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Effects of Δ⁹-tetrahydrocannabinol on reward and anxiety in rats exposed to chronic unpredictable stress

S Fokos *Laboratory of Behavioral Neuroscience, Department of Psychology, School of Social Sciences, University of Crete, Crete, Greece.*
G Panagis *Laboratory of Behavioral Neuroscience, Department of Psychology, School of Social Sciences, University of Crete, Crete, Greece.*

Abstract

Although cannabis derivatives produce clear subjective motivational responses in humans leading to drug-seeking behaviour, the reinforcing attributes of these subjective effects are difficult to define in experimental animals. The aim of this study was to examine how exposure to chronic unpredictable stress (CUS) will affect reward function and anxiety after acute administration of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) in rats. Male rats were exposed to either 10 days of CUS or no stressor. Alterations in brain reward function were assessed with the intracranial self-stimulation (ICSS) paradigm, and anxiety responses were measured with the elevated plus maze. CUS did not affect baseline brain stimulation reward thresholds. Δ⁹-THC did not exhibit reinforcing actions in the ICSS paradigm neither in nonstressed nor in stressed animals. More importantly, in nonstressed animals, both the low and the high dose of Δ⁹-THC exerted anxiolytic-like effects. In stressed animals, however, only the high dose of THC induced an anxiolytic-like response, whereas the low dose induced anxiogenic effects. The present results provide clear evidence for an anxiolytic effect of Δ⁹-THC both in stressed and in nonstressed animals, and indicate that environmental conditions, such as stressful experiences, do not alter the behavioural effects of Δ⁹-THC in the ICSS paradigm.

Key words

anxiety; cannabinoid; chronic unpredictable stress; elevated plus maze; intracranial self-stimulation; reward

Introduction

Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the main psychoactive constituent of *Cannabis sativa*, is constantly the most widely consumed illicit drug in the world (Adams and Martin, 1996). Over the last decade, great efforts have been made to understand the neurobiological basis of the behavioural effects of cannabis and other cannabinoids. Convergent data from pharmacological, neurobiological and behavioural studies indicate that cannabinoids regulate mood, emotion and anxiety in humans (Moreira and Lutz, 2008; Valverde, 2005; Viveros, *et al.*, 2005; Witkin, *et al.*, 2005). However, although in humans cannabis derivatives produce clear subjective motivational responses leading to drug-seeking behaviour and in a specific proportion to repeated drug use and dependence, the bases of the addictive properties of these compounds remain to be clearly identified.

In human users, cannabis derivatives can produce opposite effects, varying from euphoria (high) to dysphoria and from relaxation to anxiety or even panic (Green, *et al.*, 2003; Hollister and Overall, 1975). These effects appear to be dependent upon specific environmental cues, the state of the organism, the time of administration and the concentration of the psychoactive ingredient itself. Although it has been postulated that euphoria is the primary factor in maintaining cannabis use, the most commonly reported effect in naturalistic studies of cannabis users is relaxation (Green, *et al.*, 2003).

Similarly, cannabinoids modulate emotional responses and have long been known to affect anxiety in experimental animals. However, the direction of these effects has been controversial. Indeed, Δ⁹-THC and other synthetic cannabinoid agonists can induce both appetitive and aversive effects, as it has been documented in various experimental conditions using several behavioural paradigms (for a recent review see, Panagis, *et al.*, 2008).

The intracranial self-stimulation (ICSS) paradigm has been extensively used to measure the reward-related properties of various psychotropic drugs (Kornetsky, 1985; Wise, 1996). Most drugs of abuse are able to lower the brain stimulation reward threshold, an effect which supports the notion that

Corresponding author: George Panagis, University of Crete, School of Social Sciences, Department of Psychology, Laboratory of Behavioral Neuroscience, University Campus at Gallos, 74100 Rethymnon, Crete, Greece. Email: panagis@psy.soc.uoc.gr

they activate the same substrate with electrical stimulation in a synergistic manner (Wise, 1996, 1998). Recent studies from our laboratory have shown that acute administration of Δ^9-THC in Sprague-Dawley rats did not possess reward-enhancing properties in the ICSS paradigm. On the contrary, at the two highest doses tested (1 and 2 mg/kg), Δ^9-THC produced anhedonic-like effects, as it increased the brain stimulation reward threshold (Vlachou, *et al*., 2007). The above results are in agreement with the studies of Stark and Dews, 1980; Kucharski, *et al*., 1983; Vlachou, *et al*., 2005, in which Δ^9-THC or other cannabinoid analogs structurally related to Δ^9-THC did not enhance brain stimulation reward. Accordingly, controversial data also exist in the literature concerning the ability of cannabinoids to support self-administration or conditioned place preference (for a recent review see, Panagis, *et al*., 2008). Thus, it has been postulated that cannabinoids have reinforcing and reward-facilitating properties in experimental animals mostly under particular experimental conditions.

Furthermore, there is a possibility that cannabinoids possess anxiolytic actions, which may contribute to drug-taking behaviour. However, the role of cannabinoids in the modulation of anxiety in experimental animals is still a matter of controversy. More specifically, Δ^9-THC and other cannabinoid analogs can exert both anxiolytic-like and anxiogenic-like responses, depending on various variables, such as the drug dose, the strain of the animals, the experimental setup and design and the environmental context (Berrendero and Maldonado, 2002; Bortolato, *et al*., 2006; Braida, *et al*., 2007; Genn, *et al*., 2004; Giuliani, *et al*., 2000; Onaivi, *et al*., 1990; Rubino, *et al*., 2007; Rutkowska, *et al*., 2006; Schramm-Sapyta, *et al*., 2007). In rodents, cannabinoids seem to display a dose-dependent biphasic profile, with low doses producing anxiolytic-like responses, whereas higher doses produce anxiogenic-like effects (Viveros, *et al*., 2005).

Moreover, the endocannabinoid system has been implicated in the responses seen after exposure to stressors (McGregor, *et al*., 1996), and it is well established that exposure to stressors can alter the effects of drugs of abuse (Haile, *et al*., 2001). Interestingly, chronic unpredictable stress (CUS), which involves exposing rats to a variety of stressors in an unpredictable manner, has been shown to enhance sensitivity to the behavioural effects of cocaine, whereas chronic exposure to the same stressor does not (Haile, *et al*., 2001). However, recent work has demonstrated that the endocannabinoid system can modulate the effects of exposure to CUS (Bortolato, *et al*., 2007; Hill, *et al*., 2005; Martin, *et al*., 2002).

On the basis of the above findings, this study examines how CUS will affect reward and anxiety-like responses elicited by the acute administration of Δ^9-THC. The effects of Δ^9-THC on brain stimulation reward were measured using the ICSS paradigm, whereas anxiety responses to acute Δ^9-THC administration were measured in the elevated plus maze (EPM).

Materials and methods

Subjects

Male Sprague–Dawley rats ($n = 91$) weighing 270–320 g at the beginning of the study were used. Rats were bred at the Laboratory of Behavioral Neuroscience at the Department of Psychology of the University of Crete. Before surgical implantation and the CUS exposure, the animals were housed in groups of four and maintained in a temperature- and light-controlled environment on a 12:12 h light:dark cycle (lights on at 9:00 h) with free access to food and water, except for the brief periods of food deprivation specified in the experimental procedures of CUS. All animals were handled daily for a week before the beginning of the experimental sessions. All the procedures were carried out according to the National Institutes of Health public document 85–23 (1985).

Drugs

Δ^9-THC (Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in a vehicle solution that consisted of 5% dimethylsulfoxide, 5% cremophor EL and 90 % of 0.9% NaCl and injected intraperitoneally (i.p.) at a volume of 3 ml/kg of body weight. Control animals received the corresponding vehicle solution intraperitoneally in the same injection volume.

Chronic unpredictable stress procedure

The CUS sequence consisted of different types of stressors presented randomly, two per day, over a period of 10 days. The stressors varied from day to day. The CUS procedure that was used in the current study is a slight modification of those used previously in other laboratories (Gouirand and Matuszewich, 2005; Haile, *et al*., 2001; Ortiz, *et al*., 1996) and is presented in Table 1 (see Table 1).

Body weight measurement

All rats that were used in the ICSS studies ($n = 21$; animals that were stereotaxically implanted with a monopolar stimulating electrode aimed at the lateral hypothalamus) and the EPM study group of stressed animals ($n = 24$) (see below) were weighed before the CUS procedure and the subsequent stress exposure on day 11. The body weight change was calculated as percentage changes in body weight and compared with equivalent control groups, that is, animals not receiving stress exposure stereotaxically implanted with a monopolar stimulating electrode aimed at the lateral hypothalamus ($n = 10$) or intact animals not exposed to stressors ($n = 12$).

Intracranial self-stimulation procedure

The animals were anaesthetised with intramuscular injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg).

Table 1 Chronic unpredictable stress procedure sequence

Day	Time	Stressor	Duration	Time	Stressor	Duration
1st	13.00	Cage rotation	50 min	14.00	Swim stress	4 min
2nd	12.00	Cold isolation (4 °C)	60 min	20.00	Lights on	Overnight (12 h)
3rd	13.00	Lights off	3 h	16.00	Cold isolation (4 °C)	15 min
4th	20.00	Cage rotation	50 min	20.00	Food and water deprivation	Overnight (12 h)
5th	14.00	Swim stress	3 min	20.00	Cold isolation (4 °C)	60 min
6th	12.00	Restrain stress	60 min	16.00	Lights off	2 h
7th	11.00	Swim stress	4 min	17.00	Restrain stress	60 min
8th	20.00	Lights on	Overnight (12 h)	20.00	Food and water deprivation	Overnight (12 h)
9th	11.00	Cage rotation	20 min	20.00	Lights on	Overnight (12 h)
10th	20.00	Restrain stress	60 min	20.00	Food and water deprivation	Overnight (12 h)

Atropine sulphate (0.6 mg/kg, i.m.) was injected to reduce bronchial secretion. The animals were implanted with a monopolar stimulation electrode aimed at the medial forebrain bundle (MFB) at the level of the lateral hypothalamus (2.56 mm posterior to bregma, 1.8 mm lateral from midsaggital suture and 8.6 mm below the outer flat skull), according to Paxinos and Watson (2007). The electrodes were constructed from 0.25 mm stainless steel wire insulated with Epoxylite except for the conically shaped tip. The anode was an uninsulated stainless steel wire connected to an amphenol pin. Five miniature skull screws, the electrode and the anode were secured to the skull with acrylic dental cement. Following implantation and for the entire duration of the experiments, the animals were housed individually.

Following one week of recovery, the rats were tested for self-stimulation in an operant chamber made of transparent Plexiglas (25 cm wide, 25 cm deep and 30 cm high). Each chamber was equipped with a stainless-steel lever 4 cm wide and protruded 2 cm from the left side at a height of 4 cm from the bottom. Each bar-press triggered a constant current stimulator (Med Associates, St. Albans, Vermont, USA) that delivered a 0.4 s train of rectangular cathodal pulses of constant duration (0.1 ms) and intensity (250 μA) and variable frequency (15–100 Hz, i.e., 6–40 number of pulses/0.4 s). The pulse frequency, that is the number of pulses within a train, was progressively increased up to 40 per stimulation train until the subject showed vigorous self-stimulation. During the acquisition phase the animals were trained to self-stimulate for at least three consecutive days (1 h daily), using stimulation parameters that maintained near maximal bar-pressing rates. After self-stimulation had been acquired and stabilised for a given pulse frequency, rats were trained to self-stimulate using four alternating series of ascending and descending pulse frequencies. The pulse frequency was varied by steps of approximately 0.1 log units. Each frequency was tested within trials of 60 s in duration, followed by an extinction period of 30 s. For each trial, there was an initial 'priming' phase during which the animals received three trains of stimulation at the frequency, which was available for the specific trial. A rate-frequency determination session lasted about 45 min. One rate-frequency curve was established daily, for 10–14 days, depending on the period when the self-stimulation indices (i.e., curve shift and threshold measure) were stable. Drug testing began for each animal when the function relating bar-pressing rate to pulse frequency (the rate-frequency function) was stable for at least three consecutive days. The criterion for stability was met when the frequency thresholds did not vary by more than 0.1 log units. The stimulation parameters, intracranial self-stimulation sessions and data collection were controlled by a computer.

Unequivocally, intracranial self-stimulation behaviour has the advantage of not being affected by satiation or aversive effects, which are potentially modulated by various drug treatments (Wise, 1996). However, since cannabinoids seem to affect motor activity/performance capacity in a dose-dependent manner (Stark and Dews, 1980), the use of a reward selective measure, like the curve-shift, was requisite. In this method, plotting the responses of the animals against the various pulse frequencies yields a sigmoidal rate-frequency curve. Shifts to the lateral position of the curve provide a selective measure of stimulation-produced reward, as elegantly demonstrated by Edmonds and Gallistel (1974), whereas vertical shifts provide information on the motor/performance capacity of the animals. Furthermore, this method offers quantitative scaling of drug-induced changes in reward (see, Campbell, *et al*., 1985), which is useful when comparing the effects of different drugs. In other words, the curve-shift method appears to have the reward selectivity that is required in psychopharmacological research (Liebman, 1983; Markou and Koob, 1992; Miliaressis, *et al*., 1986).

The rats were then tested daily for 3 days, and the average of thresholds and asymptotes (see below) was estimated (baseline determination). After the three-day baseline sessions, the rats were randomly divided into three experimental groups [vehicle ($n = 7$), THC 0.5 mg ($n = 7$) and THC 1 mg ($n = 7$)] and tested one more time for self-stimulation behaviour (preinjection, prestress session). Then, they were immediately removed from the ICSS chamber and injected with the analogous for their group dose of THC (0.5 or 1 mg/kg, i.p.) or its vehicle. The rats were returned to the ICSS chamber and thresholds were assessed 10 min later (postinjection, prestress session). This time interval has also been used in previous

self-stimulation studies and appears to be critical for the observation of other behavioural and physiological effects of cannabinoids (Vlachou, *et al*., 2007). On the next day after the administration sessions, all animals were subjected to a 10-day regimen of CUS. Upon completion of the CUS period, self-stimulation behaviour was assessed for all animals (day 11; preinjection, poststress session). Immediately following this self-stimulation session, each animal was injected with the same dose of THC (0.5 or 1 mg/kg, i.p.) or its vehicle, and a postinjection rate-frequency function was determined for each animal (postinjection, poststress session).

Elevated plus-maze procedure

The EPM consisted of two opposite open arms (50 cm long × 10 cm wide) and two enclosed arms (50 × 10 × 40 cm) extended from a common central platform (10 × 10 cm), based on a design validated by Lister (1987). The maze was constructed of blond varnish-painted wood, elevated to a height of 74 cm above floor level. The enclosed arms were surrounded by white plexiglas. The entire apparatus was placed in the centre of a small room that was lit with fluorescent lights.

After a one week handling period, the animals were randomly divided into two groups: stress (rats were exposed to CUS for 10 days; $n = 24$) and nonstress (cage control condition; $n = 24$), and each group (stress or nonstress) into three subgroups: 1) vehicle ($n = 8$ for both stress and nonstress groups), 2) THC 0.5 mg/kg ($n = 8$ for both stress and nonstress groups) and 3) THC 1 mg/kg ($n = 8$ for both stress and nonstress groups). The day after the end of handling or CUS period, nonstressed and stressed rats, respectively, were injected intraperitoneally in the colony room and transported individually to the plus-maze laboratory to facilitate adaptation to novel surroundings for 30 min. Then, the rats were placed onto the centre of the apparatus facing an open arm, and the time spent on and entries into each arm were detected for 5 min. The maze was wiped clean with water and dried after each trial. The number of open- and closed-arm entries, the number of total entries and the time spent on each arm or centre was recorded. Data were expressed as the percentage of the open- and closed-arm entries and the time spent in each arm (open, closed or centre time/total time × 100; open or closed entries/total entries × 100) and as the value of total entries. The percentage of time spent in the open arms and the percentage of open-arm entries were used as measures of anxiety, whereas the total entries measurement as a motor activity indicator (see Hogg, 1996).

Histology

Following the completion of the experiment, the animals were given a lethal dose of sodium pentothal. The location of the terminal stimulation site was then marked according to the protocol described by Vlachou, *et al*. (2006). The brains were then removed and stored in a solution of 10% formalin until sectioned and stained for verification of the electrode tips. Only the rats in which tracks from the electrode were verified to be located in the MFB at the level of lateral hypothalamus were included in this study.

Data analysis and statistics

Body weight Data were expressed as the means ± SEM, and the significance of stress effect to body weight was evaluated using the Independent samples *t*-test Statistical Package for the Social Sciences (SPSS) v.15.0 (SPSS Inc., Chicago, Illinois, USA). Significance levels were set at an alpha value of 0.05.

Intracranial self-stimulation Data gathered from pre- and postinjection portions of each session were curve-fitted, and threshold and asymptote estimates were obtained using the Gompertz sigmoid model (Coulombe and Miliaressis, 1987):

$$f(X) = \alpha e^{-e^{b(x_i - x)}}$$

In this equation, α represents the maximum rate or asymptote (determined from the linear transition between the lower asymptote and the upper asymptote), whereas X_i (X at inflection) represents the threshold frequency. The latter is the pulse number producing 36.7% of the asymptotic rate, that is, the rate lying on the fastest-accelerating region of the curve. Parameter b represents an index of the slope, whereas e is the base of natural logarithms.

The analysis was performed on two aspects of data obtained from the rate-frequency curve, that is, the brain stimulation reward threshold and the maximum rate of responding or asymptote. The pre- and postinjection threshold and asymptote values were expressed as percentage of baseline and preinjection values, respectively. Results were expressed as the means ± SEM. The significance of drug (three levels THC 0.5, 1 mg/kg and vehicle) and condition (stress or nonstress; one level all animals were exposed to CUS) interaction was statistically evaluated using two-way mixed design analysis of variance (ANOVA). *Post hoc* analyses were performed using the least significant difference (LSD) test to examine the statistically significant differences between the different drug doses and vehicle. The stress effect significance was evaluated using the paired samples *t*-test. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) v.15.0 (SPSS Inc., Chicago, Illinois, USA). Significance levels were set at an alpha value of 0.05.

Elevated plus-maze All data were expressed as the means ± SEM. The significance of drug (three levels THC 0.5, 1 mg/kg and vehicle) and condition (two levels stress or nonstress) interaction was statistically evaluated using two-way analysis of variance (ANOVA). *Post hoc* analyses were performed using an LSD test to examine the statistically significant differences between the different drug doses and vehicle. Stress effect significance was estimated using the Independent samples *t*-test. All calculations were carried out using the SPSS v.15.0. Significance levels were set at an alpha value of 0.05.

Results

Body Weight

Rats exposed to CUS showed less body weight gain than controls in both ICSS and EPM experiments (see Figure 1(a) and (b), respectively). The body weight alteration of stressed animals used in ICSS studies was significantly lower than the body weight of control animals that were not exposed to stressors and were stereotaxically implanted with a monopolar stimulating electrode aimed at the lateral hypothalamus ($t = 5.585$, $DF = 29$, $P < 0.001$). Similarly, the group of EPM animals exposed to CUS showed significantly lower body weight than intact animals not exposed to stressors ($t = 5.311$, $DF = 34$, $P < 0.001$).

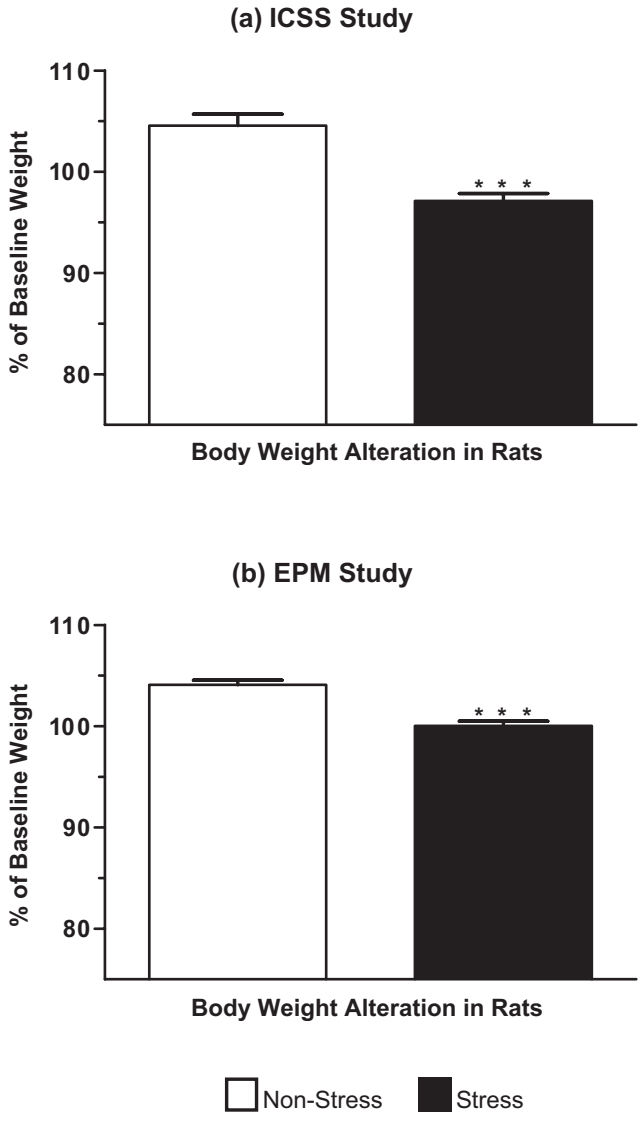

Figure 1 Effects of CUS on body weight. (a) Alteration of body weight (%) after 10 days of stress or nonstress of animals that were stereotaxically implanted with a monopolar stimulating electrode aimed at the lateral hypothalamus (LH), (b) alteration of animal's body weight (%) after 10 days stress or nonstress (cage condition). *Vertical bars* represent the SEMs. Three *asterisks* (***) signify a body weight alteration significantly different (***$P < 0.001$) from the control (nonstress) group.

ICSS studies

The changes in self-stimulation threshold and in asymptotic rate of responding, before and after stress exposure, are presented in Figure 2(a) and (c), respectively, whereas the changes after Δ^9-THC injection in self-stimulation threshold and in asymptotic rate of responding, before and after stress exposure, are presented in Figure 2(b) and (d), respectively. Two-way mixed design ANOVA indicated that there was no significant interaction between the three drug groups (THC 0.5 mg/kg, THC 1 mg/kg and vehicle) and the condition (stress or nonstress) for brain stimulation reward threshold and asymptote, either in preinjection ($F_{2,18} = 1.282$, $P = 0.302$ and $F_{2,18} = 0.332$, $P = 0.722$, respectively) or in postinjection ($F_{2,18} = 0.653$, $P = 0.533$ and $F_{2,18} = 0.238$, $P = 0.790$, respectively) sessions. Similarly, the main drug effects (postinjection/preinjection × 100; preinjection, pre- and poststress sessions) to brain stimulation reward threshold and asymptote were not significant ($F_{1,18} = 0.012$, $P = 0.982$ and $F_{1,18} = 1.451$, $P = 0.244$, respectively). However, the highest dose of THC (1 mg/kg) showed a tendency to increase brain stimulation reward threshold (see Figure 2(b)). The main effects of preinjection ICSS asymptotic rate of responding changes compared with baseline (preinjection/baseline × 100; preinjection, pre and poststress sessions) were not significant ($F_{1,18} = 3.148$, $P = 0.093$), whereas the same main effects of brain stimulation reward threshold were significant ($F_{1,18} = 13.888$, $P = 0.002$). However, post-hoc LSD test analysis showed that these effects upon the self-stimulation thresholds were not significant among the three groups, either prestress (vehicle compared with 0.5 mg/kg of THC: $P = 0.372$, vehicle compared with 1 mg/kg of THC: $P = 0.763$ and 1 mg/kg of THC compared with 0.5 mg/kg of THC: $P = 0.238$) or poststress ($P = 0.338$, $P = 0.472$ and $P = 0.103$, respectively) exposure. Similarly, paired samples *t*-test analysis showed that there were no significant differences between pre- and poststress exposure for any one of the three groups (vehicle: $t = -0.930$, $DF = 6$, $P = 0.388$; THC 0.5: $t = -0.972$, $DF = 6$, $P = 0.368$; THC 1: $t = -1.536$, $DF = 6$, $P = 0.175$).

EPM studies

Figure 3 presents the differences between stressed and nonstressed groups with respect to EPM parameters at the two doses of THC (0.5 and 1 mg/kg) and vehicle. Two-way ANOVA indicated a significant interaction between stress

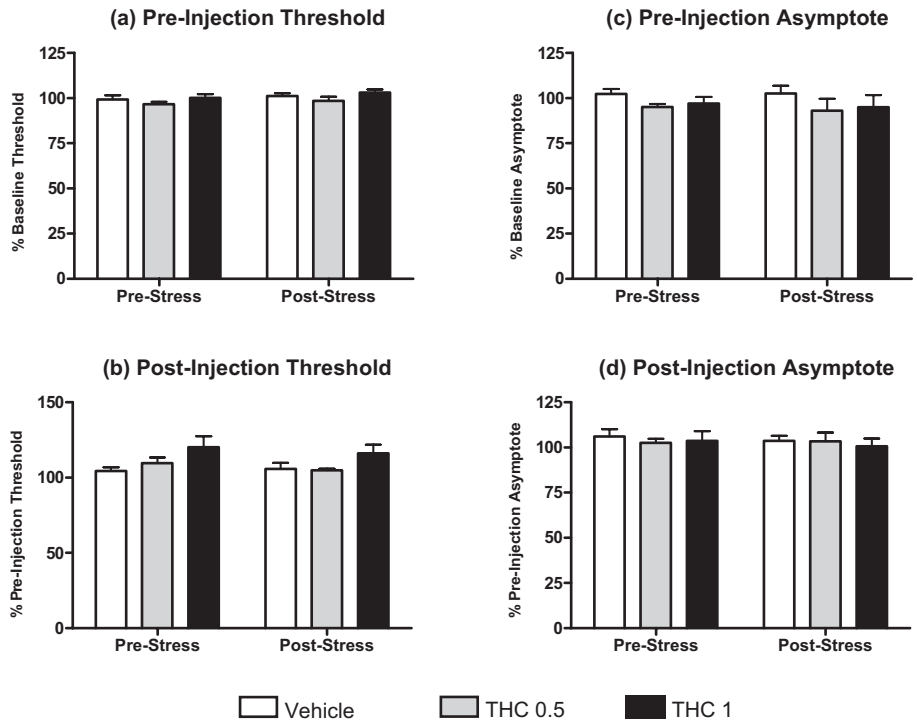

Figure 2 Pre and poststress changes in brain stimulation reward threshold (a and b) and asymptote (c and d), expressed as percentage of baseline and preinjection values. Vertical bars represent the SEMs.

treatment and drug dose on the percentage of closed-arm entries ($F_{2,42} = 3.307$, $P = 0.046$), open-arm entries ($F_{2,42} = 7.824$, $P = 0.001$), closed-arm time ($F_{2,42} = 4.503$, $P = 0.017$) and open-arm time ($F_{2,42} = 11.822$, $P < 0.001$). *Post hoc* LSD test analysis found that both doses of THC (0.5 and 1 mg/kg) significantly decreased the closed-arm entries ($P = 0.004$ and $P = 0.002$, respectively) and the time spent in closed arms ($P = 0.001$ and $P = 0.005$, respectively), whereas increased the open-arm entries ($P = 0.001$ and $P = 0.001$, respectively) and the time spent in open-arms ($P < 0.001$ and $P = 0.004$) in nonstressed animals, compared with the vehicle administration group. In stressed animals, *post hoc* comparison showed that 0.5 mg/kg of THC increased the closed-entries ($P = 0.040$) and decreased the time spent in open arms ($P = 0.001$), compared with the 1 mg/kg dose, whereas 0.5 mg/kg of THC compared with vehicle or to 1 mg/kg of THC decreased the open-entries ($P = 0.044$ and $P < 0.001$, respectively). Furthermore, in stressed animals, *post hoc* analysis demonstrated that 1 mg/kg of THC compared with vehicle increased the time spent in open arms ($P = 0.05$). Independent samples *t*-test analysis showed that in vehicle-treated animals there was no effect of stress on closed-arm entries ($t = 0.934$, $DF = 14$, $P = 0.366$) and on open-arm entries ($t = 0.934$, $DF = 14$, $P = 0.366$), whereas there was a significant difference between vehicle-treated stressed and nonstressed animals in the time spent in closed-arms ($t = 3.596$, $DF = 14$, $P = 0.003$) and in open-arms ($t = -2.571$, $DF = 14$, $P = 0.022$). There was no effect of stress on animals treated with 1 mg/kg of THC (closed-arm entries: $t = -1.297$, $DF = 14$, $P = 0.216$, open-arm entries: $t = 1.298$, $DF = 14$, $P = 0.215$, time spent in closed-arms: $t = 0.099$, $DF = 14$, $P = 0.922$, time spent in open-arms: $t = -0.589$, $DF = 14$, $P = 0.565$). However, there were significant differences in the closed-arm entries, in the open-arm entries and in the time spent in open-arms ($t = -2.127$, $DF = 14$, $P = 0.05$, $t = 4.035$, $DF = 14$, $P = 0.002$ and $t = 3.976$, $DF = 14$, $P = 0.003$, respectively) between stressed and nonstressed animals, following a dose of 0.5 mg/kg of THC. Data for closed-arm entries, open-arm entries, time spent in closed-arms and time spent in open-arms are presented in Figure 3(a), (b), (d) and (e), respectively.

Two-way ANOVA demonstrated that there was no significant interaction between stress condition and the drug dose in total-arm entries ($F_{2,42} = 2.820$, $P = 0.071$) and in the time spent in the centre ($F_{2,42} = 0.899$, $P = 0.415$). Furthermore, there were no main effects of stress on total arm entries ($F_{1,42} = 0.156$, $P = 0.695$) and on time spent in the centre ($F_{1,42} = 0.643$, $P = 0.427$). Similarly, there were no main effects of drug dose on total arm entries ($F_{2,42} = 1.530$, $P = 0.228$) and on time spent in the centre ($F_{2,42} = 0.186$, $P = 0.831$). Data for total arm entries and time spent in the centre are presented in Figure 3(c) and (f), respectively.

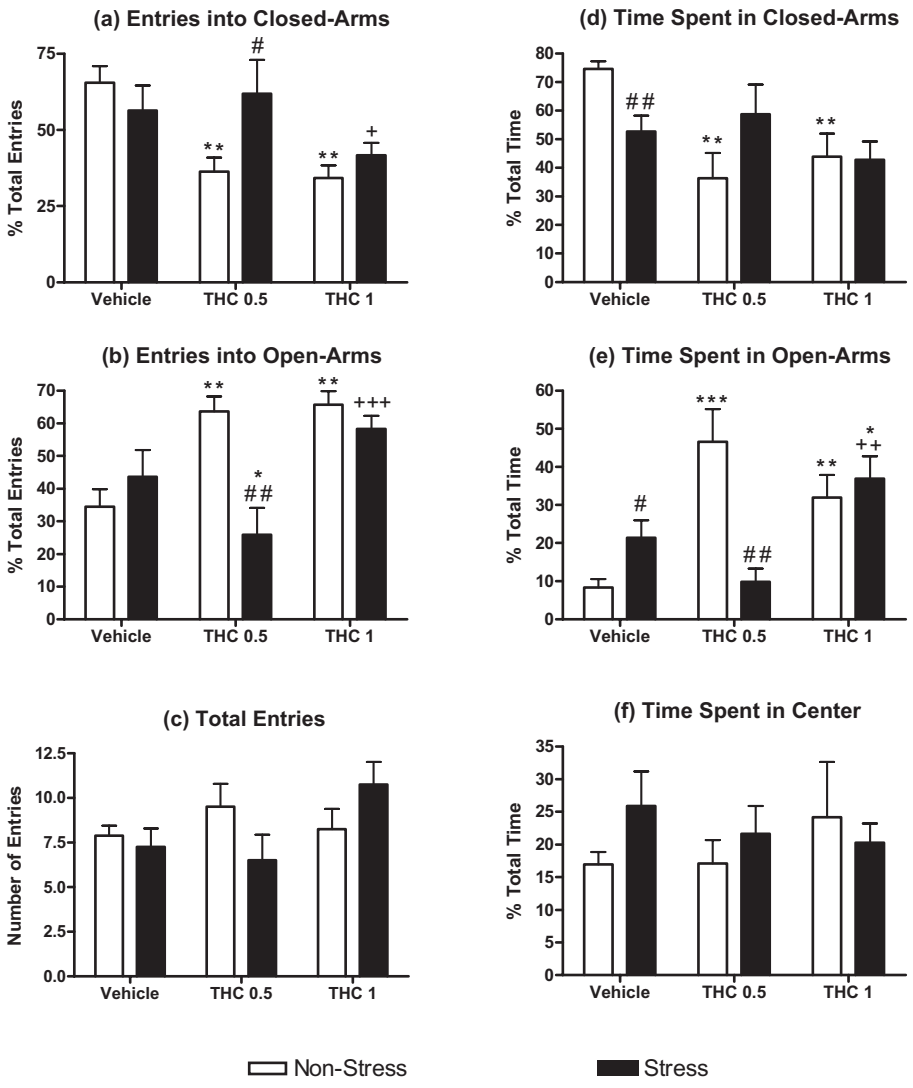

Figure 3 The effects of stress and dose of Δ⁹-THC (0, 0.5 and 1 mg/kg, i.p.) in the rat EPM test. (a) entries into closed-arms, (b) entries into open-arms, (c) total entries, (d) time spent in closed-arms, (e) time spend in open-arms, (f) time spent in centre. Vertical bars represent the SEMs. The *asterisk* (*) signifies a significant difference when compared with vehicle in the same condition (stress or nonstress) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$); The pound sign (#) indicates a significant difference between stress and nonstress condition in the same dose of drug (THC 0.5 or 1 mg/kg) or vehicle ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$); The plus sign(+) indicates a significant difference when compared with THC 0.5 mg/kg in the same condition (stress or nonstress) ($^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$).

Discussion

One purpose of the present study was to determine whether there are differences between stressed and nonstressed animals in their response to Δ⁹-THC in the ICSS paradigm. Consistent with a previous study from our research group (Vlachou, *et al*., 2007), Δ⁹-THC did not have reward-facilitating properties in the ICSS paradigm. In addition, statistical analysis suggested that there were no differences between stressed and nonstressed rats with regard to the effects of Δ⁹-THC on brain stimulation reward. The second goal of this study was to compare how exposure to CUS would affect anxiety responses elicited by acute administration of Δ⁹-THC. Results from this experiment showed that in nonstressed rats both the low (0.5 mg/kg) and the high dose (1 mg/kg) of Δ⁹-THC used in this study induced anxiolytic-like effects. Interestingly, however, the low dose of Δ⁹-THC induced anxiogenic responses, whereas the high dose induced an anxiolytic response in the animals exposed to CUS.

According to the results from the first experiment, acute administration of Δ⁹-THC in Sprague-Dawley rats did not

reveal any reward-enhancing properties in the ICSS paradigm. On the contrary, we observed a tendency towards anhedonic-like effects at the highest dose tested (1 mg/kg). Further, Δ^9-THC did not affect maximal rates of responding at any of the doses tested. Our results are consistent with previous studies, showing that Δ^9-THC and direct as well as indirect cannabinoid agonists, either do not affect or increase intracranial self-stimulation threshold, depending on the dose tested (Arnold, *et al*., 2001; Kucharski, *et al*., 1983; Stark and Dews, 1980; Vlachou, *et al*., 2003, 2005, 2006, 2007, 2008). Our results differ from those of previous studies by Gardner, *et al*. (1988); Gardner and Vorel, (1998); Lepore, *et al*. (1996), which have shown that Δ^9-THC increases the rewarding efficacy of brain stimulation. This discrepancy in results between the different studies could be due to differences in the experimental design or the strains of the animals used, as has been detailed in a recent article by Vlachou, *et al*. (2007). More specifically, Gardner and colleagues adopt a very strict criterion of stable responding (i.e., 0.01 log units for three consecutive days), which indicates that the observed effect of Δ^9-THC in their study could reflect normal baseline variation over days. However, in our study the criterion of stable responding was defined within 0.1 log units over three consecutive days, in agreement with most other studies utilising the ICSS paradigm. Furthermore, according to the studies by Gardner and colleagues, Δ^9-THC exhibits reinforcing properties in Sprague-Dawley rats only when the Θ_0 criterion, and not the M_{50} criterion for threshold measure is used. Remarkably, in our study, the criterion used resembles the M_{50} criterion. Evaluated in this manner, our data are in agreement with the data of Lepore, *et al*. (1996) in the strain of Sprague-Dawley rats. It is worth noting that other behavioural models, such as the conditioned place preference and the self-administration paradigm, have also provided inconsistent results both with Δ^9-THC and other cannabinoid agonists (for a recent review, see Panagis, *et al*., 2008).

A recent study using animal models relevant to drug abuse has shown that exposure to stress alters responses to typical drugs of abuse, such as cocaine (Goeders, 2002). Interestingly, CUS has been shown to enhance the behavioural effects of cocaine, whereas chronic predictable stress is without effect (Haile, *et al*., 2001). CUS is a stress procedure that appears to cause a moderate stress response. Rats exposed to CUS show reduced body weight gain (Gouirand and Matuszewich, 2005), an effect also observed in the present study. This effect indicates that rats exposed to CUS differ from control rats. However, the CUS procedure used in the present study could be considered to induce a moderate level of stress. According to Gouirand and Matuszewich (2005), CUS appears to mimic the pattern of daily life stressors experienced by humans and does not come to pathological levels. Thus, it is not surprising that CUS did not affect the thresholds for ICSS. Our results are in agreement with the study by Gouirand and Matuszewich (2005), in which the same stress procedure and the same strain of rats were used as in our study. According to the results of that study, no differences were detected between stressed and nonstressed rats on sucrose consumption. However, several previous studies using other CUS protocols have found decreased (Banasr, *et al*., 2007; D'Aquila, *et al*., 1994, 1997; Moreau, *et al*., 1992, 1995; Willner, *et al*., 1996) or increased (Lin, *et al*., 2002; Nielsen, *et al*., 2000) responsiveness to rewarding stimuli after the termination of stress. The disparate outcomes of these studies are difficult to reconcile. Yet, the majority of the above cited studies demonstrate a decreased or increased brain reward function following chronic stress paradigms for 2–3 weeks, such as the chronic mild stress (CMS). However, even with those paradigms, it has been shown that the effects of stress on brain reward function depend on the strain of the animal used (Pothion, *et al*., 2004) or the type of stressors included in the regimen (Forbes, *et al*., 1996). In any case, we speculate that the CUS procedure used in the current study is a mild form of chronic stress, which unlike CMS may not reflect the anhedonia observed after pathological levels of stress, such as those of depression in humans. However, such an anhedonia is not usually observed in the healthy population simply because of facing short periods of stress. The failure to observe any anhedonic-like effects in the presently used CUS adds to its suitability as an animal model of chronic mild stress that resembles the healthy population, which may use Δ^9-THC or other drugs under stress conditions.

This is the first study showing that Δ^9-THC did not increase the rewarding efficacy of lateral hypothalamic self-stimulation in rats exposed to CUS. In other words, CUS did not alter the behavioural effects of Δ^9-THC on the brain reward function. In apparent contrast to these effects, the same animal model of stress has been shown to enhance the locomotor activating and rewarding effects of cocaine (Haile, *et al*., 2001). Analogous effects have been observed using other chronic stress-inducing paradigms and various typical drugs of abuse, such as alcohol (Cruz, *et al*., 2008), morphine (Will, *et al*., 1998), cocaine (Miczek and Mutschler, 1996) and amphetamine (Herman, *et al*., 1984). Thus, the findings that Δ^9-THC did not exhibit rewarding properties in the ICSS paradigm neither in nonstressed animals nor in animals exposed to CUS, suggest that Δ^9-THC, the main psychoactive constituent of cannabis, differs from other typical drugs of abuse, such as psychostimulants, opioids and alcohol.

According to the results of the second experiment, CUS alone induced an anxiolytic effect, as indicated by the increased time spent in the open arms and the decreased time spent in the closed arms. Further, CUS did not affect motor activity, as indicated by the total arm entries. The anxiolytic effect of CUS observed in the current study is in agreement with a previous study by D'Aquila, *et al*. (1994). This finding is difficult to interpret. One possibility is that the anxiolytic effect observed after the exposure to CUS is related with homeostatic mechanisms involved in the adaptation of the organism on various environmental conditions. Accordingly, if humans are exposed to mild stressful experiences, they develop coping strategies in order to function more efficiently in a more demanding environment. Thus, we suggest that the CUS may provide the animals with the means of coping with the

environment and for this reason may be appropriate in mimicking the normal human population under stress conditions.

Results from the second experiment demonstrated that Δ^9-THC, in both doses tested (0.5 and 1 mg/kg), induced anxiolytic-like effects in the nonstressed rats, as indicated by increased entries and time spent in the open-arms of the EPM, whereas it reduced the corresponding indices in the closed-arms. Our results are in agreement with a previous study by Rubino, *et al*. (2007), who present data for anxiolytic actions of Δ^9-THC in a range of doses between 0.075 and 1.5 mg/kg in the EPM. Interestingly, a number of studies using various animal models of anxiety have shown that Δ^9-THC and other cannabinoid agonists display a dose-dependent biphasic profile in rodents, in which low doses produce anxiolytic-like responses, whereas higher doses produce anxiogenic-like responses (Arevalo, *et al*., 2001; Berrendero and Maldonado, 2002; Braida, *et al*., 2007; Genn, *et al*., 2004; Giuliani, *et al*., 2000; Haller, *et al*., 2004; Hill and Gorzalka, 2004; Marco, *et al*., 2004; Marin, *et al*., 2003; Onaivi, *et al*., 1990; Patel and Hillard, 2006; Valjent, *et al*., 2002). Ultimately, these behavioural data are in agreement with the observation that one of the most reliable factors determining the emotional response (relaxation/anxiolysis or anxiogenesis/panic reactions) to cannabis in humans is the dose (Isbell, *et al*., 1967). The mechanism by which cannabinoids would control anxiety-related behaviour is not yet elucidated. Several studies implicated the stimulation of CB_1 receptors (Berrendero and Maldonado, 2002; Haller, *et al*., 2004), μ and Δ opioid receptors (Berrendero and Maldonado, 2002) and 5-HT_{1A} receptors (Braida, *et al*., 2007) in the anxiolytic actions of cannabinoids. However, the anxiogenic effects of cannabinoids might be related with a different receptor than the CB_1 (Haller, *et al*., 2002, 2004; Rodgers, *et al*., 2005) and/or the κ opioid receptor (Marin, *et al*., 2003).

Interestingly, one finding of the second experiment is that in the animals exposed to CUS the low dose of Δ^9-THC induced anxiogenic responses, whereas the high dose induced anxiolytic responses. To our knowledge, this is the first study indicating a biphasic effect of Δ^9-THC on anxiety responses under chronic stress conditions. Our findings agree partly with Hill and Gorzalka (2004) who found that in stressed animals HU-210 produced an anxiogenic response. However, in our study, the high dose (1 mg/kg) of Δ^9-THC induced anxiolytic-like effects in the stressed rats, an effect which is in accordance with that of Δ^9-THC in the nonstressed rats. However, in the study by Hill and Gorzalka (2004) the high dose (50 μg/kg) of HU-210, which induced anxiogenic responses in the stressed rats, has also the same effect in the group of nonstressed rats. It is not very clear why the lower dose of Δ^9-THC induced anxiogenic, whereas the highest dose anxiolytic responses in the stressed rats. Apparently, these findings can not be attributed to cannabinoid- or stress-induced changes in motor activity, as we did not find any differences between the different groups in the total arm entries. Importantly, a recent study by Hill, *et al*. (2005) found that CUS is associated with robust reductions in both 2-AG content and CB_1 receptor density in the hippocampus, a region known for its associations with anxiety and stress responses. Thus, one possibility may be that the different doses of Δ^9-THC used in the current study differentially affect these down-regulated cannabinoid receptors. Further, it has been shown that two distinct cannabinoid presynaptic receptors regulate network activity in the hippocampus. The first (CB_1) is selectively expressed in a subset of inhibitory GABAergic interneurons and reduces GABA release from presynaptic terminals. The second (nonCB_1), is present in glutamatergic terminals and suppress glutamate release (Hajos and Freund, 2002). It is possible that different doses of Δ^9-THC differentially affect these discrete cannabinoid receptors with diverse behavioural outcome. Alternatively, as indicated by Tzavara, *et al*. (2003), the bimodal effects of cannabinoids may be related with distinct neuroanatomical CB_1 receptors, with a differential sensitivity to Δ^9-THC in the stressed animals.

In summary, CUS, an animal model that reflects the pattern of stressful experiences in the normal population, reduced the body weight and induced mild anxiolysis, but did not affect the rewarding efficacy of ICSS. Δ^9-THC did not affect brain stimulation reward threshold in nonstressed or in stressed animals. This effect indicates that Δ^9-THC, in contrast to typical drugs of abuse, does not exhibit reward-facilitating properties in the ICSS paradigm. However, Δ^9-THC, both in the dose of 0.5 and 1 mg/kg, induced anxiolytic-like effects in the nonstressed rats, whereas in the rats exposed to CUS the low dose induced anxiogenic-like affects and the high dose anxiolytic-like effects. The observed anxiolytic-like effects of Δ^9-THC both in stressed and nonstressed conditions may explain some aspects of the recreational use of cannabis in humans. Besides, the observed anxiogenic-like effects of Δ^9-THC under stressed conditions may partly reflect the dysphoria, and the anxiety or the panic witnessed in several cases after cannabis use. Moreover, the fact that Δ^9-THC did not exhibit reward-enhancing properties in the ICSS paradigm may, also, explain why cannabinoids do not induce the typical pattern of obsessive drug seeking and compulsive drug taking behaviour, observed in humans addicted to typical drugs of abuse, such as cocaine, heroin, alcohol and nicotine.

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