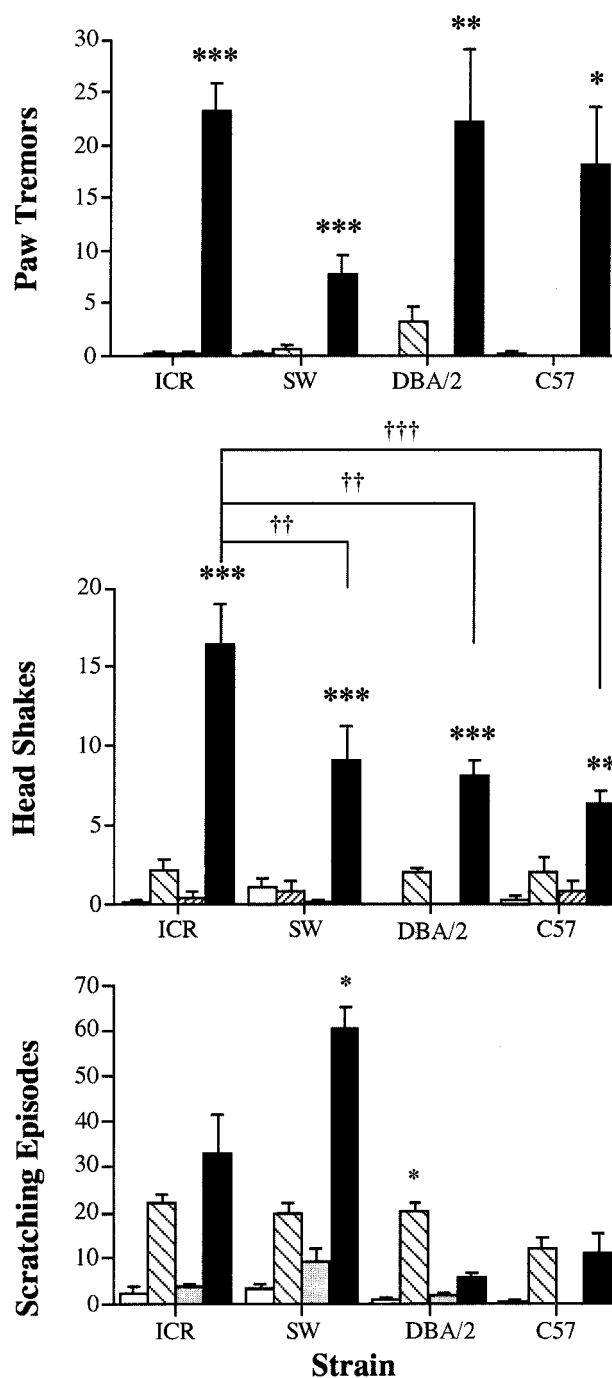
As shown in Fig. 1, top, SR 141716A precipitated increases in paw tremors in  $\Delta^9$ -THC-treated mice, regardless of strain, as indicated by a significant two-way interaction between  $\Delta^9$ -THC and SR 141716A,  $F(1, 83) = 29, p < 0.05$ . There was no main effect of mouse strain and no interactions between any of the factors and mouse strain. For each strain, the mice treated repeatedly with  $\Delta^9$ -THC and challenged with SR 141716A exhibited significantly more paw tremors than each of the other three groups (Scheffé's test,  $p < 0.05$ ).

On the other hand, the head shake data resulted in a significant three-way interaction for mouse strain by  $\Delta^9$ -THC by SR 141716A,  $F(3,83) = 4.9, p < 0.05$  (Fig. 1, middle). This interaction occurred because the ICR mice treated with  $\Delta^9$ -THC and challenged with SR 141716A exhibited significantly more head shakes than that exhibited by each of the other groups. Once again, for each strain, the  $\Delta^9$ -THC-treated mice challenged with SR 141716A exhibited significantly more head shakes than that exhibited by each of the other three groups (Scheffé's test,  $p < 0.05$ ).

A significant three-way interaction of mouse strain by  $\Delta^9$ -THC treatment by SR 141716A challenge was also found for scratching of the body and face,  $F(3,83) = 10.5, p < 0.05$  (Fig. 1, bottom). To interpret this interaction, separate two-way analyses of variance ( $\Delta^9$ -THC treatment by SR 141716A challenge) were conducted on the data from each of the four strains. In both the ICR and C57BL/6 mice, SR 141716A elicited a significant increase in scratching behavior irrespective of  $\Delta^9$ -THC treatment ( $p < 0.001$ ). For the Swiss-Webster and DBA/2 mice ( $p < 0.001$ ), however, significant two-way interactions between  $\Delta^9$ -THC treatment and SR 141716A challenge occurred. In the Swiss-Webster strain, the  $\Delta^9$ -THC-treated mice that were challenged with SR 141716A engaged in significantly more scratching behavior than each of the other three groups. SR 141716A also elicited more scratching behavior in the mice that were given repeated injections of vehicle than the two groups that were challenged acutely with vehicle. Conversely, the opposite pattern of results occurred in the DBA/2 mice, the greatest increase of scratching behavior occurred in the mice treated repeatedly with vehicle and challenged with SR 141716A.

Scratching behavior is not shown in subsequent studies because this measure did not reflect precipitated withdrawal in either ICR or C57BL/6 mice. In addition, writhing and ptosis only occurred sporadically, and diarrhea and jumping were never observed in any of the  $\Delta^9$ -THC-dependent mice. Thus, these measures are also not shown in subsequent studies.

**Evaluation of SR 141716A-Precipitated  $\Delta^9$ -THC Withdrawal in Mice Devoid of Either CB<sub>1</sub> Cannabinoid Receptors or  $\mu$ -Opioid Receptors.** The primary goal of



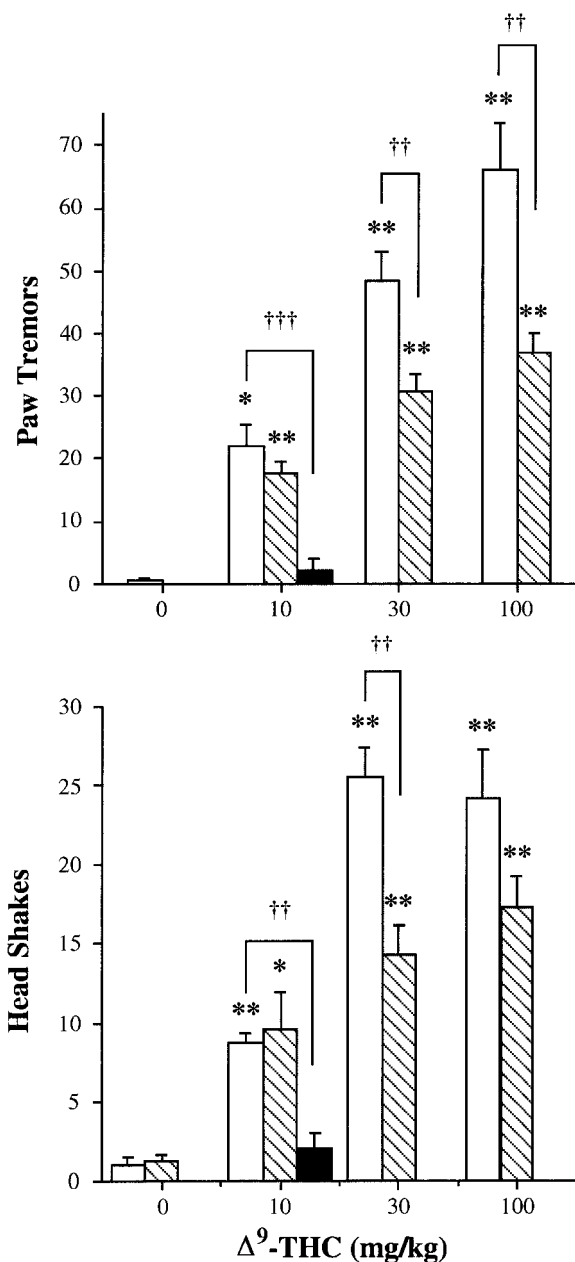
**Fig. 1.** Evaluation of SR 141716A precipitated  $\Delta^9$ -THC withdrawal in ICR, Swiss-Webster (SW), DBA/2, and C57BL/6 (C57) mice given s.c. injections of either vehicle or 10 mg/kg  $\Delta^9$ -THC for 5 days and challenged on the 5th day with an i.p. injection of either vehicle or 10 mg/kg SR 141716A. Mice were in the following groups: repeated vehicle with vehicle challenge (□), repeated vehicle with SR 141716A challenge (▨), repeated  $\Delta^9$ -THC with vehicle challenge (▩), and repeated  $\Delta^9$ -THC with SR 141716A challenge (■). The results are presented as means  $\pm$  S.E. with six mice per group. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ; Scheffé's test between group treated repeatedly with  $\Delta^9$ -THC and challenged with SR 141716A and each of the other three groups for each genotype.

these experiments was to evaluate whether SR 141716A-precipitated cannabinoid withdrawal would be altered by deletion of either the  $\mu$ -opioid receptor or the CB<sub>1</sub> cannabinoid receptor. The knockout mice or C57BL/6 wild-type controls were given repeated injections of  $\Delta^9$ -THC, challenged



with SR 141716A (10 mg/kg) on the 5th day, and physical signs of withdrawal were then measured.

Of importance, SR 141716A-precipitated cannabinoid withdrawal was significantly attenuated in the  $\mu$ -opioid receptor-deficient mice compared with the wild-type controls that received 10, 30, or 100 mg/kg  $\Delta^9$ -THC per day for 5 days (Fig. 2). Significant interactions between genotype and repeated  $\Delta^9$ -THC treatment were found for both paw tremors,  $F(3,39) = 5.8$ ,  $p < 0.05$ , and head shakes,  $F(3,39) = 5.8$ ,  $p <$



**Fig. 2.** Deletion of the  $\mu$ -opioid receptor attenuates SR 141716A-precipitated cannabinoid withdrawal signs. Wild-type (□) and  $\mu$ -opioid receptor knockout (▨) were given daily injections of vehicle or  $\Delta^9$ -THC (10, 30, or 100 mg/kg) for 5 days, and CB<sub>1</sub> cannabinoid receptor knockout mice (■) were treated with 10 mg/kg  $\Delta^9$ -THC for 5 days. All mice were challenged with SR 141716A and the numbers of paw tremor (top) and head shake (bottom) bouts were tallied for a 30-min observation period. The results are presented as means  $\pm$  S.E. with six mice per group. \* $p < 0.05$  and \*\* $p < 0.01$ ; Dunnett's test comparisons between each treatment group and the appropriate vehicle control group. †† $p \leq 0.01$  and ††† $p < 0.001$ ; planned comparisons.

0.05. For each respective genotype, SR 141716A-challenged subjects that were given repeated injections of  $\Delta^9$ -THC exhibited significantly more paw tremors and head shakes than that exhibited by the SR 141716A-challenged animals that received daily injections of vehicle (Dunnett's test,  $p < 0.05$ ). The  $\mu$ -opioid receptor knockout mice treated repeatedly with either 30 or 100 mg/kg  $\Delta^9$ -THC exhibited significantly fewer paw tremors than the respective wild-type control groups (Bonferroni  $t$  test,  $p < 0.0125$ ). Similarly, the  $\mu$ -opioid receptor knockout mice treated with daily injections of 30 mg/kg  $\Delta^9$ -THC exhibited significantly fewer SR 141716A-induced head shakes than the wild-type controls (Bonferroni  $t$  test,  $p < 0.0125$ ), while the 100-mg/kg  $\Delta^9$ -THC groups failed to differ significantly ( $p = 0.10$ ). Also shown Fig. 2 is that SR 141716A failed to elicit either paw tremors or head shakes in CB<sub>1</sub> cannabinoid receptor-deficient mice given daily injections of 10 mg/kg  $\Delta^9$ -THC for 5 days.

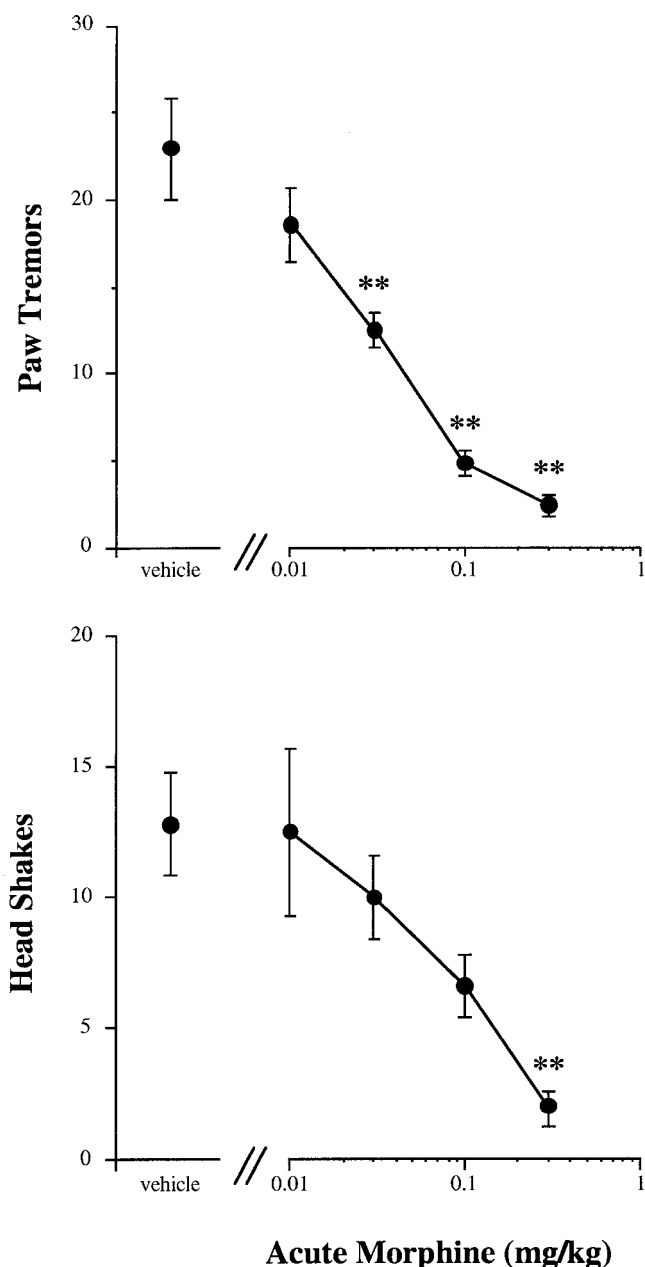
**Acute Effects of Morphine on SR 141716A-Precipitated  $\Delta^9$ -THC Withdrawal.** The goal of this experiment was to evaluate whether SR 141716A-precipitated cannabinoid withdrawal would be altered by an acute injection of morphine. Following daily administration of 10 mg/kg  $\Delta^9$ -THC for 5 days, ICR mice were given a single s.c. injection of saline or morphine (0.01–0.3 mg/kg) 30 min before challenge with SR 141716A (10 mg/kg), and observed for head shakes and paw tremors.

Morphine reduced SR 141716A-precipitated increases in paw tremors,  $F(4,33) = 21.8$ ,  $p < 0.05$ , and head shakes,  $F(4,33) = 7.69$ ,  $p < 0.05$ , in a dose-dependent manner (Fig. 3). Each dose of morphine significantly reduced the number of paw tremors, however, only the 0.3 mg/kg morphine dose reduced the number of head shakes (Dunnett's test,  $p < 0.05$ ). The morphine AD<sub>50</sub> values (95% CL) for each respective measure were 0.035 (0.03–0.04) and 0.07 (0.04–0.12) mg/kg.

**Determination of Whether Naloxone Precipitates Withdrawal in  $\Delta^9$ -THC-Dependent Mice.** To evaluate whether blockade of opioid receptors would precipitate withdrawal effects using our cannabinoid dependence protocol, ICR mice were given daily injections of either vehicle or  $\Delta^9$ -THC (10 mg/kg) for 5 days and then challenged with either saline or naloxone (1 or 5 mg/kg). As depicted in Table 1, no significant effects were found for  $\Delta^9$ -THC treatment or the interaction between  $\Delta^9$ -THC and naloxone for paw tremors, head shakes, or scratching. On the other hand, naloxone elicited small but significant increases in paw tremors,  $F(2,34) = 6.5$ ,  $p < 0.05$ , head shakes,  $F(2,34) = 3.5$ ,  $p < 0.05$ , and scratching,  $F(2,34) = 4.4$ ,  $p < 0.05$ , irrespective of  $\Delta^9$ -THC treatment. After collapsing across  $\Delta^9$ -THC treatment, 1 mg/kg naloxone differed from the vehicle treatment for each of the three measures, while 5 mg/kg naloxone only differed from the vehicle group in paw tremors. Finally, naloxone also failed to elicit any other observable withdrawal signs.

#### Influence of Cannabinoid Receptor Manipulations on Opioid Dependence

**Evaluation of Morphine Dependence in Mice Devoid of Either CB<sub>1</sub> Cannabinoid Receptors or  $\mu$ -Opioid Receptors.** The purpose of these experiments was to evaluate whether naloxone-precipitated opioid withdrawal would be altered in mice that were lacking either CB<sub>1</sub> cannabinoid receptors or  $\mu$ -opioid receptors. Both groups of knockouts as



**Fig. 3.** Effects of an acute dose of morphine on SR 141716A-precipitated paw tremors (top) and head shakes (bottom) in  $\Delta^9$ -THC-dependent mice. Morphine dose dependently decreased both measures indicative of withdrawal. The results are presented as means  $\pm$  S.E. with six mice per group. \* $p < 0.05$  and \*\* $p < 0.01$ ; Dunnett's test comparisons between each morphine group and the vehicle control group.

well as the C57BL/6 wild-type controls were implanted with 75-mg morphine sulfate pellets, challenged with 1 mg/kg naloxone on the 5th day, and physical signs of withdrawal were then measured. Significant effects of genotype were found for both paw tremors,  $F(2,19) = 39$ ,  $p < 0.05$ , and head shakes,  $F(2,19) = 9$ ,  $p < 0.05$ . In the case of paw tremors (Fig. 4, top), each of the groups significantly differed from each other, with the wild-type mice exhibiting the greatest frequency, the CB<sub>1</sub> cannabinoid receptor knockout mice had about one-third of this effect, and the  $\mu$ -opioid receptor knockout mice were virtually unaffected. Both the wild-type controls and CB<sub>1</sub> cannabinoid receptor knockouts had a mod-

erate number of head shakes and differed significantly from the  $\mu$ -opioid receptor knockout mice, which were virtually without effect (Fig. 4, bottom). Diarrhea and pedestal jumping occurred in all of the CB<sub>1</sub> cannabinoid receptor knockout mice and wild-type controls; however, the  $\mu$ -opioid receptor knockout mice failed to exhibit either of these withdrawal signs.

**Effects of Acute  $\Delta^9$ -THC on Naloxone-Precipitated Morphine Withdrawal.** ICR mice were implanted with 75-mg morphine sulfate pellets and on day 5 were given an acute dose of either vehicle or 0.1, 0.3, 1, 3, or 10 mg/kg  $\Delta^9$ -THC 30 min before challenge with 1 mg/kg naloxone. As depicted in Fig. 5, significant effects were found for paw tremors (top),  $F(5,29) = 19.2$ ,  $p < 0.05$ , and head shakes (bottom),  $F(5,29) = 14.6$ ,  $p < 0.05$ . The AD<sub>50</sub> (95% CL) values for paw tremors and head shakes were 0.5 (0.3–1.0) and 0.6 (0.57–0.74) mg/kg, respectively. Again, all the mice challenged with naloxone exhibited both jumping from the pedestal and diarrhea, regardless of acute treatment of  $\Delta^9$ -THC.

**Evaluation of SR 141716A Challenge in Morphine-Dependent Mice.** The purpose of this experiment was to determine whether SR 141716A would precipitate withdrawal effects in morphine dependent mice. C57BL/6 mice were implanted with placebo, 25-mg, or 75-mg morphine sulfate pellets. Five days later all mice were given an i.p. injection of 10 mg/kg SR 141716A and observed for 30 min. As shown in Table 1, SR 141716A failed to affect any of the measures.

## Discussion

In contrast to evidence implicating the involvement of CB<sub>1</sub> cannabinoid receptors in opioid dependence, it is unknown whether the opioid system can also influence cannabinoid dependence. The relatively recent availability of the CB<sub>1</sub> cannabinoid receptor antagonist SR 141716A has led to the development of reliable and dependable animal models of cannabinoid dependence to address this issue. The absence of SR 141716A-precipitated effects in CB<sub>1</sub> cannabinoid receptor knockout mice that were treated repeatedly with  $\Delta^9$ -THC replicates previous research (Ledent et al., 1999), demonstrating that the CB<sub>1</sub> cannabinoid receptor is a mandatory component of cannabinoid dependence. Strikingly, deletion of the  $\mu$ -opioid receptor resulted in a significant attenuation of SR 141716A-precipitated withdrawal paw tremors and head shakes compared with the wild-type controls. In addition, both withdrawal indices were completely blocked in a dose-dependent manner by an acute injection of morphine in wild-type mice. Taken together, these data implicate a role of opioid systems in the modulation of cannabinoid dependence.

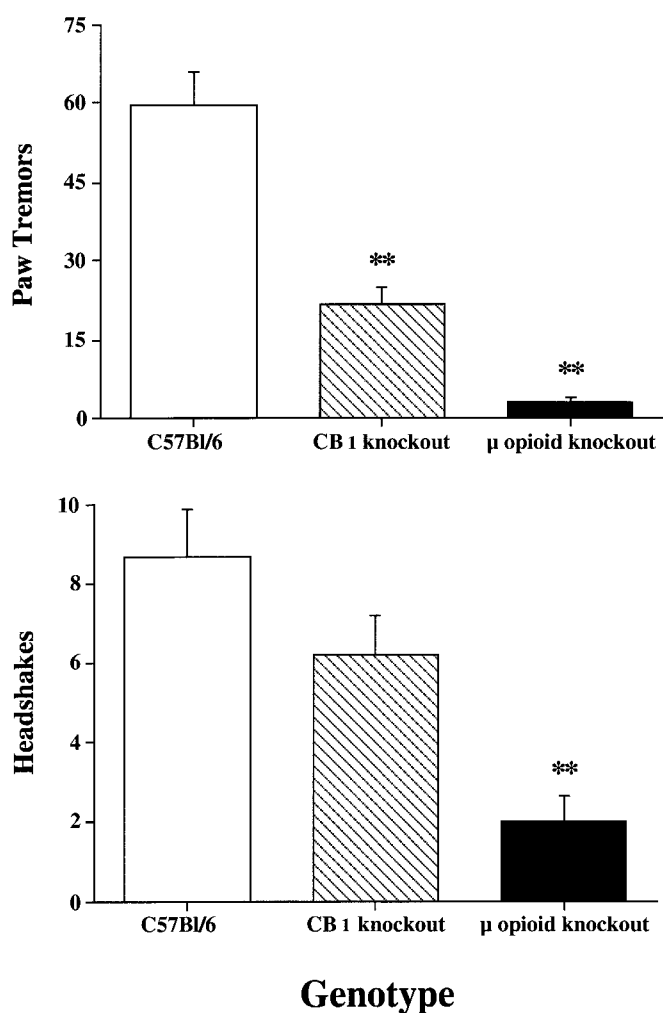
The observation that SR 141716A precipitated qualitatively similar  $\Delta^9$ -THC withdrawal effects across four different mouse strains indicates the reliability and validity of the mouse cannabinoid withdrawal model. Paw tremors and head shakes proved to be the most reliable cannabinoid withdrawal signs in ICR, C57BL/6, DBA/2, and Swiss-Webster mice. SR 141716A also precipitated scratching behavior in  $\Delta^9$ -THC-dependent Swiss-Webster mice, but not in any of the other strains. In contrast, writhing and ptosis occurred only sporadically, and diarrhea and jumping were never observed in any of the  $\Delta^9$ -THC-dependent mice. Consistent with the present results, Cook et al. (1998) made similar observations

TABLE 1

Naloxone failed to precipitate withdrawal signs in  $\Delta^9$ -THC-dependent mice, and SR 141716A failed to precipitate withdrawal signs in morphine-dependent mice

Each group consisted of six mice per group.

| Repeated Treatment             | Antagonist Treatment | Paw Tremors   | Head Shakes   | Scratching    | Writhing  | Diarrhea |
|--------------------------------|----------------------|---------------|---------------|---------------|-----------|----------|
| $\Delta^9$ -THC-dependent mice |                      |               |               |               |           |          |
| Vehicle                        | Vehicle              | 0 $\pm$ 0     | 0 $\pm$ 0     | 1.2 $\pm$ 0.4 | 0 $\pm$ 0 | no       |
| Vehicle                        | 1 mg/kg Naloxone     | 0.2 $\pm$ 0.2 | 0.8 $\pm$ 0.3 | 5.3 $\pm$ 1.9 | 0 $\pm$ 0 | no       |
| Vehicle                        | 5 mg/kg Naloxone     | 1.6 $\pm$ 1.0 | 0.6 $\pm$ 0.4 | 2.4 $\pm$ 0.9 | 0 $\pm$ 0 | no       |
| 10 mg/kg $\Delta^9$ -THC       | Vehicle              | 0.1 $\pm$ 0.1 | 0.1 $\pm$ 0.1 | 3.5 $\pm$ 1.3 | 0 $\pm$ 0 | no       |
| 10 mg/kg $\Delta^9$ -THC       | 1 mg/kg Naloxone     | 0.3 $\pm$ 0.3 | 0.3 $\pm$ 0.3 | 7.5 $\pm$ 2.3 | 0 $\pm$ 0 | no       |
| 10 mg/kg $\Delta^9$ -THC       | 5 mg/kg Naloxone     | 2.2 $\pm$ 1.6 | 0 $\pm$ 0     | 4.2 $\pm$ 1.4 | 0 $\pm$ 0 | no       |
| Morphine-dependent mice        |                      |               |               |               |           |          |
| Vehicle pellet                 | SR 141716A           | 0 $\pm$ 0     | 0 $\pm$ 0     | 10 $\pm$ 2.4  | 0 $\pm$ 0 | no       |
| 25 mg of Morphine              | SR 141716A           | 0 $\pm$ 0     | 0 $\pm$ 0     | 14 $\pm$ 5.2  | 0 $\pm$ 0 | no       |
| 75 mg of Morphine              | SR 141716A           | 0 $\pm$ 0     | 0 $\pm$ 0     | 3.5 $\pm$ 1.4 | 0 $\pm$ 0 | no       |



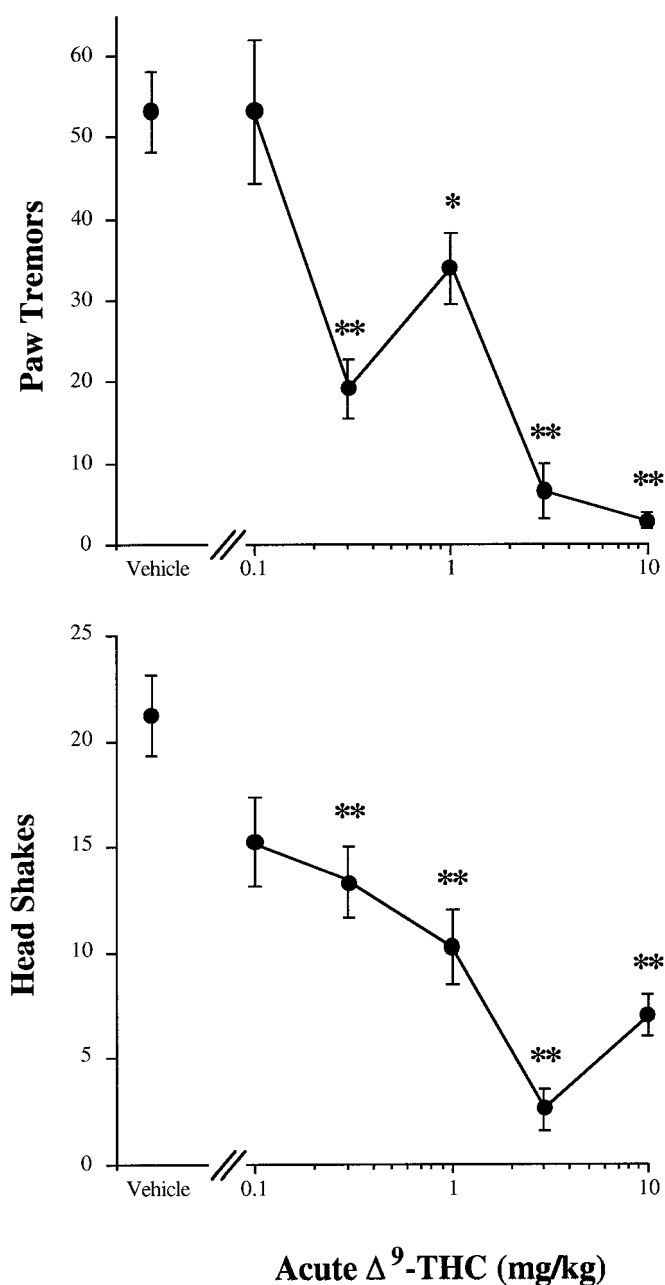
**Fig. 4.** Effects of naloxone in C57BL/6 wild-type ( $\square$ ),  $CB_1$  cannabinoid receptor knockout ( $\text{▨}$ ), and  $\mu$ -opioid receptor knockout ( $\blacksquare$ ) mice implanted with 75-mg morphine sulfate pellets. Top, number of paw tremors was significantly reduced in the  $CB_1$  cannabinoid receptor knockout and the  $\mu$ -opioid mice exhibited virtually none of these responses. Bottom,  $\mu$ -opioid receptor knockout mice exhibited significantly fewer head shake incidents than those exhibited in the wild-type mice. The results are presented as means  $\pm$  S.E. with six mice per group. \*\* $p < 0.01$ ; Dunnett's test comparisons between each genotype and the C57BL/6 wild-type control group.

in ICR mice. Interestingly, in addition to observing increases in wet dog shakes and paw tremors, Ledent et al. (1999)

reported increases in sniffing episodes and increased subjective ratings of both piloerection and body tremors in CD-1 mice. In contrast, rats exhibit a greater number of SR 141716A-precipitated cannabinoid withdrawal signs than that observed in mice. These behavioral signs range in intensity and include wet dog shakes, facial rubs, horizontal and vertical activity, forepaw fluttering, chewing, tongue rolling, paw shakes and head shakes, retropulsion, myoclonic spasms, front paw treading, and eyelid ptosis (Aceto et al., 1995; Tsou et al., 1995; Navarro et al., 1998). In  $\Delta^9$ -THC-dependent dogs, SR 141716A precipitated yet another unique pattern of withdrawal signs that included excessive salivation, vomiting, diarrhea, restless behavior, trembling, and decreases in social behavior (Lichtman et al., 1998). These results taken together indicate that the specific behavioral signs that occur during cannabinoid withdrawal are species specific.

SR 141716A also has effects of its own. As described by others (Aceto et al., 1996; Cook et al., 1998; Rubino et al., 1998), we observed that SR 141716A, by itself, elicited scratching of the face and body in ICR and C57BL/6 mice. This drug has also been reported to produce mild withdrawal-like effects in naive (Rodriguez de Fonseca et al., 1997) or vehicle-treated rats (Aceto et al., 1995, 1996). Consequently, it is not surprising that SR 141716A has been demonstrated to produce some inverse agonist effects at the  $CB_1$  cannabinoid receptor in vitro (Bouaboula et al., 1997; Landsman et al., 1997). The fact that SR 141716A possesses intrinsic activity on its own underscores the importance of including control groups that receive repeated injections of vehicle. Nonetheless, the occurrence of reliable SR 141716A-precipitated cannabinoid withdrawal models in mice, rats (Aceto et al., 1995; Tsou et al., 1995), and dogs (Lichtman et al., 1998) unequivocally supports the existence of cannabinoid withdrawal.

The results presented here also provide confirmatory support that cannabinoid receptors play a modulatory role in opioid dependence. Acute administration of  $\Delta^9$ -THC, as well as other cannabinoids, has been reported to alleviate naloxone-precipitated jumping and defecation in morphine-dependent rodents (Hine et al., 1975; Bhargava, 1976a,b). Although we show here that  $\Delta^9$ -THC was very potent in completely blocking naloxone-precipitated paw tremors ( $ED_{50} = 0.5$  mg/kg) and head shakes ( $ED_{50} = 0.6$  mg/kg) in morphine-dependent mice, it failed to ameliorate naloxone-precipitated jumping or diarrhea. Procedural differences that



**Fig. 5.** Effect of an acute dose of  $\Delta^9$ -THC on naloxone-precipitated withdrawal of morphine-dependent mice.  $\Delta^9$ -THC dose dependently decreased the number of paw tremors (top) and head shakes (bottom) in mice implanted with pellets containing 75 mg of morphine sulfate and given a s.c. injection of naloxone (1 mg/kg). The results are presented as means  $\pm$  S.E. with six mice per group. \* $p$  < 0.05 and \*\* $p$  < 0.01; Dunnett's test comparisons between each  $\Delta^9$ -THC group and the vehicle control group.

are related to naloxone dose might account for the apparent disparity between the present and earlier studies. Bhargava (1976) found that naloxone-precipitated jumping and diarrhea decreased in potency from an  $ED_{50}$  value of 0.01 mg/kg in morphine-dependent mice that were treated with vehicle to a value of 0.12 mg/kg in morphine-dependent mice that were treated with cannabinoids (Bhargava, 1976b). The high dose of naloxone (i.e., 1 mg/kg) used in our study is likely to have obscured this potency shift. In addition, the magnitude of these responses were not scored in the present study, we only assessed whether diarrhea or jumping occurred.

Other compelling evidence linking the cannabinoid system with opioid dependence is that the severity of naloxone-precipitated morphine withdrawal was decreased in mice devoid of  $CB_1$  cannabinoid receptors (Ledent et al., 1999). Although the present study replicated this effect, alternative interpretations related to the use of knockout models cannot be ruled out. Several of these potential confounds include compensatory reactions resulting from the absence of the targeted gene throughout development, hitchhiking genes that are derived from the original cell line, epistasis in which the effect of gene disruption is modified by the genetic background on which it is placed, and pleiotropic effects in which other consequences of gene disruption indirectly affect the behavior of interest (Mogil and Grisel, 1998). Nonetheless, the observations that naloxone-precipitated opioid withdrawal was attenuated in  $CB_1$  cannabinoid receptor knockout mice on two different background strains, CD-1 (Ledent et al., 1999) and C57BL/6 mice, tends to support a direct role of the  $CB_1$  cannabinoid receptor. Also consistent with this notion is the recent finding in which SR 141716A administered repeatedly to morphine-dependent rats lessened the intensity of naloxone-precipitated withdrawal (Rubino et al., 2000). The diminution of opioid withdrawal in either  $CB_1$  cannabinoid receptor knockout mice or mice treated acutely with  $\Delta^9$ -THC provides additional support to the notion that the cannabinoid system modulates opioid dependence.

A controversy exists as to whether naloxone precipitates withdrawal effects in cannabinoid-dependent animals. Early work demonstrated that naloxone precipitated withdrawal effects in rats following either 5 weeks of high doses of  $\Delta^9$ -THC (Kaymakcalan et al., 1977) or moderate doses of  $\Delta^9$ -THC (i.e., 4 mg/kg) for 2 months (Hirschhorn and Rosecrans, 1974). Similarly, naloxone precipitated withdrawal in rats following repeated injections of the potent cannabinoid analog HU-210 for 15 days (Navarro et al., 1998). It should be noted that considerable toxicity occurred following chronic high doses of  $\Delta^9$ -THC (Kaymakcalan, 1979), although no such toxicity was reported in the other studies. Conversely, naloxone was ineffective in precipitating withdrawal in  $\Delta^9$ -THC-dependent monkeys (Beardsley et al., 1986), pigeons (McMillan et al., 1971), or mice in the present study. Conflicting results on the effectiveness of SR 141716A in eliciting withdrawal effects in morphine-dependent animals are emerging as well. Whereas SR 141716A failed to elicit any responses in morphine-dependent mice in the present study, it induced withdrawal effects in morphine-dependent rats (Navarro et al., 1998). Considerable methodological differences used among the studies, including the selection of agonist, species, dosing regimen, and the dependent measures, make it difficult to account for the differential effectiveness of the antagonist in precipitating withdrawal. Nonetheless, the  $\Delta^9$ -THC dosing regimen used in the present study was considerably more mild than the regimens used in the other studies. Moreover, we found no evidence supporting the occurrence of precipitated withdrawal following either SR 141716A in morphine-dependent mice or naloxone in  $\Delta^9$ -THC-dependent mice.

In conclusion, the association between cannabinoids and opioids on dependence is bidirectional. As previously reported, administration of  $\Delta^9$ -THC (Hine et al., 1975; Bhargava, 1976b) or deletion of the  $CB_1$  cannabinoid receptor (Ledent et al., 1999) decreased the severity of naloxone-pre-



precipitated morphine withdrawal. We have demonstrated a strikingly similar pattern of results for cannabinoid dependence. Moreover, the severity of SR 141716A-precipitated  $\Delta^9$ -THC withdrawal was significantly attenuated either following administration of morphine or in  $\mu$ -opioid receptor-deficient mice. Collectively, these data indicate a reciprocal relationship between the cannabinoid and opioid systems in dependence.

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