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Behavioural and biochemical evidence for signs of abstinence in mice chronically treated with Δ-9-tetrahydrocannabinol

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1 Tolerance and dependence induced by chronic Δ-9-tetrahydrocannabinol (THC) administration were investigated in mice. The effects on body weight, analgesia and hypothermia were measured during 6 days of treatment (10 or 20 mg kg^{-1} THC twice daily). A rapid tolerance to the acute effects was observed from the second THC administration.

2 The selective CB-1 receptor antagonist SR 141716A (10 mg kg^{-1}) was administered at the end of the treatment, and somatic and vegetative manifestations of abstinence were evaluated. SR 141716A administration precipitated several somatic signs that included wet dog shakes, frontpaw tremor, ataxia, hunched posture, tremor, ptosis, piloerection, decreased locomotor activity and mastication, which can be interpreted as being part of a withdrawal syndrome.

3 Brains were removed immediately after the behavioural measures and assayed for adenylyl cyclase activity. An increase in basal, forskolin and calcium/calmodulin stimulated adenylyl cyclase activities was specifically observed in the cerebellum of these mice.

4 The motivational effects of THC administration and withdrawal were evaluated by using the place conditioning paradigm. No conditioned change in preference to withdrawal associated environment was observed. In contrast, a conditioned place aversion was produced by the repeated pairing of THC (20 mg kg^{-1}), without observing place preference at any of the doses used.

5 This study constitutes a clear behavioural and biochemical model of physical THC withdrawal with no motivational aversive consequences. This model permits an easy quantification of THC abstinence in mice and can be useful for the elucidation of the molecular mechanisms involved in cannabinoid dependence.

Keywords: Δ-9-tetrahydrocannabinol; physical dependence; SR 141716A; adenylyl cyclase; cyclic AMP; withdrawal; place conditioning; tolerance

Introduction

Preparations, derived from the plant *cannabis sativa* are among of the most commonly used illicit drugs in the United States and Europe (Kozel & Adams, 1986; Adams & Martin, 1996). The main psychoactive component of these preparations is the alkaloid Δ-9-tetrahydrocannabinol (THC), which is responsible for their euphorogenic and sedative effects (Adams & Martin, 1996).

Recently, an endogenous cannabinoid system has been characterized in the central nervous system. Endogenous cannabinoids such as anandamide (Devane *et al*., 1992; Cadas *et al*., 1996) and more recently 2-arachidonyl-glycerol (Stella *et al*., 1997) have been identified. Specific receptors for cannabinoids, namely the CB-1 receptor localized preferentially in the brain (Devane *et al*., 1988; Matsuda *et al*., 1990) and the CB-2 receptor found in the peripheral tissues (Munro *et al*., 1993), have been cloned. Activation of cannabinoid receptors leads to an inhibition of adenylyl cyclase (AC) activity (Howlett & Fleming, 1984), as well as an inhibition of N-type Ca^{2+} channels (Mackie & Hille, 1992), a stimulation of arachidonic acid release (Burstein *et al*., 1994; Shivachar *et al*., 1996) and the MAPkinase cascade (Bouaboula *et al*., 1995) that are independent of cyclic AMP. Cannabinoid research has further benefited from the synthesis of an antagonist with high affinity and selectivity for the CB-1 receptor, SR 141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxy-amide] (Rinaldi-Carmona *et al*., 1994). This antagonist blocks the effects of cannabinoid agonists both *in vitro* (Rinaldi-Carmona *et al*., 1994) and *in vivo* (Rinaldi-Carmona *et al*., 1994; Compton *et al*., 1996).

In vivo effects of acute administration of cannabinoids share common features with opiates such as analgesia (Lichtman & Martin, 1991; Raft *et al*., 1976; Dewey *et al*., 1970), hypothermia (Anderson *et al*., 1975) and reduced locomotor activity at high doses (Davis *et al*., 1972; Anderson *et al*., 1975). In more recent investigations, the antagonist SR 141716A was able to precipitate some behavioural manifestations that could be related to the expression of a withdrawal syndrome in rats chronically treated with THC (Tsou *et al*., 1995; Aceto *et al*., 1996). The cannabinoid abstinence was also associated with an increase in corticotrophin-releasing hormone in the nucleus accumbens (Rodríguez de Fonseca *et al*., 1997). These withdrawal manifestations (Tsou *et al*., 1995; Aceto *et al*., 1996) have indicated a component of chronic cannabinoid misuse in common with the cycle of dependence seen with drugs such as opiates (Koob & Le Moal, 1997). However, the behavioural and biochemical expression of cannabinoid abstinence is not as apparent as the opiate withdrawal syndrome and remains to be clearly characterized. Furthermore, the rewarding effects of cannabinoids in the different animal models currently used are

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controversial. Thus, (1) cannabinoid agonists failed to induce self-administration behaviour in monkeys (Harris *et al*., 1974; Carney *et al*., 1977; Mansbach *et al*., 1994) or rats (Leite & Carlini, 1974), but are self-administered in mice (Martellota *et al*., 1998); (2) cannabinoid administration produced conditioned place aversion (McGregor *et al*., 1996; Parker & Gillies, 1995; Sañudo-Peña *et al*., 1997) although one study reports the occurrence of place preference in some specific experimental conditions (Lepore *et al*., 1995); (3) in line with this, cannabinoids can reduce reward thresholds in the intracranial self stimulation paradigm (Gardner *et al*., 1988), as seen with other drugs of abuse (Wise, 1996). Furthermore, elevations of dopamine in the limbic system, a mechanism thought to underlie drug reinforcement, has been produced by acute cannabinoid administration (Tanda *et al*., 1997; Chen *et al*., 1990), as well as by other groups of abused drugs (Tanda *et al*., 1997; Di Chiara *et al*., 1991).

Although cannabinoids and opiates share some common signal transduction elements, such as inhibition of AC, no parallelism has been established between the adaptive modulations at the molecular level, following their repeated administration mainly because of the scarcity of biochemical studies after chronic cannabinoid treatment. Interestingly, repeated cannabinoid administration has been shown to induce a receptor desensitization, concomitant with a phenomenon of tolerance (Dill & Howlett, 1987), but no studies have investigated the effects of chronic cannabinoid treatment and withdrawal on AC. Biochemical adaptations to chronic opioid treatment involve a long lasting upregulation of the cyclic AMP pathway after repeated exposure to the drug, as revealed by the marked increase in AC activity during morphine withdrawal (Duman *et al*., 1988; Matthes *et al*., 1996). This compensatory response to the acute inhibitory effects of opioids was shown to be a major mechanism involved in the expression of physical dependence to these drugs (Maldonado *et al*., 1996; Valverde *et al*., 1996; Lane-Ladd *et al*., 1997).

Thus, controversy over the classification of cannabinoid drugs for recreational and medical use still remains. This study aims to investigate the behavioural and biochemical expression of SR 141716A-induced withdrawal from THC in mice. For this purpose, we have studied several somatic and vegetative signs, the aversive/dysphoric effects on the place conditioning paradigm and the changes in AC in distinct brain regions, during THC abstinence. Tolerance to some well-known pharmacological responses of THC, such as antinociception and hypothermia, and the motivational effects induced by repeated administration of THC were also evaluated. This detailed study will permit comparison between consequences of THC repeated administration and abstinence to those previously reported under similar experimental conditions with other drugs of abuse such as opiates.

Methods

Animals

Male CD-1 mice (Charles River, France) weighing 22–26 g at the start of the study, were housed in perspex cages in groups of two or four, and maintained at a controlled temperature of $21 \pm 1^{\circ}C$. The mice were given access to food and water *ad libitum* except during behavioural observations. Lighting was maintained at 12 h cycles (on at 08.00 h and off at 20.00 h). Behavioural tests and animal husbandry were conducted in accordance with standard ethical guidelines (NIH, 1995) and were approved by the local ethical committee. All the behavioural tests were performed by an observer who did not know the treatment of the animals.

Drugs

All drugs were administered by intraperitoneal injection except morphine which was given by subcutaneous injection. THC (Sigma, France) was purchased as 100 mg/ml alcohol and dissolved into a final concentration of 5% ethanol, 5% cremophor EL (Sigma, France) and 90% distilled water, and injected in a volume of 0.1 ml per 10 g of body weight. The antagonist SR 141716A [N-piperidin-1-yl)-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxyamide] generously provided by Sanofi Recherche (France), was dissolved in a solution of 10% alcohol, 10% cremophor EL (Sigma, France) and 80% distilled water, and injected in a final volume of 0.2 ml per 10 g of body weight. Morphine HCl (Sigma, France) was dissolved in saline (0.9% sodium chloride in water) and injected in a final volume of 0.1 ml per 10 g of body weight. ATP, cyclic AMP, phosphocreatine, creatine phosphokinase, forskolin (FSK), N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulphonamide HCl (W7) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (France). [α-^{32}P]ATP (30 Ci mmol^{-1}) and [8-^{3}H] cyclic AMP (30 Ci mmol^{-1}) were purchased from Amersham (France). Ready Safe liquid scintillant was purchased from Beckman (France).

Measurement of THC tolerance and dependence

Two groups of mice ($n = 16$ per group) were injected i.p. twice daily at 09.00 h and 19.00 h during 5 days with THC (20 mg kg^{-1}) or vehicle. On day 6 the mice only received the morning injection. In a second experiment, two groups of mice ($n = 10$ per group) were injected with THC (10 mg kg^{-1}) or vehicle under the same conditions.

Tolerance to THC effects

Three different responses were measured during THC treatment: body weight, rectal temperature and antinociception. Body weights were recorded for each animal, using an electronic balance (Mettler PM4800, sensitive to 0.01 g), twice a day before all morning and evening injections.

Rectal temperature was measured in each mouse using an electronic thermocouple flexible rectal probe (Bioblock Scientific, France, Model MT 140). The probe was placed 3 cm into the rectum of the mice for 30 s before the temperature was recorded. On days 1 and 2, measures were taken immediately prior to, and 20 min after, each injection. On days 3, 4 and 5 rectal temperature was evaluated before and 20 min after the evening injection only.

Antinociceptive responses were measured using the tail immersion assay, as described previously (Janssen *et al*., 1963). The water used for immersing the tail was maintained at a constant temperature of $50 \pm 0.5^{\circ}C$. The time (seconds) to withdraw the tail from the bath was registered, with a cut off latency of 15 s to prevent tissue damage. Nociceptive measurements for each individual were taken just after rectal temperature measurements, prior to and 20 min after, morning (days 1 and 2) and evening (everyday) injections.

Somatic expression of THC withdrawal

Four hours after the last THC or vehicle injections, mice were placed in a circular clear plastic observation area (30 mm diameter × 35 mm height) for a 15 min period of habituation.

Immediately after habituation animals were observed for a further period of 15 min, followed by administration of SR 141716A (10 mg kg^{-1} i.p.). The mice were then observed for a 45 min period. At the end of this observation period, each mouse was placed into individual locomotor activity chambers for 45 min. Body weight, rectal temperature and tail immersion latency were measured immediately before and after the habituation period, as well as 45 and 90 min after SR 141716A injection. The experimental procedure is summarized in Figure 1. Observations of somatic signs before and after SR 141716A challenge were divided in 5 min time intervals. The number of bouts of writhing, wet dog shake and frontpaw tremor were counted. Penile licking or erection, ataxia, hunched posture, tremor and piloerection were scored 1 for appearance and 0 for non-appearance within each 5 min time epoch. Scores for the level of activity were made by giving in each 5 min period a value of 0 = low activity, 1 = normal activity, or 2 = increased activity. A three level score was also applied to evaluate ptosis (0 = normal, 1 = eye lids half closed, and 2 = eye lids fully closed). A quantitative value was calculated in each animal for the different checked signs by adding the scores obtained in each 5 min time epoch.

Locomotor activity was evaluated in separate rectangular activity boxes (255 mm × 205 mm) held within dark (less than 3 lux) and sound attenuating housing. Two crossed photobeams transected the middle of each activity box. Photobeam counts were recorded at 15 min intervals.

Biochemical expression of THC withdrawal

Basal and forskolin stimulated AC activities were evaluated in several brain structures during THC withdrawal. For this purpose, mice were killed by cervical dislocation immediately after the last antinociceptive measurement. Brains were rapidly removed and dissected at 4°C to isolate the following brain regions: frontal cortex, hippocampus, striatum, cerebellum and periaqueductal grey matter. Each region was homogenized in ten times the volume of an ice-cold homogenization buffer of the following composition: 20 mM Tris-HCl pH:8, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF. Homogenates were centrifuged at 500 × *g* for 5 min at 4°C. Pellets were discarded and the supernatants were centrifuged at 15,000 × *g* at 4°C for another 30 min. The resulting pellets, which represented the crude membrane fractions, were resuspended in the above buffer to a final protein concentration of 1–5 mg/ml.

To evaluate AC activity, membranes (15–40 μg $protein^{-1}$) were incubated at 35°C for 10 min in 60 μl of the following assay medium : 50 mM Tris pH:7.6, 5 mM $MgCl_2$, 1 mM cyclic AMP, 100 mM ATP containing 10^6 c.p.m. [α-^{32}P]ATP, in a regenerating system consisting of 5 mM creatine phosphate and 250 μg ml^{-1} creatine kinase. Forskolin was added in a final concentration of 100 μM in some of the assays. The reaction started with the addition of the membranes and was stopped in 500 mM HCl. Assays were neutralized with 1.5 M imidazol followed by separation of the cyclic AMP from the ATP on alumina columns. The amount of the [α-^{32}P]cyclic AMP formed was measured and corrected for the recovery of added [^{3}H]cyclic AMP. In order to assess the calcium and calmodulin sensitivity of AC, 1 mM EGTA was added in all assay mixtures. Cyclase activity was assayed in the presence or absence of 950 μM calcium and 2.4 μM calmodulin, or of the calmodulin inhibitor W-7 (100 μM). These conditions have been previously shown to be optimal for assessing calcium effects on AC from brain preparations (Tzavara *et al*., 1996). In all experiments, protein amount was determined using the Bio-Rad reactif assay (Bio-Rad, FRG).

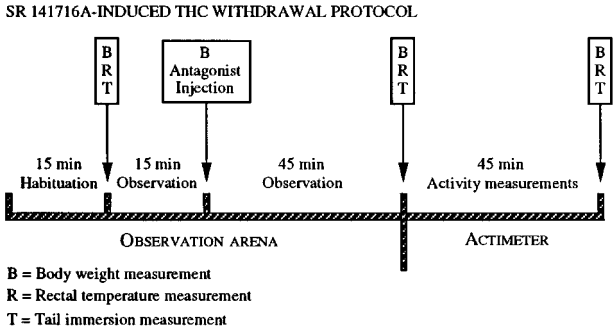

Figure 1 Summary of the protocol for SR 141716A-induced THC withdrawal measurements.

Place conditioning paradigm

To evaluate the motivational consequences of THC administration and withdrawal an unbiased place conditioning methodology was used, as described previously (Maldonado *et al*., 1997). Briefly, the apparatus consisted of two main square conditioning compartments (15 × 15 × 15 cm) separated by a triangular central division. The light intensity within the conditioning chambers was 6 ± 0.5 Lux. The movement and location of the mice were monitored by computerized monitoring software (Videotrack®, View Point, Lyon) with images relayed from a camera placed above the conditioning apparatus. The protocol consisted of three phases. (1) Habituation phase: drug naïve mice had free access to both compartments of the conditioning apparatus for 18 min with the time spent in each compartment and the number of compartment entries recorded. (2) Conditioning phase: this phase consisted of 2 (experiments 1 and 2) or 6 (experiment 3) days, where each conditioning chamber was closed by inserting partitions at the entrance to each of the two main conditioning chambers. On the first conditioning day, mice were injected with the drug or the vehicle and immediately placed in one of the conditioning environments individually for 30 min. The following day, mice were injected with vehicle and placed in the opposite compartment. (3) Test phase: the day following the conditioning phase, mice were allowed free access to all of the apparatus. The time of occupancy in each compartment and the number of compartment entries were recorded.

Experiment 1 – SR 141716A place conditioning in THC naive mice The motivational responses of SR 141716A when given under acute conditions were first determined in naïve animals in order to discard any intrinsic effect that would be of relevance for the interpretation of the THC withdrawal place conditioning experiment (experiment 2). Thus, separate groups of mice (n = 10 per group) received SR 141716A at the dose of 2.5, 5.0 and 10 mg kg^{-1}, i.p. or vehicle injection on the first conditioning day. On the second conditioning day all mice received vehicle injections.

Experiment 2 – SR 141716A-induced withdrawal place conditioning in THC dependent mice On the day following the habituation phase, mice were treated with THC 20 mg kg^{-1}, i.p. (group 1) or vehicle (groups 2 and 3) twice daily (n = 10 per group), on an identical schedule to that described in the tolerance and dependence study. However the treatment schedule was continued through the conditioning phase with last injections given on the evening of the last conditioning day (day 7). Four hours after the THC or vehicle morning injection

of day 6, mice received SR 141716A 10 mg kg^{-1}, i.p. (groups 1 and 2) or vehicle (group 3) and were placed in the conditioning chambers. On the second conditioning day, all mice received vehicle injections before being placed in the opposite conditioning chamber. Only two pairings were used in the withdrawal place conditioning experiment, as previously reported (Mucha *et al*., 1986; Valverde *et al*., 1996) since (1) the negative motivational effects produced by antagonist administration, at least in the case of opiate-dependent animals, is strong enough to produce a clear place conditioned aversion after one single pairing; (2) the severity of withdrawal and their motivational effects in this paradigm could be decreased when repeating the systemic administration of the antagonist.

Experiment 3 – THC and morphine place conditioning in drug naïve mice Separate groups of mice ($n=10$ per group) received vehicle or THC at the dose 0.5, 1, 2.5, 5, 10 and 20 mg kg^{-1}, i.p. on the first conditioning day, and vehicle injection on the second day. This sequence of drug and vehicle administration was repeated three times. To assess the validity of the place conditioning protocol a positive control group was performed under the same experimental conditions, excepting the conditioning time used in this case was 20 min per day. This control consisted of two groups of mice ($n=10$) receiving saline or morphine (5 mg kg^{-1}, s.c.) on the first conditioning day and all of them saline on the second day. This sequence was repeated three times, as previously reported (Carr *et al*., 1989; Maldonado *et al*., 1997) in order to optimize the conditioning procedure.

Statistics

Data from the tolerance study were analysed by using a two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were treatment (between subjects) and time (within subjects). Individual group comparisons at each time point were made using one-way ANOVA.

Somatic signs of withdrawal were compared by using a one-way ANOVA (between subjects). A first comparison between the different groups was made with data obtained during the 15 min preceding SR 141716A injection. A second analysis compared data obtained in each group during the 45 min post-antagonist administration.

Values obtained in place conditioning experiments were compared using two-way ANOVA for repeated measures with factors of variation being treatment (between subjects) and day (within subjects). One-way ANOVA was conducted separately for pre-test and test days with significance for group effects identified using *post hoc* Dunnett's *t*-test. Identical analysis was used for compartment entry data. This analysis was conducted separately for drug and vehicle associated compartments. Within group analysis was also made for each group compartment times before and after conditioning using a two-tailed Student's paired *t*-test.

Data obtained from the AC assay were analysed using linear regression analysis to measure effects in comparison with control AC activity. Separate linear regression analysis was conducted for THC values pooled from both 10 and 20 mg kg^{-1} groups, in comparison with AC activity in chronic vehicle controls. The two-tailed unpaired Student *t*-test was used for comparisons between chronic vehicle and THC groups, after antagonist administration, for each brain region. Identical separate analyses were conducted for basal and forskolin stimulated AC activity experiments. Significance for all analysis was accepted at $P<0.05$.

Results

Tolerance

Rectal temperature Two-way ANOVA showed for rectal temperature significant effects of treatment (10 mg kg^{-1} schedule, $F_{(1,126)}=37.38$, $P<0.001$, 20 mg kg^{-1} schedule, $F_{(1,194)}=27.48$, $P<0.001$), time (10 mg kg^{-1} schedule, $F_{(6,126)}=6.28$, $P<0.001$, 20 mg kg^{-1} schedule, $F_{(6,194)}=14.21$, $P<0.001$) and interaction between these two factors (10 mg kg^{-1} schedule, $F_{(6,126)}=6.30$, $P<0.001$, 20 mg kg^{-1} schedule, $F_{(6,194)}=7.33$, $P<0.001$).

The administration of THC at the dose of 10 mg kg^{-1} produced significant hypothermic effects at day 1 morning ($F_{(1,18)}=42.91$, $P<0.001$) and evening measurements ($F_{(1,18)}=6.41$, $P<0.05$). No significant effect of THC (10 mg kg^{-1}) was observed after the day 2 morning injection nor at all the subsequent measurements, revealing a tolerance to THC hypothermic effects (Figure 2A).

Administration of THC at the highest dose (20 mg kg^{-1}) resulted in significant decreases in rectal temperature on day 1 (morning, $F_{(1,22)}=29.05$, $P<0.001$; evening, $F_{(1,30)}=6.63$, $P<0.05$) and day 2 morning ($F_{(1,22)}=5.82$, $P<0.05$), but not at the day 2 evening measurement (Figure 2B). Tolerance to the hypothermic effects was therefore reached by the fourth injection and remained at all subsequent measurements.

Between groups analysis of basal rectal temperatures before each day injection showed no significant difference in the lower dependence schedule (10 mg kg^{-1}). However in mice receiving the higher dose of THC (20 mg kg^{-1}), there was a significant difference in the basal control values of vehicle (38.18 ± 0.14°C) and THC (37.77 ± 0.24°C) groups before day 1 evening injections ($F_{(1,22)}=4.88$, $P<0.05$), but not at subsequent basal measurements.

Nociception Two-way ANOVA of the latency values showed significant effects of treatment ($F_{(1,194)}=15.76$, $P<0.001$), time ($F_{(6,194)}=2.71$, $P<0.05$) and interaction between time and treatment ($F_{(6,194)}=5.41$, $P<0.001$) at the higher dose schedule (THC 20 mg kg^{-1}), but not with the dose of 10 mg kg^{-1} of THC. A significant analgesic response was observed after the first administration of the lower dose of THC (10 mg kg^{-1} schedule, $F_{(1,18)}=5.36$, $P<0.05$). A tolerance to the analgesic effects of THC was already seen at the evening measurement on the first day (Figure 3A). THC administration at the higher

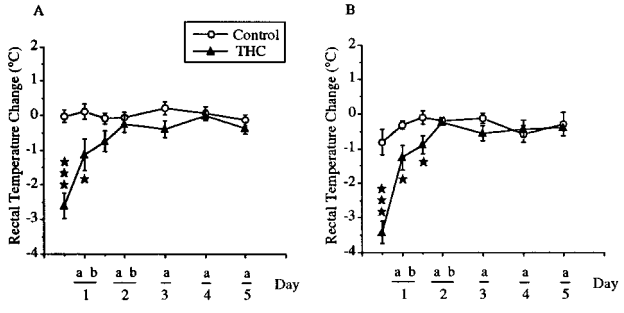

Figure 2 Effects of chronic THC treatment on mice rectal temperature. A = Mice received vehicle or THC (10 mg kg^{-1}) twice daily; B = Mice received vehicle or THC (20 mg kg^{-1}) twice daily. Measurements were performed during the 5 days of treatment as indicated on the horizontal axis. a = measurement performed at 09.00 h; b = measurement performed at 19.00 h. Values are expressed as means ± s.e.mean rectal temperature change calculated by subtracting the values at 20 min after from the value obtained just before THC administration. $^{*}P<0.05$, $^{***}P<0.001$ versus vehicle group (one-way ANOVA).

dose (20 mg kg^{-1} schedule) resulted in large significant increases in tail flick latency when compared to the control group after the day 1 morning ($F_{(1,22)}=17.60$, $P<0.001$) and evening injections ($F_{(1,30)}=13.60$, $P<0.001$). A tolerance to the analgesic effect of THC, was seen at day 2, with no significant differences between THC and vehicle groups in all the following measurements (Figure 3B). Analysis of tail immersion latencies before THC or vehicle injections revealed no significant differences between the basal latencies of these groups at any of the measurement points performed during the different chronic treatments (data not shown).

Body weight Two-way ANOVA showed a significant effect of treatment (10 mg kg^{-1} schedule, $F_{(1,18)}=7.48$, $P<0.05$, 20 mg kg^{-1} schedule, $F_{(1,30)}=13.65$, $P<0.001$), time (10 mg kg^{-1} schedule, $F_{(4,72)}=19.98$, $P<0.001$, 20 mg kg^{-1} schedule, $F_{(4,120)}=8.52$, $P<0.001$) and interaction between these two factors (10 mg kg^{-1} schedule, $F_{(4,72)}=3.93$, $P<0.01$, 20 mg kg^{-1} schedule, $F_{(4,120)}=6.35$, $P<0.001$).

Body weights of the different groups of mice were similar before starting the treatment. Body weight changes data were calculated by subtracting each morning body weight from the preceding morning's value (Figure 4A,B). A significant decrease in body weight was observed in THC treated mice from day 1 to day 2, (10 mg kg^{-1} schedule, $F_{(1,18)}=9.26$, $P<0.05$, 20 mg kg^{-1} schedule, $F_{(1,30)}=21.02$, $P<0.001$). No significant differences were revealed at the subsequent time points.

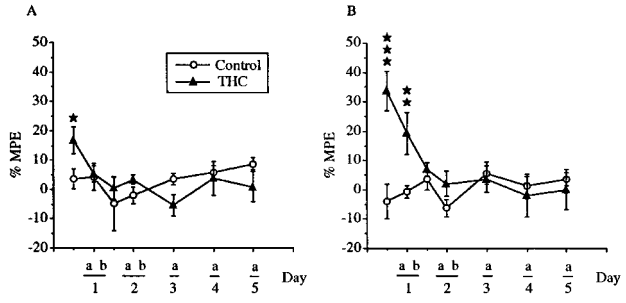

Figure 3 Effects of chronic THC treatment on nociceptive responses (tail withdrawal latencies) evaluated in the tail immersion test in mice, and expressed as means ± s.e.mean of the percentage of the maximum possible effect (MPE), calculated using the following formula: (test latency − control mean latency) / (cut-off time − control mean latency) × 100. A = Mice received vehicle or THC (10 mg kg^{-1}) twice daily; B = Mice received vehicle or THC (20 mg kg^{-1}) twice daily. Measurements were performed during the 5 days of treatment as indicated on the horizontal axis. a = measurement performed at 09.00 h; b = measurement performed at 19.00 h. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus vehicle group (one-way ANOVA).

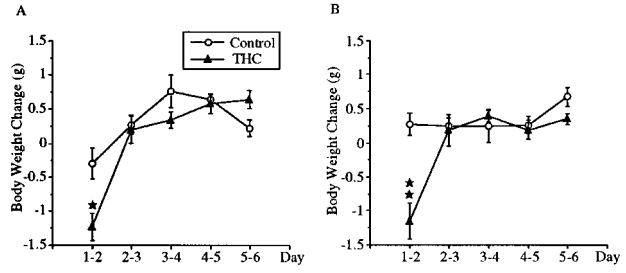

Figure 4 Effect of THC on daily body weight change of mice during the chronic dependence schedule. A = Mice received vehicle or THC (10 mg kg^{-1}) twice daily; B = Mice received vehicle or THC (20 mg kg^{-1}) twice daily. Measurements were performed during the 5 days of treatment as indicated on the horizontal axis. Values are expressed as means ± s.e.mean body weight change. $^{*}P<0.05$, $^{**}P<0.01$ versus vehicle group (one-way ANOVA).

Dependence

Several biochemical and behavioural manifestations of withdrawal were evaluated after SR-141716A administration in mice chronically receiving THC: somatic signs, AC activity in various brain regions, vegetative manifestations, and changes-induced on nociceptive threshold and locomotor activity.

Somatic signs of withdrawal Before the administration of the CB-1 antagonist SR 141716A, there were no significant differences between groups in the incidence of the different counted (wet dog shakes, frontpaw tremor) and checked (ataxia, hunched posture, tremor, ptosis, piloerection, locomotor activity and mastication) somatic signs of withdrawal in any of the experiments. Only a significant difference between chronic vehicle and THC 20 mg kg^{-1} treated mice before antagonist was observed in locomotor activity with values of 4.0 ± 0.47 and 2.2 ± 0.38 respectively ($F_{(1,30)}=8.92$, $P<0.01$).

Counted somatic withdrawal signs Wet dog shakes: Between group comparisons after antagonist administration showed a higher incidence of wet dog shakes in the groups receiving THC 10 mg kg^{-1} ($F_{(1,18)}$ 5.97, $P<0.05$) and 20 mg kg^{-1} ($F_{(1,30)}=36.00$, $P<0.001$) than their respective controls (Figure 5).

Frontpaw tremor: Frontpaw tremor values post-antagonist were significantly higher in groups treated chronically with THC 10 mg kg^{-1} ($F_{(1,18)}=28.32$, $P<0.001$) and 20 mg kg^{-1} ($F_{(1,30)}=15.36$, $P<0.001$), as compared to their respective controls (Figure 5).

Checked somatic withdrawal signs Ataxia: Mice treated with THC 20 mg kg^{-1}, but not with THC 10 mg kg^{-1}, showed significantly elevated episodes of ataxia versus their control groups ($F_{(1,30)}=22.99$, $P<0.001$) (Figure 5).

Hunched posture: After SR 141716A injection, significantly higher incidences of this sign were observed in groups treated chronically with THC 10 mg kg^{-1} ($F_{(1,18)}=11.17$, $P<0.01$) and 20 mg kg^{-1} ($F_{(1,30)}=29.40$, $P<0.001$), compared to their respective controls (Figure 5).

Tremor: Significantly higher tremor were observed in mice treated chronically with THC 10 mg kg^{-1} ($F_{(1,18)}=62.02$, $P<0.001$) and 20 mg kg^{-1} ($F_{(1,30)}=69.78$, $P<0.001$) after antagonist administration in comparison with their controls (Figure 5).

Ptosis: Mice treated with THC 20 mg kg^{-1}, but not the group receiving 10 mg kg^{-1} of THC, showed significantly elevated incidence of ptosis compared to control mice after SR 141716A injection ($F_{(1,30)}=18.54$, $P<0.001$) (Figure 5).

Piloerection: There were significantly more incidents of this parameter in mice treated chronically with THC 20 mg kg^{-1} when compared to their controls after antagonist administration ($F_{(1,30)}=5.86$, $P<0.05$), but not in mice receiving THC 10 mg kg^{-1} (Figure 5B).

Locomotor activity: After antagonist administration, mice treated with 20 mg kg^{-1} of THC, but not with THC 10 mg kg^{-1}, showed a significantly lower activity compared to their controls ($F_{(1,30)}=12.16$, $P<0.01$) (Figure 5).

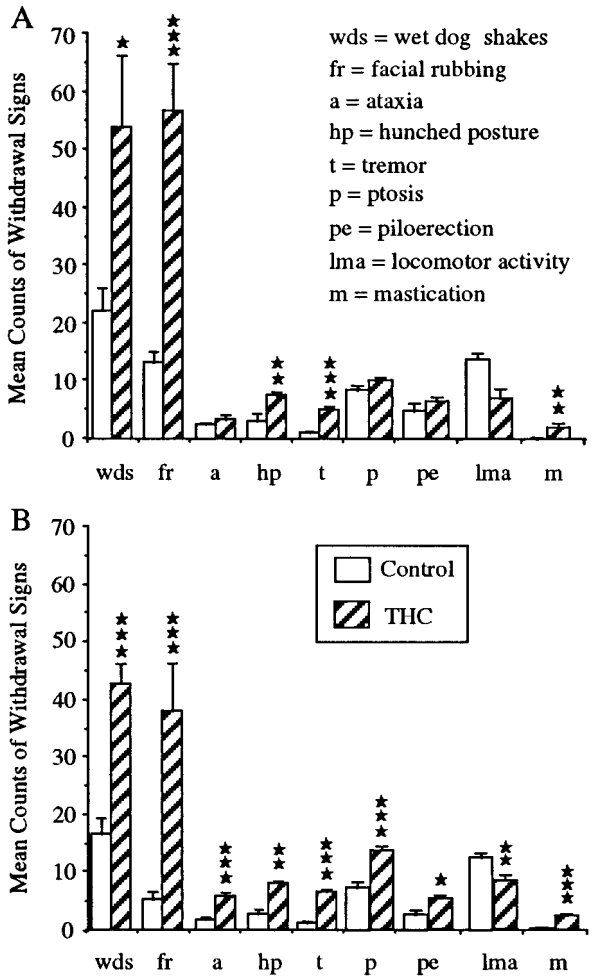

Figure 5 Effects of SR 141716A (10 mg kg^{-1}) after administration to mice after chronic THC or vehicle injection schedule. A = Mice received vehicle or THC (10 mg kg^{-1}) twice daily; B = Mice received vehicle or THC (20 mg kg^{-1}) twice daily. Data are expressed as means ± s.e.mean incidents of counted and checked physical withdrawal signs observed during the 45 min immediately after SR 141716A administration. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus vehicle group (one-way ANOVA).

Table 1 Region specific increase in adenylyl cyclase activity after SR 141716A administration in THC-dependent mice

		Vehicle + SR 141716A	*THC + SR 141716A*
Cortex	Basal	90 ± 3	90 ± 3
	Forskolin	680 ± 19	510 ± 19
Hippocampus	Basal	142 ± 17	162 ± 8
	Forskolin	939 ± 76	1046 ± 48
Striatum	Basal	76 ± 23	87 ± 13
	Forskolin	1197 ± 290	1389 ± 223
Periaqueductal	Basal	38 ± 10	68 ± 23
Grey matter	Forskolin	289 ± 71	323 ± 108
Cerebellum	Basal	59 ± 5	107 ± 17**
	Forskolin	388 ± 40	652 ± 56***

Analysis of basal and forskolin stimulated adenylyl cyclase activity in various brain regions after administration of SR 141716A (10 mg kg^{-1}) in mice treated chronically with THC (20 mg kg^{-1}, twice a day) or vehicle. Results are expressed in pmoles cyclic AMP formed/min mg^{-1} $protein^{-1}$ and represent means ± s.e.mean of at least four individual experiments run in triplicate. $^{**}P<0.01$, $^{***}P<0.001$, compared with vehicle + SR 141716A.

Table 2 Effects of SR 141716A on basal and forskolin-stimulated adenylyl cyclase activity in the cerebellum of mice receiving THC chronic treatment at different doses

		THC	
	Vehicle	*10 mg kg^{-1}*	*20 mg kg^{-1}*
(A) Basal adenylyl cyclase activity			
Vehicle	50 ± 3	46 ± 0.6	57 ± 11
SR 141716A	59 ± 5	88 ± 20	107 ± 17*
(B) Forskolin stimulated adenylyl cyclase activity			
Vehicle	263 ± 27	159 ± 13	176 ± 20
SR 141716A	388 ± 40	504 ± 70*	652 ± 56*

Mice treated chronically with THC (10 or 20 mg kg^{-1}, twice a day) or vehicle were administered SR 141716A (10 mg kg^{-1}) or vehicle. Table shows basal (A) and forskolin stimulated (B) adenylyl cyclase activity assessed in cerebellar membrane preparations. Results are expressed in pmoles cyclic AMP formed/min mg^{-1} $protein^{-1}$ and represent means ± s.e.mean of at least four individual experiments run in triplicate. $^{*}P<0.01$ compared to chronic vehicle/vehicle treated control (Student's unpaired *t*-test).

Mastication: After antagonist administration, significant increases in mastication were recorded in the groups treated chronically with THC 10 mg kg^{-1} ($F_{(1,18)}=10.06$, $P<0.01$) and 20 mg kg^{-1} ($F_{(1,30)}=20.81$, $P<0.001$) groups in comparison with their respective controls (Figure 5).

Other behaviours: The presence of other behavioural signs, such as writhing and penile erection was not significant in any group of animals before or after SR 141716A administration.

Adaptive changes in the cyclic AMP pathway Under THC withdrawal, a pronounced increase in both, basal and forskolin stimulated AC activity was specifically observed in the cerebellum. In this region, AC activity was increased 1.7 fold after SR 141716A (10 mg kg^{-1}) administration in mice treated chronically with 20 mg kg^{-1} THC, compared to controls chronically receiving vehicle ($t_{(1,24)}=2.7$, $P<0.05$ for basal, $t_{(1,24)}=3.8$, $P<0.001$ for forskolin). No significant change was found in other brain regions such as the cortex, hippocampus, striatum or periaqueductal grey matter (Table 1).

Basal AC activity significant main effects of THC ($F_{(2,51)}=3.8$, $P<0.05$) and SR 141716A ($F_{(1,51)}=7.7$, $P<0.001$) were observed in the cerebellum (Table 2A). There was also a significant interaction between SR 141716A and THC treatments when both THC treated group values were pooled ($F_{(1,51)}=4.6$, $P<0.05$). In untreated mice no significant main effects of SR 141716A administration on AC activity was shown. However, in THC treated animals antagonist administration specifically increased AC activity.

The same pattern of results was observed for forskolin stimulated AC activity with significant elevations for main effect of THC ($F_{(2,51)}=3.7$, $P<0.05$), SR 141716A ($F_{(1,51)}=39.6$, $P<0.001$) and interaction of these two factors ($F_{(2,49)}=8.0$, $P<0.001$), and when both THC group values were pooled ($F_{(1,51)}=17.3$, $P<0.001$) (Table 2B). The increase in AC activity after SR 141716A administration was more elevated in THC treated than in untreated mice.

Figure 6 shows that (1) after SR 141716A administration, the AC activity in the cerebellum doubles in THC treated mice as compared to THC untreated controls for basal ($t_{(1,20)}=4.85$, $P<0.001$) and for calcium/calmodulin stimulated activity ($t_{(1,20)}=3.39$, $P<0.01$), (2) the calcium/calmodulin sensitivity

of the AC activity is the same in mice treated chronically THC and vehicle groups with a 4 fold increase in AC activity seen in both.

Vegetative signs of withdrawal Lack of body weight changes during withdrawal: No significant difference between controls and group receiving 10 mg kg^{-1} of THC was found before SR 141716A administration. However, a significant difference in body weight between mice receiving THC 20 mg kg^{-1} (23.8 ± 0.4 g) and their controls (25.8 ± 0.5 g) occurred before antagonist administration as a consequence of the chronic THC treatment ($F_{(1,30)} = 9.99$, $P < 0.01$).

Effects of antagonist-induced withdrawal on body weight were assessed using the change in this parameter between each measurement point during abstinence. There were no significant differences between control and THC groups in body weight change before (time $-15-0$ min) or after antagonist administration (0–45 min and 45–90 min) for both THC chronic treatment groups (10 and 20 mg kg^{-1}) in comparison with their respective controls (data not shown).

Lack of temperature changes during withdrawal: There were no significant differences in rectal temperature between vehicle and THC (10 and 20 mg kg^{-1}) treated mice before (-15 min) or after antagonist injection (time 45 and 90 min) in any of the experiments (data not shown).

Nociception (tail immersion test) and locomotor activity during withdrawal No significant differences were observed between controls and mice treated with THC (10 and 20 mg kg^{-1}) in nociception or locomotor activity values in any of the experiments (data not shown).

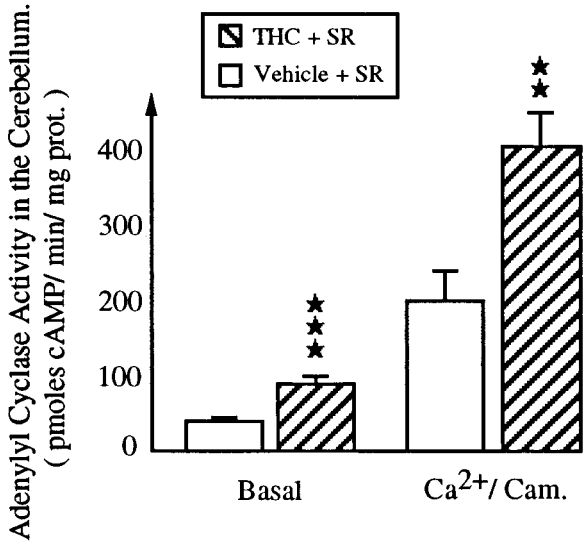

Figure 6 Effects of SR 141716A in control and THC dependent mice on basal and calcium-calmodulin stimulated AC activity in the cerebellum. Mice treated chronically with THC (20 mg kg^{-1}) twice daily) or vehicle were administered SR 141617A (10 mg kg^{-1}). AC activity was assayed in cerebellar membranes in the presence or absence of calcium-calmodulin (see Methods). Results are expressed in pmoles cyclic AMP formed/min mg^{-1} $protein^{-1}$ and represent means ± s.e.mean of at least ten individual experiments run in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle + SR 141716A under the same assay conditions.

Place conditioning

SR 141716A place conditioning in THC naïve mice (experiment 1) Mice showed no initial preferences for the two conditioning compartments indicating the unbiased nature of the protocol. No significant change in compartment occupancy time or number of visits to each compartment was observed after SR 141716A 0, 2.5, 5.0 or 10 mg kg^{-1} conditioning (repeated measures ANOVA). However, within group comparisons, of pre-conditioning and testing days showed a significant decrease in the time spent in the compartment associated with the dose of 2.5 mg kg^{-1} SR 141716A. In this group, the time spent in the drug paired compartment was 353.7 ± 34.2 during the pre-conditioning day and 239.7 ± 23.2 during the testing day ($t_{(1,8)} = 4.39$, $P < 0.01$), whereas in the vehicle paired side values during pre-conditioning and testing days were 339.0 ± 46.9 and 302.9 ± 31.1, respectively (data not shown).

SR141716A-induced withdrawal place conditioning in THC dependent mice (experiment 2) There was no initial preference in pre-conditioning for assigned SR 141716A-induced withdrawal and vehicle compartments. Analysis of data showed no significant effects for treatment, conditioning, occupancy time nor number of visits to either the drug or vehicle associated compartments (data not shown).

THC conditioning in THC-naïve mice (experiment 3) Before conditioning, mice showed no initial preference for the two conditioning environments indicating the unbiased nature of

Table 3 Effects induced by THC in the place conditioning paradigm in drug naïve mice

	n	*Pre-test compartment time*		*Test day compartment time*	
		Drug paired side	*Vehicle paired side*	*Drug paired side*	*Vehicle paired side*
Dose of THC					
0 mg kg^{-1}	10	352.3 ± 15.1	413.1 ± 32.7	436.2 ± 73.1	336.0 ± 28.5
0.5 mg kg^{-1}	10	369.0 ± 21.2	394.5 ± 30.5	351.5 ± 22.7	373.1 ± 18.7
1.0 mg kg^{-1}	10	370.0 ± 23.3	371.8 ± 21.2	363.0 ± 24.4	352.4 ± 25.5
2.5 mg kg^{-1}	10	399.3 ± 23.9	349.0 ± 24.4	352.8 ± 34.8	400.4 ± 39.1
5.0 mg kg^{-1}	10	373.3 ± 31.9	366.6 ± 15.8	418.2 ± 37.9	407.3 ± 48.5
10 mg kg^{-1}	10	394.0 ± 39.4	353.4 ± 32.2	340.3 ± 29.2	393.5 ± 29.5
20 mg kg^{-1}	10	324.6 ± 28.9	334.2 ± 24.1	239.3 ± 20.5*	430.4 ± 33.9
Dose of MP					
0 mg kg^{-1}	10	334.5 ± 26.8	317.8 ± 31.2	368.7 ± 24.9	311.5 ± 27.2
10 mg kg^{-1}	9	295.2 ± 27.9	390.8 ± 43.2	416.3 ± 12.2**	256.8 ± 23.3*

Values represent time in seconds spent in each compartment during the pre-test and test day measurements (means ± s.e.mean). Mice received THC or its vehicle immediately before place conditioning. $^{*}P < 0.05$, $^{**}P < 0.01$, in comparison with pre-test value in the same compartment (two-tailed Student's paired *t*-test).

the protocol. Repeated measures ANOVA of the time spent in the drug associated compartment showed a significant effect of treatment ($F_{(6,63)}=2.59$, $P<0.05$), but not of day, nor interaction between these factors. Similar analysis of the vehicle associated compartments did not show any significant effect of treatment, day, nor interaction between these two factors (Table 3). Analysis of the number of compartment visits into the drug and non-drug associated compartments did not show any significant effect (data not shown). One-way ANOVA for test day values showed a significant effect of treatment ($F_{(6,63)}=2.73$, $P<0.05$), which *post hoc* analysis showed to be significant only at the dose of 20 mg kg^{-1} of THC ($t_{(1,18)}=3.62$, $P<0.05$). No significant effect of treatment using the same analysis was seen with the test day values from the vehicle paired side. Within group analysis of the time spent in each compartment during pre-conditioning and test days showed a significant aversion for the THC 20 mg kg^{-1} associated compartment ($t_{(1,9)}=3.17$, $P<0.05$). Similar within group analysis for the vehicle compartment time at pre-conditioning and test days did not show any significant differences in any of the other groups (Table 3).

The same analysis was used for the morphine positive control. Briefly, a significant increase occurred for the time spent in the compartment paired with morphine ($t_{(1,8)}=3.70$, $P<0.01$), associated with a significant decrease in the time spent in the vehicle paired compartment ($t_{(1,8)}=2.99$, $P<0.05$).

Discussion

The present study shows that administration of SR 141716A in mice chronically treated with THC (20 mg kg^{-1} twice a day) precipitated several signs that could be interpreted as a withdrawal syndrome. These signs included wet dog shakes, frontpaw tremor, ataxia, hunched posture, tremor, ptosis, piloerection, decreased locomotor activity and mastication. In mice receiving the lower dose of THC dependence schedule (10 mg kg^{-1} twice a day), administration of SR 141716A resulted in significant increases in five of these nine withdrawal signs including wet dog shakes, frontpaw tremor, hunched posture, tremor, and mastication. Not one of these responses was significantly observed before antagonist administration. Some of these signs are in common with those previously reported during SR 141716A-induced THC withdrawal in rats, such as wet dog shakes, ptosis and hunched posture (described as arched back) showing the similarity of THC withdrawal across these species (Tsou *et al*., 1995; Aceto *et al*., 1996). In agreement with these previous studies, intense grooming was observed after antagonist administration in both chronic vehicle and THC groups. However, there were not significant differences in the intensity of grooming between the two groups (data not shown). Therefore, these results validate for the first time a behavioural model in mice of the physical withdrawal syndrome to THC.

SR 141716A-precipitated THC withdrawal was expressed, at the cellular level, by a pronounced increase of AC activity that was selectively observed in the cerebellum but not in other brain structures also containing CB-1 receptors. This upregulation of the AC activity is reported for the first time in the present study and could be due to a compensatory response to the persistent inhibition of the AC during the chronic THC treatment, which is abruptly unmasked by the antagonist. This 'rebound' phenomenon has been well documented for other agents that inhibit AC *via* Gi proteins such as α_2-adrenergic agonists and opioids (Sharma *et al*., 1975). In the case of opioid administration, the upregulation of the cyclic AMP pathway has been directly related to the behavioural expression of antagonist-precipitated morphine withdrawal (Rasmussen *et al*., 1990).

The administration of SR 141716A in control mice receiving vehicle chronically induced a slight presence of some somatic signs similar to those observed during THC withdrawal, suggesting an intrinsic activity of this compound as has been previously indicated (Compton *et al*., 1996; Mansbach *et al*., 1996; Sañudo-Peña *et al*., 1997; Rubino *et al*., 1998). A proposed explanation for the apparent pharmacological activity of SR 141716A has been through antagonism of an endogenous cannabinoid tone (Sañudo-Peña *et al*., 1997) which may be a possibility at high antagonist doses (Rinaldi-Carmona *et al*., 1994). This may also account for the small increase in forskolin stimulated AC activity in cerebellum observed in the present investigation after SR 141716A (see Table 3).

In agreement with previous studies, acute administration of THC (10 and 20 mg kg^{-1}) resulted in significant hypothermia, antinociception and decreases in body weight gain (Carlini *et al*., 1970; Pertwee *et al*., 1993; Aceto *et al*., 1996), as well as in a cataleptic-like state where mice still responded to external stimuli (Adams & Martin, 1996), and may relate to the initial decreases in activity disrupting normal feeding behaviour (Stark & Dews, 1980). Thus, tolerance to the cataleptic response to THC on subsequent administrations may explain the lack of effect of THC on body weight gain after the first day of its chronic administration. The rapid onset of tolerance to the hypothermic and antinociceptive effects of THC seen in this study has been documented before in mice (Anderson, 1975; Fan *et al*., 1996) and other species (Martin *et al*., 1976). Analysis of basal analgesia and body temperatures before each daily THC (or vehicle) injection ensured that there was no conditioned or behavioural component to the tolerance and that residual effects of the previous THC administration were not evident. Indeed, only a residual hypothermic effect of THC was observed before day 1 evening injections. Therefore, this chronic THC administration schedule induced a clear tolerance to the main pharmacological responses of THC in mice.

The abrupt precipitation of a THC withdrawal syndrome by use of an antagonist has been proposed as evidence for physical dependence to THC. Under spontaneous conditions, this is not clearly observed probably due to the extended activity profile of THC (Adams & Martin, 1996). The behavioural expression of THC-withdrawal syndrome was different from that seen with antagonist-induced withdrawal from opiates. Thus, opiate withdrawal is normally associated with motoric behaviours such as jumping and hyperactivity, and autonomic signs including lacrimation, rhinorrhoea, diarrhoea (contributing to body weight loss), hypothermia and hyperalgesia (Marshall & Weinstock, 1971). Motoric and autonomic signs of abstinence were not observed during THC withdrawal in this study. However, the expression during THC-withdrawal of behaviours such as wet dog shakes, hunched posture, ptosis, tremor, piloerection and mastication is in common with opiate withdrawal. Evidence indicates that the diversity of behavioural events during opiate withdrawal are mediated by several neural substrates (Maldonado *et al*., 1992; Pothos *et al*., 1991). It seems possible therefore that the large range of behaviours observed during antagonist-precipitated THC withdrawal is also mediated by several neural mechanisms, subsequent to cessation of cannabinoid receptor activation, a proposal which is reinforced by the changes in AC activity observed in the present study.

Numerous studies have previously demonstrated that the CB receptors are coupled to different effector systems, associated to Gi/Go proteins, which can operate in a different manner in distinct physiological systems (Childers *et al*., 1993). Among the second messenger systems coupled to CB receptors, the most widely studied is the cyclic AMP dependent pathway. Thus, the acute administration of cannabinoid agonists produces an inhibition of AC that has been well documented in various models, including cell culture systems (Howlett & Fleming, 1984) and brain preparations (Bidaut-Russel *et al*., 1990). Although cannabinoid-induced tolerance has been already described in cultured neuroblastoma cells (Dill & Howlett, 1987), the present study is to our knowledge, the first report of an upregulation of the cyclic AMP pathway following chronic THC treatment. Interestingly, this occurred mainly in the cerebellum, which is one of the richest regions in CB receptor density (Herkenham *et al*., 1990), contains the highest concentrations of cannabinoid-inhibited AC (Pacheco *et al*., 1992) and displays higher values for SR 141716A binding than any other brain structures (M. Rinaldi, personal communication).

Upregulation of the AC activity in cerebellum after SR 141716A-precipitated withdrawal in THC treated mice strongly resembles that observed after naloxone-precipitated opioid withdrawal in brain regions with high opioid receptor populations such as the locus coeruleus. In this region, increased levels of the calcium/calmodulin activated AC type VIII and AC type I, have been reported after chronic morphine treatment (Matsuoka *et al*., 1994; Lane-Ladd *et al*., 1997). Interestingly, the upregulated AC activity in the cerebellum of the THC abstinent mice, reported in the present study, is also stimulated by calcium/calmodulin. In this brain region the major AC isoform seems to be AC type I, although AC VIII and the calcium/calmodulin insensitive AC type II, have also been detected (Mons *et al*., 1993). Furthermore, AC type I is expressed only in granule cells, in the same cellular population as the CB1 receptor. Therefore, in analogy with morphine, a cyclic AMP dependent homeostatic cascade may be activated in the cerebellum of mice chronically treated with THC, resulting in an increase in AC activity probably as a consequence of long-term upregulation of AC types I and/or VIII. These adaptive changes could account for the manifestations of some of the reported somatic signs of THC withdrawal, such as ataxia. It is important to point out that although common cellular mechanisms may underlie the actions of opioids and THC, the effects produced *in vivo* by the chronic administration of these agents are completely different. Indeed, the AC upregulation after THC withdrawal concerns selectively the cerebellum whereas structures involved in morphine dependence, such as the striatum and the PAG are unaffected by chronic THC treatment (Matthes *et al*., 1996).

The absence of adaptive changes in the AC system in other brain structures, such as the mesolimbic system or the autonomic areas, correlates with the lack of vegetative signs and aversive/dysphoric properties of THC withdrawal which are observed in opiate abstinence. This is clearly demonstrated by the absence of negative effects of SR 141716A-induced THC withdrawal in the place conditioning paradigm. Using place conditioning protocols similar to this study (one pairing to the antagonist-induced withdrawal) naloxone-induced opiate withdrawal results in a clear aversion to its associated environment (Mucha *et al*., 1986; Hand *et al*., 1988; Valverde *et al*., 1996). Recently, place preferences for environments associated with SR 141716A (0.5 and 5 mg kg^{-1}) administration have been reported in rats after at least two drug paired conditioning periods (Sañudo-Peña *et al*., 1997). However, the possibility that the antagonist blocked any aversive consequences of withdrawal from chronic THC in the present study through an opposing rewarding action is unlikely since the dose of SR 141716A selected (10 mg kg^{-1}), has no motivational effects in this paradigm after one pairing. Furthermore, the lowest dose of SR 141716A used in the present study (2.5 mg kg^{-1}) was found to result in an aversion in THC naive mice, expressed as a decrease in time spent in the compartment associated with its administration. This finding is opposite to the positive reinforcing effects of SR 141716A found after several place conditioning pairings (Sañudo-Peña *et al*., 1997), but this probably relates to methodological and species differences.

A recent study has shown elevations in corticotrophin-releasing factor and cFos expression in the amygdala during THC withdrawal (Rodriguez de Fonseca *et al*., 1997) in common with withdrawal from other drugs of abuse such as alcohol (Merlo Pich *et al*., 1995) and opiates (Beckmann *et al*., 1995; Tanda *et al*., 1997). It has been suggested therefore, that the adaptive neurophysiological responses to THC treatment in this brain structure are therefore similar to those produced by drugs exerting a negative motivational drive during withdrawal, which could account for the continued drug intake as part of the cycle of drug dependence. The current study confirms the existence of a clear physical withdrawal syndrome to THC in mice, expressed by several behaviours and biochemical signs. However, motivational consequences of THC abstinence are lacking since no place aversion to environment paired with THC withdrawal was observed, as measured for the first time in the present study. The place conditioning paradigm utilized in this investigation is sensitive to the motivational stimuli since the aversive and rewarding effects of THC and morphine respectively, were demonstrated in drug naïve mice (Table 3). Furthermore, the possible positive motivational effects of THC in naïve mice were not seen in the present study in agreement with previous studies using rats that have also shown no effect and conditioned place aversions to THC (McGregor *et al*., 1996; Parker & Gillies, 1995; Sañudo-Peña *et al*., 1997). The lack of place preference to THC may relate to the long duration of its pharmacological response such that possible THC rewarding effects continue after conditioning pairings on return to the home cage thus reducing the salient association of drug and environment stimuli. In addition, THC self-administration in animals has not been demonstrated under normal conditions (Harris *et al*., 1974; Leite & Carlini, 1974; Carney *et al*., 1977; Mansbach *et al*., 1994), although a recent study reported self-administration of the cannabinoid receptor agonist WIN 55,212-2 in mice (Martellota *et al*., 1998). The possibility still remains however, that the misuse of cannabinoid drugs by humans are related to a rewarding action which could be difficult to reveal in the currently used animal models as a consequence of the long duration of cannabinoid action and their anxiogenic effects (Zuardi *et al*., 1982).

In conclusion, this study provides a clear behavioural and biochemical model of THC withdrawal in mice. Some of the behavioural signs of THC withdrawal are similar to those observed during opiate abstinence, but taken together, all these results indicate that most of the somatic, vegetative, motivational and biochemical consequences of abstinence from both drugs of abuse are different. In particular, we demonstrated that physical withdrawal from THC is less intense in comparison with that of opiates and lacks aversive/dysphoric properties. The present model permits an easy quantification of THC abstinence in mice and can be very useful to elucidate the neurobiochemical mechanisms involved in cannabinoid dependence.

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References

ACETO, M.D., SCATES, S.M., LOWE, J. & MARTIN, B.R. (1996). Dependence on Δ-9-tetrahydrocannabinol: studies on precipitated and abrupt withdrawal. *J. Pharmacol. Exp. Ther.,* **278,** 1290 – 1295.

ADAMS, I.B. & MARTIN, B.R. (1996). Cannabis: pharmacology and toxicology in animals and humans. *Addiction,* **91,** 1585 – 1614.

ANDERSON, P.F., JACKSON, D.M., CHESHER, G.B. & MALOR, R. (1975). Tolerance to the effects of delta-9-tetrahydrocannabinol in mice on intestinal motility, temperature and locomotor activity. *Psychopharmacologia,* **48,** 31 – 36.

BECKMANN, A.M., MATSUMOTO, I. & WILCE, P.A. (1995). Immediate early gene expression during morphine withdrawal. *Neuropharmacology,* **34,** 1183 – 1189.

BIDAUT-RUSSELL, M., DEVANE, W.A. & HOWLETT, A.C. (1990). Cannabinoid receptors and modulation of cAMP accumulation in the rat brain. *J. Neurochem.,* **55,** 21 – 26.

BOUABOULA, M., POINOT-CHAZEL, C., BOURRIE, B., CANAT, X., CALANDRA, B., RINALDI-CARMONA, M., LE FUR, G. & CASEL LAS, P. (1995). Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem. J.,* **312,** 637 – 641.

BURSTEIN, S., BUDROW, J., DEBATIS, M., HUNTER, S.A. & SUBRAMANIAN, A. (1994). Phospholipase participation in cannabinoid-induced release of free arachidonic acid. *Biochem. Pharmacol.,* **48,** 1253 – 1264.

CADAS, H., GAILLET, S., BELTRAMO, M., VENANCE, L. & PIOMELLI, D. (1996). Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J. Neurosci.,* **16,** 3934 – 3942.

CARLINI, E.A., SANTOS, M., CLAUSSEN, U., BIENIEK, D. & KORTE, F. (1970). Structure activity relationship of four tetrahydrocannabinols and the pharmacological activity of five semi-purified extracts. *Psychopharmacologia,* **18,** 82 – 93.

CARNEY, J.M., UWAYDAH, I.M. & BALSTER, R.L. (1977). Evaluation of a suspension system for intravenous self-administration studies of water-insoluble compounds in the rhesus monkey. *Pharmacol. Biochem. Behav.,* **7,** 357 – 364.

CARR, G.D., FIBIGER, H.C. & PHILIPS, A.G. (1989). Conditioned place preference as a measure of drug reward. In: *The Neuropharmacological Basis of Reward.* eds. Leibman, J.M. & Cooper, S.J. pp. 264 – 319. New-York: Clarendon Press.

CHEN, J., PAREDES, W., LI, J., SMITH, D., LOWINSON, J. & GARDNER, E.L. (1990). Δ-9-Tetrahydrocannabinol produces naloxone-blockable enhancement of presynaptic basal dopamine efflux in nucleus accumbens of conscious, freely-moving rats as measured by intracerebral microdialysis. *Psychopharmacology,* **102,** 156 – 162.

CHILDERS, S.R., PACHECO, M.A., BENNETT, M.A., EDWARDS, T.A., HAMPSON, R.E., MU, J. & DEADWYLER, S.A. (1993). Cannabinoid receptors: G-protein-mediated signal transduction mechanisms. *Biochem. Soc. Symp.,* **59,** 27 – 50.

COMPTON, D.R., ACETO, M.D., LOWE, J. & MARTIN, B.R. (1996). In vivo characterisation of a specific cannabinoid receptor antagonist (SR141716A): Inhibition of Δ-9-tetrahydrocannabinol-induced responses and apparent agonist activity. *J. Pharmacol. Exp. Ther.,* **277,** 586 – 594.

DAVIS, W.M., MORETON, J.E., KING, W.T. & PACE, H.B. (1972). Marihuana on locomotor activity: biphasic effect and tolerance development. *Res. Commun. Chem. Pathol. Pharmacol.,* **3**(1), 29 – 35.

DEVANE, W.A., DYSARZ III, F.A., JOHNSON, M.R., MELVIN, L.S. & HOWLETT, A.C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.,* **34,** 605 – 613.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* **258,** 1946 – 1949.

DEWEY, W.L., HARRIS, L.S., HOWES, J.F., KENNEDY, J.S., GRANCHELLI, F.E., PARS, H.G. & RAZDAN, R.K. (1970). Pharmacology of some marijuana constituents and two heterocyclic analogues. *Nature,* **226,** 1265 – 1267.

DI CHIARA, G., ACQUAS, E. & CARBONI, E. (1991). Role of mesolimbic dopamine in the motivational effects of drugs: brain dialysis and place preference studies. In *The Mesolimbic Dopamine System: From Motivation to Action.* eds. Willner, P. & Scheel-Kruger J. pp. 331 – 366. London: John Wiley & Sons Ltd.

DILL, G.A. & HOWLETT, A.C. (1987). Regulation of adenylate cyclase by chronic exposure to cannabimimetic drugs. *J. Pharmacol. Exp. Ther.,* **244,** 1157 – 1163.

DUMAN, R.S., TALLMAN, J.F. & NESTLER, E.J. (1988). Acute and chronic opiate regulation of adenylyl cyclase in brain: specific and acute effects in locus coeruleus. *J. Pharmacol. Exp. Ther.,* **246,** 1033 – 1039.

FAN, F., TAO, Q., ABOOD, M. & MARTIN, B.R. (1996). Cannabinoid receptor down-regulation without alteration of the inhibitory effect of CP 55,940 on adenylyl cyclase in the cerebellum of CP 55,940-tolerant mice. *Brain Res.,* **706,** 13 – 20.

GARDNER, E.L., PAREDES, W., SMITH, D., DONNER, A., MILLING, C., COHEN, D. & MORRISON, D. (1988). Facilitation of brain stimulation reward by Δ-9-tetrahydrocannabinol. *Psychopharmacology,* **96,** 142 – 144.

HAND, T.H., KOOB, G.F., STINUS, L. & LE MOAL, M. (1988). Aversive properties of opiate receptor blockade: evidence for exclusively central mediation in naive and morphine-dependent rats. *Brain Res.,* **474,** 364 – 368.

HARRIS, R.T., WATERS, W. & McLENDON, D. (1974). Evaluation of reinforcing capability of delta-9-tetrahydrocannabinol in rhesus monkeys. *Psychopharmacologia,* **37,** 23 – 29.

HERKENHAM, M., LYNNE, A.B., LITTLE, M.D., JOHNSON, M.R., MELVIN, L.S., DE COSTA, B.R. & RICE, K.C. (1990). Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 1932 – 1936.

HOWLETT, A.C. & FLEMING, R.M. (1984). Cannabinoid inhibition of adenylyl cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol. Pharmacol.,* **27,** 429 – 439.

JANSSEN, P., NEIMEGEERS, C. & DONY, J. (1963). The inhibitory effects of fentanyl (R4263) and other morphine like analgesics, on the warm water induced tail reflex in rats. *Arzneimittel-Forsh.,* **13,** 502 – 507.

KOOB, G.F. & LE MOAL, M. (1997). Drug abuse: hedonic homeostatic dysregulation. *Science,* **278,** 52 – 58.

KOZEL, N.J. & ADAMS, E.H. (1986). Epidemiology of drug abuse: an overview. *Science,* **234,** 970 – 974.

LANE-LADD, S.B., PINEDA, J., BOUNDY, V.A., PFEUFFER, T., KRUPINSKI, J., AGHAJANIAN, G.K. & NESTLER, E.J. (1997). CREB (cAMP responsive element binding protein) in the locus coeruleus: biochemical, physiological and behavioral evidence for a role in opiate dependence. *J. Neurosci.,* **17,** 7890 – 7901.

LEITE, J.R. & CARLINI, E.A. (1974). Failure to obtain 'cannabis-directed behavior' and abstinence syndrome in rats chronically treated with cannabis sativa extracts. *Psychopharmacologia,* **36,** 133 – 145.

LEPORE, M., VOREL, S.R., LOWINSON, J. & GARDNER, E.L. (1995). Conditioned place preference induced by Δ-9-tetrahydrocannabinol comparison with cocaine, morphine, and food reward. *Life Sci.,* **56,** 2073 – 2080.

LICHTMAN, A.H. & MARTIN, B.R. (1991). Spinal and supraspinal components of cannabinoid-induced antinociception. *J. Pharmacol. Exp. Ther.,* **258,** 517 – 523.

MACKIE, K. & HILLE, B. (1992). Cannabinoids inhibit N-type calcium channels in neuroblastoma x glioma cells. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 3825 – 3829.

MALDONADO, R., BLENDY, J.A., TZAVARA, E., GASS, P., ROQUES, B.P., HANOUNE, J. & SCHUTZ, G. (1996). Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science,* **273,** 657 – 659.

MALDONADO, R., SAIARDI, A., VALVERDE, O., SAMAD, T.A., ROQUES, B.P. & BORRELLI, E. (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature,* **388,** 586 – 589.

MALDONADO, R., STINUS, L., GOLD, L.H. & KOOB, G.F. (1992). Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *J. Pharmacol. Exp. Ther.,* **261,** 669 – 677.

MANSBACH, R.S., NICHOLSON, K.L., MARTIN, B.R. & BALSTER, R.L. (1994). Failure of Δ-9-tetrahydrocannabinol and CPP 55,940 to maintain intravenous self-administration under a fixed-interval schedule in rhesus monkeys. *Behav. Pharmacol.,* **5,** 219 – 225.

MANSBACH, R.S., ROVETTI, C.C., WINSTON, E.N. & LOWE III, J.A. (1996). Effects of the cannabinoid CB1 receptor antagonist SR141716A on the behavior of pigeons and rats. *Psychopharmacology,* **124,** 315 – 322.

MARSHALL, I. & WEINSTOCK, M. (1971). Quantitative method for assessing one symptom of the withdrawal syndrome in mice after chronic morphine administration. *Nature,* **234,** 223 – 224.

MARTELLOTA, M.C., COSSU, G., GESSA, G.L. & FRATTA, W. (1998). Self-administration of the cannabinoid receptor agonist WIN 55,212-2 in drug-naive mice. *Neuroscience,* **85,** 327 – 330.

MARTIN, B.R., DEWEY, W.L., HARRIS, L.S. & BECKNER, J.S. (1976). ^{3}H-Δ-9-tetrahydrocannabinol tissue and subcellular distribution in the central nervous system and tissue distribution in peripheral organs of tolerant and nontolerant dogs. *J. Pharmacol. Exp. Ther.,* **196,** 128 – 144.

MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, M.J., YOUNG, A.C. & BONNER, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature,* **346,** 561 – 564.

MATSUOKA, I., MALDONADO, R., DEFER, N., NOEL, F., HANOUNE, J. & ROQUES, B.P. (1994). Chronic morphine administration causes region-specific increase of brain type VIII adenylyl cyclase mRNA. *Eur. J. Pharmacol.,* **268,** 215 – 221.

MATTHES, H.W.D., MALDONADO, R., SIMONININ, F., VALVERDE, O., SLOWE, S., KITCHEN, I., BEFORT, K., DIERICH, A., LE MEUR, M., DOLLE, P., TZAVARA, E., HANOUNE, J., ROQUES, B.P. & KEIFFER, B.L. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature,* **383,** 819 – 823.

McGREGOR, I.S., ISSAKIDIS, C.N. & PRIOR, G. (1996). Aversive effects of the synthetic cannabinoid CP 55,940 in rats. *Pharmacol. Biochem. Behav.,* **53,** 657 – 664.

MERLO PICH, E.M., LORANG, M., YEGANEH, M., RODRIGUEZ DE FONSECA, F., RABER, J., KOOB, G.F. & WEISS, F. (1995). Increase in extracellular corticotrophin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. *J. Neurosci.,* **15**(8), 5439 – 5447.

MONS, N., YOSHIMURA, M. & COOPER, D.M.F. (1993). Discrete expression of Ca^{2+}/calmodulin-sensitive and Ca^{2+} insensitive adenylyl cyclases in the rat brain. *Synapse,* **14,** 51 – 59.

MUCHA, R.F., GRITTI, M.D. & KIM, C. (1986). Aversive properties of opiate withdrawal studied in rats. *NIDA Res. Monog.,* **75,** 567 – 570.

MUNRO, S., THOMAS, K.L. & ABU-SHAAR, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* **365,** 61 – 65.

N.I.H. (1995). Guide for the care and use of laboratory animals. *N.I.H. Publication,* 85/23.

PACHECO, M., CHILDERS, S.R., ARNOLD, R., CASIANO, F. & WARD, S.J. (1992). Aminoalkylindoles: actions of specific G-protein-linked receptors. *J. Pharmacol. Exp. Ther.,* **257,** 170 – 183.

PARKER, L.A. & GILLIES, T. (1995). THC-induced place and taste aversions in Lewis and Sprague-Dawley rats. *Behav. Neurosci.,* **109,** 71 – 78.

PERTWEE, R.G., STEVENSON, L.A. & GRIFFIN, G. (1993). Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide. *Br. J. Pharmacol.,* **110,** 1483 – 1490.

POTHOS, E., RADA, P., MARK, G.P. & HOEBEL, B.G. (1991). Dopamine microdialysis in the nucleus accumbens during acute and chronic morphine, naloxone-precipitated withdrawal and clonidine treatment. *Brain Res.,* **566,** 348 – 350.

RAFT, D., GREGG, J., GHIA, J. & HARRIS, L. (1976). Effects of intravenous tetrahydrocannabinol on experimental and surgical pain. *Clin. Pharmacol. Ther.,* **21**(1), 26 – 33.

RASMUSSEN, K., BEITNER-JOHNSON, D., KRYSTAL, J.H., AGHAJANIAN, G.K. & NESTLER, E.J. (1990). Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates. *J. Neurosci.,* **10**(7), 2308 – 2317.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.,* **350,** 240 – 244.

RODRIGUEZ DE FONSECA, F., CARRERA, M.R.A., NAVARRO, M., KOOB, G.F. & WEISS, F. (1997). Activation of corticotropin-releasing factor in the limbic system during cannabinoid withdrawal. *Science,* **276,** 2050 – 2054.

RUBINO, T., PATRINI, G., MASSI, P., FUZIO, D., VIGANO, D., GIAGNONI, G. & PAROLARO, D. (1998). Cannabinoid-precipitated withdrawal: a time-course study of the behavioral aspect and its correlation with cannabinoid receptors and G-protein expression. *J. Pharmacol. Exp. Ther.,* **285,** 813 – 819.

SANUDO-PENA, M.C., TSOU, K., DELAY, E.R., HOHMAN, A.G., FORCE, M. & WALKER, J.M. (1997). Endogenous canabinnoids as an aversive or counter-rewarding system in the rat. *Neuroscience Lett.,* **223,** 125 – 128.

SHARMA, S.K., KLEE, W.A. & NIRENBERG, M. (1975). Dual regulation of adenylyl cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. U.S.A.,* **72,** 3092 – 3096.

SHIVACHAR, A.C., MARTIN, B.R. & ELLIS, E.F. (1996). Anandamide and Δ-9-tetrahydrocannabinol-evoked arachidonic acid mobilisation and blockade by SR141716A. *Biochem. Pharmacol.,* **51,** 669 – 676.

STARK, P. & DEWS, P.B. (1980). Cannabinoids. I. Behavioral effects. *J. Pharmacol. Exp. Ther.,* **214,** 124 – 130.

STELLA, N., SCHWEITZER, P. & PIOMELLI, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature,* **388,** 733 – 778.

TANDA, G., PONTIERI, F.E. & DI CHIARA, G. (1997). Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common μ_1-opioid receptor mechanism. *Science,* **276,** 2048 – 2050.

TSOU, K., PATRICK, S.L. & WALKER, J.M. (1995). Physical withdrawal in rats tolerant to Δ-9-tetrahydrocannabinol precipitated by a cannabinoid receptor antagonist. *Eur. J. Pharmacol.,* **280,** R13 – R15.

TZAVARA, E., POUILLE, Y., DEFER, N. & HANOUNE, J. (1996). Diurnal variations of adenylyl cyclase type 1 in the rat pineal gland. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 11208 – 11212.

VALVERDE, O., TZAVARA, E., HANOUNE, J., ROQUES, B.P. & MALDONADO, R. (1996). Protein kinases in the rat nucleus accumbens are involved in the aversive component of opiate withdrawal. *Eur. J. Neurosci.,* **8,** 2671 – 2678.

WISE, R.A. (1996). Addictive drugs and brain stimulation reward. *Ann. Rev. Neurosci.,* **19,** 319 – 340.

ZUARDI, A.W., SHIRAKAWA, I., FINKELFARB, E. & KARNIOL, I.G. (1982). Action of cannabidiol on the anxiety and other effects produced by Δ-9-tetrahydrocannabinol in normal subjects. *Psychopharmacology,* **76,** 245 – 250.

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