Psychopharm

Journal of Psychopharmacology
18(1) (2004) 59–65

ISSN 0269-8811
SAGE Publications Ltd, London, Thousand Oaks, CA and New Delhi
10.1177/0269881104040238

Spontaneous cannabinoid withdrawal produces a differential time-related responsiveness in cannabinoid CB_1 receptor gene expression in the mouse brain

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Abstract

This study aimed to examine the behavioural and neurochemical (cannabinoid CB_1 receptor gene expression) changes induced by spontaneous cannabinoid withdrawal in mice. Tolerance was assessed by measuring rectal temperature and motor activity in the open-field test after CP-55, 940 administration. Cannabinoid withdrawal symptoms were determined by measuring motor activity and behavioural signs of abstinence. Cessation of CP-55, 940 treatment in tolerant mice induced a spontaneous time-dependent behavioural withdrawal syndrome consisting of marked increases (140%) in motor activity, number of rearings (170%), decreases in grooming (57%), wet dog shakes (73%) and rubbing behaviours (74%) on day 1, progressively reaching values similar to vehicle-treated mice on day 3. Interestingly, this spontaneous cannabinoid withdrawal resulted in CB_1 gene expression upregulation (20–30%) in caudate-putamen, ventromedial hypothalamic nucleus, central amygdaloid nucleus and CA1, whereas in the CA3 field of hippocampus, a significant decrease (15–20%) was detected. Taken together, the results of this study suggest that cessation of CP-55, 940 administration in tolerant mice produces a behavioural cannabinoid withdrawal syndrome and a selective and differential responsiveness in CB_1 receptor gene expression in several brain regions of the mice. These findings further suggest a time and regional differential role for cannabinoid receptors in short- and long-term neuroadaptations that occur after exposure to cannabis derivatives.

Keywords
cannabinoids, gene expression, mRNA, withdrawal

Introduction

Cannabis sativa plant preparations, such as hashish or marijuana, are the most abused substances for recreational use. In recent years, the promotion of pharmaceutical preparations of cannabis for therapeutical purposes has stimulated further research of behavioural and neurochemical alterations associated with a potential spontaneous cannabinoid withdrawal syndrome. A number of reports have described that in humans abstinence from continuous marijuana use produces spontaneous withdrawal signs, including hyperirritability, tremor, dysphoria and sleep disturbances (Jones *et al.*, 1976, 1981; Wiesbeck *et al.*, 1996). However, in rodents, interruption of cannabinoid administration has produced contradictory withdrawal behavioural effects, depending upon the regimen of cannabinoid drug administration (dose, duration of treatment, etc.). Administration of the cannabinoid receptor antagonist SR-141 716A to animals treated with cannabinoid receptor agonists precipitated a withdrawal syndrome that includes alterations in motor activity, head shakes and tremor (Tsou *et al.*, 1995; Aceto *et al.*, 1996; Cook *et al.*, 1998; Diana *et al.*, 1998; Hutcheson *et al.*, 1998; Rubino *et al.*, 1998; Beardsley and Martin, 2000; Costa *et al.*, 2000; Rubino *et al.*, 2000; Aceto *et al.*, 2001; Lichtman *et al.*, 2001). While most of these reports described behavioural alterations produced after spontaneous- or SR-141, 716A-precipitated

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withdrawal from cannabinoids, the neurochemical alterations in functional activity of the cannabinoid CB_1 receptor associated with these behavioural signs remain to be determined. A few reports indicate that chronic administration of tetrahydrocannabinol or cannabinoid receptor agonists significantly reduces agonist-stimulated [^{35}S]GTPγS-binding in brain regions, as revealed by autoradiography, including caudate-putamen, cortex, hippocampus, substantia nigra and cerebellum (Sim *et al.*, 1996; Rubino *et al.*, 2000), suggesting tolerance induced down-regulation of cannabinoid receptor activity. However, little is known about the cannabinoid receptor alterations and the key brain regions underlying the molecular mechanisms involved in spontaneous cannabinoid withdrawal. This study aimed to evaluate behavioural (motor activity and somatic signs) and neurochemical (cannabinoid CB_1 receptor gene expression) changes induced by spontaneous cannabinoid withdrawal in selected brain regions of mice.

Materials and methods

Animals

Male Swiss albino mice, weighing 20–25 g, were obtained from Harlan (Barcelona, Spain) and were maintained in a temperature and light (23 ± 1 °C, light on between 08.00 h and 20.00 h) controlled environment. Food and tap water were provided *ad libitum*. All experiments included in this study were performed following the highest standards of human animal care, monitoring health care and minimizing pain and suffering, in accordance to National and International Laws for the Care and Use of Laboratory Animals.

Drug and treatments

Cannabinoid CB_1 receptor agonist CP-55, 940 [(–)-*cis*-3-[2-hydroxy-4-(1,1,-dimethylheptyl)-phenyl]-*trans*-4(-3-hydroxypropyl)cyclohexanol] was dissolved in ethanol:cremophor: saline (1:1:18) and administered (0.5 mg/kg/12 h; i.p.) for 6 days. On days 1, 3, 7 and 14 after the final injection, mice were killed by decapitation, and their brains quickly removed, frozen over dry ice and stored at –80 °C.

Assessment of tolerance to cannabinoid administration

Rectal temperature Rectal temperature was measured in each mouse using a lubricated digital thermistoprobe which was inserted into the rectum 2.5 cm for 30 s. Rectal temperature was determined 30 min before and after each morning injection on days 0, 1, 3, and 6.

Body weight measurement Body weight was measured in each mouse using an animal balance (Pacisa, Madrid, Spain) (precision ± 0.1 g). Body weight was determined 30 min after each morning injection on days 0, 1, 3, and 6.

Motor behaviour One hour after the administration of CP-55, 940 on days 0, 1, 3 and 6, mice were placed into individual motor activity chambers (20 × 20 × 25 cm) for 5 min of habituation. Immediately after habituation, behaviour was recorded on a videotape for an additional 30 min and motor behaviour analysis was subsequently performed from the recording. The time of motor activity (when a mouse is moving three or more paws horizontally) was counted.

Behavioural assessment after cannabinoid withdrawal

Motor behaviour and somatic expression On days 1 and 3 after the last administration, mice were placed in a square clear plastic observation area (20 × 20 × 25 cm) for 5 min of habituation. Immediately after habituation, animals were observed for a further 15 min. At the end of this observation period, each mouse was placed into an individual motor activity chamber (25 × 25 × 25 cm) for 15 min. The time of motor activity, number of groomings (measured as number of lickings of body and forepaws), rubbings (measured as forepaws movements over ears and nose), wet dog shakes, scratchings, diggings (action of scratching the surface of the cage floor with two or more paws) and penile licking/erection were scored by counting the number of events during the period of behavioural evaluation.

In situ hybridization histochemistry (ISHH)

Brain sections (six slices/level) were cut at 12 μm at two different levels containing the regions of interest. The first level includes the caudate-putamen (CPu) and the second level contains the ventromedial hypothalamic nucleus (VMN), central amygdaloid nucleus (Ce) and CA1, CA2, CA3 fields of hippocampus. All these sections were obtained according to Paxinos and Franklin (2001), mounted onto gelatin-coated slides and stored at –80 °C until the day of the assay.

ISHH was performed as described previously (Young *et al.*, 1986) using three synthetic oligonucleotide probes complementary to cannabinoid CB_1 receptor mRNA (bases 4–51, 349–396 and 952–999). Oligonucleotide probes were labelled using terminal deoxytransferase (Boehringer, Madrid, Spain) to add a ^{35}S-labelled deoxyATP (1000 Ci/mmol; Amersham, Madrid, Spain) tail to the 3′ end of the probes. The probe (in 50 μl of hybridization buffer) was applied to each section and left overnight at 37 °C for hybridization. Following hybridization, sections were washed four times for 15 min each in 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2 (1 × saline sodium citrate, SSC) at 55 °C, followed by two 30-min washes in 1 × SSC at room temperature and one brief water dip, and were then air-dried. To control for imaging enhancement variables, each set of slides was apposed to the same film (Kodak BioMax MR-1, Amersham, Madrid, Spain) in individualized cassettes at 10 days for CPu and 15 days for VMN, Ce, CA1, CA2 and CA3.

Image analyses quantification

Autoradiograms from ISHH studies (two slices per level in each animal) were analysed on a personal computer using the public domain NIH Image program (developed at the US National

Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Previous experiments in our laboratory have found that the selected times of exposure to film in these brains regions, and under our experimental conditions (oligonucleotide probe, radioactivity added to each slide, incubation conditions, type of film selected), renders a hybridization signal whose grey levels are linear with the optical density, according to the NIH Image Program. Therefore, optical densities were calculated from the uncalibrated mode of the NIH Image Program by substracting its corresponding background from each measurement and expressing in grey-scale values. The background measurement was taken from an area of the slice with the lowest non-specific hybridization signal and substracted from the hybridization signal measurement in the same slice. Measurements were pooled from brain sections and the values were averaged. The results are presented considering mean control values as 100%. Additional brain sections were cohybridized with a 100-fold excess of cold probes or with RNAse to establish the specificity of the signal. As expected, no hybridization signal was detected in these sections (data not shown).

Statistical analysis

The analysis of motor activity in the tolerance stage was performed using a two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were treatment and days of treatment as independent variables. One-way ANOVA followed by Student–Newman–Keul's test was performed for each individual group comparison. Data of rectal temperature and body weight and those obtained during withdrawal stage, including somatic signs and CB_1 gene expression, were evaluated using one-way ANOVA followed by Student–Newman–Keul's test. $p < 0.05$ was considered statistically significant.

Results

Assessment of tolerance to cannabinoid administration

Tolerance to cannabinoid administration was evaluated by measuring motor behaviour, rectal temperature and body weight.

A two-way ANOVA was performed to evaluate differences in motor activity during the administration of 0.5 mg/kg/12 h of CP-55, 940. The results showed that cannabinoid treatment produced a significant decrease in motor activity on day 1 (62%) and 3 (28%) but was without effect on day 7. These results revealed tolerance produced by CP-55, 940 administration after 3 days of treatment (Fig. 1) [treatment: $F(1,68) = 78.851$, $p < 0.001$; time: $F(2,68) = 11.791$, $p < 0.001$]; and interactions between treatment and time [$F(2,68) = 20.399$, $p < 0.001$].

One-way ANOVA of the results revealed that CP-55, 940 administration produced a marked decreased in rectal temperature on days 0, 1 and 3, reaching values similar to vehicle-treated mice by day 6 (Fig. 2) [$F(7,144) = 17.991$, $p < 0.001$].

One-way ANOVA was performed to evaluate differences in body weight during the administration of 0.5 mg/kg/12 h of CP-55, 940. The results showed that cannabinoid treatment produced a significant decreased in body weight on days 1 and 3 reaching values similar to vehicle-treated mice by day 6 (Fig. 2) [$F(7,162) = 4.945$ $p < 0.001$].

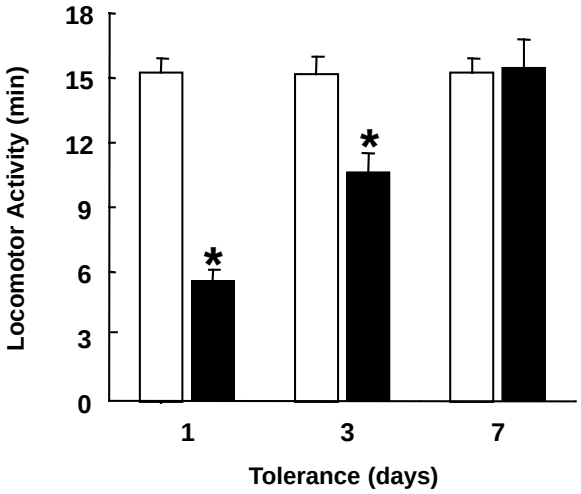

Figure 1 Effects of CP-55, 940 administration on motor behaviour. Mice were injected with CP-55, 940 (0.5 mg/kg/12 h; 7 days; i. p.) or with its vehicle (saline : ethanol : cremophor; 18 : 1 : 1, 1 ml/kg). On days 0, 1, 3 and 6, motor behaviour was measured (1 h after the morning CP-55, 940 administration) in a 30-min session for evaluation of tolerance due to cannabinoid treatment. Columns and vertical lines represent the mean ± SEM of time of activity in eight mice. *Values from CP-55, 940-treated animals (black columns) that are significantly different ($p < 0.05$) from vehicle-treated mice (white columns)

Behavioural assessment of spontaneous cannabinoid withdrawal

Behavioural assessment of spontaneous cannabinoid withdrawal was carried out by measuring motor behaviour, exploratory behaviour (number of rearings) and a number of somatic signs including groomings, rubbings, wet dog shakes, scratchings, penile erections or lickings and diggings, on days 1 and 3 after cessation of treatment with CP-55, 940 (Fig. 3).

The one-way ANOVA revealed that cessation of cannabinoid treatment produced a significant increase in motor activity [$F(2,24) = 17.95$, $p < 0.001$] on day 1 (140%) and 3 (75%) compared to vehicle. The number of rearings markedly increased [$F(2,19) = 54.72$, $p < 0.001$] on day 1 (170%) and day 3 (95%) after cessation of cannabinoid treatment compared to vehicle-treated mice. One-way ANOVA showed significant alterations in most of the somatic signs examined: number of groomings [$F(2,21) = 38.32$, $p < 0.001$], rubbings [$F(2,24) = 79.92$, $p < 0.001$], wet dog shakes [$F(2,27) = 6.68$, $p < 0.001$] and diggings [$F(2,21) = 6.184$, $p < 0.001$]. No significant differences were found in scratching or penile erections or lickings during cannabinoid withdrawal.

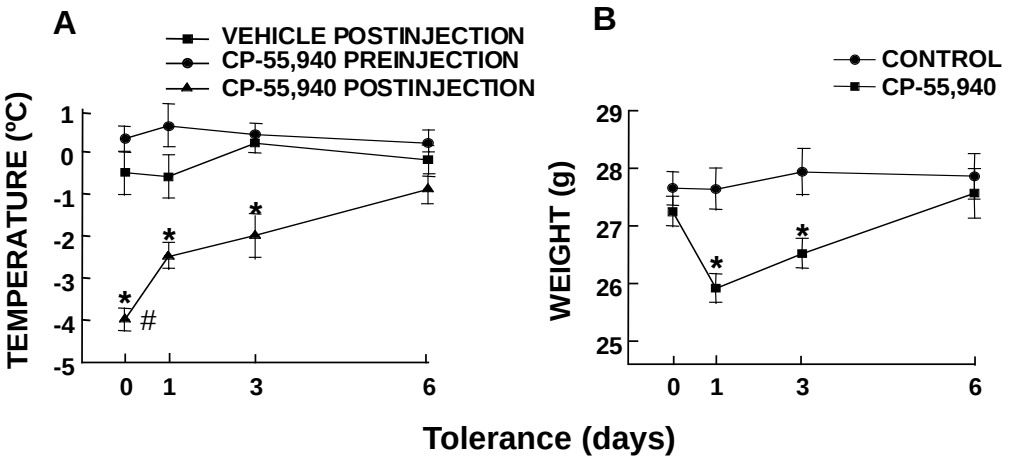

Figure 2 Effects of CP-55, 940 treatment on rectal temperature (A) and weight (g) (B). Mice were injected with CP-55, 940 (0.5 mg/kg/12 h; 7 days; i. p.) or with its vehicle (saline : ethanol : cremophor; 18 : 1 : 1, 1 ml/kg). On days 0, 1, 3 and 6, rectal temperature was measured 30 min before and after the morning CP-55, 940 administration. Weight was measured 30 min after CP-55, 940 administration. Rectal temperature and weight were evaluated for tolerance due to cannabinoid treatment. Symbols and vertical lines represent the means ± SEM of differences in rectal temperature in 6–8 mice. *Values from CP-55, 940-treated animals that are significantly different ($p < 0.05$) from vehicle-treated mice; #Values from CP-55, 940-treated animals on day 0 that are significantly different ($p < 0.05$) from mice treated on days 1, 3 and 6

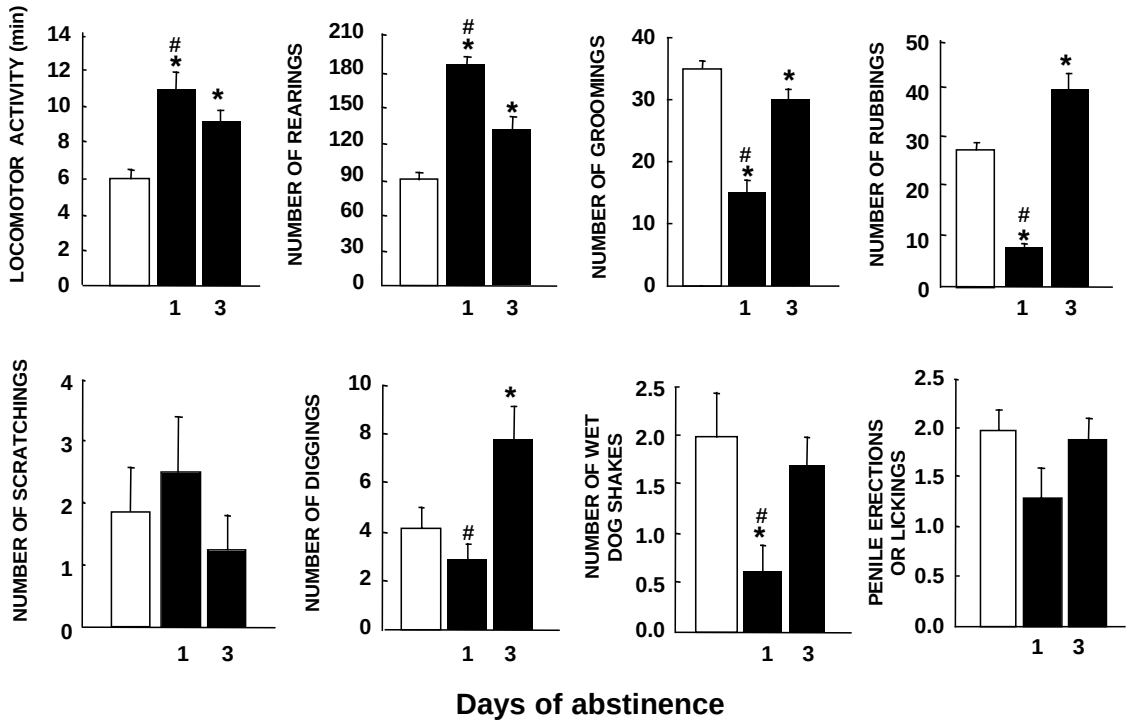

Figure 3 Time course effects of spontaneous cannabinoid withdrawal on motor activity and behavioural signs of abstinence (number of rearings, groomings, rubbings, scratchings, wet dog shakes, penile erections or lickings and diggings). Mice were injected with CP-55, 940 (0.5 mg/kg/12 h; 7 days; i. p.) or with its vehicle (saline : ethanol : cremophor; 18 : 1 : 1, 1 ml/kg) and motor activity and behavioural signs were evaluated on days 1 and 3 after the last administration. Motor activity and behavioural signs analyses were performed in a 15-min session. Columns and vertical lines represent the mean ± SEM of motor activity, number of rearings, groomings, rubbings, scratchings, wet dog shakes, diggings and penile erections or lickings. *Values from CP-55, 940-treated animals (black columns) that are significantly different ($p < 0.05$) from vehicle-treated mice (white columns). #Values from CP-55, 940-treated animals that are significantly different ($p < 0,05$) from mice treated on day 3

Cannabinoid CB_1 receptor gene expression

Spontaneous cannabinoid withdrawal produced a differential time related response in CB_1 mRNA levels in the CPu, VMN, Ce, and in the CA1 and CA3 fields of hippocampus (Figs 4 and 5). The one way ANOVA indicated that cessation of CP-55, 940 treatment increases CB_1 gene expression in most of the studied regions. In CPu [$F(4,33) = 11.806$, $p < 0.001$] and Ce [$F(4,31) = 4.95$, $p = 0.004$], the increase in CB_1 gene expression began on day 1 and remained elevated up to day 14 whereas in VMN [$F(4,33) = 3.93$, $p < 0.011$] and CA1 [$F(4,32) = 3{,}59$, $p = 0.011$], the increased of CB_1 gene expression occurred only on day 1, returning to

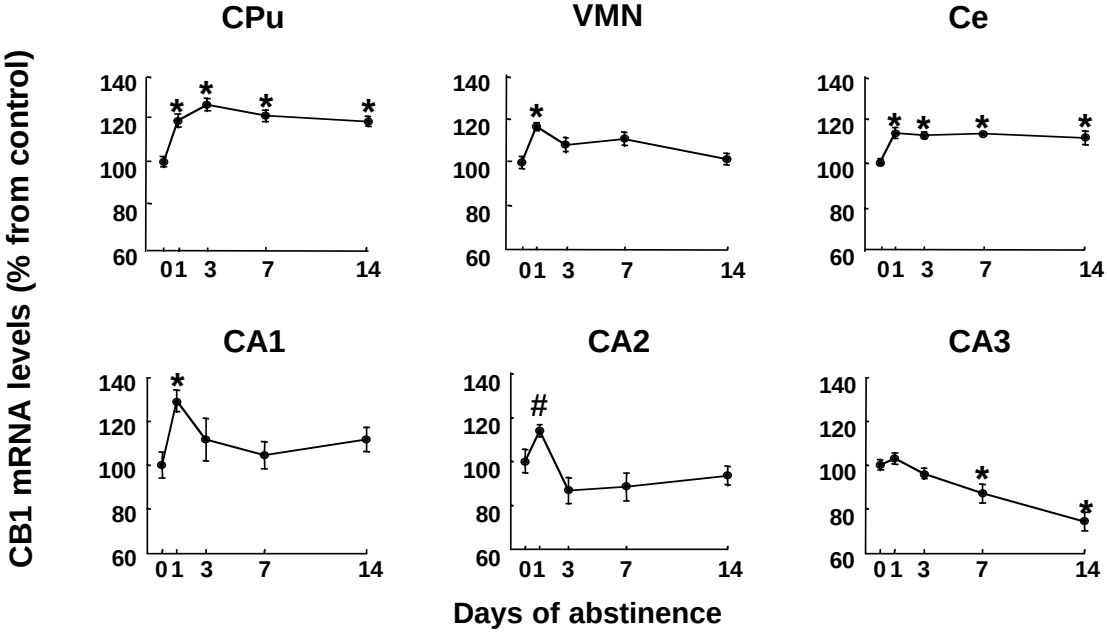

Figure 4 Time course of spontaneous cannabinoid withdrawal of CB_1 gene expression in forebrain regions. Mice injected with CP-55, 940 (0.5 mg/kg/12 h; 7 days; i. p.) or with its vehicle (saline : ethanol : cremophor; 18 : 1 : 1, 1 ml/kg) were killed by decapitation 1, 3, 7 and 14 days after the last administration. Symbols and vertical lines represent the mean ± SEM of CB_1 mRNA levels in eight mice. *Values from CP-55, 940-treated animals that are significantly different ($p < 0.05$) from vehicle-treated mice. #Values from CP-55, 940-treated animals that are significantly different ($p < 0.05$) from animals treated on days 3, 7 and 14

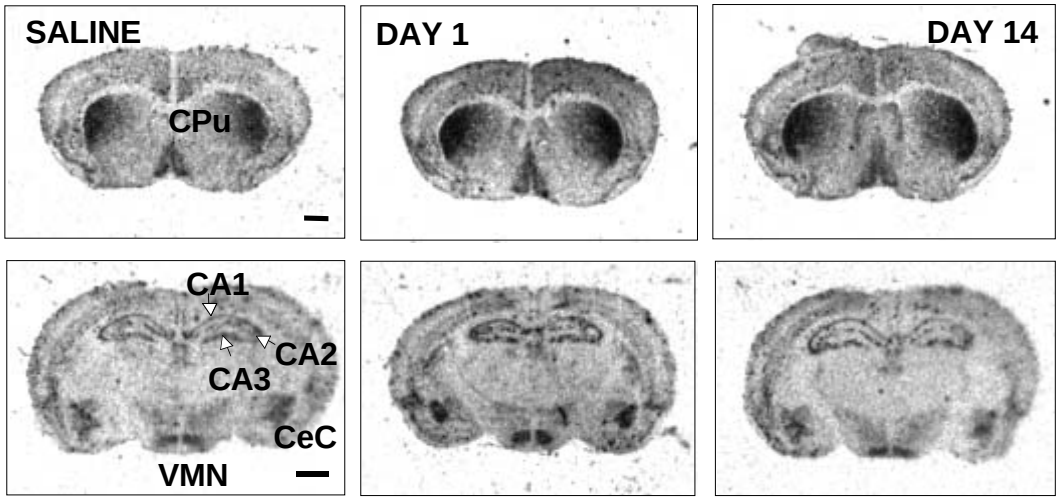

Figure 5 Representative autoradiograms of coronal brain sections at the level of caudate-putamen and ventromedial nucleus. Mice injected with CP-55, 940 (0.5 mg/kg/12 h; 7 days; i. p.) or with its vehicle (saline : ethanol : cremophor; 18 : 1 : 1, 1 ml/kg) were killed by decapitation 1, 3, 7 and 14 days after the last administration. Cpu, caudate-putamen; VMN, ventromedial hypothalamic nucleus; Ce, central amygdaloid nucleus; CA1–3, fields CA1, CA2 and CA3 of hippocampus. Sale bar = 1 mm

vehicle-treated values by days 3, 7 and 14. By contrast, in the CA3 field of hippocampus [$F(4,29) = 9.45$, $p < 0.001$], CB_1 receptor gene expression decreased on days 7 and 14. In the CA2 field of hippocampus, no differences were found at any time compared to day 0. However, an increase was found on day 1 compared to days 3, 7 and 14 [$F(4,31) = 4.33$, $p < 0.008$].

Discussion

Cessation of CP-55, 940 treatment in mice that are made tolerant to this cannabinoid agonist results in a spontaneous withdrawal syndrome consisting of increased motor and exploratory behaviour accompanied by long-lasting alterations in cannabinoid CB_1 receptor gene expression in several brain regions.

In agreement with previous reports (Abood and Martin, 1992; Aceto *et al.*, 2001; Hutcheson *et al.*, 1998; Aceto *et al.*, 2001) examining the effects of repeated administration of cannabinoid receptor agonists, treatment with CP-55, 940 produced tolerance to its effects on rectal temperature, motor activity and weight of the animals. Rectal temperature, time of activity in the open field and weight decreased on days 1 and 3 after CP-55, 940 administration and returned to values similar to those observed in vehicle-treated mice after 7 days of treatment.

Although abrupt cessation of treatment with this cannabinoid receptor agonist does not produce the somatic signs of abstinence (tremor, ptosis, ataxia, mastication) observed after cannabinoid antagonist-precipitated withdrawal (Cook *et al.*, 1998; Diana *et al.*, 1998; Hutcheson *et al.*, 1998; Rubino *et al.*, 1998; Beardsley and Martin, 2000), a spontaneous behavioural 'withdrawal syndrome' was clearly shown in this study. This syndrome may be characterized by pronounced increases in motor activity and number of rearings and decreases in the number of groomings, rubbings and wet dog shakes.

These behavioural changes induced by cessation of treatment with CP-55, 940 were accompanied by significant and time-related alterations in cannabinoid CB_1 receptor gene expression in various brain regions of mice. The increases in CB_1 receptor mRNA levels during cannabinoid withdrawal may be part of a compensatory neuroadaptative response to down-regulation of cannabinoid receptor gene expression found after repeated treatment with a cannabinoid agonist (Rodriguez de Fonseca *et al.*, 1994; Sim *et al.*, 1996; Romero *et al.*, 1998; González *et al.*, 1999). In general, a rapid increase in the expression of the CB_1 receptor was observed in most of the areas studied; however, the duration and the magnitude of this effect is different depending upon the brain region. For example, in areas closely involved in the regulation of drug dependence, motor activity and emotional behaviour responses, such as the CPu or Ce, the up-regulation in the expression of the CB_1 receptor is maintained even 2 weeks after interruption of administration with CP-55, 940. The prolonged increase in the expression of the cannabinoid receptor gene in these brain areas may possibly alter the vulnerability to drug dependence or to the appearance of anxiety-related disorders.

A rapid increase in CB_1 receptor gene expression also occurs in the ventromedial nucleus of the hypothalamus and the CA1 field of hippocampus; however, these effects were no longer present on days 3, 7 or 14 after cessation of treatment with CP-55, 940. By contrast, in the CA3 field of hippocampus, spontaneous cannabinoid withdrawal significantly decreased cannabinoid receptor gene expression even on days 7 and 14 of abstinence, whereas no significant changes were found in the CA2 field. The nature by which spontaneous cannabinoid withdrawal produces a differential regulation in CB_1 receptor gene expression in the different hippocampal fields remains to be elucidated. The hippocampus, through numerous synaptic and non-synaptic interactions between neurotransmitters (Vizi and Kiss, 1998), plays an important role in the regulation of learning and memory. Activation of cannabinoid receptors in the hippocampus inhibits neurotransmitter release (acetylcholine, GABA, glutamate and norepinephrine) (Doherty and Dingledine, 2003) which presumably contributes to short- and long-term plasticity underlying memory alterations that often occur after prolonged exposure to cannabis derivatives. The results of prolonged alterations in cannabinoid receptor gene expression during spontaneous cannabinoid withdrawal in hippocampal fields relate long-lasting changes produced by drug withdrawal to alterations in learning and memory. Recently, common molecular and cellular substrates of addiction and memory have been proposed, suggesting a close relationship between brain circuits involved in the regulation of drug dependence and learning and memory (Nestler, 2002).

In summary, the results of the present study reveal that a spontaneous cannabinoid withdrawal may occur after cessation of cannabinoid receptor agonist treatment. This withdrawal syndrome produced behavioural alterations and short and long-term changes in cannabinoid receptor gene expression in various brain areas of mice. Overall, our findings suggest a time and regional differential role for cannabinoid receptors in the short and long-term neuroadaptations that occur after exposure to cannabinoid derivatives.

Acknowledgements

This research was supported by Grant of 'Fondo de Investigaciones Sanitarias' (FISS 01/1438) to J. Manzanares.

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