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ORIGINAL INVESTIGATION

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Cannabinoid CB_1 receptors are involved in motivational effects of nicotine in rats

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Abstract *Rationale:* The endocannabinoid system plays a role in mediating the appetitive value of a variety of reinforcing compounds, either natural rewards or drugs of abuse, but little is known about its involvement in the incentive properties of nicotine. *Objectives:* The objective of the study is to evaluate whether activation of CB_1 cannabinoid receptors is necessary for the establishment and the short- and long-term expression of nicotine-induced conditioned place preference (CPP). This was studied in rats subjected to an unbiased, one-compartment place conditioning procedure, using the selective CB_1 receptor antagonist, rimonabant, as a pharmacological tool. *Methods:* Wistar rats, given previous experience with nicotine in their home cage, were subjected to eight alternating nicotine (0.006–0.6 mg/kg s.c.) and saline pairings with distinct floor textures in an open field and given a test session, with no nicotine injection, in the open field whose floor was covered by two quadrants of the saline-paired texture and two quadrants of the nicotine-paired texture. Rimonabant (0.3–3 mg/kg i.p.) was administered 30 min before each nicotine (0.06 mg/kg) pairing to assess its effect on the establishment of nicotine-CPP. To study the effects of CB_1 receptor blockade on short- and long-term expression of nicotine-CPP, rimonabant was administered as a single injection 30 min before the test session, conducted either 24 h, 3 weeks or 12 weeks after the last conditioning session. *Results:* Rats developed reliable and robust CPP to the 0.06- and 0.125-mg/kg doses of nicotine. Once established, CPP persisted for at least 12 weeks without additional exposure to nicotine and the test apparatus. Pre-pairing injections of rimonabant (3 mg/kg, but not lower doses) prevented the acquisition of nicotine-CPP, and a single pretest administration of rimonabant (3 mg/kg) abolished the expression of nicotine-CPP when the test session took place 24 h after the last conditioning session. However, rimonabant (3 mg/kg) did not antagonize the expression of nicotine-CPP when the test session was conducted 3 or 12 weeks after the acquisition phase. *Conclusions:* The endocannabinoids are a necessary component in both the perception by rats of the motivational value of nicotine and the short-term capacity of nicotine-paired conditioned stimuli to elicit approach behaviour. In contrast, the acute blockade of CB_1 receptors no longer impairs the long-term control of behaviour by nicotine-associated environmental cues. These data provide support to the notion that the blockade of CB_1 receptors can oppose tobacco dependence, withdrawal and even relapse, though the time window of efficacy and/or the schedule of administration remain to be established.

Keywords Nicotine · Cannabinoid receptor · Rimonabant · Conditioned place preference · Incentive learning · Rat

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Introduction

Tobacco and marijuana (*Cannabis sativa*) smoking represent worldwide public health problems. Nicotine is the principal component in tobacco smoke that leads to addiction, and Δ^9-tetrahydrocannabinol (Δ^9-THC) is the major psychoactive component of cannabis. They exert their initial effect at different receptors, highly expressed in the central nervous system (CNS). Nicotine activates neuronal nicotinic acetylcholine (nACh) ion channel receptors, whereas Δ^9-THC, as well as synthetic compounds (WIN 55,212-2, CP 55,940, HU 210), are agonists at CB_1 and CB2 cannabinoid G-protein-coupled receptors (Pertwee 1999). Central effects of cannabinoids, including their reinforcing properties, are thought to be mediated by the CB_1 receptor since most of them are counteracted by the potent and selective CB_1 receptor antagonist, rimonabant (Rinaldi-Carmona et al. 1995), or are not observed in CB_1 receptor knockout (KO) mice (Ledent et al. 1999; Zimmer et al. 1999).

Nicotine and Δ^9-THC exhibit the main features of addictive drugs. In particular, in animals, they produce discriminative effects and support self-administration behaviour (for reviews, see Chaperon and Thiébot 1999; Stolerman 1999; Di Chiara 2000; Tanda and Goldberg 2003). Nicotine and Δ^9-THC facilitate intracranial self-stimulation (Schaefer and Michael 1986; Herberg et al. 1993; Lepore et al. 1996), though synthetic CB receptor agonists seem inactive in this respect (Arnold et al. 2001; Vlachou et al. 2003). In addition, an extensive literature indicates that nicotine and CB receptor agonists share with other drugs of abuse the property of activating the mesolimbic dopaminergic (DA) system (for reviews, see Balfour et al. 2000; Di Chiara 2000; Tanda and Goldberg 2003), and this is considered as central to their rewarding motivational properties. Interestingly, nicotine as well as Δ^9-THC and WIN 55,212-2 increased extracellular levels of DA preferentially in the shell of the nucleus accumbens (NAcc), while having little or no effect in the core (Pontieri et al. 1996; Tanda et al. 1997; Iyaniwura et al. 2001), an effect that is claimed to correlate with the abuse liability of drugs (Di Chiara et al. 1999).

On the other hand, accumulating evidence indicates that an endocannabinoid system exists in the CNS, which is involved in brain reward processes, and behavioural studies suggest that this system plays a pivotal role not only in the motivational effects of cannabinoids, but also in the action of non-cannabinoid reinforcers. In particular, genetic deletion and pharmacological blockade of CB_1 receptors attenuate the motivational properties of various classes of drugs of abuse, in addition to cannabis. Thus, impairment in CB_1 receptor-mediated transmission reduces reinforcing effects of opiates, as evaluated by (1) self-administration procedures (Fratta et al. 1998; Ledent et al. 1999; Cossu et al. 2001; Navarro et al. 2001; Caillé and Parsons 2003; Solinas et al. 2003), (2) reinstatement of seeking behaviour after long-term extinction of self-administration (Fattore et al. 2003), and (3) conditioned place preference (CPP) (Chaperon et al. 1998; Martin et al. 2000; Mas-Nieto et al. 2001; Navarro et al. 2001). The motivational effects of alcohol also appear controlled by endogenous cannabinoid processes (Arnone et al. 1997; Gallate and McGregor 1999; Freedland et al. 2001; Serra et al. 2001; Hungund et al. 2003; Poncelet et al. 2003; Wang et al. 2003; Colombo et al. 2004), and rimonabant blocks the rebound of alcohol intake after transient deprivation (Serra et al. 2002). However, investigations on the role of endocannabinoids in the appetitive effects of psychostimulants have yielded contradictory results. Indeed, rimonabant has been reported to prevent the establishment of cocaine-CPP (Chaperon et al. 1998), to reduce self-administration of methamphetamine (Vinklerová et al. 2002) and to attenuate cocaine seeking induced by cocaine or cocaine-associated cues (De Vries et al. 2001). By contrast, the blockade of CB_1 receptors did not impair cocaine self-administration in rats and monkeys (Fattore et al. 1999; Tanda et al. 2000), and the ability of cocaine or D-amphetamine to sustain self-administration behaviour, as well as cocaine-CPP, was not abolished in CB_1 KO mice (Martin et al. 2000; Cossu et al. 2001). Finally, several studies indicated that the endocannabinoid system plays a role in the processes which control the motivation to obtain natural reinforcers such as food or palatable solutions (Arnone et al. 1997; Gallate et al. 1999; Kirkham and Williams 2001; Higgs et al. 2003; Sanchis-Segura et al. 2004). Collectively, these studies suggest that the endocannabinoid system could be differentially involved in the motivation for a variety of natural or drug reinforcers.

Surprisingly, there is only scarce information about the possible role of the endocannabinoid system in the motivational effects of nicotine, and behavioural studies on this issue led to contradictory results. CPP supported by nicotine (Castañé et al. 2002), but not nicotine self-administration (Cossu et al. 2001), appeared to be abolished in CB_1 KO mice, although in rats, once established, both behaviours were reduced by rimonabant (Cohen et al. 2002; Le Foll and Goldberg 2004). To the best of our knowledge, there are no other studies investigating the involvement of CB_1 receptors in nicotine incentive properties. This might be due to the actual difficulties to demonstrate reliable motivational effects of nicotine by place conditioning procedures. Indeed, in rats and mice, some studies indicated that nicotine does not support place conditioning (Clarke and Fibiger 1987; Parker 1992; Rogers et al. 2004), whereas others reported either modest place preference (Fudala et al. 1985; Fudala and Iwamoto 1986; Shoaib et al. 1994; Horan et al. 1997; Dewey et al. 1999; Castañé et al. 2002; Vastola et al. 2002; Zarrindast et al. 2003; Belluzzi et al. 2004), place aversion (Jorenby et al. 1990; Horan et al. 1997) or biphasic effects (Risinger and Oakes 1995; Philibin et al. 2005), depending on the experimental design (biased or unbiased), the doses, the strain and even the age of the animals.

The main objective of the present study was to investigate the involvement of CB_1 receptor-mediated endocannabinoid processes in the reinforcing properties of nicotine, as assessed by place conditioning. The first experiment was designed to determine experimental conditions suitable for nicotine to support reproducible CPP. This was conducted in rats subjected to a one-compartment procedure, according to an unbiased design in current use in the laboratory (Guyon et al. 1993; Chaperon et al. 1998; Duarte et al. 2003). Since either positive or aversive effects of nicotine have been reported in mice, depending on the dose (Risinger and Oakes 1995), a large range of doses (6 μg/kg–0.6 mg/kg) was studied. Furthermore, in order to prevent association of the test apparatus with aversive reactions to the first injections of nicotine (Shoaib et al. 1994, 2002; Laviolette and van der Kooy 2004), rats were given prior experience with nicotine in the home cage. In a second set of experiments, the influence of CB_1 receptor blockade by rimonabant (0.3–3 mg/kg, i.p.) was investigated on (1) the establishment of CPP supported by nicotine, i.e. on the perception by rats of the intrinsic incentive value of nicotine, and (2) the short- and long-term expression of nicotine-CPP, i.e. on the perception by animals of the appetitive value acquired by initially neutral environmental stimuli that have been paired with nicotine

during the conditioning phase. The doses of rimonabant were chosen in the range of those found active to reverse completely the in vivo effects, including place conditioning, induced by the CB receptor agonist WIN 55,212-2 (Rinaldi-Carmona et al. 1995; Chaperon et al. 1998).

Materials and methods

Animals

The experiments were carried out on drug- and test-naive male Wistar AF rats (CERJ, Le Genest, France). They were 5 weeks old (150–160 g) upon their arrival in the laboratory. They weighed 225–245 g at the beginning of the experiments, 2 weeks later. Rats were housed eight per cage (40×40×18 cm) under standard conditions (12-h light–dark cycle with lights on at 0730 h; room temperature 21±1°C) with free access to water in their home cage. One week prior to the beginning of the experiments, rats were brought daily from the animal housing facility to the laboratory; they were handled, weighed and given on Monday, Wednesday and Friday a subcutaneous (s.c.) injection of nicotine (at the dose to be tested) or its vehicle; on alternate days, they received an intraperitoneal (i.p.) injection of saline to be also habituated to this route of administration. From this first week onwards, they were placed on a daily schedule of mild food restriction (150 g of standard chow per day for eight rats, given as a single meal in the evening), which was maintained until the end of the experiments; indeed, food restriction has been reported to enhance the rewarding effects of drugs of abuse (Bell et al. 1997; Carr 2002). Experiments were performed in agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies (Council directives no. 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission nos. 75-116 to M.H. and 75-118 to M.H.T.).

Place conditioning paradigm

Apparatus

Experiments were conducted in a one-compartment apparatus, using an unbiased experimental design, as previously described (Guyon et al. 1993; Chaperon et al. 1998). Briefly, the rats were trained and tested in four black wooden open fields (76×76×50 cm) located in a room dimly lit with four 15-W red light bulbs, positioned 150 cm above each open field (1 lx at floor level), supplied with continuous masking noise (60 dB). The floor of each open field was covered with removable quadrants made from one of two textures, wire mesh or rough Plexiglas. These textures were chosen on the basis of previous studies indicating that naive rats exhibited no unconditioned preference for one of them. Video cameras, mounted above each open field, were connected with controlling equipment located in the adjacent room.

Experimental procedure

The general procedure consisted of two phases: conditioning and testing. Each rat was subjected to eight 30-min conditioning sessions (one session per day) in one open field (always the same for one rat) whose four floor quadrants were of the same texture. Nicotine was administered immediately before the "odd"-numbered sessions (i.e. 1, 3, 5 and 7) paired with one floor texture. Saline was injected (same administration schedule) before the "even"-numbered sessions (i.e. 2, 4, 6 and 8) paired with the other floor texture. Nicotine–texture pairings were counterbalanced so that, within each treatment group, nicotine was associated with the wire mesh floor for half of the rats and with the Plexiglas floor for the other half. Depending on the experiment (see below), one or three 20-min test sessions were conducted in the same open field whose floor was covered by two quadrants of the saline-paired texture and two quadrants of the nicotine-paired texture. Quadrants of the same texture were positioned diagonally opposite to each other.

The time (in s) spent on each texture and the distance travelled (in cm) were automatically recorded by means of the video system and analyzed by appropriate software (SuperG Software, Hans C. Neijt; Novartis Pharma, Basel, Switzerland). Briefly, the cameras were connected to a frame grabber (type DT3155; Data Translation Inc., Marlboro, MA). Every second, the digitized frame was compared with the previously stored frame, whereby the pixels with altered intensity were identified and used to compute the position of the rat and the distance travelled. Half of the time spent on the dividing lines was added to the total time spent on the nicotine- and the saline-paired textures.

Experiment 1: ability of nicotine to support place conditioning Naive rats were given nicotine (0.006, 0.06, 0.125 and 0.6 mg/kg s.c.), or its vehicle for the control group, immediately before the "odd" conditioning sessions. All of the rats received saline before the "even" sessions. A single test session took place the day following the last conditioning session, i.e. 48 h after the last injection of nicotine. Rats were given no injection before this session.

Additional experiments:

(1) To examine the influence of the number of conditioning trials on the ability of nicotine to establish CPP, on two occasions, independent groups of naive rats were subjected to only four 30-min conditioning sessions. They were given nicotine (0.06 mg/kg s.c.) and saline, immediately before the two "odd" and the two "even" conditioning sessions, respectively. The test session was conducted 1 day later, as described above.
(2) The influence of food restriction on the ability of nicotine (0.06 mg/kg, s.c.) to support CPP was in-

vestigated using two independent groups of rats, either subjected to the standard food regimen (150 g/day for eight rats) or fed ad libitum. Conditioning and test sessions were conducted as described above.

(3) In order to assess whether CPP induced by nicotine could be accounted for by some delayed rebound dysphoric effect of nicotine, naive rats were subjected to eight 30-min conditioning sessions. Nicotine (0.06 mg/kg s.c.) was administered either immediately *before* (conditioned group) or immediately *after* (pseudo-conditioned group), the "odd" conditioning sessions, and saline was injected according to the same time schedule, on "even" sessions. The test session was conducted 24 h later, as described above.

Experiment 2: effect of rimonabant on the establishment of nicotine-induced conditioned place preference During the conditioning phase, rats of all groups were given nicotine (0.06 mg/kg s.c.) immediately before the "odd" conditioning sessions. Rimonabant (0.3, 1 and 3 mg/kg i.p.), or its vehicle for the paired control group, was injected 30 min before nicotine. All rats were given vehicle and saline according to the same injection schedule, before each "even" conditioning session. A single test session was conducted the day following the last conditioning session, i.e. 48 h after the last injection of nicotine. Rats received no injection before the test session.

Experiment 3: effect of rimonabant on short-term expression of nicotine-induced conditioned place preference During the conditioning phase, rats of all groups were given nicotine (0.06 mg/kg s.c.) and saline immediately before the four "odd" and the four "even" conditioning sessions, respectively. Rimonabant (0.3, 1 and 3 mg/kg i.p.), or its vehicle for the associated control group, was administered only once, 30 min before a single test session, which was conducted the day following the last conditioning session, i.e. 48 h after the last nicotine injection.

Experiment 4: effect of rimonabant on long-term expression of nicotine-induced conditioned place preference During the conditioning phase, all rats received nicotine (0.06 mg/kg s.c.) and saline immediately before the four "odd" and the four "even" conditioning sessions, respectively. One day after the last conditioning session, they were subjected to a first 20-min test session, without treatment, to assess the establishment of nicotine-induced conditioned place preference (probe session). During the next 12 weeks, the animals were regularly handled but subjected neither to nicotine administration nor to placement in the open fields. At the end of this period, they were given two additional 20-min test sessions, conducted 24 h apart. Rats received no injection before the second test session, which was conducted to evaluate whether they still expressed preference for the floor texture previously paired with nicotine. Then, the rats, divided into two groups matched according to the time spent on the nicotine-paired texture during the second test session, were given rimonabant (3 mg/kg i.p.) or its vehicle 30 min before the third test session.

Drugs

Rimonabant [SR 141716, *N*-piperidino-5-(4-chlorophenyl)-4-methylpyrazole-3-carboxamide, base] (Sanofi-Aventis, Montpellier, France) was suspended with one drop of Tween 80 in saline (0.9% NaCl). (−)Nicotine bitartrate (Sigma Chemicals, St. Louis, MO, USA) was dissolved in saline, and the pH was adjusted between 7.3 and 7.5 with a few drops of 0.1 M NaOH. Doses are expressed as the free base. Drugs or their respective vehicle were injected in a volume of 5 ml/kg body weight. The number of animals in each treatment group is indicated in Results.

Statistical analyses

The results are expressed as mean (±SEM) time (s) spent on the nicotine-paired floor texture during the test session. Place preference was assessed by testing whether the time spent on the nicotine-paired texture was longer than the time spent on the unpaired texture, using one-tailed, paired Student's *t*-test. Drug effects on paired minus unpaired time differences were analyzed by one-way analysis of variance (ANOVA), followed, where appropriate, by planned pairwise comparisons with controls by two-tailed Dunnett's *t*-test, using the error variance term from the ANOVA. Motor activity expressed as mean (±SEM) distance (cm) travelled during the two experimental phases was analyzed by two-way ANOVA (treatment and sessions as repeated measures) for the conditioning sessions and one-way ANOVA (treatment) for the test sessions. Planned pairwise comparisons with controls were made by two-tailed Dunnett's *t*-test. The entire dose range of nicotine (experiment 1) has been studied in the course of two independent experiments. A vehicle control group of rats was associated with each individual experiment, and the 0.06- and 0.125-mg/kg doses were also tested twice. There were no statistically significant differences between the two experiments with regard to performance of control rats, and corresponding data were pooled for global analysis.

Results

Experiment 1: ability of nicotine to support place conditioning

Control rats (given saline and vehicle before the "even" and "odd" conditioning sessions, respectively) spent 611±35 s on the vehicle-paired floor texture. This time, not statistically different from the time spent on the saline-paired texture (t_{24}=0.31; NS), gives evidence that there was no unconditioned preference for either floor texture (Fig. 1).

Rats given nicotine at the 0.06- and 0.125-mg/kg doses during the conditioning phase spent significantly more time on the paired than on the unpaired texture during the test session (one-tailed, paired t_{15}=5.20, p<0.0001 and t_{15}=3.13, p<0.005, respectively), indicating that these doses of nicotine supported conditioned place preference.

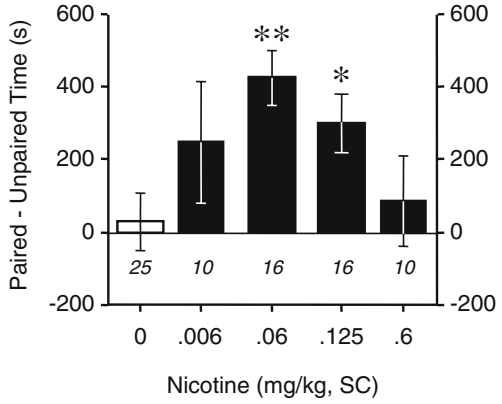

Fig. 1 Place conditioning supported by nicotine (experiment 1). *Histograms* represent the mean (±SEM) difference between the time (s) spent on the floor texture previously paired with nicotine and the time spent on the unpaired texture, during the 20-min test session. Positive values indicate preference for the paired texture. Rats with prior experience with nicotine in the home cage (see Materials and methods) were given nicotine (or its vehicle, 0) and saline (s.c.) immediately before each of the four "odd" and "even" conditioning sessions, respectively. Animals were drug-free during the test session, conducted 24 h after the last conditioning session. The number of rats per group is indicated under each *histogram*. *p<0.005, **p<0.0001, time spent on the nicotine-paired vs unpaired texture (paired Student's *t*-test)

Additional experiments

(1) Two groups of rats (n=14/group) were independently subjected to only two nicotine-paired and two saline-paired conditioning sessions. During the test session, they spent 710±67 and 683±55 s, respectively, on the floor texture previously associated with nicotine (0.06 mg/kg). These times were not significantly longer than those spent on the unpaired texture (one-tailed, paired t_{13}=1.64, p=0.063 and t_{13}=1.51, p=0.078). Therefore, two nicotine pairings were insufficient to induce CPP.

(2) Food-restricted rats (mean body weight at the time of the test session=275±5 g, n=16) and rats fed ad libitum (311±4 g, n=16) spent 744±43 and 687±51 s, respectively, on the texture paired with nicotine (0.06 mg/kg). Though these two values did not significantly differ (two-tailed, unpaired t_{30}=0.69, NS), food-restricted rats spent more time on the paired than on the unpaired texture, as in experiment 1 (one-tailed, paired t_{15}=3.89, p<0.002), whereas in rats fed ad libitum, this comparison fell short of statistical significance (t_{15}=1.70; p=0.055). Thus, moderately food-restricted rats developed a preference for nicotine-associated texture which was more pronounced than that of unrestricted animals.

(3) As in experiment 1, rats given nicotine (0.06 mg/kg) immediately *before* the four "odd" conditioning sessions exhibited a preference for the floor texture previously paired with nicotine (times spent on the paired and unpaired textures 756±32 and 444±32 s, respectively; t_{11}=5.66, p<0.0001, n=12). Rats of the pseudo-conditioned group, which received nicotine (0.06 mg/kg) immediately *after* the "odd" conditioning sessions, spent 620±73 s on the floor texture present during these sessions. This duration was not significantly longer than the corresponding time on the texture present during the "even" sessions (t_{11}=0.38, NS, n=12), indicating that nicotine did not induce 24-h delayed aversive effects.

All subsequent experiments were conducted using the paradigm with eight conditioning sessions and 0.06 mg/kg as the standard conditioning dose of nicotine.

Experiment 2: effect of rimonabant on the establishment of nicotine-induced conditioned place preference

Control rats (vehicle i.p., 30 min before nicotine) spent 796 ±37 s on the texture previously paired with nicotine (0.06 mg/kg). This time was significantly longer than that spent on the unpaired texture (one-tailed paired t_{17}=5.33, p< 0.0001). The ANOVA showed a main effect of rimonabant on the time differences (paired vs unpaired textures) ($F_{3,67}$=4.51, p<0.006), and pairwise comparisons with the control group indicated that pre-pairing injections of the 3-mg/kg dose of rimonabant antagonized the *establishment* of nicotine-CPP ($t_{4,67}$=3.10, p<0.01). Accordingly, rats of this 3-mg/kg group no longer developed preference for the texture previously associated with nicotine (t_{17}= 0.26, NS), whereas rats given the lower doses of rimonabant exhibited nicotine-CPP, as shown by significant differences

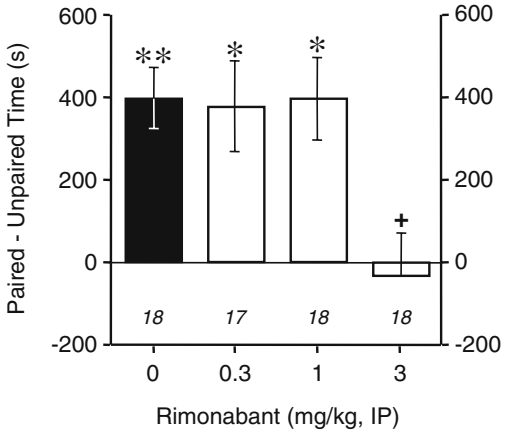

Fig. 2 Effect of rimonabant on the establishment of conditioned place preference induced by nicotine (experiment 2). Histograms represent the mean (±SEM) difference between the time (s) spent on the floor texture previously paired with nicotine (0.06 mg/kg s.c.) and the time spent on the unpaired texture, during the 20-min test session. Positive and negative values indicate preference and aversion for the paired texture, respectively. Rimonabant, or its vehicle (0), was administered i.p., 30 min before each of the four nicotine-paired conditioning sessions. Animals were drug-free during the test session, conducted 24 h after the last conditioning session. The number of rats per group is indicated under each *histogram*. *p<0.005, **p<0.0001, time spent on the nicotine-paired vs unpaired texture (paired Student's *t*-test); ^{+}p<0.01, vs control group given nicotine alone (0) (Dunnett's *t*-test after ANOVA)

between the times spent on paired vs unpaired textures (0.3 mg/kg, t_{16}=3.37, p<0.005; 1 mg/kg, t_{17}=3.88, p<0.001) (Fig. 2).

Rats' locomotor activity during the test session was not modified by the administration of rimonabant during the conditioning phase ($F_{3,67}$=0.74, NS) (not shown). The distance travelled during the four nicotine-paired conditioning sessions is reported in Table 1. Vehicle-injected control rats exhibited no change in locomotor activity in response to repeated nicotine administrations (regression, $F_{1,70}$=1.90, p=0.17). The ANOVA for repeated measures revealed significant effects for rimonabant ($F_{3,67}$=12.10, p<0.0001) and rank of injection ($F_{3,201}$=3.00, p=0.034), but no treatment×rank interaction ($F_{9,201}$=1.20, NS). The results of independent statistical analyses for each nicotine conditioning session are indicated in Table 1. Globally, rimonabant dose-dependently reduced the distance travelled by rats given nicotine, whatever the nicotine conditioning session considered.

Experiment 3: effect of rimonabant on short-term expression of nicotine-induced conditioned place preference

During the test session conducted 24 h after the last conditioning session, i.e. 48 h after the last injection of nicotine (0.06 mg/kg), the time spent by control rats (vehicle i.p., 30 min before testing) on the nicotine-paired floor texture was significantly longer than for the unpaired texture (819 ±68 vs 381±68 s; t_{11}=3.21, p<0.005), indicating the expected development of nicotine-CPP. The ANOVA revealed that rimonabant administered as a single injection before the test session exerted an overall effect on the time differences ($F_{3,43}$=3.59, p=0.02), and subsequent comparisons with the control group indicated that this was due to the 3-mg/kg dose which blocked the *expression* of nicotine-CPP measured 24 h after the last conditioning session ($t_{4,43}$=2.29, p<0.05). Accordingly, the 3-mg/kg-injected rats no longer exhibited preference for the nicotine-paired texture (t_{10}=0.32, NS), whereas animals given rimonabant at 0.3 or 1 mg/kg spent more time on the paired than the unpaired texture (t_{11}=6.51, p<0.0001 and t_{11}=3.13, p< 0.005, respectively) (Fig. 3).

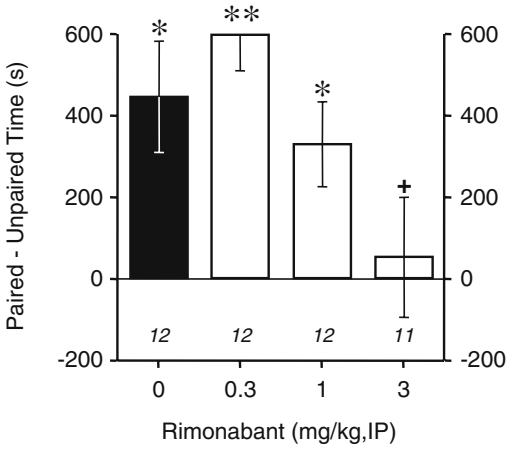

Fig. 3 Effect of rimonabant on short-term (24 h) expression of conditioned place preference induced by nicotine (experiment 3). *Histograms* represent the mean (±SEM) difference between the time (s) spent on the floor texture previously paired with nicotine (0.06 mg/kg s.c.) and the time spent on the unpaired texture, during the 20-min test session. Positive values indicate preference for the paired texture. Rimonabant, or its vehicle (0), was administered i.p., only once, 30 min before the test session conducted 24 h after the last conditioning session. Animals were drug-free during the test session. The number of rats per group is indicated under each *histogram*. *p<0.005, **p<0.0001, time spent on the nicotine-paired vs unpaired texture (paired Student's t-test); ^{+}p<0.05, vs control group given nicotine alone (0) (Dunnett's t-test after ANOVA)

The administration of rimonabant before the test session had no significant effect on the distance travelled by rats during this session ($F_{3,43}$=1.89, NS) (not shown). Table 2 reports the locomotor activity of the whole group of rats, recorded during the four conditioning sessions "with nicotine" and the four conditioning sessions "with saline." Whatever the rank of injection, nicotine induced small (+15 to +40%), but significant, increases of the distance travelled, as compared with saline (lowest paired t_{46}=3.86,

Table 1 Effects of rimonabant on rats' locomotor activity during the four nicotine-paired conditioning sessions (experiment 2)

Rimonabant (mg/kg, i.p.)	*N*	Mean (±SEM) distance travelled (cm/30 min) after successive administrations of nicotine			
		First injection (conditioning session no. 1)	Second injection (conditioning session no. 3)	Third injection (conditioning session no. 5)	Fourth injection (conditioning session no. 7)
Vehicle	18	6,372±424	6,622±498	6,169±378	7,489±558
0.3	17	6,108±427	4,958±530**	4,987±530	5,111±491**
1	18	5,092±430	4,247±472**	4,544±413*	4,603±613**
3	18	4,367±385*	3,570±303**	3,898±402**	4,203±409**
-	$F_{3,67}$=	5.20; p<0.003	8.45; p<0.0001	4.63; p<0.006	8.00; p<0.0001

Rimonabant (0.3, 1, 3 mg/kg i.p.), or its vehicle, was injected 30 min before nicotine (0.06 mg/kg s.c.). Rats were placed in the open field immediately after nicotine administration for a 30-min conditioning session. The results of independent one-way ANOVAs calculated on the distance travelled during each session are indicated in the table

n Number of rats per group

*p<0.05, vs corresponding score of the vehicle-injected rats (Dunnett's t-test)

**p<0.01, vs corresponding score of the vehicle-injected rats (Dunnett's t-test)

Table 2 Locomotor activity during alternating conditioning sessions with and without nicotine (experiment 3)

Subcutaneous injection	Mean (±SEM) distance travelled (cm/30 min) during successive conditioning sessions			
	Rank of injection			
	First	Second	Third	Fourth
Saline	5,629±267	5,217±241	5,362±203	5,167±234
Nicotine	6,442 ±215* (114%)	7,295 ±287** (140%)	6,243 ±264** (116%)	7,189 ±318** (139%)

Nicotine (0.06 mg/kg s.c.) was administered immediately before conditioning session nos. 1, 3, 5 and 7 and saline immediately before conditioning session nos. 2, 4, 6 and 8. n=47 rats
*p<0.001, vs corresponding saline injection (paired Student's t-test)
**p<0.0001, vs corresponding saline injection (paired Student's t-test)

p<0.001), but, as in the previous experiment, rats exhibited no change in locomotor activity in response to repeated nicotine administrations (regression, $F_{1,186}$<1, NS).

Experiment 4: effect of rimonabant on the long-term expression of nicotine-induced conditioned place preference

The results are reported in Table 3. During the first test session (probe session) conducted 24 h after the last conditioning session, i.e. 48 h after the last injection of nicotine (0.06 mg/kg), the 46 tested rats spent 762±22 s on the floor texture previously paired with nicotine (paired vs unpaired time: t_{45}=5.75, p<0.0001). Thus, as expected, rats developed a preference for the nicotine-associated texture. Seven of these animals exhibiting a bias for the unpaired texture (time spent on the paired texture, 424±28 s, range 343–518 s) were discarded. The remaining 39 rats, which showed individual positive difference in time spent on the paired vs unpaired textures, were subjected to a second test session, 12 weeks later, without additional nicotine injection or placement in the open fields. During this second test session, the mean time spent on the nicotine-paired texture (735±41 s) was still significantly longer than the corresponding time on the unpaired texture (t_{38}=3.33, p<0.001), showing that preference for the nicotine-paired floor texture was still present 12 weeks after the last nicotine injection. Twenty-eight of these rats, i.e. showing positive time differences (paired vs unpaired textures), were divided into two matched groups and subjected to a third test session, 24 h later, 30 min after acute administration of rimonabant (3 mg/kg) or vehicle. During the third test session, vehicle- and rimonabant-injected rats spent 782±76 and 758±53 s, respectively, on the texture previously paired with nicotine. These two values were not statistically different (t_{26}=0.21, NS) and were significantly above that for the unpaired texture (one-tailed, paired t_{13}=2.40, p<0.02 and t_{13}=1.79, p<0.05, respectively), indicating that a preference for the nicotine-paired texture was still present. Rimonabant marginally reduced the distance travelled during the third test session (Table 3).

In an independent experiment, the effect of rimonabant (3 mg/kg) on the delayed expression of nicotine (0.06 mg/kg)-induced CPP was assessed 3 weeks after the end of the conditioning phase. As in the previous 12-week experiment, rats were subjected to eight conditioning sessions and two non-drug test sessions (conducted 48 h after the last injection of nicotine and 24 h before the rimonabant test session). During the third test session, the time spent on the texture previously paired with nicotine was 737±51 s in vehicle controls and 781±68 s in rimonabant-injected rats (n=10/group). These values were significantly higher than the respective times spent on the unpaired texture (one-tailed, paired t_9=2.70 and t_9=2.57, respectively, p<0.02) and did not significantly differ from each other (t_{18}=0.53, NS).

Table 3 Lack of effect of rimonabant on long-term (12 weeks) expression of conditioned place preference induced by nicotine (experiment 4)

Test sessions (delay after the last conditioning session)	Number	Injection before test session	Paired–unpaired time (s)	Distance traveled (cm/20 min)
First test session (24 h)	46	–	324±44**	3,560±207
→paired > unpaired time	39	–	(458±40)[a]	(3,709±228)
Second test session (12 weeks)	39	–	270±82**	3,757±149
→paired > unpaired time	28	–	(548±56)[a]	(3,810±193)
Third test session (12 weeks + 24 h)	14	Vehicle	364±152*	3,735±397
	14	SR 3 mg/kg	316±106*	2,691±536***

Rats were subjected to three successive test sessions conducted 24 h, 12 weeks and 12 weeks + 24 h after the last conditioning session. They were drug free during test sessions 1 and 2. Rimonabant (SR 3 mg/kg i.p.), or its vehicle, was administered only once, 30 min before the third test session. The results are the mean (±SEM) difference between the time (s) spent on the floor texture previously paired with nicotine (0.06 mg/kg s.c.) and the time spent on the unpaired texture, during three successive 20-min test sessions, for all of the tested rats. Positive values indicate preference for the nicotine-paired texture. Locomotor activity is reported as the mean (±SEM) distance (cm) travelled during the test sessions
[a]Mean performance of rats that individually exhibited a preference for the nicotine-paired texture and were subjected to the next test session. These values were not subjected to statistical analyses since they were selected a posteriori
*p<0.05, time spent on the nicotine-paired vs unpaired texture (paired Student's t-test)
**p<0.001, time spent on the nicotine-paired vs unpaired texture (paired Student's t-test)
***p<0.10, distance travelled: rimonabant vs vehicle (unpaired Students' t-test)

Thus, rimonabant did not abolish the expression of nicotine-CPP 3 or 12 weeks after the conditioning phase.

Discussion

This study demonstrates that nicotine supports reliable and robust CPP in rats given previous experience with nicotine in their home cage. Once developed, place preference persisted for at least 12 weeks in rats given no additional exposure to the drug and the test apparatus. The CB_1 receptor antagonist, rimonabant, impaired both the establishment and the short-term expression of CPP induced by nicotine, indicating that the motivational effects of this drug are controlled by endogenous cannabinoids. However, the long-term expression of such incentive learning was not affected by acute CB_1 receptor blockade, suggesting underlying mechanisms independent of endocannabinoid processes.

The dose-response curve of nicotine to support CPP was bell-shaped, as usually observed with drugs of abuse. The largest response occurred at doses (0.06 and 0.125 mg/kg) lower than those tested in most place conditioning studies (see references in Introduction) and was of similar magnitude as that induced by cocaine (2 mg/kg s.c.), morphine (4 mg/kg s.c.) or amphetamine (1 mg/kg i.p.), using the same experimental procedure (Chaperon 1997; Chaperon et al. 1998; Duarte et al. 2003). However, the effect of the 0.06-mg/kg dose shortly failed to achieve statistical significance in animals subjected to only two nicotine and two saline conditioning sessions and in rats fed ad libitum. Therefore, more than two pairings were necessary for nicotine to establish CPP, and chronic mild food restriction was found to increase the incentive value of nicotine, as already reported for cocaine-induced CPP (Bell et al. 1997).

A drug discrimination study suggested that a negative rebound cue occurred within 16–24 h after a single injection of nicotine (Barrett et al. 2001). Though this phenomenon was described with rather high doses (0.25 and 0.5 mg/kg), it might constitute a confounding factor to the present results. However, rats conditioned with 0.6 mg/kg of nicotine, and rats given the 0.06-mg/kg dose immediately *after* conditioning sessions, did not develop place conditioning. Hence, the motivational effects of nicotine observed in the present study cannot be accounted for by a delayed aversion for the saline-paired texture, and clearly, nicotine induced subjective effects that were very probably perceived as pleasant by rats.

Once established, nicotine-CPP endured at least 12 weeks, providing evidence that the passage of time alone is insufficient to disrupt the incentive value acquired by the environmental stimuli previously paired with nicotine, as already reported for morphine and cocaine (Mueller and Stewart 2000; Mueller et al. 2002). Accordingly, it has been shown recently that the contingent presentation of stimuli previously paired with nicotine self-administration maintained non-reinforced responding for at least 3 months after nicotine withdrawal (Cohen et al. 2005), though rapid extinction of nicotine self-administration behaviour has been also reported under similar conditions (Shaham et al. 1997). These results emphasize the notion that nicotine-associated cues (either contextual or contingent) may exert a persistent control over animals' behaviour, even a long time after drug discontinuation.

The present study also provides clear-cut evidence that rimonabant prevents acquisition and abolishes short-term expression of nicotine-CPP. These findings suggest that the blockade of CB_1 receptors impairs the perception by rats of both the incentive value of nicotine during the conditioning phase and the attractiveness acquired by the floor texture previously paired with nicotine. However, a single injection of rimonabant no longer antagonized the expression of nicotine-CPP when rats were tested 12 or even 3 weeks after conditioning. Although the possibility cannot be discounted that, in these experiments, the two prior test sessions might have changed the nature of nicotine-CPP, these results indicate that an acute blockade of endocannabinoid-related processes does not impair the long-term ability of conditioned stimuli to elicit approach behaviour.

These results deserve several points of discussion. First, in line with the relevant literature (Ksir 1994; Domino 2001), nicotine stimulated locomotor activity during the conditioning sessions. This effect was modest and did not increase with the repetition of nicotine administrations, suggesting that no sensitization occurred. On the other hand, since stimulation of motor activity might contribute to incentive learning (Carr et al. 1989), the reduction of nicotine-CPP by rimonabant could be secondary to the dose-dependent reversal by the CB_1 receptor antagonist of such a motor activation. However, the lowest tested doses of rimonabant (0.3 and 1 mg/kg) reduced nicotine-induced motor stimulation while they were inactive on CPP supported by nicotine. Thus, the prevention of the establishment of nicotine-CPP by the 3-mg/kg dose of rimonabant was unlikely the consequence of a primary action on motor activity. Second, the suppression of nicotine-CPP cannot be related to a counterbalancing aversive action of rimonabant since previous studies have shown that, on its own, this CB_1 receptor antagonist does not support conditioned place avoidance (Chaperon et al. 1998; Braida et al. 2001, 2004; and see also Sañudo-Peña et al. 1997; Cheer et al. 2000). Third, it is also unlikely that the blockade of CB_1 receptors prevented the CPP response to nicotine by impairing associative processes necessary for such Pavlovian approach behaviour. Indeed, although as yet, a role for CB_1 receptors in contextual memory formation has not been established, rimonabant has never been reported to induce amnestic-like effects, but rather seems to enhance memory in rodents (Terranova et al. 1996; Lichtman 2000). Finally, as underlined above, nicotine-induced CPP was facilitated by mild food restriction. This observation is reminiscent of the enhanced ability of drugs of abuse to lower intracranial self-stimulation threshold and to maintain self-administration behaviour in food deprived rats, two augmenting effects that have been linked to changes in DA neurotransmission within the NAcc (see Carr 2002). In this context, since rimonabant reduces food intake (Arnone et al. 1997; Verty et al. 2004), it cannot be excluded that an

attenuation of restriction-induced motivational state might account for the blockade of nicotine-CPP. However, such a possibility seems unlikely since rimonabant failed to counteract the long-term expression of nicotine-CPP, at a time when rats were still food restricted.

On the other hand, a potential confound in the present study is that rats were given three nicotine injections prior to the conditioning phase and could have experienced some kind of rimonabant-precipitated nicotine withdrawal during the conditioning or the test sessions. Such a possibility seems very unlikely for several reasons. First, an acute administration of rimonabant (1–10 mg/kg) did not induce somatic manifestations of withdrawal in mice given a 6-day continuous infusion of nicotine (25 mg kg^{-1} day^{-1}) (Balerio et al. 2004). Second, the small dose of nicotine and the paced schedule of injections used in the present study cannot be compared with the more than 150-fold larger doses, infused for several days or weeks, that are necessary for conditioned place avoidance to be induced by nicotine withdrawal precipitated by the nicotine antagonist, mecamylamine, or the opioid antagonist, naloxone (see, e.g. Suzuki et al. 1996; Balerio et al. 2004). Third, rimonabant did not abolish the acquired ability of rats to discriminate nicotine from saline (Cohen et al. 2002; Le Foll and Goldberg 2004). Thus, rats which have been given the CB_1 receptor antagonist still perceived at least some of the subjective effects of nicotine, arguing against the hypothesis that a rimonabant-precipitated nicotine withdrawal might otherwise account for the impairment of the establishment or the expression of nicotine-CPP. In addition, drug discrimination studies (Cohen et al. 2002; Le Foll and Goldberg 2004) also indicate that the failure of nicotine to support CPP in rats given rimonabant during the conditioning phase could hardly be accounted for by the disappearance of its discriminative stimulus effects or some pharmacokinetic interactions during the conditioning sessions.

Interestingly, previous studies conducted by our group using the same experimental paradigm showed that rimonabant also prevented the establishment of CPP induced by cocaine, morphine and food (Chaperon et al. 1998). On the contrary, rimonabant did not prevent conditioned place avoidance induced by a variety of aversive drugs (Chaperon and Thiébot 1999). Thus, the blockade of CB_1 receptors can reduce the incentive value of positive reinforcers such as drugs of abuse and natural rewards while having no effect on negative incentives.

Although there is strong evidence linking NAcc DA neurotransmission and reward, several studies suggest that DA signals novelty or reward expectation, rather than reward itself (see references in Mansvelder and McGehee 2002). In this regard, the NAcc shell may be more critically involved in strengthening the learning processes underlying context–drug associations than in mediating the direct rewarding effects of drugs (Di Chiara 1999). In keeping with a nicotine-induced DA overflow in the NAcc shell, and its reduction by rimonabant (Cohen et al. 2002), such a mechanism is likely to play a role in the development of place preference to nicotine and account for the ability of the CB_1 receptor antagonist to prevent the establishment of nicotine-CPP. On the other hand, it has been shown that *Pavlovian* responding to cues repetitively paired with drugs increased DA selectively within the NAcc core, whereas this effect was apparently unnecessary for *operant* responding maintained by the contingent presentation of a conditioned stimulus (Ito et al. 2000; Balfour 2002). Therefore, this mechanism can hypothetically account for a role of DA in the incentive value acquired by contextual cues explicitly paired with drugs during the conditioning phase. Indeed, a single injection of rimonabant suppressed the expression of nicotine-CPP when the test took place 48 h after the last injection of nicotine. However, it remains to study directly in such conditions the release of DA within the NAcc shell and/or core and the effects of rimonabant thereon.

The blockade of expression of nicotine-CPP no longer occurred when 3 months—and even 3 weeks—elapsed between the conditioning phase and the test session. This finding stands in apparent contrast with a recent study showing that, in nicotine self-administering rats, 1 month after drug discontinuation, rimonabant decreased operant responding maintained by stimuli previously paired with nicotine infusion (Cohen et al. 2005). In both studies, rats were subjected to chronic mild food restriction until the end of the experiments; thus, putative differences in baseline DA levels due to the feeding status (Pothos et al. 1995) cannot explain this discrepancy. However, several other factors, non-mutually exclusive, might account for this different sensibility to CB_1 receptor blockade. The number of contacts with nicotine and the daily dose received during the conditioning phase were clearly different: seven injections of 0.06 mg/kg, 2–3 days apart in the present study; 0.6 mg/kg for a preconditioning evaluation of rats' response to nicotine and then ca. 1 mg kg^{-1} day^{-1}, 5 days a week, during ten daily self-administration sessions in Cohen et al.'s (2005) study. Such schedules of administration might result in differences in the highly complex regulatory processes undergone by the diverse neuronal nACh receptor subtypes (for a review, see Mansvelder and McGehee 2002) and, consequently, on the net effect of nicotine on DA neurotransmission. This might be of crucial importance since nicotine switches the firing pattern of DA neurones from tonic activity to burst firing (Rice and Cragg 2004; Zhang and Sulzer 2004), the latter mode being claimed to convey reward-related signalling for both primary and conditioned reinforcers (Hyland et al. 2002; Schultz 2002; Phillips et al. 2003). The observed differences may also relate to the type of conditioning used in the two studies (operant vs Pavlovian), the response requirement (active and predetermined vs no explicitly learned responses) and/or the characteristics of the stimuli subserving rats' behaviour (discrete, response-contingent stimuli vs passive exposure to contextual cues), all factors exerting pivotal influence on reward-related neurochemical and behavioural processes. In addition, the fact that self-administering rats were regularly re-exposed to discrete response-contingent stimuli after nicotine withdrawal (Cohen et al. 2005), whereas in the present study, animals were exposed to the

contextual cues only twice between the last nicotine injection and the rimonabant test session, might account for such differences in the effect of the CB_1 receptor antagonist on long-term nicotine-seeking behaviour. Together, these results indicate that the mechanisms allowing the perception by animals of the motivational strength of explicit stimuli previously paired with nicotine differed depending on the dose, the number and the rhythm of nicotine administration and/or the time elapsed from the last drug exposure and/or the behavioural procedure used to assess nicotine seeking. Clearly, the complete treatment of this specific question is beyond the scope of this paper, but several possibilities can be considered. Long-term changes in synaptic influences might have developed, as synapses may appear or disappear in response to changing patterns of use (see Alger 2002). For instance, sensitization of rats' locomotor activity, claimed to play a role in the acquisition and maintenance of addictive behaviour, almost completely disappeared 2 weeks after the last of six injections of nicotine (0.3 mg/kg, each other day) (Villégier et al. 2003). The age of rats at the time of the test sessions (9 vs 12 or 21 weeks) may also contribute to these differences, as cannabinoids seem to readily suppress excitatory postsynaptic currents (EPSCs) in tissues from young animals, whereas they seem to have no effect in adults (see Alger 2002). On the other hand, novelty appears also as an important determinant of the activation of DA neurons (Spanagel and Weiss 1999). Since the effect of rimonabant on long-term expression of nicotine-CPP has been investigated in rats already subjected to two test sessions in a non-drugged state (24 h after the end of the conditioning phase and 24 h before the rimonabant test session), it cannot be excluded that familiarization with the test conditions (two textures present in the open field) might have reduced the endocannabinoid signalling that participates in the feedback control of synaptic efficacy in the reward circuit.

To conclude, the present study clearly shows that nicotine has strong incentive properties. The environmental stimuli previously paired with its neurobiological (rewarding) effects come to elicit approach response on test sessions, and there is no spontaneous extinction within 3 months after the conditioning phase. These results are consonant with recent data showing that nicotine enhances the control over behaviour by reward-associated cues, and that such inability to inhibit reward-elicited behaviour may extend over long periods after drug discontinuation (see Olausson et al. 2003). The blockade by rimonabant of nicotine-induced CPP suggests that endocannabinoids are necessary to the perception by rats of the motivational value of nicotine during conditioning and participate in the neurobiological processes underlying the capacity of nicotine-paired stimuli to elicit approach behaviour during the short-term expression phase. On the contrary, the long-term "trace" (or "memory") of incentive learning was not impaired by a single pretest injection of rimonabant. It cannot be excluded, however, that rimonabant could be effective earlier after drug discontinuation and/or on repeated administrations. As a matter of fact, a 10-week rimonabant treatment, beginning while subjects were still smoking, has been found active in Phase III clinical trials for smoking cessation (Dale et al. 2004). Therefore, the present results suggest that the mechanisms which ensure short-term motivational valence to nicotine-paired stimuli are likely different, at least in part, from those taking place later, and that an acute activation of CB_1 receptors is no longer essential for the control of behaviour by environmental incentives after a given time interval (to be determined, but of less than 3 weeks) after exposure to nicotine.

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