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Δ9-tetrahydrocannabinol decreases somatic and motivational manifestations of nicotine withdrawal in mice

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Abstract

The possible interactions between Δ9-tetrahydrocannabinol (THC) and nicotine remain unclear in spite of the current association of cannabis and tobacco in humans. The aim of the present study was to explore the interactions between these two drugs of abuse by evaluating the consequences of THC administration on the somatic manifestations and the aversive motivational state associated to nicotine withdrawal in mice. Acute THC administration significantly decreased the incidence of several nicotine withdrawal signs precipitated by mecamylamine or naloxone, such as wet-dog-shakes, paw tremor and scratches. In both experimental conditions, the global withdrawal score was also significantly attenuated by acute THC administration. THC also reversed conditioned place aversion associated to naloxone precipitated nicotine withdrawal. We have then evaluated whether this effect of THC was due to possible adaptive changes induced by chronic nicotine on CB1 cannabinoid receptors. The stimulation of GTPγS-binding proteins by the cannabinoid agonist WIN 55,212-2 and the density of CB1 cannabinoid receptor binding labelled with [^{3}H] CP-55,940 were not modified by chronic nicotine treatment in the different brain structures investigated. Finally, we evaluated the consequences of THC administration on c-Fos expression in several brain structures after chronic nicotine administration and withdrawal. c-Fos was decreased in the caudate putamen and the dentate gyrus after mecamylamine precipitated nicotine withdrawal. However, acute THC administration did not modify c-Fos expression under these experimental conditions. Taken together, these results indicate that THC administration attenuated somatic **signs of nicotine withdrawal and this effect was not** associated to compensatory changes on CB1 cannabinoid receptors during chronic nicotine administration. **In addition, THC also ameliorated the aversive motivational consequences of nicotine withdrawal.**

Introduction

Nicotine and cannabinoids are among the most widely consumed drugs of abuse in humans. Both types of drugs share multiple common pharmacological properties including antinociception, hypothermia, impairment of locomotion, rewarding properties and dependence (Cook *et al.* 1998; Hutcheson *et al.*, 1998; Hildebrand *et al.*, 1999; Valjent & Maldonado, 2000; Watkins *et al.*, 2000). In both cases, these effects are mediated by the activation of receptors highly expressed in the central nervous system (CNS): the CB1 cannabinoid receptors (Herkenham *et al.*, 1990; Tsou *et al.*, 1998), which are metabotropic receptors (Matsuda *et al.*, 1990), and nicotinic acetylcholine (Ach) receptors (Martin & Aceto, 1981; Luetje *et al.*, 1990), which are ionotropic pentamer receptors made up of different subunits (Cordero-Erausquin *et al.*, 2000). Recent studies carried out with mutant mice lacking the CB1 cannabinoid receptor gene have demonstrated that this receptor mediates central pharmacological effects of cannabinoids (Ledent *et al.*, 1999; Zimmer *et al.*, 1999). Interestingly, an overlapping distribution of CB1 cannabinoid receptors and ACh receptors has been reported in several brain structures, such as the hippocampus and the amygdala (Piccioto *et al.*, 2000), which suggests the possibility of functional interactions between these two systems. Both cannabinoid and cholinergic systems are modulatory pathways in the CNS (Ameri, 1999; Calabresi *et al.*, 2000 for review). In addition, cannabinoid receptor activation modulates the release and turnover of Ach in various brain areas. Thus, cannabinoid agonists enhaced Ach release in the hippocampus, cortex and striatum (Tripathi *et al.*, 1987; Acquas *et al.*, 2000), and decreased Ach turnover in these structures (Revuelta *et al.*, 1978; Tripathi *et al.*, 1987). **In the same way, Gessa *et al.* (1998) showed that cannabinoids decrease Ach release in the medial-prefrontal**

cortex and hippocampus. Moreover, a recent study suggested a novel role of Ach as a potent enhancer of endocannabinoid signalling that would act retrogradely from postsynaptic to presynaptic neurons (Kim *et al.*, 2002).

The specific behavioural and biochemical consequences of the interaction between Δ9-tetrahydrocannabinol (THC) and nicotine are poorly documented in animal models in spite of the current association of these two substances in humans. Recently, it has been reported that nicotine strongly facilitated hypothermia, antinociception, hypolocomotion and anxiolytic-like responses induced by acute administration of THC (Valjent *et al.*, 2002). Acute THC and nicotine co-administration also facilitated the effects of these drugs on c-Fos expression in several brain structures (Valjent *et al.*, 2002). Furthermore, the co-administration of sub-threshold doses of THC and nicotine produced a significant conditioned place preference (Valjent *et al.*, 2002), while the rewarding effects of nicotine were absent in CB1 cannabinoid receptor knockout mice (Castañé *et al.*, 2002). Nicotine and THC co-treatment also attenuated the development of THC tolerance and enhaced the somatic expression of cannabinoid antagonist-precipitated THC withdrawal (Valjent *et al.*, 2002). However, no differences in the severity of nicotine withdrawal were observed in CB1 knockout mice (Castañé *et al.*, 2002).

Taken together, these results suggest that nicotine is able to facilitate several pharmacological responses of THC related to its addictive properties. However the possible effects of THC on the different manifestations of nicotine dependence remain to be clarified. The aim of the present study was to analyse the consequences of THC administration on the somatic manifestations. **In a next step, we evaluated whether this THC effect was due to possible adaptive changes induced by chronic nicotine treatment on CB1 cannabinoid receptors. Another purpose was to study the**

consequences of THC administration on the aversive motivational state associated to nicotine withdrawal in mice. Finally, we investigated the consequences of THC administration on the changes induced in CNS c-Fos expression by chronic nicotine treatment and withdrawal.

Methods

Animals

Male CD-1 mice (Charles River, France) weighing 20-22 g were housed five per cage, acclimated to the laboratory conditions (12 h light – dark cycle, 21 ± 1ºC room temperature, 65 ± 10% humidity) and manipulated by the investigators for 1 week prior to the experiment. Food and water were avaible *ad libitum*. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (NIH, publication no. 85-23, revised 1985; European Communities Council Directive 86/609/EEC) and approved by the Local Ethical Committee. All experiments were performed with the investigators being blind to the treatment conditions.

Drugs

THC (THC Pharm, Franckfurt, Germany) was dissolved in a solution of 5% ethanol, 5% cremophor EL (Sigma Chemical Co., Madrid Spain) and 90% distilled water and was administered by intraperitoneal route. (-)-Nicotine hydrogen tartrate salt ([-]-1-methyl-2-[3-pyridyl]pyrrolidine), mecamylamine hydrochloride and naloxone hydrochloride (Sigma Chemical Co., Madrid Spain) were dissolved in physiological saline (0.9%) and administered by subcutaneously route. THC, mecamylamine and naloxone were administered in a volume of 10 ml/kg. Nicotine was perfused by using Alzet minipumps. The selective CB1 receptor antagonist SR141716A [(N-piperidin-1-yl)-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxyamide] (Sanofi Recherche, Montpellier, France) was dissolved in a solution of 10% ethanol, 10% cremophor EL and 80% distilled water and administered by intraperitoneal route in a volume of 20 ml/kg.

Nicotine dependence and withdrawal

Induction of nicotine dependence

Mice were implanted subcutaneously with Alzet osmotic minipumps (Model, 2001) (Alzet^{R} , Cupertino, CA), under brief diethyl ether anaesthesia and were divided in two groups. The minipumps contained saline or nicotine solutions and delivered a constant subcutaneous flow in a rate of 1 μ/h. The concentration of nicotine was adjusted to compensate for differences in the body weight of the subjects. Thus, average-weight mouse received a dose of approximately 25 mg/kg/day nicotine hydrogen tartrate salt over 6 days.

Mecamylamine, naloxone and SR 141716A-precipitated nicotine withdrawal syndrome

Six days after minipump implantation, mice were placed inside a circular clear plastic observation area and the withdrawal syndrome was precipitated by injection of the nicotinic receptor antagonist mecamylamine, the opioid receptor antagonist naloxone or the CB1 cannabinoid receptor antagonist SR 141716A. The dose of mecamylamine (1 mg/kg, sc) was chosen taking into account the results obtained in previous studies in our laboratory (Castañé *et al.*, 2002). Two doses of naloxone, 3 and 5 mg/kg (sc) were tested, and a similar severity of nicotine withdrawal was observed with both doses. Therefore, the lowest dose of naloxone (3 mg/kg) was chosen for further studies. Finally, three doses of SR 141716A (1, 3 and 10 mg/kg, i.p.) were used to challenge nicotine dependent mice. The following abstinence signs were evaluated during a period of 15 min before and 30 min after mecamylamine, naloxone or SR 141716A injection: locomotor activity, ptosis, wet dog shakes, teeth chattering, front paw tremor, scratching, genital licks, and piloerection. The number of wet dog shakes, front paw tremor, and scratches was counted. Ptosis, genital licks, piloerection and teeth chattering

were scored 1 for appearance or 0 for non-appearance within each 5 min time. The locomotor activity over 5 min periods was rated 0, 1 or 2 (0 for inactivity, 1 for low activity and 2 for normal activity). A quantitative value was obtained for these checked signs by adding the individual values obtained each 5 min period during the whole time of observation. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight: 0.5 for each episode of wet dog shake, front paw tremor and scratching; and 1 for the presence of ptosis, genital lick, piloerection and teeth chattering during each observation period of 5 min. The relative weight of locomotor activity for each 5 min period was 0 normal activity, 0.5 low activity and 1 inactivity. THC (0.3, 1 and 3 mg/kg, i.p.) or vehicle were administered 15 min before mecamylamine or naloxone injection, and the effects on nicotine withdrawal were evaluated.

Place Aversion Conditioning Procedure

The place conditioning paradigm was used to evaluate the effect of THC (0.3 mg/kg) on the dysphoric manifestations associated to nicotine withdrawal. The apparatus consisted of two main square conditioning compartments separated by a triangular neutral area (Maldonado *et al.*, 1997). The time spent by the mouse in each compartment was recorded by computerized monitoring software (Videotrack®, View Point, France). In order to induce nicotine dependence in animals, mice were implanted subcutaneously with osmotic minipumps (Alzet®, Model 2002, Cupertino, CA) under brief diethyl ether anaesthesia and were divided in two groups. The minipumps contained saline or nicotine hydrogen tartrate salt and delivered a constant flow in a rate of 0.5 μl/h. The concentration of nicotine will be adjusted to compensate for differences in the body weight of the mice, and average-weight mouse received a dose of approximately 10

mg/kg/day nicotine hydrogen tartrate salt over 14 days. In a preliminary experiment, different doses of mecamylamine were not able to produce an aversive response associated to nicotine withdrawal. Considering this negative result, only naloxone was used to precipitate the aversive manifestations of nicotine withdrawal in this experiment.

Five days after minipumps implantation, the pre-conditioning phase was performed. Each animal was placed in the neutral area, allowing to freely explore the conditioning compartments, and the time spent in each compartment was recorded during 18 min. Animals did not show any initial preference or aversion for the two compartments. Mice were distributed in the different treatment groups counterbalancing the drug-paired compartments. In the conditioning phase, animals received THC (0.3 mg/kg, i.p.) or vehicle 15 min before the withdrawal induction by the administration of naloxone (0.12 mg/kg, s.c.) or saline (control group) on days 7, 9, 11 and 13 after minipump implantation. Then, they were immediately confined in the withdrawal-paired comparment by using guillotine doors matching walls for 30 min. On days 8, 10, 12 and 14 after minipump implantation, all mice received vehicle 15 min before saline injection, and were then immediately confined in the saline-paired compartment for 30 min. The testing phase was conducted on days 15 and 18 after minipump implantation, i.e., one and fourth days after the end of the nicotine infusions, respectively. This phase was performed as the preconditioning phase, i.e., animals were placed in the neutral area and had free access to the conditioning compartments during 18 min. The time spent in the central area was proportionally distributed and added to the time spent in each compartment, as previously reported (Maldonado *et al*, 1997). A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment.

Cannabinoid receptor and GTPγS studies

Brain slicing

Six days after saline or nicotine minipump implantation, animals received saline or mecamylamine injection, and were sacrified one hour later by cervical dislocation. Brains were removed and quickly frozen by immersion in 2-methyl-butane surrounded by dry ice. All samples were stored at –80 ºC before use. Coronal sections 20 μm-thick were cut in a cryostat, thaw-mounted on gelatin/chrome-coated slides, dried briefly at 30 ºC and stored at –80 ºC until used. Cannabinoid receptor and GTPγS binding experiments were performed no longer than one month after mouse was sacrified. For the identification of the different brain nuclei, adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet and analyzed according to the Paxinos and Franklin atlas (1997).

Autoradiography of cannabinoid receptor binding

The protocol used was based on the method described by Herkenham *et al.* (1991). Briefly, slide-mounted brain sections were incubated for 2.5 h at 37 ºC in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), pH 7.4, and 10 nM [^{3}H]CP-55, 940 (NEN, Boston, MA) prepared in the same buffer, in the absence or presence of 10 μM nonlabeled CP-55,940 (Tocris) to determine total and nonspecific binding, respectively. Following this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2 x 2 h) at 0 ºC, dipped in ice-cold distilled water, then dried under a stream of cool dry air. Autoradiograms were generated by apposing the labelled tissues, together with autoradiographic standards ([^{3}H]micro-scales, Amersham), to tritium-sensitive film (Biomax MS, Amersham) for a period of 4 weeks and developed for 4 min at 20 ºC.

Developed films were analyzed and quantitated in a computerized image analysis system (MCID, St. Catharines, Ontario, Canada) using the standard curve generated from [^{3}H]-standards.

Analysis of WIN 55,212-2-stimulated [^{35}S]-GTPγS binding

The protocol used was based on the method described by Sim *et al.* (1995). Briefly, slide-mounted brain sections were rinsed in assay buffer (50 mM TRIS, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 0.5% bovine serum albumin fatty acid-free, pH 7.4) at 25º C for 10 min, then pretreated for 15 min with an excess concentration (2 mM) of GDP (Sigma Chemical Co., Madrid Spain) in assay buffer. Afterwards, sections were incubated at 25 ºC for 2 h in assay buffer containing 0.04 nM [^{35}S]-GTPγS (Amersham), 2 mM GDP, and 5 μM WIN-55,212-2 (Sigma Chemical Co., Madrid Spain). Basal activity was assessed in the absence of agonist, whereas nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. In pilot experiments, additional brain sections were incubated in the presence of the cannabinoid receptor antagonist SR 141716A (1.5 μM) (Sanofi Recherche, France) (Rinaldi-Carmona et al., 1994) in addition to 0.04 nM [^{35}S]-GTPγS, 2 mM GDP, and 5 μM WIN-55,212-2. SR 141716A significantly antagonized the increase in WIN-55,212-2-stimulated [^{35}S]-GTPγS binding, thus supporting that this increase was specifically caused through activation of CB_1 cannabinoid receptors (data not shown). Slices were rinsed twice in 50 mM TRIS buffer, pH 7.4, at 4 ºC and deionized once in water, then dried under a stream of cool dry air. Autoradiograms were generated by apposing the labelled tissues to film (Hyperfilm-β-max, Amersham) for a period of 3 days and developed for 4 min at 20 ºC. Developed film were analyzed and quantitated in a computerized image analysis system (MCID, St. Catharines, Ontario, Canada).

C-fos experiments

Tissue preparation

Six days after saline or nicotine minipump implantation, animals received saline or mecamylamine injection, and 15 min before they received an acute THC (3 mg/kg, i.p.) or vehicle administration. One hour after the last injection, animals were deeply anesthetized using a mixture of ketamine (50 mg/kg, Rhone Merieux, France), ethanol 5%, xylazine (10 mg/kg, Sigma, Spain) and sterile water 95%. They were then perfused transcardially with heparinized PB (0.1 M phosphate buffer, pH 7.4), followed by a cold solution of 4% paraformaldehyde delivered with a peristaltic pump at 10 ml/min during 5 min. Brains were removed and postfixed for 3 hr in the same fixative, and cryoprotected overnight in a 30% sucrose solution. Coronal frozen sections were made at 40 μm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4 ºC.

C-fos immunohistochemistry

The immunocytochemical procedure was adapted from previously described protocols (Bester *et al.*, 2001). All reactions were performed at room temperature on floating sections agitated on a shaker. Sections were incubated for 2 hr in 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB (NGS-T-PB). Then, sections were incubated overnight in a rabbit polyclonal antibody anti-C-fos (Ab-5; Calbiochem, La Jolla, CA) diluted in NGS-T-PB (1:100,000). Sections were washed in 0.1 M PB and incubated for 1 hr in a goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA): 1:500 in NGS-T-PB. Sections were washed again in 0.1 M PB and incubated for 2 hr in avidin-biotin peroxidase complex (ABC Elite; Vectastain, Vector Laboratories). After washes in Tris buffer (0.15 M, pH 7.6), sections were incubated for 2 min in a solution

containing 0.05% of 3,3' diaminobenzidine (DAB; Sigma) and 0.2% ammonium nickel sulfate in 0.15 M Tris buffer. Increasing doses of H_2O_2 were added every 5 min. Finally, the reaction was stopped by washes in 0.1 M PB. The sections were mounted onto glass slides coated with gelatine, air dried, dehydrated through graded alcohols, cleared in xylene and cover-slipped using Eukitt mounting medium.

Data quantification

For quantitative analysis of cells positive for fos-like immunoreactivity, every third section of the brain region of interest was taken from each animal. In each section, the number of fos-positive cells was counted in a tissue area of 0.0025 mm^2 by an observer blind to the drug treatment. The average of these cell counts was calculated for each animal. Results in each brain structure are presented as means ± S.E.M for each treatment group.

Statistical analysis

Somatic signs of withdrawal were analysed using two-way ANOVA with chronic treatment (saline or nicotine) and acute treatment (THC or vehicle) as between-subjects factors of variation, followed by corresponding one-way ANOVAs and Dunnett´s *post-hoc* test. For the place conditioning experiments, score values were compared using one way ANOVA for each chronic treatment (saline and nicotine) followed by the Fisher´s *post-hoc* test. A Student's paired *t*-test was used to compare within each group the time spent during the preconditioning and testing phases in the withdrawal-associated compartment. Receptor and GTPγS binding values were compared by Student's *t*-test (saline *vs* nicotine). The number of c-Fos immunoreactive neurones were compared using one-way ANOVA (between subjects) followed by Dunnett´s *post-hoc* test.

Results

THC attenuates the somatic expression of nicotine withdrawal

In order to investigate whether THC could affect the expression of the somatic signs of nicotine withdrawal, mice chronically perfused with nicotine (25 mg/kg/día) received on day 6 an acute injection of mecamylamine or naloxone 15 min after THC (0, 0.3, 1 and 3 mg/kg) administration.

Mecamylamine-precipitated nicotine withdrawal

The following somatic signs were significantly revealed in the different experimental groups: paw-tremor, wet-dog shakes, genital licks, locomotor activity, ptosis, teeth chattering and scratches (see table 1 for two-way ANOVA and one-way ANOVA). Comparisons between saline and nicotine treated groups (one-way ANOVA) showed that paw tremor [$F(1,17) = 23.828$, $p < 0.001$], wet-dog shakes [$F(1,17) = 22.072$, $p < 0.001$], genital licks [$F(1,17) = 25.667$, $p < 0.001$] and scratches [$F(1,17) = 4.566$, $p < 0.05$] were significantly higher in mice receiving acute vehicle. Dunnett´s *post-hoc* test showed that the three doses of THC significantly decreased paw tremor and wet-dog shakes in nicotine treated mice ($p < 0.001$) compared to vehicle, and the highest dose of THC also significantly decreased genital licks ($p < 0.001$) and locomotor activity ($p < 0.01$). The doses of 0.3 and 3 mg/kg of THC also significantly decreased scratches in nicotine treated mice compared to vehicle ($p < 0.01$).

The analysis of the global withdrawal score (see table 1 for two-way ANOVA and one-way ANOVA) confirmed that the three doses of THC significantly decreased this global score in nicotine treated mice ($p < 0.001$) (Dunnett´s *post-hoc* test) compared to vehicle. However, comparisons between saline and nicotine treated groups (one-way ANOVA)

still revealed a significant manifestation of withdrawal in mice receiving acute THC at the dose of 0.3 ($p < 0.01$), 1 ($p < 0.001$) and 3 mg/kg ($p < 0.05$) (Fig. 1).

Naloxone-precipitated nicotine withdrawal

The following somatic signs were significantly revealed in the different experimental groups: paw-tremor, wet-dog shakes, genital licks, locomotor activity, ptosis, teeth chattering and scratches (see table 2 for two-way ANOVA and one-way ANOVA). Comparisons between saline and nicotine treated groups (one-way ANOVA) showed that paw tremor [$F(1,18) = 15.346$, $p < 0.001$] and wet-dog shakes [$F(1,18) = 10.236$, $p < 0.01$] were significantly higher in mice receiving acute vehicle. Dunnett´s *post-hoc* test showed that the doses of 1 and 3 mg/kg of THC significantly decreased paw tremor ($p < 0.001$) and wet-dog shakes ($p < 0.01$) in nicotine treated mice compared to vehicle.

The analysis of the global withdrawal score (see table 2 for two-way ANOVA and one-way ANOVA) confirmed that the doses of 1 and 3 mg/kg of THC significantly decreased the global withdrawal score in nicotine treated mice ($p < 0.001$) (Dunnett´s *post-hoc* test) compared to vehicle. However, comparisons between saline and nicotine treated groups (one-way ANOVA) still revealed a significant manifestation of withdrawal in mice receiving acute THC at the dose of 0.3 ($p < 0.001$), 1 ($p < 0.01$) and 3 mg/kg ($p < 0.05$) (Fig. 2).

SR 141716A-precipitated nicotine withdrawal

In order to investigate whether SR 141716A (1, 3 and 10 mg/kg) could precipitate a nicotine withdrawal syndrome, mice chronically perfused with nicotine received on day 6 an acute injection of this CB1 receptor antagonist, and the behavioural effects were compared to those produced by mecamylamine.

The following somatic signs were significantly revealed in the different experimental groups: paw-tremor, wet-dog shakes, genital licks, locomotor activity, ptosis, teeth chattering and scratches (see table 3 for two-way ANOVA and one-way ANOVA). Comparisons between saline and nicotine treated groups (one-way ANOVA) showed that paw tremor [$F(1,18) = 85.510$, $p < 0.001$] and teeth chattering [$F(1,18) = 12.950$, $p < 0.01$] were significantly higher in mice receiving acute mecamylamine, but this comparison did not reveal any significant effect in mice receiving SR 141716A in the different individual signs evaluated. Dunnett´*s post-hoc* test revealed a significantly lower value of paw tremor ($p < 0.001$) and teeth chattering ($p < 0.05$, $p < 0.05$ and $p < 0.01$) in nicotine treated mice receiving the three doses of SR 141716A compared to the group challenged with mecamylamine.

The analysis of the global withdrawal score (see table 3 for two-way ANOVA and one-way ANOVA) confirmed that the effects induced by the three doses of SR 141716A were significantly lower ($p < 0.001$, $p < 0.001$ and 0.05 respectively) than those elicited by mecamylamine in nicotine treated mice (Dunnett´s *post-hoc* test). In addition SR 141716A showed intrinsic behavioural effects in saline treated mice at all the doses used ($p < 0.05$, $p < 0.001$ and $p < 0.01$). However, comparisons between saline and nicotine treated groups (one-way ANOVA) revealed that the effects of SR 141716A administration at the dose of 10 mg/kg were significantly higher ($p < 0.01$) in the nicotine group indicating a selective behavioural response of the cannabinoid antagonist in nicotine-dependent mice (Fig. 3).

THC attenuates the dysphoric manifestations of nicotine withdrawal

The effect of the administration of THC (0.3 mg/kg, i.p.) on the dysphoric properties of nicotine withdrawal was evaluated. This dose of THC was chosen since it does not

produce any intrinsic behavioural response in the place conditioning paradigm (Valjent & Maldonado, 2000). Time spent in the withdrawal-paired compartment during pre-test was similar in the different experimental groups, which ensures the use of an unbiased procedure (one-way ANOVA, $F(5, 59) = 0.179$, $p = 0.970$). No significant differences among the different experimental groups were observed on the first day after the end of nicotine treatment (data not shown) which indicates that naloxone-induced conditioned place aversion could not be revealed in nicotine treated mice at this time point. However, significant differences were revealed by one-way ANOVA, [$F(2,30) = 3.358$, $p < 0.05$]) four days after nicotine administration. *Post hoc* analysis (Fisher's test) showed a significant decrease in the score of vehicle treated nicotine-dependent animals conditioned to naloxone-precipitated withdrawal as compared to vehicle treated nicotine-dependent animals conditioned with saline ($p = 0.05$). In addition, THC significantly blocked naloxone-precipitated aversive effect in nicotine-dependent animals ($p < 0.05$) (Fig. 4A). A significant decrease in the time spent in the withdrawal-paired compartment during the testing phase with respect to the time spent during the pre-conditioning phase was observed in the nicotine group treated with vehicle and naloxone (Student's *t* test, $p < 0.05$), but not in any other group (Fig. 4B). These results indicate that conditioned place aversion induced by naloxone in nicotine treated mice was reversed by THC pretreatment.

Autoradiography of CB1 cannabinoid receptor binding and WIN 55,212-2-stimulated [^{35}S]-GTPγS binding in chronic nicotine-treated mice

The qualitative and quantitative distribution of CB1 cannabinoid receptors labelled with [^{3}H]CP-55,940 in coronal sections of brains from control mice was similar to those previously reported (Herkenham *et al*., 1991; Mailleux & Vanderhaeghen, 1992). In this

line, the anatomical distribution of cannabinoid receptor-activated G-proteins showed a good correlation with the presence of cannabinoid receptor binding sites in the same brain structures.

As shown in tables 4 and 5, chronic nicotine treatment did not produce any significant effect in the levels of CB1 cannabinoid receptors nor in the activation of G-proteins by CB1 receptor estimulation in any of the brain structures analysed.

C-Fos expression following mecamylamine-precipitated nicotine withdrawal

One-way ANOVA revealed significant differences between groups in the number of Fos-positive nuclei in the caudate putamen [$F(3,16) = 3.083$, $p < 0.05$], dentate gyrus [$F(3,16) = 5.719$, $p < 0.01$] and basolateral nucleus of the amygdala [$F(3,16) = 4.250$, $p < 0.05$]. *Post-hoc* Dunnett's test showed a significant decrease on c-Fos expression in nicotine withdrawal group (nicotine-vehicle-mecamylamine) compared to control group (saline-vehicle-saline) in the caudate putamen and the dentate gyrus ($p < 0.05$ and $p < 0.01$, respectively). The number of Fos-positive nuclei was not significantly modified by chronic nicotine treatment and withdrawal in the other brain structures investigated: core and shell of the nucleus accumbens, CA1 and CA3 of the hippocampus, central nucleus of the amygdala and cingulate cortex (Fig. 5). In a second group of experiments, we investigated the effects of THC administration on these changes induced by nicotine withdrawal on c-Fos expression in the different brain regions. The administration of THC did not modify the expression of c-Fos neither in chronic nicotine treated mice nor in nicotine withdrawal animals in any of the brain areas investigated (data not shown).

Discussion

We have investigated the possible interaction between THC and nicotine in the manifestations of physical dependence. Thus, we have previously demonstrated that nicotine co-administration attenuated the development of THC tolerance and enhanced the somatic expression of cannabinoid antagonist-precipitated THC withdrawal (Valjent *et al.*, 2002). In the present study, we have evaluated the effects of acute THC administration on the somatic and motivational manifestations of nicotine withdrawal. Our results clearly show that acute THC administration attenuates the somatic expression and the dysphoric manifestations of nicotine withdrawal. This is revealed by a significant decrease of the somatic signs precipitated by mecamylamine and naloxone in nicotine-dependent mice, and a significant attenuation of the aversive response associated to naloxone-precipitated nicotine withdrawal.

Few studies have previously investigated the possible interactions between nicotine and THC. Thus, an early study showed that THC's acute depressant effects in the conditioned avoidance paradigm, locomotor activity, heart rate, body temperature and rotarod performance were potentiated by nicotine co-administration in rats (Pryor *et al.*, 1978). However, the responses evaluated in this previous study do not provide any information about the possible consequences of the association of these two compounds on addictive related behaviours. Acute THC effects on locomotor activity, body temperature and nociception were also reported to be facilitated by nicotine in a recent study (Valjent *et al.*, 2002). Furthermore, the co-administration of sub-threshold doses of THC and nicotine produced anxiolytic-like responses and conditioned place preference (Valjent *et al.*, 2002), whereas nicotine rewarding effects were absent in CB1 knockout mice (Castañé *et al.*, 2002). These findings could be explained by a possible interaction between cannabinoid and nicotine receptor/neurotransmitter systems

(Tripathi *et al.*,1987; Acquas *et al.*, 2000). In addition, a possible modulation in the activity of heterologous systems such as the dopaminergic and the opioid system could also be involved in these interactions (Di Chiara and Imperato, 1988; Szabo *et al.*, 1999; Valjent & Maldonado, 2000).

We have first evaluated the effects of THC on the somatic manifestations of nicotine abstinence. Chronic nicotine administration has been shown to induce physical dependence revealed by the incidence of several somatic signs after nicotine withdrawal in rodents (Epping-Jordan *et al.*, 1998; Hildebrand *et al.*, 1998; Isola *et al.*, 1999; Castañé *et al.*, 2002), and by irritability and stress in humans (Hughes *et al.*, 1991; West *et al.*, 1989). In our study the nicotine antagonist mecamylamine (1 mg/kg) and the opioid antagonist naloxone (3 mg/kg) were able to precipitate several somatic signs of withdrawal in mice chronically perfused with nicotine, in agreement with previous reports (Castané *et al.*, 2002; Malin *et al.*, 1993). The severity of withdrawal was higher in animals receiving mecamylamine than in those treated with naloxone, as expected (Watkins *et al.*, 2000). Acute THC pretreatment significantly decreased the severity of the different somatic manifestations of nicotine withdrawal precipitated by mecamylamine and naloxone. This result shows that the somatic expression of nicotine withdrawal can be modulated by acting on the cannabinoid system. One possible explanation for this behavioural effect could be the existence of an interaction between cannabinoids and nicotine neurotransmitter systems. Thus, cannabinoid agonists modulate the release and the turnover of Ach in various brain areas involved in nicotine behavioural responses. In this way, cannabinoid agonists cause an elevation of Ach release in hippocampus, cortex and striatum (Tripathi *et al*., 1987; Acquas *et al.*, 2000), and decreased Ach turnover in these structures (Revuelta *et al.*, 1978; Tripathi *et al.*, 1987).

To further clarify this interaction, we analysed whether the cannabinoid CB1 receptor antagonist SR 141716A was able to precipitate a withdrawal syndrome in nicotine-dependent mice. We have observed that SR 141716A induced intrinsic behavioural effects that could represent a bias for the interpretation of the results, mainly when administered at the doses of 1 and 3 mg/kg, that produced a similar effect in saline and nicotine treated mice. At the dose of 10 mg/kg, SR 141716A produced a significantly higher response in nicotine treated mice than in saline treated animals. However, this effect on the withdrawal score in nicotine treated mice could be due, at least in part, to the inverse agonist action of SR 141716A in CB1cannabinoid receptor (MacLennan et al., 1998; Pan et al., 1998; Sim-Selley et al., 2001; Mato et al., 2002). Therefore these results suggest that the endogenous cannabinoid system does not seem to play a major role in the physiological control of the somatic manifestations of nicotine withdrawal. In agreement, the severity of nicotine abstinence was not modified in knockout mice lacking the CB1 receptor (Castañé *et al.*, 2002).

In a second group of experiments, the effects of THC administration were evaluated on the aversive manifestations of nicotine withdrawal. In a preliminary experiment, different doses of mecamylamine were not able to produce an aversive response associated to nicotine withdrawal. It is important to point out that the doses of mecamylamine previously reported to produce aversive manifestations in nicotine-dependent rats also induced aversive effects in chronically saline treated rats (Watkins *et al.*, 2000; Kenny & Markou, 2001). Other studies did not reveal such an aversive effect of mecamylamine in saline treated rats (Suzuki *et al.*, 1996; Suzuki *et al.*, 1999), but drug motivational responses in this paradigm can be influenced by multiple factors such as species, gender, strain and environment (Milton *et al.*, 1995). To our knowledge, aversive manifestations of nicotine withdrawal have not been yet reported

after mecamylamine administration in mice. The aversive manifestations of nicotine withdrawal precipitated by naloxone administration were also attenuated by pretreatment with THC. The dose of THC (0.3 mg /kg, i.p.) was chosen in this study considering the alleviatory effects observed on the somatic expression of nicotine withdrawal in the previous experiment and the results of former studies on the motivational effects of THC in the place conditioning paradigm. Indeed, we have previously reported that THC at the dose of 1 mg/kg produces a conditioned place preference in mice, whereas higher doses of THC (5 mg/kg) are able to produce a conditioned place aversion in this animal species (Valjent & Maldonado, 2000). These intrinsic behavioural effects of THC would prevent the use of higher doses in this particular paradigm.

The aversive manifestations of drug withdrawal have been related to the inhibition of the dopamine release in the nucleus accumbens (Schechter & Meechan, 1994). Acute cannabinoid administration has been reported to increase dopamine extracellular levels in the nucleus accumbens (Di Chiara & Imperato, 1988; Dani & Heinemann, 1996; Tanda *et al.*, 1997). Thus, activation of CB1 cannabinoid receptors presynaptically inhibits GABAergic neurotransmission in the ventral tegmental area, which would increase dopamine neurons firing rate and the release of dopamine in the nucleus accumbens (Szabo *et al.*, 2002). This sequence of events might explain the ability of THC to prevent the aversive manifestations of nicotine withdrawal in the present work.

In order to investigate whether **the behavioural changes induced by THC on the somatic expression of nicotine withdrawal could be due to possible adaptive changes induced by** nicotine exposure on CB1 cannabinoid receptors, we analysed the density and the functional activity of these receptors during chronic nicotine treatment. The number and distribution of CB1 cannabinoid receptor binding sites, and the

stimulation of GTPγS-binding proteins by WIN 55,212-2 were not modified by chronic nicotine treatment in the different brain structures studied. Accordingly with our results, González *et al.* (2002) have shown using different experimental conditions that chronic nicotine exposure in rats did not modify CB1 cannabinoid receptor binding in cortical, limbic, motor and hippocampal regions. However, a significant increase in the content of the two main endocannabinoids, arachidonoylethanolamide and 2-arachidonoyl-glycerol, was observed in the brainstem of these animals (González *et al.*, 2002), a region that contains nuclei directly related to the manifestations of drug withdrawal symptoms (Maldonado, 1997). This increase does not seem to play an important role on the somatic expression of nicotine withdrawal since only the highest dose of SR 141716A (10 mg/kg) increased the withdrawal score in nicotine treated mice, and the severity of nicotine abstinence was not modified in CB1 knockout mice (Castañé *et al.*, 2002).

Addictive related behaviours are linked to different molecular adaptations, such as gene regulation, that are observed in discrete brain areas (Berke & Hyman, 2000; Nestler, 2000). Thus, nicotine treatment and self-administration have been shown to increase Fos-like immunoreactivity in several brain regions, including areas which receive dopaminergic innervation from the ventral tegmental area (Matta *et al.*, 1993; Nisell *et al.*, 1997; Pagliusi *et al.*, 1996; Panagis *et al.*, 1996; Pang *et al.*, 1993; Ren & Sagar, 1992; Salminen *et al.*, 1996). Our histochemical analysis demonstrated that nicotine withdrawal decreases the number of Fos-positive nuclei in the caudate putamen and the dentate gyrus of the hippocampus, whereas no significant changes in c-Fos expression were observed in the other areas. Few reports have previously studied the immediate early gene induction following nicotine withdrawal. Panagis *et al.* (2000) demonstrated that nicotine withdrawal increases the number of Fos-positive nuclei selectively in the

central nucleus of the amygdala, but not in other brain structures. In the present work, the acute administration of THC did not modify the effects of mecamylamine-precipitated nicotine withdrawal on c-Fos expression in the different brain areas investigated. Previous studies have reported a facilitatory effect induced by the acute co-administration of nicotine and THC on c-Fos expression in several brain structures (Valjent *et al.*, 2002). In the present work, THC administration did not modify c-Fos expression after chronic nicotine administration and withdrawal in any of the brain regions investigated, suggesting a different interaction between these two drugs when nicotine dependence has been developed.

In summary, the present results demonstrate that THC administration attenuates the somatic manifestations **of nicotine withdrawal. This response was not due to** compensatory changes on CB1 cannabinoid receptors density or functional activity during chronic nicotine exposure**. In addition, THC decreased the aversive effects associated to nicotine withdrawal.** This behavioural finding provides important insights to better understand the interactions that may occur when tobacco and cannabis consumption overlaps in humans.

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Abbreviations

CNS: central nervous system; THC: Δ^9-tetrahydrocannabinol.

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Figure legends

Fig. 1. Attenuation of the severity of mecamylamine-precipitated nicotine withdrawal syndrome by acute Δ^9-tetrahydrocannabinol (THC) administration. Counted (wet dog shakes, paw tremor and scratches) and checked (genital licks, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed during 30 min immediately after mecamylamine (1 mg/kg, sc) administration. THC (0.3, 1 and 3 mg/kg, i.p.) or vehicle (VEH) were administered 15 min before withdrawal. A global withdrawal score was calculated for each animal as described in the methods. Data are expressed as mean ± S.E.M. (n = 9-10 mice for each group). ☆☆ $p < 0.01$; ☆☆☆ $p < 0.001$ compared to vehicle group (Dunnett's test). ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$ comparisons between similar groups receiving chronic saline or nicotine (One-way ANOVA).

Fig. 2. Attenuation of the severity of naloxone-precipitated nicotine withdrawal syndrome by acute Δ^9-tetrahydrocannabinol (THC) administration. Counted (wet dog shakes, paw tremor and scratches) and checked (genital licks, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed during 30 min immediately after naloxone (3 mg/kg, sc) administration. THC (0.3, 1 and 3 mg/kg, i.p.) or vehicle (VEH) were administered 15 min before withdrawal. A global withdrawal score was calculated for each animal as described in the methods. Data are expressed as mean ± S.E.M. (n = 10-11 mice for each group). ☆☆ $p < 0.01$; ☆☆☆ $p < 0.001$ when compared to vehicle group (Dunnett's test). ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$ comparisons between similar groups receiving chronic saline or nicotine (One-way ANOVA).

Fig. 3. Severity of nicotine withdrawal precipitated by acute administration of mecamylamine (MEC) or the CB1 receptor antagonist SR 141716A. Counted (wet dog shakes, paw tremor and scratches) and checked (genital licks, ptosis, teeth chattering and piloerection) somatic signs of withdrawal were observed during 30 min immediately after SR 141716A (1, 3 and 10 mg/kg, i.p.) or MEC (1 mg/kg, sc) administration. A global withdrawal score was calculated for each animal as described in the methods. Data are expressed as mean ± S.E.M. (n = 10 mice for each group). ⋆ $p < 0.05$; ⋆⋆ $p < 0.01$; ⋆⋆⋆ $p < 0.001$ compared to nicotine-mecamylamine group ; △ $p < 0.05$; △△ $p < 0.01$; △△△ $p < 0.001$ compared to saline-mecamylamine group (Dunnett's test). ****** $p < 0.01$; ******* $p < 0.001$ comparisons between similar groups receiving chronic saline or nicotine (One-way ANOVA).

Fig. 4. Δ^9-tetrahydrocannabinol (THC) prevented naloxone (NLX) induced nicotine (NIC) withdrawal place aversion. The place aversion score is shown in (A) and was calculated as the difference between the time (sec) spent in the withdrawal associated compartment during the testing and the pre-conditioning phase (see methods for details). Absolute values of the time spent in the withdrawal-associated compartment during the pre-conditioning (white bars) and the testing phase (black bars) are shown in (B). Data are expressed as mean ± S.E.M. (n = 10-11 mice for each group). ***** $p = 0.05$ compared to vehicle-saline (VEH-SAL) group (Fischer's test). ⋆ $p < 0.05$ compared to VEH-NLX group (Fischer's test). △ $p < 0.05$ comparison within each group of the time spent during the pre-conditioning and testing phase (Student's t-test).

Fig. 5. C-Fos expression following chronic nicotine (NIC) treatment and mecamylamine (MEC)-precipitated nicotine withdrawal in the caudate putamen (Cpu); core of the nucleus accumbens (Nac); shell of the nucleus accumbens (Nas); dentate gyrus (DG); CA1 and CA3 of the hippocampus; basolateral nucleus of the amygdala (BLA); central nucleus of the amygdala (CeA); cingulate cortex (CgC). Data are expressed as mean ± S.E.M. (n = 5 for each group).* $p < 0.05$ and** $p < 0.01$ compared to saline-vehicle-saline (SAL-VEH-SAL) group (Dunnett´s test).