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Behavioural and biochemical evidence for interactions between Δ9-tetrahydrocannabinol and nicotine

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1 Behavioural and pharmacological effects of Δ9-tetrahydrocannabinol (THC) and nicotine are well known. However, the possible interactions between these two drugs of abuse remain unclear in spite of the current association of cannabis and tobacco in humans.

2 The present study was designed to analyse the consequences of nicotine administration on THC-induced acute behavioural and biochemical responses, tolerance and physical dependence.

3 Nicotine strongly facilitated hypothermia, antinociception and hypolocomotion induced by the acute administration of THC. Furthermore, the co-administration of sub-threshold doses of THC and nicotine produced an anxiolytic-like response in the light–dark box and in the open-field test as well as a significant conditioned place preference. Animals co-treated with nicotine and THC displayed an attenuation in THC tolerance and an enhancement in the somatic expression of cannabinoid antagonist-precipitated THC withdrawal.

4 THC and nicotine administration induced c-Fos expression in several brain structures. Co-administration of both compounds enhanced c-Fos expression in the shell of the nucleus accumbens, central and basolateral nucleus of the amygdala, dorso-lateral bed nucleus of the stria terminalis, cingular and piriform cortex, and paraventricular nucleus of the hypothalamus.

5 These results clearly demonstrate the existence of a functional interaction between THC and nicotine. The facilitation of THC-induced acute pharmacological and biochemical responses, tolerance and physical dependence by nicotine could play an important role in the development of addictive processes.

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Abbreviations: Ach, acetylcholine; CNS, central nervous system; THC, Δ9-tetrahydrocannabinol

Introduction

Δ9-tetrahydrocannabinol (THC) is the main psychoactive component of *Cannabis sativa*, the most widely consumed illicit drug in humans (Adams & Martin, 1996). The consumption of cannabis is highly associated with tobacco, which contains nicotine, another important psychoactive compound (Wise, 1996 for review). The administration of THC and nicotine in rodents produces multiple common pharmacological responses 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 cannabinoid CB1 receptors (Herkenham *et al*., 1990; Tsou *et al*., 1998), which are metabotropic receptors (Matsuda *et al*., 1990), and the nicotinic acetylcholine (Ach) receptors (Martin & Aceto, 1981; Luetje *et al*., 1990), which are pentamers made up of various subunits (Cordero-Erausquin *et al*., 2000 for review). The use of pharmacological antagonists and knock-out mice for CB1 receptors and for specific subunits of the nicotinic Ach receptors have shown the exclusive role of these receptors in the behavioural responses induced by cannabinoids (Ledent *et al*., 1999; Zimmer *et al*., 1999) and nicotine (Orr-Urtreger *et al*., 1997; Picciotto *et al*., 1998; Marubio *et al*., 1999; Xu *et al*., 1999a, b), respectively.

Both endogenous cannabinoid and cholinergic systems are crucial modulatory pathways in the CNS (Ameri, 1999; Calabresi *et al*., 2000 for review), and several studies have suggested a possible functional interaction between these two systems. Interestingly, cannabinoid agonists modulate the release and the turnover of Ach in various brain areas. Thus, 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). However, this modulation remains controversial since cannabinoid agonists have been also reported to produce an inhibition of the electrically evoked release of Ach in hippocampal slices and in hippocampal and cortical synaptosomes (Gifford & Ashby, 1996, Gifford *et al*., 1997; 2000),

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and to decrease *in vivo* Ach release in the prefrontal cortex and hippocampus (Carta *et al*., 1998; Gessa *et al*., 1998a; Nava *et al*., 2000).

The specific behavioural and biochemical consequences of the interaction between THC and nicotine are poorly documented in animal models in spite of the high frequency of association of these two substances in humans. Only one early study has reported an acute behavioural interaction in rats between these two compounds on locomotor activity, heart rate and body temperature (Pryor *et al*., 1978). Furthermore, the cataleptic effects induced by THC have been reported to be facilitated by muscarinic agonists (Pertwee & Ross, 1991).

The present study was designed to analyse the consequences of nicotine on THC-induced acute behavioural and biochemical responses, tolerance and physical dependence. For this purpose, we have first evaluated the acute effects of the co-administration of nicotine and THC on locomotion, nociception and body temperature, as well as the development of tolerance and dependence induced by the chronic co-administration of both compounds. In a second set of experiments, we have investigated the effects of the co-administration of low doses of nicotine and THC on anxiolytic-like responses and rewarding properties. It is important to point out that doses of THC (Cook *et al*., 1998; Hutcheson *et al*., 1998; Ledent *et al*., 1999; Lichtman *et al*., 2001) and nicotine (Costall *et al*., 1989; Rissinger & Oakes, 1995; Hildebrand *et al*., 1999; Watkins *et al*., 2000) required to induce these anxiolytic and rewarding effects are much lower than those needed to develop tolerance and dependence. Taking into account these complex dose/response effects induced by both THC and nicotine, a different range of doses is required to perform these two independent groups of experiments. Therefore, the interpretation of these results must be limited to the particular experimental conditions used in each case. Finally, we investigated the consequences of the co-administration of THC and nicotine on c-Fos expression in several brain structures. We clearly demonstrate the existence of an interaction between THC and nicotine that could play an important role in the development of addictive properties.

Methods

Animals and drugs

Male CD-1 mice (Charles River, France) weighing 22–24 g were housed ten per cage, acclimated to the laboratory conditions (12 h light–dark cycle, $21 \pm 1^{\circ}C$ room temperature) and manipulated by the investigators for 1 week prior to the experiment. Food and water were available *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 directive 86/609/EEC) and approved by the local ethical committees. All experiments were performed with the investigators being blind to the treatment conditions. THC (Sigma, U.K.) and the selective CB1 receptor antagonist SR 141716A [(N-piperidin-1-yl)-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxyamide] (Sanofi Recherche, France) (Rinaldi-Carmona *et al*., 1994) were administrated by intraperitoneal route. THC was dissolved in a solution of 5% ethanol, 5% cremophor El and 90% distilled water, and SR 141716A was dissolved in a solution of 10% ethanol, 10% cremophor El and 80% distilled water. (−)-Nicotine (Sigma, France) was administrated subcutaneously and dissolved in saline 0.9%.

THC-induced antinociception, hypolocomotion and hypothermia

Spontaneous locomotor activity was measured by using individual locomotor activity boxes ($9 \times 20 \times 11$ cm, Imetronic, France). Each box contained a line of photocells 2 cm above the floor to measure horizontal activity, and another line located 6 cm above the floor to measure vertical activity (rears). Mice were placed in the boxes 5 min after drug injection and locomotion was recorded during 15 min in a low luminosity environment (20–25 lux). Rectal temperature was measured 20 min after drug injection using an electronic thermocouple flexible probe (Panlab, Spain) which was placed 3 cm into the rectum for 30 s before the temperature was recorded. The tail-immersion test was measured 20 min after drug administration as previously described (Simonin *et al*., 1998). Mice were loosely restrained inside a clear plexiglass cylinder prior to immersion of the tail in hot water ($50 \pm 0.5^{\circ}C$). The trial was terminated once the animal flicked its tail. In the absence of tail-flick, a 10 s cut-off was used to prevent tissue damage. The hot plate test was based on that described (Simonin *et al*., 1998). A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mice on the heated surface of the plate, which was kept at a temperature of $50 \pm 0.5^{\circ}C$. The nociceptive threshold evaluated was the jumping response. In the absence of jumps, a 240 s cut-off was used to prevent tissue damage.

Measurement of THC tolerance and physical dependence

THC (0, 5 and 10 mg kg^{-1}, i.p.) and nicotine (0 and 0.5 mg kg^{-1}, s.c.) were given alone or co-administrated twice a day for 5 days (0900 and 1900). On day 6, mice only received the morning injection (0900) ($n = 10$ mice per group).

During chronic treatment, three different responses were measured: body weight, antinociception and rectal temperature. Body weight was recorded for each mouse using an electronic balance (Metter PM 4800, sensitive to 0.01 g), twice a day before each morning and evening injection. Rectal temperature was measured on days 1 and 2, prior to and 20 min after each injection. On days 3, 4, 5 and 6 rectal temperature was evaluated before and 20 min after the morning injection only. Nociceptive threshold was evaluated immediately after rectal temperature measurements, (days 1 and 2: prior to and 20 min after each injection; days 3, 4, 5 and 6: prior to and 20 min after morning injection).

Four hours after the last chronic THC injection on day 6, mice were placed inside a circular clear plastic observation area for a 15 min period of habituation. Immediately after habituation, animals received an acute administration of the selective CB1 receptor antagonist, SR 141716A (10 mg kg^{-1}, i.p.) (Rinaldi-Carmona *et al*., 1994) and were then returned to the chamber for an additional 45 min observation period. Behavioural observations before and after SR 141716A challenge were divided into 5 min time intervals. Somatic

signs of withdrawal were quantified as previously described (Hutcheson *et al*., 1998; Ledent *et al*., 1999). The number of bouts of wet dog shakes, paw tremors and sniffing were counted during each period of observation. Piloerection, tremor, hunched posture, ptosis and mastication were scored as 1 if present, and as a 0 if absent, during each 5 min interval. A quantitative value was calculated for these different checked signs by adding the scores obtained for each of the 5 min period. A global withdrawal score was calculated for each animal by giving at each sign a proportional weight as previously reported (Valverde *et al*., 2000). Values for the global score ranged from 0 to 100.

Emotional-like responses

The possible acute interaction between THC (0, 0.03, 0.1, 0.3, 1 and 5 mg kg^{-1}) and nicotine (0 and 0.12 mg kg^{-1}) on emotional-like responses was evaluated in the light–dark box (Filliol *et al*., 2000) and in the open-field test (Simonin *et al*., 1998).

The light–dark box was composed by a small, dimly lit (5 lux) black compartment ($15 \times 20 \times 25$ cm) connected *via* a 4 cm long tunnel to a large, brightly lit (500 lux) white compartment ($30 \times 20 \times 25$ cm). Lines on the floors of both compartments permitted the measurement of locomotor activity (number of squares crossed). Each animal was placed in the dark compartment facing the tunnel at the beginning of each session which start 30 min after the acute injection of THC and/or nicotine. Locomotor activity and time spent in each compartment were then recorded for a period of 5 min.

The open-field was a rectangular area (70 cm wide, 90 cm long and 60 cm high) brightly illuminated from the top (500 lux). A total of 63 squares (10×10 cm) were drawn with black lines on the white floor of the field. Four events were recorded during an observation period of 5 min: total number of squares crossed, total number of rears, number of entries in the central area, and time spent in the central area. Mice were exposed to the test 30 min after THC and/or nicotine administration.

Conditioned place preference

An unbiased place conditioning procedure was used to evaluate the motivational consequences of the co-administration of THC (0.3 and 1 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}). The apparatus consisted of two main square conditioning compartments ($15 \times 15 \times 15$ cm) separated by a triangular central division (Maldonado *et al*., 1997). The movement and the location of the mice were monitored by computerized monitoring software (Videotrack, View Point, Lyon, France). During the preconditioning phase, drug naive mice were placed in the middle of the central division and had free access to both compartments (striped and dotted compartment) of the conditioning apparatus for 20 min, with the time spent in each compartment recorded. Time spent by all the mice in the two conditioning compartments of the apparatus was similar during the preconditioning phase (striped compartment: 438.6 ± 23.8 s, dotted compartment: 445.4 ± 22.0 s). No initial place preference or aversion was observed in any of the experimental groups. For conditioning phase, an elevated number of pairings (five pairings with drug plus five pairings with vehicle) and a long conditioning time (45 min) were used, as previously described (Valjent & Maldonado, 2000). Care was taken to ensure that treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The test phase was conducted exactly as in the preconditioning phase, i.e., free access to each compartment for 20 min. Mice were exposed only once to the preconditioning and test phases. All mice received the first injection of drug or vehicle on the first day of conditioning, excepting the group treated with the dose of 1 mg kg^{-1} of THC which received a single drug injection in the home cage 24 h before starting the place preference conditioning procedure. A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment.

Tissue preparation and immunocytochemistry technique

Mouse brains were fixed by intracardial perfusion of 4% paraformaldehyde (PFA) in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5, delivered with a peristaltic pump at 10 ml min^{-1} during 5 min. Brains were removed and post-fixed overnight in the same fixative solution. Sections (30 μm) were cut on a vibratome (Leica, Germany) and then kept in a solution containing 30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, and 0.1% diethyl pyrocarbonate (Sigma, Deisenhofen, Germany) at -20°C until they were processed for immunocytochemistry. The immunocytochemical procedure was adapted from previously described protocols (Valjent *et al*., 2000). Day 1: Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), and incubated successively in TBS containing 3% H_2O_2 and 10% methanol (5 min), and then incubated in 0.2% Triton X-100 in TBS (15 min). After three rinses they were incubated overnight with the primary antibody (see below) at 4°C. Day 2: After three rinses in TBS, the sections were incubated for 2 h at room temperature with the secondary biotinylated antibody (anti-IgG), using a dilution twice that of the first antibody in TBS. After being washed, the sections were incubated for 90 min in avidin–biotin–peroxidase complex solution (Vector Laboratories. Peterborough, U.K.). Then the sections were washed in TBS and two times in TB (0.25 M Tris, pH 7.5) for 10 min each, placed in a solution of TB containing 0.1% 3,3′-diaminobenzidine (50 mg 100 ml^{-1}), and developed by H_2O_2 addition (0.02%). After processing, the tissue sections were mounted onto gelatin-coated slices and dehydrated through alcohol to xylene for light microscopic examination. The c-Fos antibody was a polyclonal antibody directed against residues 3–16 of human c-Fos (Santa Cruz, U.S.A.). The dilution used for immuno-staining was 1 : 500 for c-Fos. Fos immunoreactive neurons were plotted using an image analyser (Biocom, France).

Statistical analysis

Acute behavioural measurements, somatic signs of withdrawal and number of c-Fos immunoreactive neurones were compared using one-way ANOVA (between subjects) followed by a Newman–Keuls *post-hoc* comparison. Values from the tolerance study were analysed by using a two-way ANOVA with repeated measures. The factors of variation were treatment (between subjects) and time (within subjects).

Individual group comparisons were then conducted for each time point using one-way ANOVA (between subjects) followed by a Newman–Keuls *post-hoc* comparison. For the place conditioning experiment, score values were compared using one-way ANOVA (between subjects) followed by a Newman–Keuls *post-hoc* comparison. Values for the time spent for each group of mice in drug-paired compartment during the preconditioning and post-conditioning measurements were compared by using a 2-tailed Student's paired *t*-test.

Results

Nicotine potentiates acute responses induced by THC

The interaction between THC (0, 5 and 10 mg kg^{-1}) and nicotine (0 and 0.5 mg kg^{-1}) was first studied on the classical acute effects induced by cannabinoid agonists in mice (Dewey *et al*., 1970; Anderson *et al*., 1975; Lichtman & Martin, 1991). One-way ANOVA (between subjects) revealed significant effects of treatment on spontaneous locomotor activity ($F_{(5,\ 54)}=28.58$, $P<0.001$) (Figure 1A), rectal temperature ($F_{(5,\ 54)}=28.56$, $P<0.001$) (Figure 1B) and antinociceptive responses in the hot plate ($F_{(5,\ 54)}=14.95$, $P<0.001$) (Figure 1C) and tail-immersion tests ($F_{(5,\ 54)}=39.62$, $P<0.001$) (Figure 1D). *Post hoc* comparisons (Newman–Keuls) showed that acute injection of a high dose of THC (10 mg kg^{-1}) induced hypolocomotion ($P<0.01$), hypothermia ($P<0.01$) and antinociceptive responses in the hot plate and tail-immersion tests ($P<0.01$) (Figure 1A–D). At lower dose, THC (5 mg kg^{-1}) slightly decreased the locomotor activity but failed to reveal any effect in the other responses. When given alone, nicotine (0.5 mg kg^{-1}) failed to induce any response. However, the co-administration of THC (5 and 10 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) markedly enhanced the responses induced by THC alone. Indeed, *post hoc* comparisons (Newman–Keuls) revealed that THC (5 or

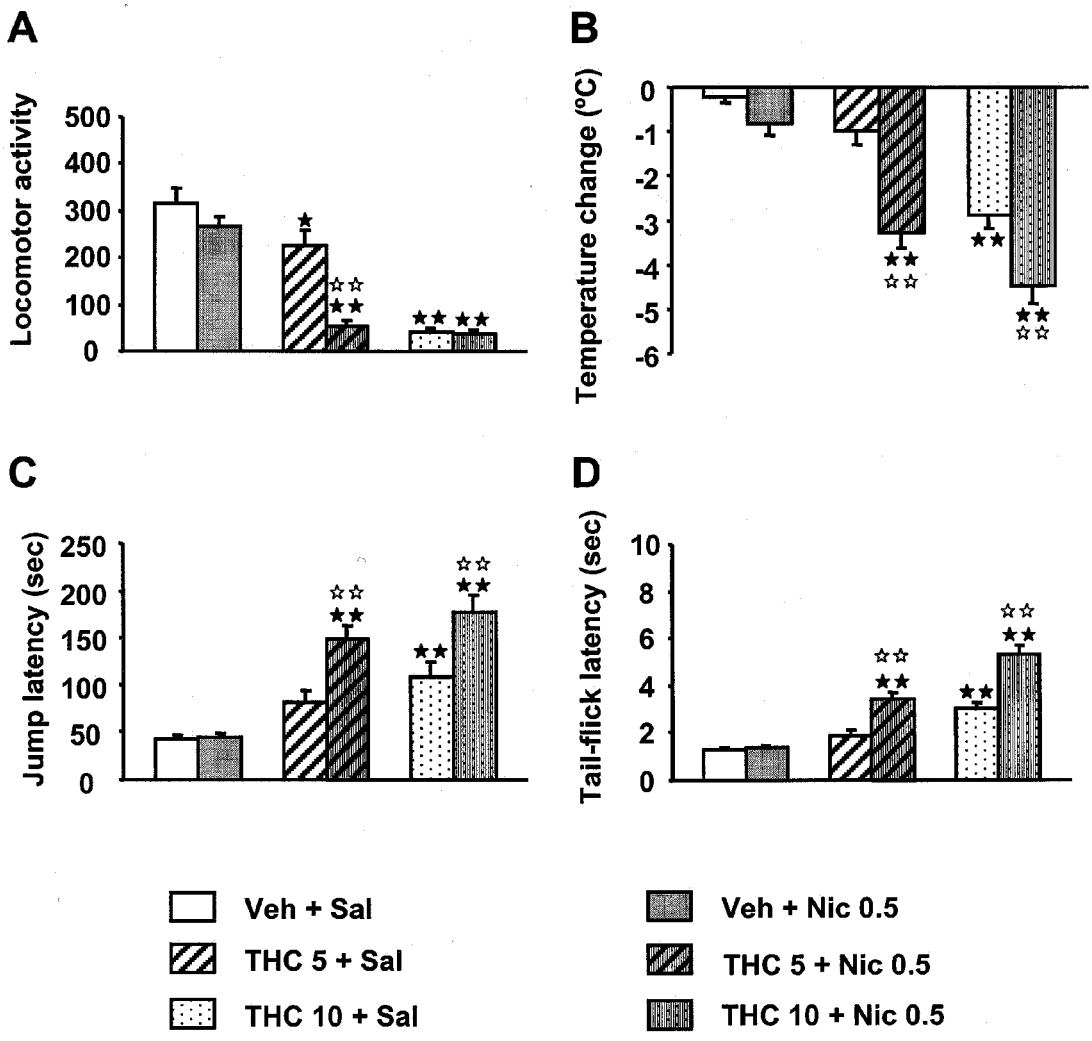

Figure 1 Nicotine facilitates acute responses induced by THC. Locomotor activity (A), body temperature (B), antinociceptive responses in the hot plate (C) and tail-immersion test (D) were measured after acute administration of THC (0, 5 and 10 mg kg^{-1}) and nicotine (0 and 0.5 mg kg^{-1}) given alone or in association. Note the strong potentiation of THC-induced acute effects when nicotine is added. Data are expressed as mean ± s.e.mean ($n=10$ mice for each group). Statistical analysis were performed using one-way ANOVA (between subjects) followed by *post-hoc* comparisons using the Newman–Keuls test. ★ $P<0.05$; ★★ $P<0.01$ when comparing with vehicle group. ☆☆ $P<0.01$ when comparing with THC group (Newman–Keuls test).

10 mg kg^{-1}) in association with nicotine produced a response that was significantly higher than the one observed in mice receiving only THC ($P<0.01$) (Figure 1A–D).

Nicotine attenuates the development of tolerance to antinociceptive and hypothermic effects of THC

Tolerance to antinociceptive effects Upon repeated treatment, THC (5 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) alone failed to produce antinociceptive responses in the tail-immersion test (Figure 2A). When co-administrated, a significant increase in the tail-flick latency was observed as compared to saline ($P<0.01$) and THC alone ($P<0.01$). This antinociceptive effect was observed during the first four days of chronic treatment ($P<0.01$) (Table 1, Figure 2A). At the dose of 10 mg kg^{-1}, THC alone produced significant antinociception on the first day (morning and evening) ($P<0.01$) (Table 1, Figure 2B). Then, a rapid tolerance to this THC response was

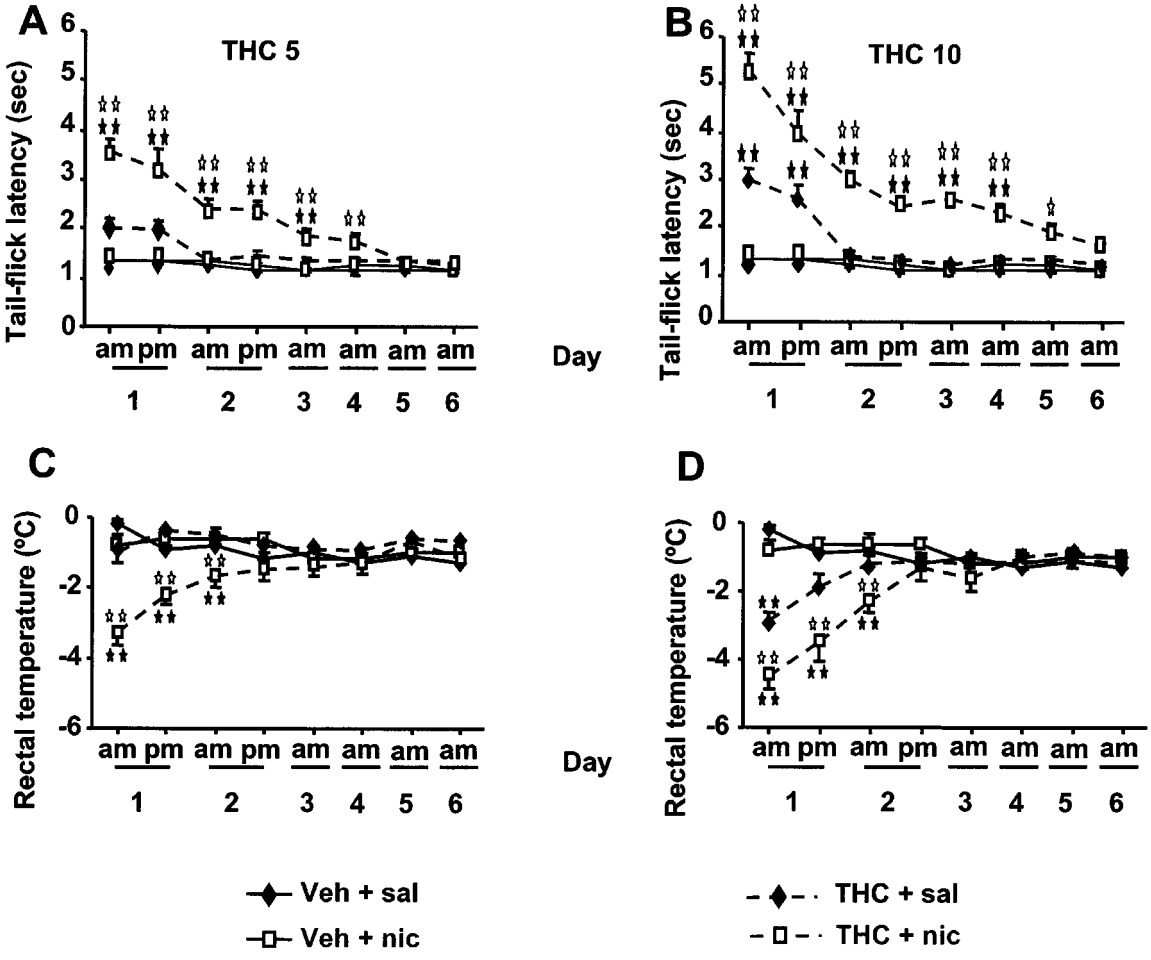

Figure 2 Decrease of tolerance to THC effects by chronic co-administration of THC and nicotine. Tolerance to antinociceptive (A and B) and hypothermic effects (C and D) were measured for 6 consecutive days. These measurements were performed twice daily on day 1 and 2, and once on day 3, 4, 5 and 6. Note that the development of tolerance is slower when THC is co-administrated with nicotine than with THC alone. Data are expressed as mean ± s.e.mean ($n=10$ mice for each group). Statistical analysis were performed using two-way ANOVA with treatment (between subjects) and days (within subjects) as factor of variation, followed by one-way ANOVA and *post-hoc* comparisons using the Newman–Keuls test. ★★$P<0.01$ when comparing with vehicle group. ☆$P<0.05$; ☆☆$P<0.01$ when comparing with THC group (Newman–Keuls test).

Table 1 Tolerance to THC-induced hypothermia and antinociception after chronic co-administration of THC and nicotine

			Two-way ANOVA			
	Treatment	P-*value*	*Days*	P-*value*	*Interaction*	P-*value*
Rectal temperature						
THC 5 mg/kg	F(3, 36) = 6.025	<0.001	F(7, 252) = 3.388	<0.001	F(21, 252) = 8.193	<0.001
THC 10 mg/kg	F(3, 36) = 4.429	<0.001	F(7, 252) = 10.854	<0.001	F(21, 252) = 10.996	<0.001
Antinociception						
THC 5 mg/kg	F(3, 36) = 65.136	<0.001	F(7, 252) = 23.772	<0.001	F(21, 252) = 8.225	<0.001
THC 10 mg/kg	F(3, 36) = 132.936	<0.001	F(7, 252) = 47.035	<0.001	F(21, 252) = 14.957	<0.001

Two-way ANOVA with treatment (between subjects) and days (within subjects) as factor of variations. See Methods for details.

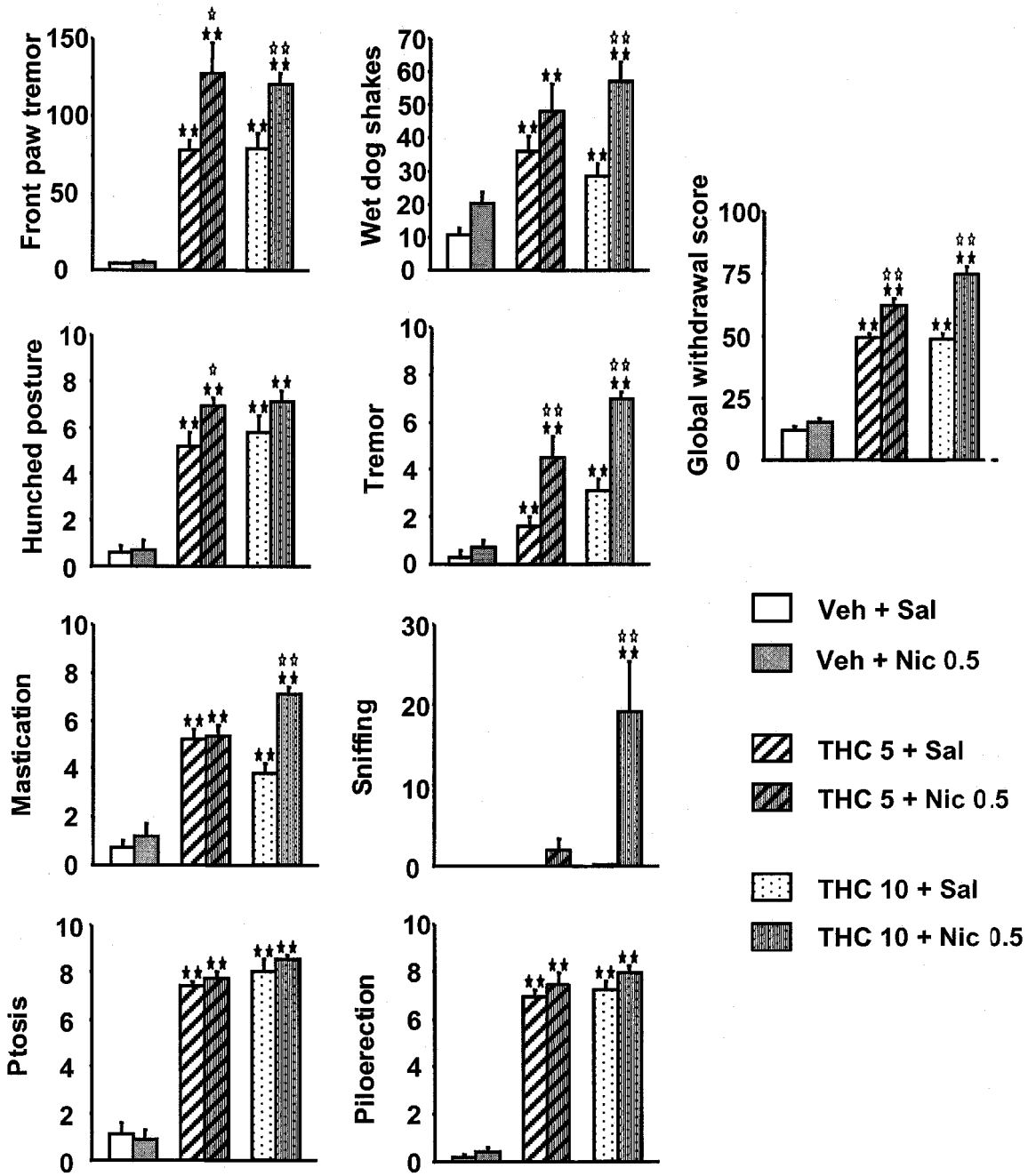

Figure 3 Severity of THC withdrawal syndrome is enhanced in mice co-administrated with THC and nicotine. Abstinence was precipitated by acute administration of the CB1 receptor antagonist SR 141716A after chronic THC treatment during 6 days. Counted (wet dog shakes, front paw tremor and sniffing) and checked (ptosis, hunched posture, tremor, piloerection and mastication) somatic signs of withdrawal were observed for 45 min immediately after SR 141716A administration. Six over eight abstinence signs were significantly enhanced in mice co-treated with THC and nicotine. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight. Values for global withdrawal score ranged from 0 to 100. Data are expressed as mean ± s.e.mean ($n = 10$ mice for each group). Statistical analysis were performed as described in Figure 1. ★★$P < 0.01$ when comparing with vehicle group. ☆$P < 0.05$; ☆☆$P < 0.01$ when comparing with THC group (Newman–Keuls test).

developed since no significant effects were found during the remaining chronic treatment. Interestingly, when THC (10 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) were associated, the antinociceptive effects induced by THC were strongly enhanced in amplitude and duration. Thus, the effects of the association were significantly higher than the corresponding THC and saline groups during the five days of co-administration ($P < 0.01$) (Table 1, Figure 2B).

Tolerance to hypothermic effects When given alone, THC (5 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) failed to modify body temperature during the repeated administration (Figure

2C). At higher dose, THC (10 mg kg^{-1}) produced a significant hypothermia only the first day of injection (morning $P<0.01$, evening $P<0.05$) (Table 1, Figure 2D). The co-administration of THC (5 mg kg^{-1} or 10 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) produced a longer (day 1 morning and evening $P<0.01$, day 2 morning $P<0.01$) and more robust hypothermia than THC alone (Table 1, Figure 2C,D).

Nicotine potentiates the somatic expression of THC abstinence

In order to investigate whether nicotine could affect the expression of the somatic signs of THC withdrawal syndrome, mice chronically treated with THC (5 or 10 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) received on day 6 an acute injection of the selective CB1 receptor antagonist SR 141716A (10 mg kg^{-1}). One-way ANOVA (between subjects) revealed a significant incidence of the following somatic signs of THC withdrawal: front-paw tremor ($F_{(5,53)}=29.914$, $P<0.001$), wet dog shakes ($F_{(5,53)}=11.327$, $P<0.001$), ptosis ($F_{(5,53)}=95.553$, $P<0.001$), hunched posture ($F_{(5,53)}=34.285$, $P<0.001$), tremor ($F_{(5,53)}=27.264$, $P<0.001$), piloerection ($F_{(5,53)}=122.21$, $P<0.001$), mastication ($F_{(5,53)}=38.697$, $P<0.001$) and sniffing ($F_{(5,53)}=8.432$, $P<0.01$) (Figure 3). *Post hoc* comparisons (Newman–Keuls) showed a significant expression of front-paw tremor, wet dog shakes, ptosis, hunched posture, mastication, piloerection and tremor in THC-treated mice, and in THC and nicotine co-treated animals. The severity of several somatic signs of withdrawal observed in mice receiving THC alone was significantly enhanced by the co-administration of THC and nicotine, as revealed by *post hoc* comparisons between these two groups (Newman–Keuls): front-paw tremor ($P<0.01$), wet dog shakes ($P<0.01$), tremor ($P<0.01$), mastication ($P<0.01$) and sniffing ($P<0.01$). The incidence of piloerection and ptosis was similar in mice treated with THC alone or associated with nicotine. SR 141716A injection in chronically nicotine-treated mice failed to induce any behavioural sign of withdrawal (Figure 3).

The analysis of the global withdrawal scores confirmed that the administration of SR 141716A precipitated a significant withdrawal syndrome in all the mice receiving chronic THC ($F_{(5,53)}=121.171$, $P<0.001$) and that the association of nicotine and THC significantly enhanced the severity of THC withdrawal ($P<0.01$) (Figure 3).

The association of THC and nicotine produces anxiolytic-like responses

Previous studies have reported that cannabinoid agonists can induce both anxiolytic-and anxiogenic-like behavioural reactions in rodents depending on the dose used and the context (Rodriguez de Fonseca *et al*., 1996; Onaivi *et al*., 1990). We used the light–dark box and the open field test to investigate the

Table 2 Effects induced by the administration of different doses of THC in the light–dark box

Dose of THC	*Time spent in the lit compartment* (%)	*Crossing squares in the lit compartment* (%)
Vehicle	47±3.2	36±3.2
0.03 mg kg^{-1}	44±3.1	35±3.1
0.1 mg kg^{-1}	51±3.7	40±2.3
0.3 mg kg^{-1}	56±1.9*	45±2.2*
1.0 mg kg^{-1}	40±3.8	31±2.7
2.5 mg kg^{-1}	39±2.5	30±1.8
5.0 mg kg^{-1}	35±3.1**	18±2.3**

Data are expressed as percentage of total values (mean± s.e.mean). Newman–Keuls *post-hoc* test after ANOVA: *$P<0.05$, **$P<0.01$, in comparison with vehicle treated group.

Table 3 Distribution of c-Fos immunopositive nuclei after an acute injection of THC (5 mg kg^{-1}), nicotine (0.5 mg kg^{-1}) or association of both compounds.

Brain region		*Vehicle* (n = 4)	*THC* (n = 4)	*Nicotine* (n = 4)	*THC/nicotine* (n = 4)
Nucleus Accumbens	core	20±2.7	84±2.6**	35±3.4*	61±7.4
	shell	23±3.7	86±15.0**	79±2.6	**151±12.1**^{a}**
Dorsal Striatum		11±1	179±29.3**	62±20.3	136±9.9**
Ventral Pallidum		1±1.4	0±0	1±0.8	2±1.9
Globus Pallidus		0±0	0±0	0±0	0±0
Cortex	cingular	31±6.7	58±8.9	97±11.5**	**207±16.9**^{b}**
	piriform	33±5.3	73±12.0*	96±10.3*	**141±11.2**^{b}**
Lateral Septal Area		13±3	75±9.5**	69±8.5*	94±5.1**
Bed Nucleus Stria	medial anterior	5±0.9	19±3.6*	10±2.2*	17±2.3*
Terminalis	lateral ventral	6±1.3	16±3.7*	5±1.3	16±2.3*
	lateral dorsal	8±2.1	61±6.7**	28±5.7*	**89±2.5**^{a}**
Amygdala	central	2±1.3	37±8.2**	22±4.3**	**117±3.5**^{b}**
	baso-lateral	6±2	18±5.3	14±4.7	**36±6**^{a}**
Hippocampus	gentate gyrus	14±2.3	29±4.2*	16±3.5	18±2.7
	CA3	6±0.7	15±3.1	13±2.1	12±2
	CA1	12±2.4	35±12.5	24±4.6	20±5
Hypothalamus	ventromedial	0±0.3	20±6.6*	21±7.5*	22±3.2*
	dorsomedial	16±2.1	36±3.4*	18±1.1	30±4.7*
	paraventricular	1±1	47±19.2	4±1.6	**112±15.8**^{b}**
Habenula		4±1	11±2.1	8±4.2	5±2.7
Thalamus	paraventricular	18±2	38±3**	30±3.6**	4.3±3.7**

Data are expressed as number of c-Fos immunopositive nuclei (mean±s.e.mean). Newman-Keuls *post-hoc* test after ANOVA: *$P<0.05$, **$P<0.01$, in comparison with vehicle group; $^{a}P<0.05$, $^{b}P<0.01$, in comparison with THC-treated group. Bold indicates brain regions showing a significant potentiation of c-Fos expression after co-administration of THC and nicotine.

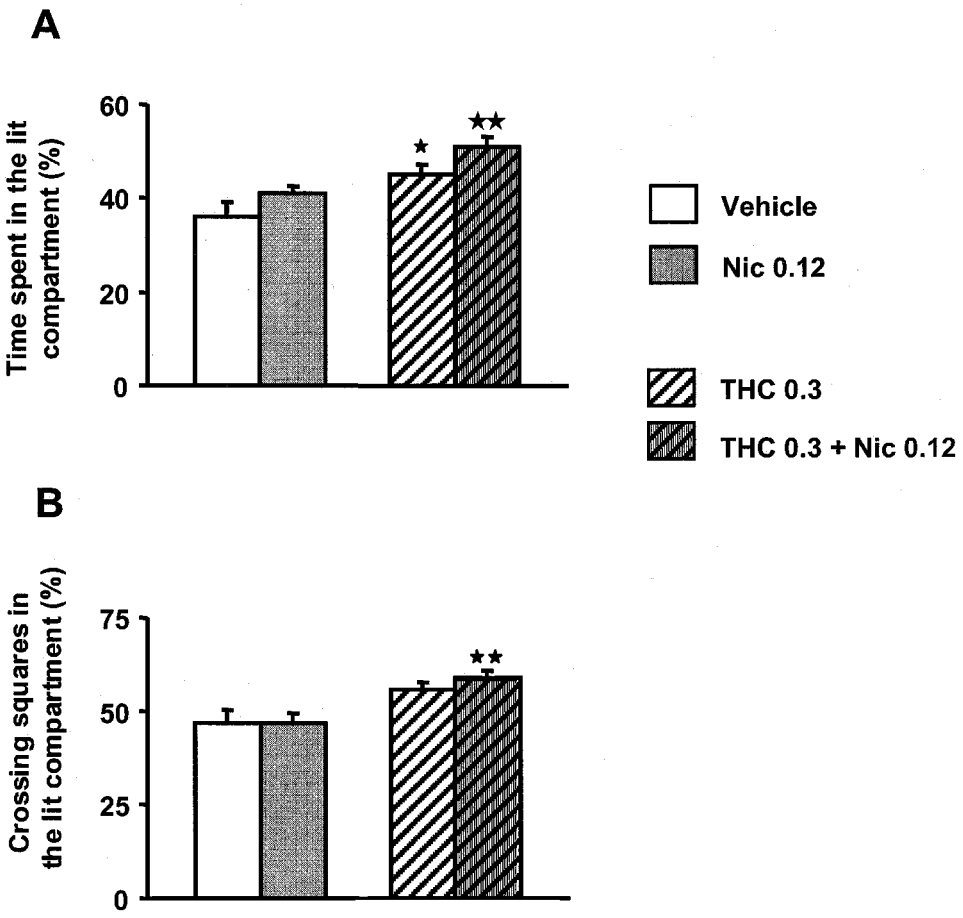

Figure 4 Anxiolytic-like effects of THC alone or associated with nicotine in the light–dark box. The behavioural response in the lit compartment is shown in the figure. The following parameters were evaluated: squares crossed (A) and time spent in the lit compartment (B) expressed as a percentage of total values (lit + dark compartment). Note the increase in crossing squares and time spent in the lit compartment after acute administration of either THC alone or associated with nicotine. Data are expressed as mean ± s.e.mean ($n = 10$ mice for each group). Data were analysed as described in Figure 1. ★$P<0.05$; ★★$P<0.01$ when comparing with vehicle group (Newman–Keuls test).

emotional-like responses induced by THC alone or associated with nicotine. In a first experiment, a dose response curve was performed in the light–dark box in order to determine a dose of THC producing clear anxiolytic-like responses in these experimental conditions. Mice were administered with saline or THC at the doses of 0.03, 0.1, 0.3, 1, 2.5 and 5 mg kg^{-1}. One-way ANOVA (between subjects) revealed a significant effect of treatment on the time spent in the lit compartment ($F_{(6,63)} = 9.842$, $P<0.001$) and the percentage of crossing squares ($F_{(6,63)} = 4.669$, $P<0.001$). A significant increase in the time spent ($P<0.05$) and crossing squares ($P<0.05$) in the lit compartment was observed after the administration of THC at the dose of 0.3 mg kg^{-1}. In contrast, the administration of 5 mg kg^{-1} of THC produced the opposite response, i.e., a significant decrease in the time spent ($P<0.01$) and crossing squares ($P<0.01$) in the lit compartment. No significant effects were observed after the administration of THC at 0.03, 0.1, 1 and 2.5 mg kg^{-1} (Table 2).

The effects produced by the association of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) were then evaluated in the light–dark box test. One-way ANOVA (between subjects) revealed a significant effect of treatment on the time spent in the lit compartment ($F_{(3,35)} = 8.558$, $P<0.001$) (Figure 4A) and the percentage of crossing squares ($F_{(3,35)} = 6.854$, $P<0.01$) (Figure 4B). *Post hoc* comparisons (Newman–Keuls) revealed a significant increase in the time spent ($P<0.01$) and number of crossed squares ($P<0.01$) in the lit compartment when THC (0.3 mg kg^{-1}) was given alone. However, nicotine (0.12 mg kg^{-1}) failed to induce any effect when given alone and to potentiate the effects induced by THC in this test (Figure 4A,B).

Anxiolytic effects of THC (0.3 mg kg^{-1}) were also revealed by the open field test (Figure 5A–D). One-way ANOVA showed a significant effect of drug treatment for crossing squares ($F_{(3,36)} = 6.552$, $P<0.01$) (Figure 5A), rearing ($F_{(3,36)} = 4.156$, $P<0.05$) ($P<0.01$) (Figure 5B), as well as for the number of entries ($F_{(3,36)} = 7.836$, $P<0.01$) (Figure 5C) and time spent in the inner squares ($F_{(3,36)} = 8.679$, $P<0.01$) (Figure 5D). *Post-hoc* comparisons showed that nicotine (0.12 mg kg^{-1}) given in association with THC only potentiates the effects of this compound on the number of rears ($P<0.05$) (Figure 5B).

Of interest, no responses were found in the light–dark box and open field test when lower doses of THC (0.03 and 0.1 mg kg^{-1}) were associated with nicotine (0.12 mg kg^{-1}) (data not shown).

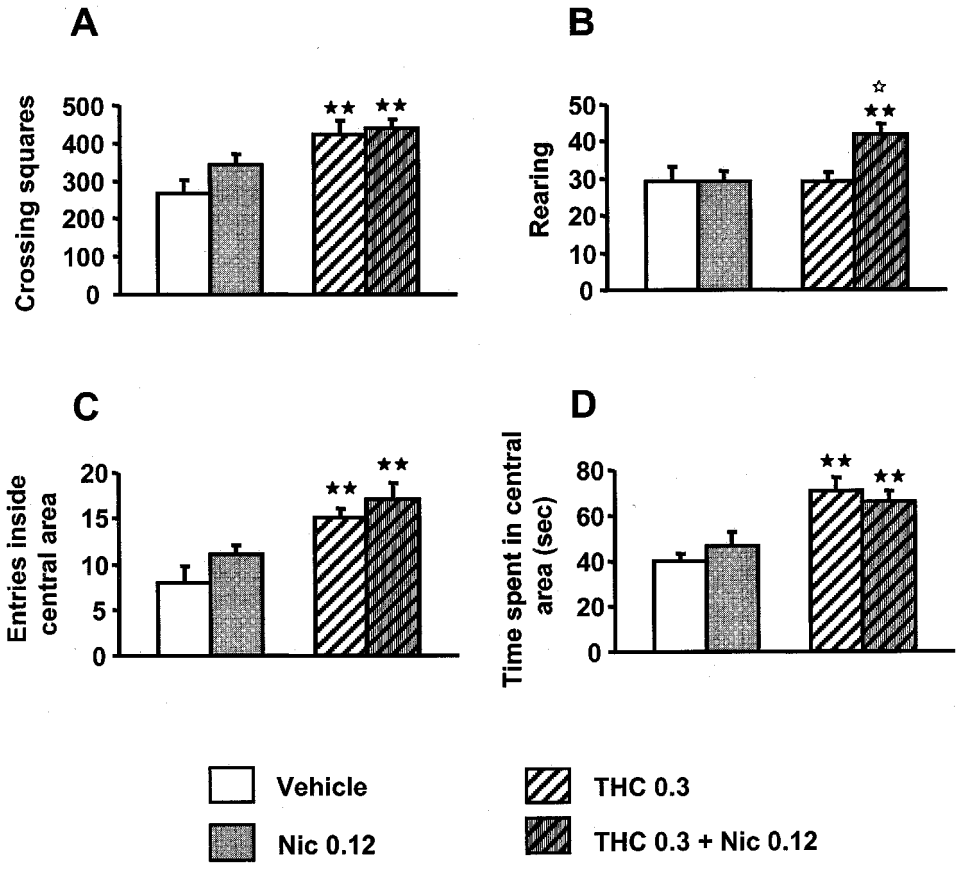

Figure 5 Anxiolytic-like effects of THC alone or associated with nicotine in the open field test. The following parameters were measured in the open field after administration of THC and/or nicotine: number of squares crossed (A), rears (B), and entries (C) and time spent inside the central area (D). Note the increase in locomotion and visits to central area observed after THC alone or co-administrated with nicotine. Data are expressed as mean ± s.e.mean ($n = 10$ mice for each group). Data are analysed as described in Figure 1. ★★$P < 0.01$ when comparing with vehicle group. ☆$P < 0.05$ when comparing with THC group (Newman – Keuls test).

Association of sub-threshold doses of THC and nicotine induces rewarding effects in the place preference paradigm

Using a long period of conditioning, a high number of pairings and a previous single THC injection in the home cage, THC (1 mg kg^{-1}) has been reported to induce place preference in mice (Valjent & Maldonado, 2000). We investigated whether the association of sub-threshold doses of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) could induce rewarding effects in the place conditioning paradigm. A positive control consisting in mice conditioned with THC (1 mg kg^{-1}) after a single injection in the home cage (Valjent & Maldonado, 2000) was included in this experiment. Time spent in the drug-paired compartment during pre-test by the different groups was compared by a one-way ANOVA to ensure use of an unbiased procedure ($F_{(4,40)} = 0.421$, $p = 0.792$). One-way ANOVA of score values revealed a significant effect of treatment ($F_{(4,41)} = 8.155$, $P < 0.001$) (Figure 6A). *Post hoc* comparisons (Newman – Keuls) showed that the dose of 1 mg kg^{-1} of THC induced a clear place preference ($P < 0.01$), while nicotine (0.12 mg kg^{-1}) or THC (0.3 mg kg^{-1}) given alone failed to reveal rewarding effects. However, the co-administration of these non-effective doses of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) induced a robust place preference ($P < 0.01$) (Figure 6A). In agreement, within-group comparisons for time spent in the drug-paired side during the pre-test and test days revealed a significant place preference in groups receiving 1 mg kg^{-1} of THC ($t_{(1,8)} = -8.225$, $P < 0.001$) and the association of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) ($t_{(1,8)} = -8.12$, $P < 0.001$) (Figure 6B).

Nicotine enhances the effects of THC on c-Fos expression in various brain areas

It is now generally admitted that c-Fos expression is a good index of neuronal activity upon drug administration. Cannabinoid agonists (Mailleux *et al*., 1994; Rodriguez de Fonseca *et al*., 1997; McGregor *et al*., 1998) and nicotine (Ren & Sagar, 1992; Salminen *et al*., 1996; Mathieu-Kia *et al*., 1998) have been reported to induce c-Fos expression in various brain areas. We investigated the consequences of the co-administration of THC and nicotine on c-Fos expression in several brain structures. Immunocytochemical analysis of c-Fos expression, analysed 1 h after administration of THC (5 mg kg^{-1}) or nicotine (0.5 mg kg^{-1}) showed a strong up-regulation of c-Fos immunoreactive cells in numerous common brain areas such as the core and shell of the nucleus accumbens, piriform cortex, lateral septal area, medial anterior and lateral dorsal nucleus of the bed nucleus stria terminalis, central amygdala, ventromedial nucleus of hypothalamus and paraventricular nucleus of the thalamus (Table 3 and Figure 7). In addition,

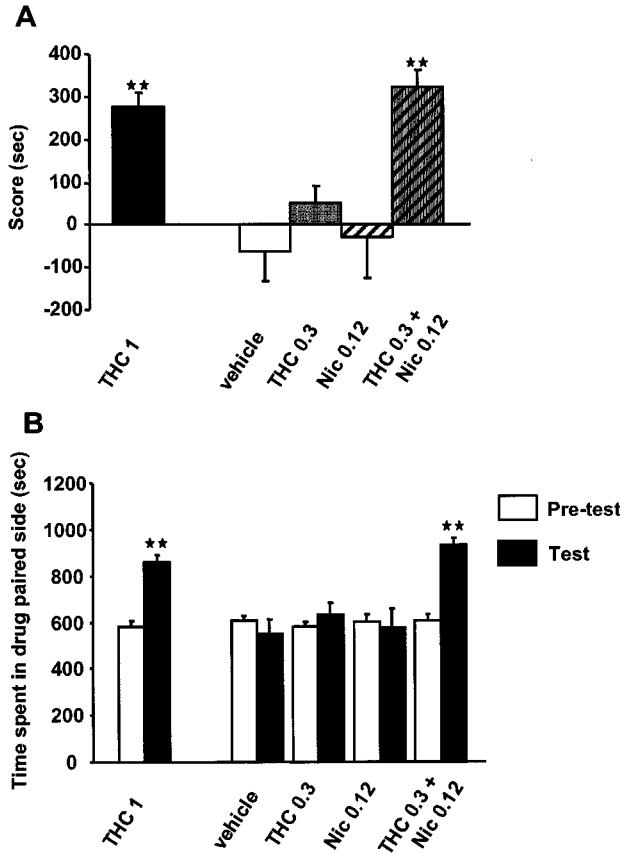

Figure 6 Association of sub-threshold doses of THC and nicotine induces place preference in mice. All mice received the first injection of drug or vehicle on the first day of conditioning, excepting the group treated with the dose of 1 mg kg^{-1} of THC which received a single drug injection in the home cage 24 h before starting the place preference conditioning procedure. Scores calculated as the difference between post-conditioning and pre-conditioning time spent in the compartment associated with the drugs are presented in (A). Time spent in drug-associated compartment during the pre-conditioning (white bars) and the testing phase (black bars) is shown in (B). Data are expressed as mean ± s.e.mean (n = nine mice for each group). Score values were analysed as Figure 1. ★★$P<0.01$ when comparing vehicle group. (Newman–Keuls test). A Student's t-test was used to compare within each group the time spent during the preconditioning and testing phases in the drug-associated compartment: ★★ $P<0.01$.

THC but not nicotine induced c-Fos expression in dorsal striatum, lateral ventral part of the bed nucleus stria terminalis, dentate gyrus and dorsomedial nucleus of the hypothalamus (Table 3, Figure 7). Interestingly, the co-administration of both drugs strongly potentiated c-Fos immunoreactivity in the shell of the nucleus accumbens ($P<0.05$), central ($P<0.01$) and basolateral ($P<0.05$) nucleus of the amygdala, lateral dorsal part of the bed nucleus stria terminalis ($P<0.05$), cingular ($P<0.01$) and piriform ($P<0.01$) cortex, and paraventricular nucleus of the hypothalamus ($P<0.01$) (Table 3 and Figure 7).

Discussion

The present results show that association of THC and nicotine clearly facilitates several acute pharmacological responses induced by THC. This is illustrated by the strong hypothermia, antinociception and hypolocomotion observed after co-treatment of non-effective doses of nicotine and THC. At this moment, only one early study has reported a possible interaction between these two drugs of abuse (Pryor *et al*., 1978). Thus, the acute depressant effects induced by THC in the conditioned avoidance response, locomotor activity, heart rate, body temperature and rotarod performance were potentiated in rats by nicotine co-administration (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.

Different hypothesis can be postulated to explain the acute behavioural interactions between THC and nicotine. A first possibility would be an additive behavioural effect between these two compounds. Indeed, both THC and nicotine can induce similar responses on body temperature, locomotion and nociception. However, the hypothermia, antinociception and hypolocomotion induced by the co-administration of nicotine and THC was greater than the sum of the intrinsic effects of each drug alone. A more likely explanation could be an interaction between cannabinoid and nicotine receptor/neurotransmitter systems. Thus, cannabinoid agonist administration modulates Ach release in several brain structures, such as hippocampus, cortex and striatum (Revuelta *et al*., 1978; Tripathi *et al*., 1987; Acquas *et al*., 2000), which participate in some behavioural effects induced by THC. In agreement with this hypothesis, the cholinesterase inhibitor physostigmine has been reported to potentiate the cataleptic effects of THC, suggesting the involvement of central Ach-release in this behavioural response induced by cannabinoids (Pertwee & Ross, 1991). Besides a possible participation of nicotinic receptors in the facilitatory interaction between THC and nicotine, muscarinic receptors seem to be also involved in some THC-induced behaviours. In this way, muscarinic agonists such as oxotremorine synergistically interact with THC to produce behavioural responses in mice (Pertwee & Ross, 1991). The interaction between cannabinoid and nicotine systems could also depend on a common mechanism separately activated. Thus, both THC and nicotine administration are able to increase the activity of the endogenous dopaminergic (Calabresi *et al*., 1989; Pidoplichko *et al*., 1997; French *et al*., 1997; Gessa *et al*., 1998b) and opioid systems (Dhatt *et al*., 1995; Valverde *et al*., 2001), which could account for some specific behavioural interactions, as further discussed. Finally, this interactive response could be explained by changes at the drug dispositional level. In this way, the magnitude or the duration of action of THC could be influenced by changes in their absorption, plasma binding sites, distribution, metabolism or elimination caused by nicotine administration.

The rapid onset of tolerance to hypothermic and antinociceptive responses of THC is in agreement with previous studies showing that most of the behavioural effects disappear rapidly after the second THC injection (Anderson *et al*., 1975; Fan *et al*., 1996; Hutcheson *et al*., 1998). Interestingly, the development of tolerance to the antinociceptive and hypothermic effects was slower in mice chronically co-treated with THC and nicotine. Furthermore, the severity of CB1 receptor antagonist-precipitated THC withdrawal was increased in mice receiving the association of

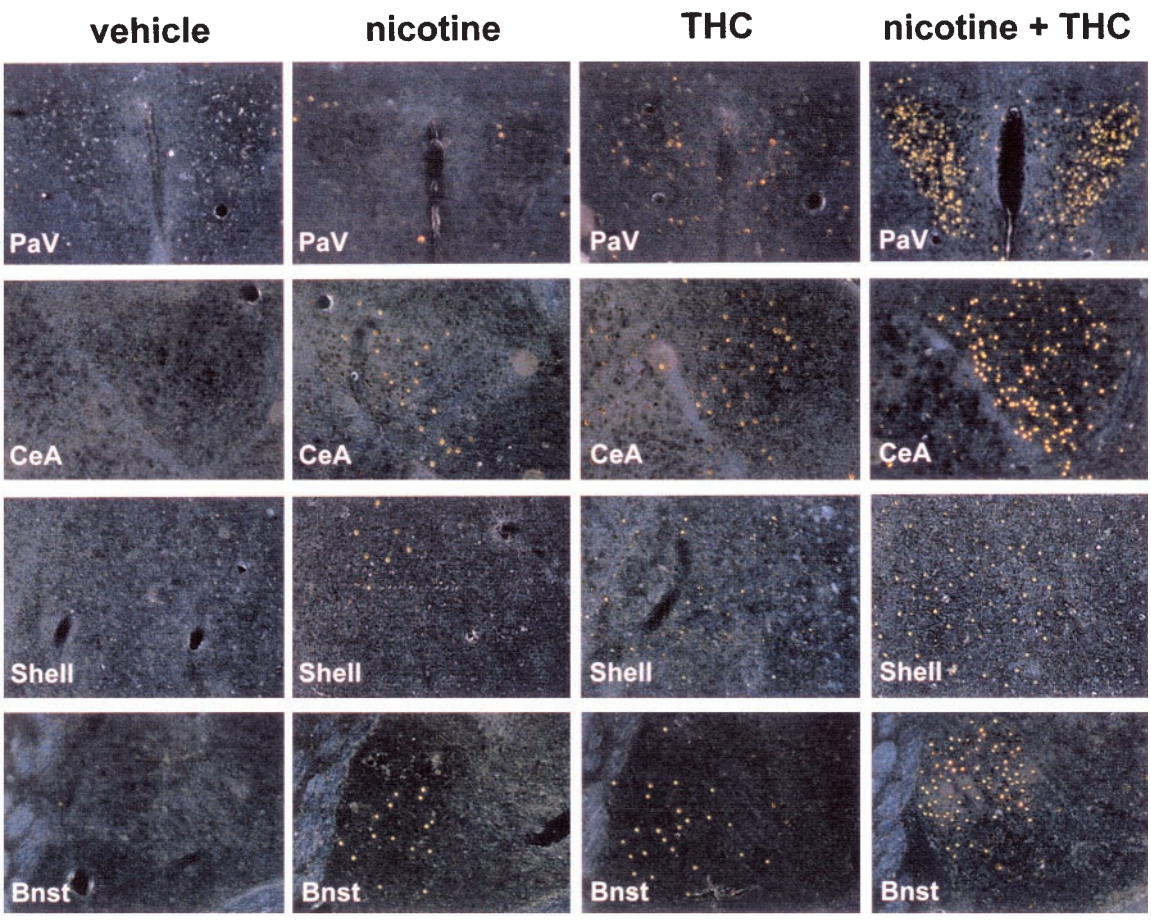

Figure 7 Nicotine potentiates THC-induced c-Fos expression. c-Fos immunoreactivity after injection of vehicle, THC and/or nicotine. Note the potentiation of c-Fos expression by co-administration of THC and nicotine in the paraventricular nucleus of the hypothalamus (PaV), central amygdala (CeA), shell of the nucleus accumbens (Shell) and dorsolateral bed nucleus stria terminalis (Bnst).

THC and nicotine. Thus, co-stimulation of nicotinic and cannabinoid receptors decreases the development of tolerance and intensifies the expression of THC physical dependence. Tolerance to THC is accompanied by down-regulation of CB1 cannabinoid receptors (Rodriguez de Fonseca *et al*., 1994) and a decrease in $G\alpha_i$ mRNA levels (Rubino *et al*., 1998). In contrast, repeated exposure to nicotine can reduce the turnover of nicotinic receptors and increase its number on the membrane surface. Depending on cholinergic activity and nicotine concentration in the brain, these nicotinic receptors can change their functional states (Wonnacott, 1990; Dani & Heinemann, 1996). These different pharmacodynamic events induced by nicotine can contribute to the changes observed in THC tolerance and physical dependence. Chronic nicotine administration has also been reported to induce and up-regulation of μ-opioid receptors in the striatum (Wewers *et al*., 1999), and to modify the expression (Dhatt *et al*., 1995; Mathieu *et al*., 1996; Mathieu-Kia & Besson, 1998) and levels (Wewers *et al*., 1999) of endogenous opioid peptides. These endogenous opioid peptides play an important role in the development and expression of cannabinoid tolerance and dependence (Valverde *et al*., 2000). On the other hand, the endogenous cannabinoid system has also been reported to participate in the somatic expression of morphine withdrawal and opioid rewarding properties (Ledent *et al*., 1999; Martin *et al*., 2000). However, SR 141716A administration did not precipitate any behavioural sign of withdrawal in mice chronically receiving nicotine alone. Although nicotine can induce an important degree of physical dependence in rodents (Hildebrand *et al*., 1999; Watkins *et al*., 2000), further studies must be performed to clarify the possible involvement of the endogenous cannabinoid system in this nicotine response. Indeed, dose and route of nicotine administration were choosen to evaluate a possible interaction with the expression of the somatic signs of THC withdrawal. Therefore, nicotine and THC were chronically administered twice daily, which represents an optimal protocol to induce cannabinoid physical dependence but not nicotine dependence. It must be pointed out that doses of THC (5 and 10 mg kg^{-1}) and nicotine (0.5 mg kg^{-1}) required to induce physical dependence in these experiments produce anxiogenic effects and are higher than those showing cannabinoid anxiolytic and rewarding properties. Therefore, THC physical dependence in these animal models do not seem to be associated to other motivational properties of THC that could potentially be related to abuse liability. Similar high doses of THC have also been reported in previous studies to be required to induce physical cannabinoid dependence (Cook *et al*., 1988; Hutcheson *et al*., 1998; Ledent *et al*., 1999; Lichtman *et al*., 2001).

In a second group of experiments, interactions between THC and nicotine have been evaluated on anxiolytic-like responses and rewarding properties. As previously reported, both nicotine and THC induce complex dose/response effects on these behavioural models. Thus, depending on the THC and nicotine dose, both anxiolytic/anxiogenic and rewarding/aversive effects can be observed (Costall *et al*., 1989; Rissinger & Oakes, 1995). The range of dose required to evaluate anxiolytic and rewarding effects is therefore different from those used in the first set of experiments, and a single lower dose of nicotine (0.12 mg kg^{-1}) has been co-administered with different lower doses of THC (0.03, 0.1 and 0.3 mg kg^{-1}) in these new experiments. These limitations must be taken into consideration for the interpretation of these results, which must be limited to the particular experimental conditions used in each case. Emotional-like responses measured in the light–dark box and the openfield test revealed that THC alone is able to induce both anxiolytic- and anxiogenic-like reactions depending on the dose. Thus, at low dose (0.3 mg kg^{-1}), THC produced a clear anxiolytic-like response, whereas at high dose (5 mg kg^{-1}) an anxiogenic reaction was observed. This result is in agreement with previous studies showing that the cannabinoid agonist HU-210 produces biphasic effects in the defensive withdrawal test in rats (Rodriguez de Fonseca *et al*., 1996). Thus, a low dose of HU-210 produced anxiolytic effects in a novel environment, whereas under familiar conditions, HU-210 administration resulted in dose-dependent anxiogenic and motor depressing effects (Rodriguez de Fonseca *et al*., 1996). Mice co-treated with low doses of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) revealed also an anxiolytic-like effect in the light–dark box and the open field test, although no clear facilitation of these responses was observed by the association of both compounds. These anxiolytic-like effects may account for the relaxation action reported after acute marijuana exposure in humans whereas anxiogenic-like effects could reflect the panic reactions, paranoia and anxiety also observed (Hollister, 1986).

THC induces rewarding properties in rodents and, interestingly, opposite motivational responses can be measured in the place preference paradigm depending on the dose of THC and the experimental design used (Lepore *et al*., 1995; Mallet & Beninger, 1998; Hutcheson *et al*., 1998; Valjent & Maldonado, 2000). While low doses of THC (0.3 mg kg^{-1}) and nicotine (0.12 mg kg^{-1}) given alone failed to reveal any conditioned response, the co-administration of both drugs induced a clear place preference. The rewarding effects induced by this co-administration was comparable to those induced by 1 mg kg^{-1} of THC in mice receiving a single THC injection in the home cage 24 h before starting the place conditioning procedure. Interestingly, this previous single injection was not required to induce conditioned place preference by the co-administration of low doses of THC and nicotine. Therefore, the first exposure to THC (0.3 mg kg^{-1}) plus nicotine (0.12 mg kg^{-1}) seems to be devoid of the dysphoric effects presumably produced by the first administration of higher doses of THC alone (Valjent & Maldonado, 2000). Taking into account these findings, low doses of cannabinoids associated with nicotine could have a higher capability to induce behavioural responses related to addictive processes than THC administration alone. These motivational responses could be due to the neurochemical changes induced by these drugs in brain areas related to addictive behaviours. Indeed, both drugs are able to stimulate the firing of dopaminergic neurones in the ventral tegmental area (Calabresi *et al*., 1989; Pidoplichko *et al*., 1997; French *et al*., 1997; Gessa *et al*., 1998b), and to induce release of DA in the shell of the nucleus accumbens (Di Chiara & Imperato, 1988; Dani & Heinemann, 1996; Tanda *et al*., 1997; Malone & Taylor, 1999). Therefore, the conditioned place preference observed here could result from a potentiation of the effects on dopaminergic mesolimbic activity induced by the co-administration of THC and nicotine. In this way, it has been reported that nicotine could enhance cocaine- and heroin-induced dopamine overflow in the shell of the nucleus accumbens, suggesting that nicotine could also enhance the rewarding effects of these two other drugs of abuse (Zernig *et al*., 1997).

It is now well established that some addictive related behaviours are strongly linked to molecular adaptations, such as gene regulation, observed in discrete brain areas (Berke & Hyman, 2000; Nestler, 2000). As previously described (Salminen *et al*., 1996; McGregor *et al*., 1998), acute administration of cannabinoids and nicotine induces c-Fos immunoreactivity preferentially in the terminal fields of neurones of the ventral tegmental area (nucleus accumbens, central amygdala, lateral septum, dorsal-lateral bed nucleus stria terminalis), which are highly involved in the rewarding properties induced by drugs of abuse (Di Chiara & Imperato, 1988; Koob *et al*., 1998) and stress-related responses (Rodriguez de Fonseca *et al*., 1997; Mathieu-Kia & Besson, 1998). Furthermore, THC also induces a strong c-Fos expression in other structures linked to stress responses such as the paraventricular nucleus of the hypothalamus and the paraventricular anterior nucleus of the thalamus. In agreement with previous studies (Mathieu-Kia & Besson, 1998), nicotine, and in a lower extent THC, also activate c-Fos expression in cortical areas. The co-administration of THC and nicotine produced a strong potentiation of c-Fos immunoreactivity in various limbic and cortical structures, including the shell of the nucleus accumbens, central and basolateral nucleus of amygdala, dorsolateral bed nucleus stria terminalis, cingular and piriform cortex and paraventricular nucleus of the hypothalamus. Interestingly, most of these areas are highly innervated by DA inputs, suggesting that the interaction between nicotine and cannabinoids could occur *via* the stimulation of mesolimbic and mesocortical dopaminergic system.

In conclusion, our data provide the first *in vivo* evidence for facilitatory effects of nicotine on acute and chronic behavioural responses induced by THC. This provides important insights for better understanding the consequences of the habits of cannabis consumption in humans. Indeed, the presence of nicotine and THC in the preparations currently used to smoke cannabis in Europe can likely increase the motivational effects of cannabis derivatives and therefore facilitate the possible abuse of these preparations. Furthermore, the association of tobacco and cannabis can also modify other somatic consequences of chronic consumption of these derivatives.

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