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Cellular Mechanisms Underlying the Anxiolytic Effect of Low Doses of Peripheral Δ^9-Tetrahydrocannabinol in Rats

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We investigated the effect of low doses of intraperitoneal Δ^9-tetrahydrocannabinol (THC) on anxiety behavior in rats using the elevated plus maze (EPM). An anxiolytic effect was obtained in a range of doses between 0.075 and 1.5 mg/kg, the 0.75 dose being the most effective. Pretreatment with the CB1 receptor antagonist AM251 fully reversed THC's effect, suggesting CB1 receptors were involved. In order to elucidate the neuroanatomical substrates underlying the effect of the maximal effective dose of THC, we investigated cFos expression in anxiety-related brain regions (prefrontal cortex, nucleus accumbens, amygdala, and hippocampus) of rats exposed to the EPM. THC significantly lowered the amount of cFos in prefrontal cortex and amygdala without affecting the other cerebral areas. As there is increasing evidence that CREB function regulates anxiety-like behavior in rats, the second biochemical parameter we measured was phosphorylated CREB in the same brain areas. Rats treated with THC showed a significant increase in CREB activation in the prefrontal cortex and hippocampus. In the prefrontal cortex this increased activation was linked to an increase in ERK activation, whereas in the hippocampus there was a drop in the activity of CAMKII, a kinase with inhibitory effect on CREB activation. All these effects were reversed by AM251 pretreatment, suggesting that stimulation of CB1 receptors is fundamental for triggering the biochemical events. Our results suggest that the stimulation of these receptors in the prefrontal cortex, amygdala, and hippocampus with the subsequent activation of different signaling pathways is the first event underlying the effects of cannabinoids on anxious states.
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INTRODUCTION

The role of cannabinoids in the modulation of anxious states is still a matter of controversy. In humans, cannabis use could promote either relaxation and euphoria, thus relieving anxious state, or anxiety and panic attacks, depending on subjects, and, within the same subject, on the emotional state prior the use (Iversen, 2003). Similarly, animal experiments with different cannabinoid agonists revealed both anxiolytic-like and anxiogenic-like responses (for a review, see Viveros *et al*, 2005) depending on the doses and the familiarity of the environment. Natural and synthetic cannabinoids seem to display a dose-dependent biphasic profile in rats and mice when using standard anxiety models. Low doses of these compounds produced anxiolytic-like responses (Valjent *et al*, 2002; Berrendero and Maldonado, 2002; Genn *et al*, 2004; Marco *et al*, 2004; Rodriguez de Fonseca *et al*, 1996), whereas higher doses resulted in anxiogenic-like effects in several models of anxiety (Onaivi *et al*, 1990; Arevalo *et al*, 2001; Marin *et al*, 2003; Genn *et al*, 2004; Marco *et al*, 2004). Recent data obtained with pharmacological agents that enhance the endocannabinoid tone join this complex picture suggesting that anandamide too has a role in the regulation of anxiety states (Bortolato *et al*, 2006; Kathuria *et al*, 2003). Peripheral injection of the endocannabinoid transport inhibitor AM404 or the active inhibitor of fatty acid amide hydrolase URB597 produced anxiolytic-like effects in different rat models of anxiety. These effects were accompanied by an increase in the brain level of anandamide and were prevented by CB1 receptor blockade.

Besides the confusing picture from behavioral experiments, the mechanisms of the effects of cannabinoids on anxiety-related responses still need further investigation. Although it has been proposed that the corticotropin-releasing hormone (CRH) system as well as CCK and serotonin (see for review Viveros *et al*, 2005) may participate in cannabinoids' effects on anxiety behavior, the intracellular pathways activated by the stimulation of CB1 receptors, involved too, are still unknown.

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The present study investigated the effect of low doses of THC on anxiety behavior in rats, measured in the EPM. To clarify the neuroanatomical substrates of the effect of intraperitoneally injected THC, we also investigated cFos expression in anxiety-related brain regions (prefrontal cortex, nucleus accumbens, amygdala, and hippocampus) of rats exposed to the EPM. Assessment of cFos expression is the most widely used functional anatomical mapping tool to identify cells and extended circuitries that become activated in response to various stimuli including anxiogenic environment (Kovacs, 1998). The second biochemical parameter we measured was phosphorylated CREB (ie activated CREB) in nuclear extracts from the prefrontal cortex, nucleus accumbens, amygdala, and hippocampus of rats treated with THC and exposed to EPM, as CREB function probably regulates anxiety-like behavior in rats (Barrot *et al*, 2002; Pandey, 2003). In the positive brain regions we then investigated the CB1-dependent intracellular pathways activated by THC that might account for the CREB modulation. There are numerous protein kinases that regulate CREB activity differently: protein kinase A (PKA) and ERK-activated ribosomal S6 kinases (RSKs) phosphorylate CREB and lead to activation of gene transcription (Carlezon *et al*, 2005); by contrast, Ca^{2+}/calmodulin-dependent kinase (CAMK) II reduces CREB-mediated gene transcription (Wu and McMurray, 2001). *In vivo* stimulation of CB1 receptors can affect PKA and ERK activity (Rubino *et al*, 2000, 2004; Valjent *et al*, 2001; Hampson *et al*, 1995), but there is no evidence of their ability to alter CAMK II *in vivo*, although the inhibition of voltage-gated calcium channels is an established cannabinoid transduction pathway (Howlett and Mukhopadhyay, 2000). We therefore investigated the activity of PKA, ERK, and CAMKII.

METHODS

Subjects

All the experimental procedures followed the guidelines of the Italian Council on Animal Care, and were approved by the Italian Government decree No. 33/2004. All efforts were made to minimize the number of animals used and their suffering.

At the beginning of the experiment, maze-naïve male Sprague–Dawley rats (Charles-River, Calco, Italy), weighing 150–175 g, were housed in cages (10 per cage) in a climatically controlled colony room under a 12-h light–dark cycle (lights on at 0800 hours). Food and water were continuously available and each animal was handled daily during the 7 days preceding the experiment. The day before the experiment animals were individually housed and randomly assigned to each experimental group (10 rats per group). Rats were used only once.

Drugs and Treatments

The following drugs were used: THC (kindly supplied by GW Pharma, Salisbury, England), in a range of doses between 0.015 and 3 mg/kg and dissolved in cremophor, ethanol, and saline (1 : 1 : 18). AM251 (3 mg/kg) was obtained from Sigma-Aldrich MO (St Louis, USA), and dissolved in DMSO 10% and saline 90%. THC was given acutely i.p. 30 min before, whereas AM251 was given 40 min before the test. SL327 (Tocris, Bristol, UK) was dissolved in 30% DMSO. The doses of the drugs were calculated as salt.

Surgery

For intracerebral surgery, rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and guide cannulae for prefrontal cortex microinjections were stereotaxically implanted unilaterally and cemented to the skull (coordinates with references to bregma according to stereotaxic atlas of Paxinos and Watson: prefrontal cortex AP + 2.7 mm, ML 0.8 mm, DV 3 mm). Immediately after surgery, rats were given an injection of amoxicilline (20 mg/kg) and then allowed 7 days to recover from surgery before any behavioral testing.

Behavioral Studies

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

Upon completion of injection made in the colony room, animals were transported to plus-maze laboratory to facilitate adaptation to novel surroundings for 30 min. Then, rats were placed individually onto the center of the apparatus facing an open arm, and the time spent on and entries onto each arm were detected for 5 min. The maze was wiped clean with water and dried after each trial. All experimental sessions were conducted during the morning time of the light cycle (1000–1400 hours). An arm entry was recorded when all four paws of the rat were in the arm. The number of open- and closed-arm entries and the time spent in each arm were recorded. Data were expressed as the percentage (open or closed time/total time × 100; open or closed entries/total entries × 100). The percentage of time spent in the open arms and the percentage of open-arm entries were used as measures of anxiety (Hogg, 1996).

Spontaneous motor activity. Spontaneous motor activity was evaluated as previously described () in an activity cage (43 × 43 × 32 cm) (Ugo Basile, Varese, Italy), placed in a sound-attenuating room. The cage was fitted with two parallel horizontal and vertical infrared beams located 2 and 6 cm from the floor. Cumulative horizontal and vertical movement counts were recorded 30 min after treatment with vehicle or THC for 15 min.

Biochemical Studies

After the EPM session, rats were killed and brains quickly removed. In these studies, a group of animals handled as the ones exposed to EPM but not tested (naïve) was also present to check the level of the biochemical parameters in basal conditions. The cerebral areas (prefrontal cortex, nucleus accumbens, amygdala, and hippocampus) were obtained within a few minutes by regional dissection on ice according

to Heffner *et al* (1980), and immediately frozen in liquid nitrogen and stored at −80°C until used.

Tissue preparation. Each brain region was homogenized in an appropriate volume of ice-cold Buffer A (10 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosfate, 0.5% Triton, 5 mg/ml aprotinin, and 5 mg/ml leupeptin) and centrifuged at 13 000 rpm at 4°C for 3 min. The supernatant was used for cytosolic preparation and pellet for nuclear extracts. Cytosolic preparation: the supernatant was saved, centrifuged at 11 200 rpm at 4°C for 20 min and the resulting supernatant was used as soluble fraction. Nuclear extracts preparation: the pellet was resuspended in an appropriate volume of ice-cold Buffer C (20 mM Hepes pH 7.5, 400 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10 μM Na_2MoO_4, 0.1 mM EDTA, 1 mM sodium orthovanadate, 10 mM PNPP, 10 mM β-glycerophosphate, 20% glycerol, 2 mM DTT, and protease inhibitors as above) and homogenized. After 30 min incubation on ice with gently rocking, samples were centrifuged at 13 000 rpm at 4°C for 10 min and the resulting supernatant was saved and used as nuclear extract. Protein concentrations in the respective fractions were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL, USA).

Western blotting. Equivalent amounts of protein from the soluble fraction for each sample were resolved by 10% SDS-PAGE, blotted electrophoretically to nitrocellulose membrane, blocked and then incubated overnight at 4°C with a rabbit polyclonal antibody that selectively recognized phospho-ERK 1-2 (Cell Signalling Technology Inc., Beverly, MA, USA; diluted 1 : 1000) or phospho-CAMKII (Chemicon International, Temecula, CA, USA; diluted 1 : 750). Bound antibodies were detected with horseradish peroxidase-linked anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; diluted 1 : 3000) and developed using an enhanced chemiluminescence reagent (Amersham Biosciences, Milan, Italy). To control for protein loading, the membranes were then stripped with T-PBS for 1 h at 60°C and reprobed with a rabbit polyclonal antibody raised against total ERK (Santa Cruz Biotechnology Inc., diluted 1 : 5000) or total CAMKII (Chemicon International, diluted 1 : 1000). The relevant immunoreactive bands were quantified with scanning densitometry using Scion Image software. To allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of control. The value of active phospho-proteins was normalized to the amount of total proteins in the same sample and expressed as a percentage of controls.

PKA activity. The amount of PKA activated *in vivo* was assayed according to the instructions of the SignaTECT PKA assay system (Promega, Madison, WI, USA). In brief, the isolated brain regions were homogenized in ice-cold buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 0.1% Triton X-100, 10 g/ml leupeptin, and 10 g/ml aprotinin. The homogenized tissues were briefly centrifuged at 13 000 g at 4°C, and the supernatant was used as cellular extract. Lysate (5 μl) was added to a reaction mixture containing 10 μCi [^{32}P]ATP, 5 μM cAMP, 100 μM PKA biotinylated peptide substrate, 40 mM Tris-HCl (pH 7.4), 25 mM $MgCl_2$, and 100 μg/ml bovine serum albumin. Each reaction was immediately incubated at 30°C for 5 min and then terminated by adding 12.5 μl of 7.5 M guanidine hydrochloride. An aliquot of the terminated reactions was spotted onto a phosphocellulose filter. After the filters were washed with 2 M NaCl in 1% H_3PO_4, and deionized water, respectively, the amount of ^{32}P-labeled substrate in the filter was measured by scintillation counting.

Transcription factors evaluation. The level of activated transcription factors was measured in the nuclear extracts using transcription factor assay kits (Active Motif, Rixensart, Belgium) based on ELISA method to detect and quantify pCREB and c-Fos activation. Briefly, oligonucleotides containing a cAMP-responsive element (CRE) or a TPA-responsive element (TRE) have been immobilized in a 96-well plate. CREB or cFos contained in nuclear cell extracts bind specifically to these oligonucleotides. By using an antibody directed against phosphorylated CREB or c-Fos, the equivalent transcription factors bound to the oligonucleotide are detected. Addition of a secondary antibody conjugated to horseradish peroxidase provides sensitive colorimetric readout that is quantified by spectrophotometry at 450 nm.

Statistical Analysis

Data were expressed as mean ± SEM and analyzed with one-way ANOVA or Kruskal–Wallis test followed by *post hoc* Tukey's or Dunnett's test. A $p < 0.05$ was considered to be significant throughout. All statistical calculations were carried out using Prism, version 4 software (GraphPad, San Diego, CA, USA).

RESULTS

Behavioral Findings

Figure 1 shows the differences between groups with respect to the percentage of EPM parameters at all THC doses, administered 30 min before the test. One-way ANOVA indicated a significant effect of treatment on the percentage of open-arm entries ($F_{(6,63)} = 14.34$, $p < 0.0001$), closed-arm entries ($F_{(6,63)} = 18.26$, $p < 0.0001$), open-arm time ($F_{(6,63)} = 8.01$, $p < 0.001$), and closed-arm time ($F_{(6,63)} = 6.58$, $p = 0.0003$). *Post hoc* analysis found significant differences between THC-treated groups and the vehicle group for doses between 0.075 and 1.5 mg/kg. The anxiolytic effect of THC was dose dependent in the range between 0.015 and 0.75 mg/kg ($r^2_{\text{open-arm entries}} = 0.95$, $r^2_{\text{open-arm time}} = 0.96$, $p < 0.02$). The maximal anxiolytic effect was obtained with the dose of 0.75 mg/kg. The highest dose (3 mg/kg) was ineffective.

The effect of the CB1 receptor antagonist AM251 given alone or with THC (0.75 mg/kg) is reported in Figure 2. One-way ANOVA showed that treatment significantly affected the percentage of open-arm entries ($F_{(3,36)} =$

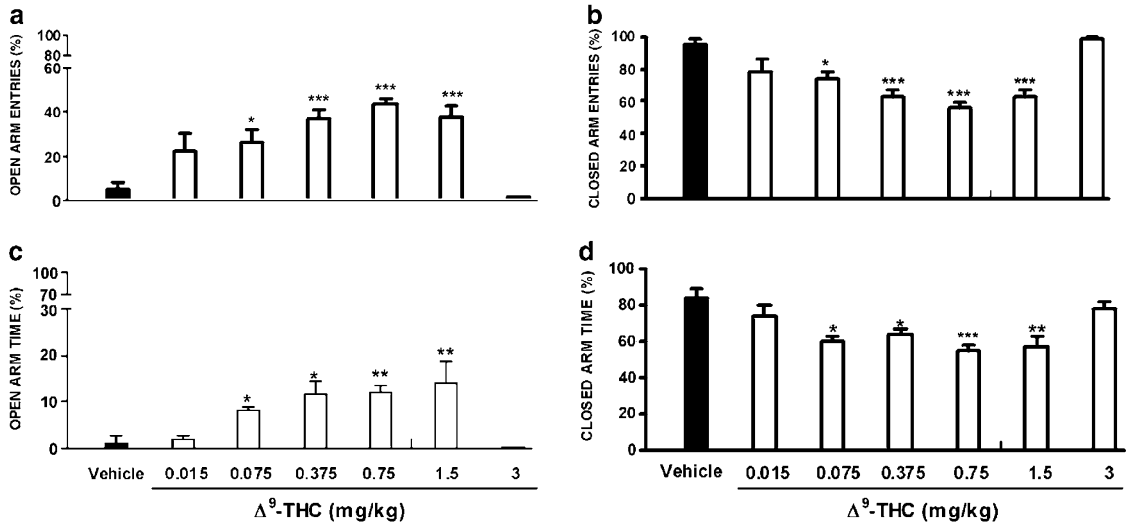

Figure 1 Effect of systemic THC on behavior in the elevated plus maze (EPM). Male adult Sprague–Dawley rats were injected with vehicle or THC (0.015–3 mg/kg, i.p.) 30 min before testing on the EPM for 5 min. Percentage of entries into open (a) and closed (b) arms, percentage of time spent in the open (c) and closed (d) arms. Each column represents the mean ± SEM of 10 animals. *$p<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle group (one-way ANOVA followed by Tukey's test).

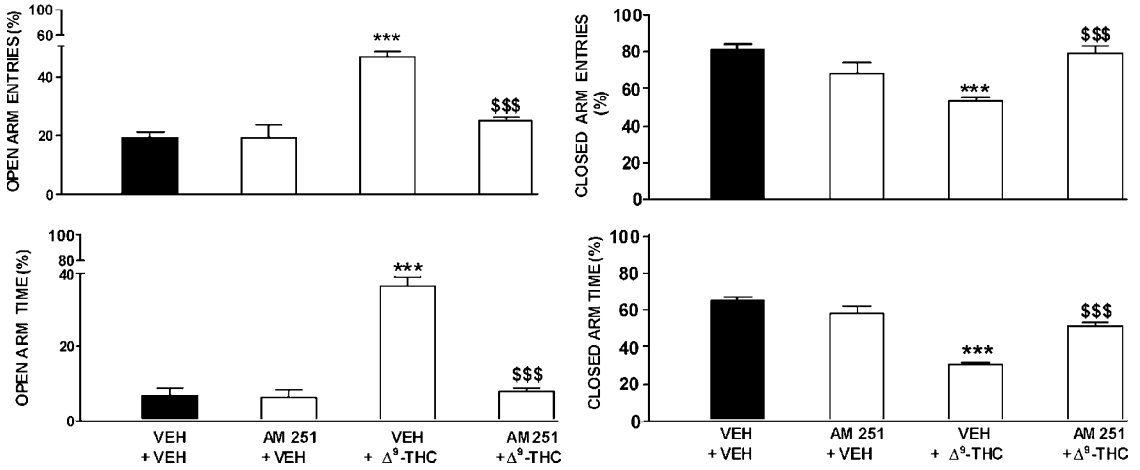

Figure 2 Effect of the CB1 cannabinoid receptor antagonist, AM251, on THC-induced anxiolytic effects in the EPM. AM251 was given i.p. at a dose of 3 mg/kg, 10 min before THC (0.75 mg/kg, i.p.), which was given 30 min before the test. Each column represents the mean ± SEM of 10 animals. VEH = vehicle. ***$p<0.001$ compared to vehicle and AM251 alone; $^{\$\$\$}p<0.001$ compared to THC alone (one-way ANOVA followed by Tukey's test).

26.06, $p<0.0001$), closed-arm entries ($F_{(3,36)}=9.82$, $p<0.0001$), open-arm time ($F_{(3,36)}=68.52$, $p<0.0001$), and closed-arm time ($F_{(3,36)}=35.17$, $p<0.0001$). *Post hoc* analysis showed that the cannabinoid antagonist *per se* did not change emotional reactivity. Pretreatment with AM251 completely blocked the effect of THC on the EPM parameters.

Figure 3 illustrates the effect of the maximal anxiolytic and the highest dose of THC, in the EPM, on spontaneous motor activity. One-way ANOVA indicated a significant effect of drug treatment on horizontal ($F_{(2,27)}=6.47$, $p=0.005$) and vertical counts ($F_{(2,27)}=6.56$, $p=0.004$). *Post hoc* comparison showed that spontaneous motor activity was not affected by the maximal anxiolytic dose of THC. The highest dose significantly reduced both the horizontal and vertical counts.

Biochemical Findings

All the biochemical experiments were carried out with the THC dose that had maximal anxiolytic effects, that is 0.75 mg/kg.

First of all we checked whether cFos expression was altered in rats exposed to the EPM compared to naïve animals. As shown in Figure 4, cFos levels were significantly higher in the prefrontal cortex ($F_{(4,32)}=4.259$, $p<0.01$; $p<0.05$ for *post hoc* comparison between naïve and vehicle) and amygdala ($F_{(4,30)}=6.363$, $p<0.001$; $p<0.05$ for *post hoc* comparison between naïve and vehicle) of EPM-tested rats. THC significantly reduced the amounts of cFos in the prefrontal cortex ($F_{(4,32)}=4.259$, $p<0.01$; $p<0.01$ for *post hoc* comparison between vehicle and THC) and amygdala ($F_{(4,30)}=6.363$, $p<0.001$; $p<0.01$ for *post hoc* comparison

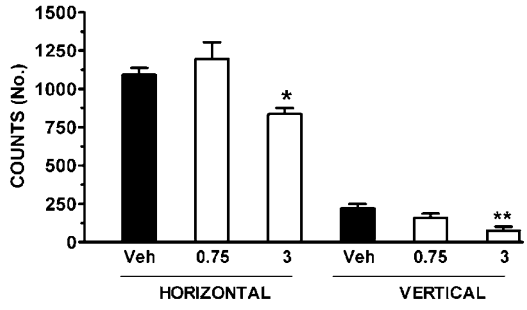

Figure 3 Effect of THC (0.75 and 3 mg/kg, i.p.), given 30 min before the test, on spontaneous motor activity evaluated in terms of horizontal and vertical counts. Each column represents the mean ± SEM of 10 animals. VEH = vehicle. $^{*}p<0.05$, $^{**}p<0.01$ compared to corresponding vehicle (one-way ANOVA followed by Tukey's test).

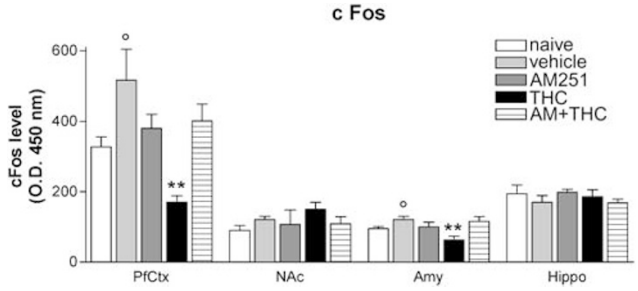

Figure 4 Effect of THC (0.75 mg/kg ip) and AM251 pretreatment (3 mg/kg i.p., 10 min before THC) on the cFos protein level in the nuclear extracts of different brain areas. cFos in naive animals is also reported. The results are expressed as optical density and are the mean ± SEM of at least four animals. PfCtx, prefrontal cortex; NAc, nucleus accumbens; Amy, amygdala; Hippo, hippocampus. $^{\circ}p<0.05$ *vs* naïve; $^{**}p<0.01$ *vs* vehicle (one-way ANOVA followed by Tukey's test).

between vehicle and THC) without affecting the other cerebral areas. When AM251 was injected before THC, the decrease was reversed, suggesting the effect was CB1 dependent.

Figure 5 shows the levels of phosphorylated CREB in the different rat cerebral areas. As for cFos experiments, first of all we checked whether pCREB level was altered in rats exposed to the EPM compared to naïve animals. No significant alterations were observed between the two groups. In rats treated with THC, only the prefrontal cortex and hippocampus showed a significant increase in the level of phosphorylated CREB. The raise was about 90% in the prefrontal cortex ($F_{(4,23)}=11.85$, $p<0.0001$; $p<0.001$ for *post hoc* comparison between vehicle and THC) and 75% in the hippocampus ($F_{(4,28)}=3.125$, $p<0.05$; $p<0.05$ for *post hoc* comparison between vehicle and THC). AM251 pretreatment fully reversed the increase, indicating that CB1 receptor stimulation was involved.

We next studied the cellular events involved in CREB regulation that could be triggered by CB1 receptor, studying the activity of PKA, CAMKII, and ERK in the prefrontal cortex and hippocampus of rats treated with THC and then exposed to EPM. As for all the other biochemical experiments, we checked these pathways in the naïve group too. EPM exposure *per se* did not modify PKA activity in the two brain areas, and THC administration did not alter it (Figure 6).

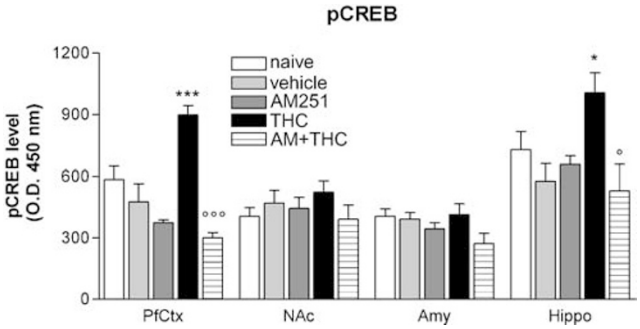

Figure 5 Effect of THC (0.75 mg/kg i.p.) and AM251 pretreatment (3 mg/kg i.p., 10 min before THC) on phosphorylated CREB protein level in the nuclear extracts of different brain areas. pCREB in naive animals is also reported. The results are expressed as optical density and are the mean ± SEM of at least four animals. PfCtx, prefrontal cortex; NAc, nucleus accumbens; Amy, amygdala; Hippo, hippocampus. $^{*}p<0.05$; $^{***}p<0.001$ *vs* vehicle $^{\circ}p<0.05$; $^{***}p<0.001$ *vs* vehicle $^{\circ}p<0.05$; $^{\circ\circ\circ}p<0.001$ THC (one-way ANOVA followed by Tukey's test).

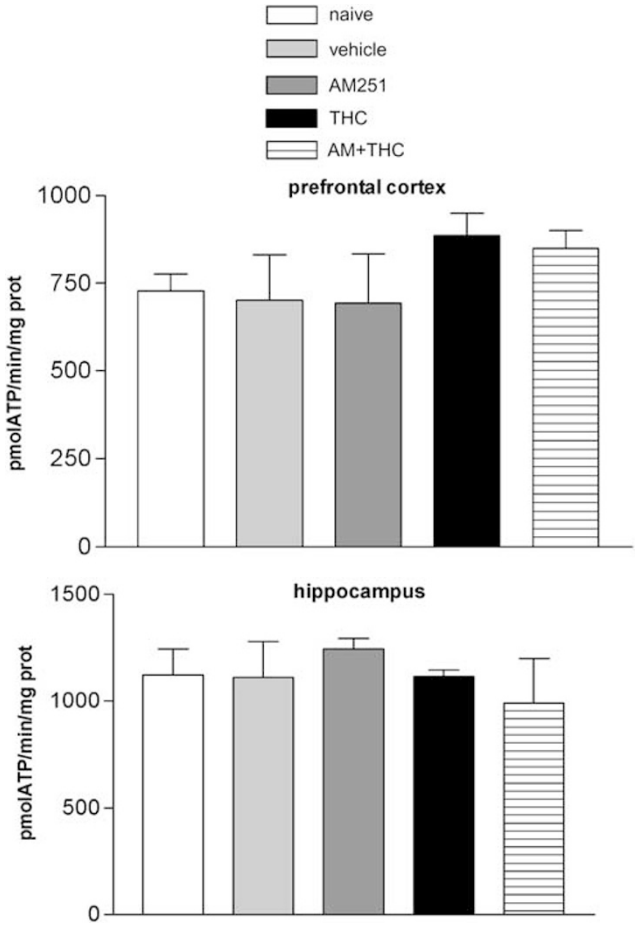

Figure 6 Effect of THC (0.75 mg/kg i.p.) and AM251 pretreatment (3 mg/kg i.p., 10 min before THC) on PKA activity in the prefrontal cortex and hippocampus. PKA activity in naive animals is also reported. The results are expressed as pmol ATP/min/mg protein and are the mean ± SEM of at least four animals.

EPM exposure *per se* induced a significant reduction in pCAMKII immunoreactivity in the prefrontal cortex ($F_{(4,15)}=9.982$, $p<0.001$; $p<0.01$ for *post hoc* comparison between naïve and vehicle), and THC administration did

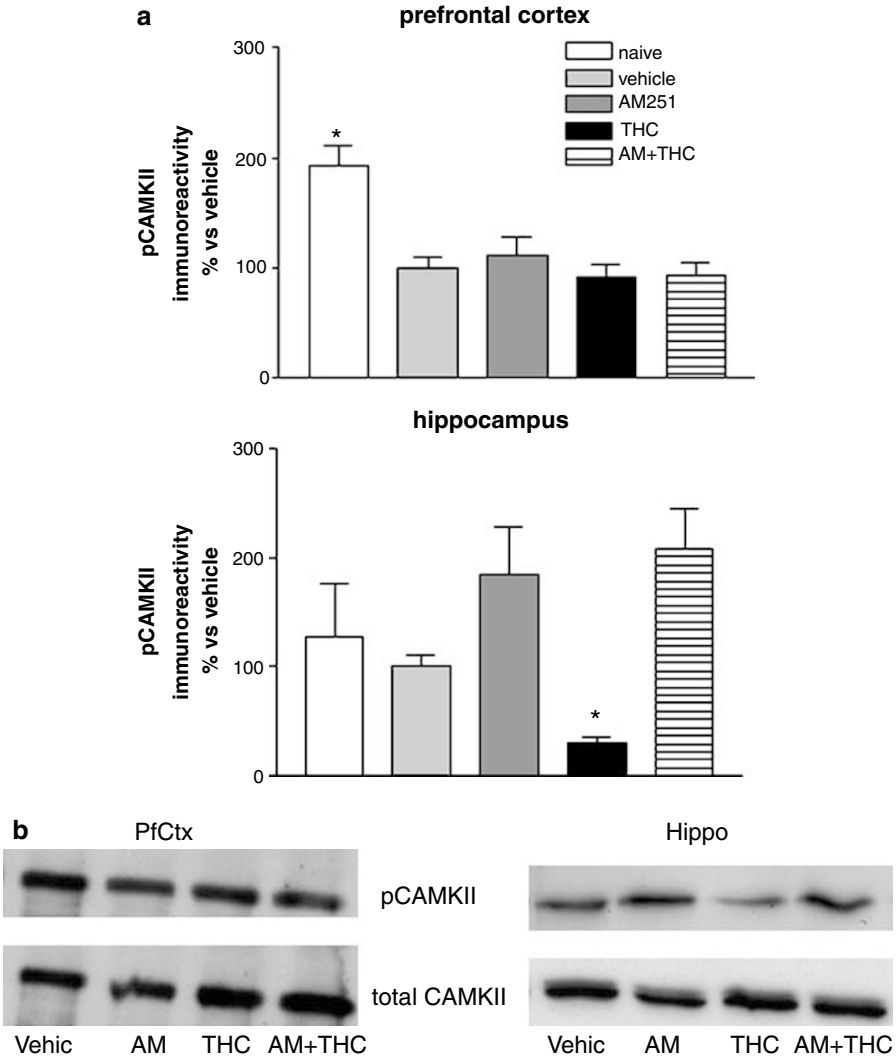

Figure 7 Effect of THC (0.75 mg/kg i.p.) and AM251 pretreatment (3 mg/kg i.p., 10 min before THC) on CAMKII activation in the prefrontal cortex (PfCtx) and hippocampus (Hippo). CAMKII activation in naive animals is also reported. (a) Densitometric analysis; (b) representative autoradiograms. The results are expressed as the percentage pCAMKII immunoreactivity *vs* vehicle and are the mean ± SEM of at least three animals. *$p < 0.05$ *vs* vehicle (one-way ANOVA followed by Tukey's test).

not modify further this picture. In the hippocampus, exposure to EPM did not modify the level of phospho-CAMKII, THC instead, significantly lowered it ($F_{(4,15)} = 4.354$, $p < 0.05$; $p < 0.05$ for *post hoc* comparison between vehicle and THC) in a CB1-dependent manner, as it was fully reversed by the AM251 pretreatment (Figure 7).

Concerning pERK immunoreactivity in the prefrontal cortex, EPM exposure *per se* induced a trend to increase pERK levels, but it did not reach the statistical significance (Figure 8). THC treatment produced an increase in pERK immunoreactivity significantly different from that induced by EPM ($F_{(4,11)} = 7.110$, $p < 0.01$; $p < 0.05$ for *post hoc* comparison between vehicle and THC) which was prevented by the CB1 antagonist (Figure 8). When the same analysis were performed in the hippocampus, no significant changes were observed (Figure 8).

The causative link between the ERK pathway and THC anxiolytic effect was tested in rats by microinjecting in the prefrontal cortex SL327, a drug that prevents the activation of ERK by inhibiting MEK, the upstream kinase of ERK (Atkins *et al*, 1998; Rubino *et al*, 2005). SL327 microinjection (30 μg) lowered ERK phosphorylation in rats prefrontal cortex ($p < 0.01$, Student's *t*-test; Figure 9a). When injected 5 min before THC, it prevented THC anxiolytic effect both in term of percentage of open-arm time ($F_{(3,11)} = 7.189$, $p < 0.01$; $p < 0.01$ for *post hoc* comparison between THC and SL327 + THC; Figure 9b) and open-arm entries ($F_{(3,11)} = 11.0$, $p < 0.01$; $p < 0.001$ for *post hoc* comparison between THC and SL327 + THC; Figure 9b). Finally, SL327 pretreatment fully reversed THC-induced CREB activation in the prefrontal cortex ($F_{(3,18)} = 18.14$, $p < 0.0001$; $p < 0.001$ for *post hoc* comparison between THC and SL327 + THC; Figure 9c).

DISCUSSION

The present study provides evidence of a dose-dependent anxiolytic effect of THC obtained at doses between 0.075 and 1.5 mg/kg, 0.75 mg/kg being the most effective. The lack

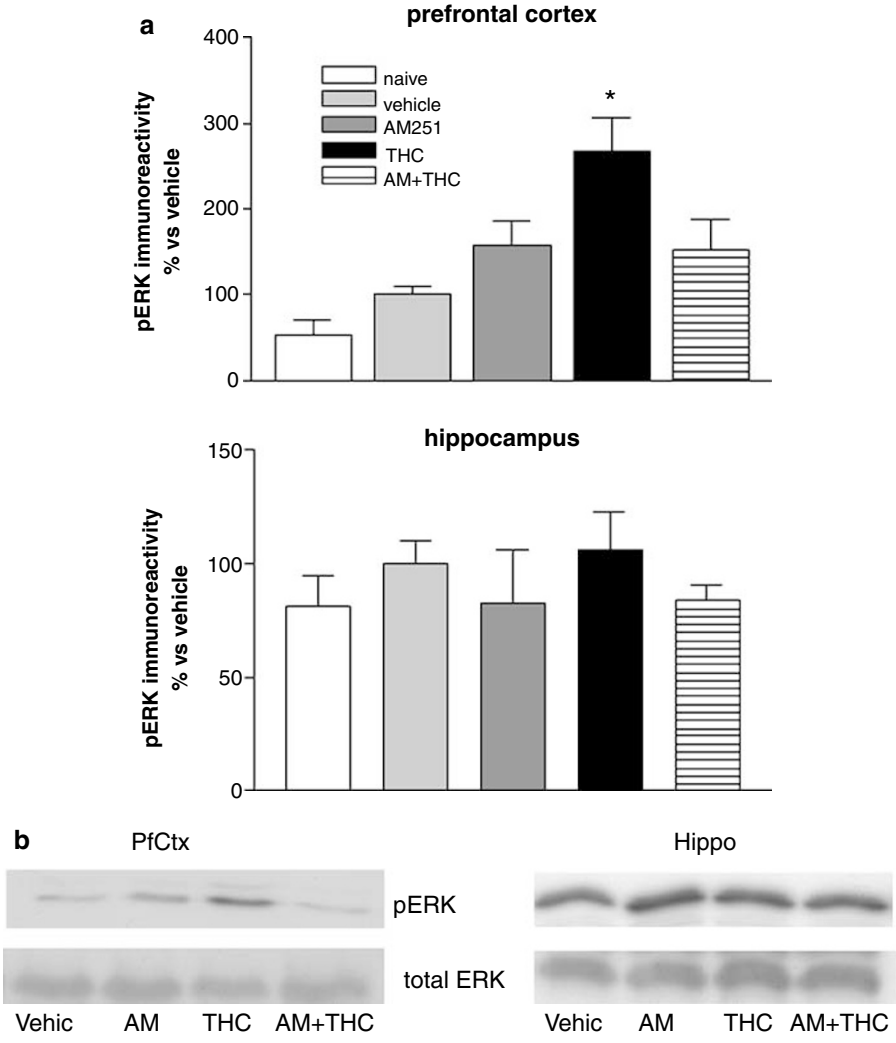

Figure 8 Effect of THC (0.75 mg/kg i.p.) and AM251 pretreatment (3 mg/kg i.p., 10 min before THC) on ERK activation in the prefrontal cortex (PfCtx) and hippocampus (Hippo). ERK activation in naive animals is also reported. (a) Densitometric analysis; (b) representative autoradiograms. The results are expressed as the percentage pERK immunoreactivity *vs* vehicle and are the mean ± SEM of at least three animals. $^{*}p < 0.05$ *vs* vehicle (one-way ANOVA followed by Tukey's test).

of anxiolytic effect with the highest dose (3 mg/kg) may be explained by the motor impairment which could have interfered with the behavior used to measure anxiolytic effects.

Our findings agree with Berrendero and Maldonado (2002) who found an anxiolytic effect of THC in mice, at a dose of 0.3 mg/kg, using the light–dark box. Onaivi *et al* (1990) using the EPM found either an anxiogenic or no effect of THC using a range of doses higher than ours. Previous studies have shown that cannabinoid agonists have a dose-dependent biphasic effect in anxiety-like responses (Onaivi *et al*, 1990; Rodriguez de Fonseca *et al*, 1996; Valjent *et al*, 2002), as already observed for other pharmacological actions induced by cannabinoids (Sanudo-Pena *et al*, 2000; Sulcova *et al*, 1998; Braida *et al*, 2004).

The CB1 antagonist AM251 reversed the anxiolytic-like effect of THC, providing evidence of the specific involvement of CB1 cannabinoid receptors in this behavior.

While we were drafting this manuscript, the paper of Patel and Hillard (2006) appeared reporting different results after injection of THC in mice. THC (0.25–10 mg/kg i.p.) did not produce anxiolytic-like response in the EPM. This disagrees with our data but the different sensitivity to THC might be due to species differences (mouse *vs* rat).

However, the most original finding of the present study concerns the neuroanatomical and cellular correlates of THC's anxiolytic effect. First of all using c-Fos strategy to map neural circuitries underlying behavioral responses, we saw that the prefrontal cortex and the amygdala were the main cerebral regions involved in the anxiolytic effect. In both areas, we found a significant decrease in cFos activation, suggesting neural deactivation that was fully reversed by pretreatment with AM251, thus indicating the CB1 dependence of this effect.

The responsiveness of these brain regions is not surprising since the prefrontal cortex and the amygdala are anatomically connected and strongly implicated in anxiety mechanisms. Activation of the amygdala results in behavioral and physiological responses associated with anxiety and panic-like behavior, whereas lesions reduce

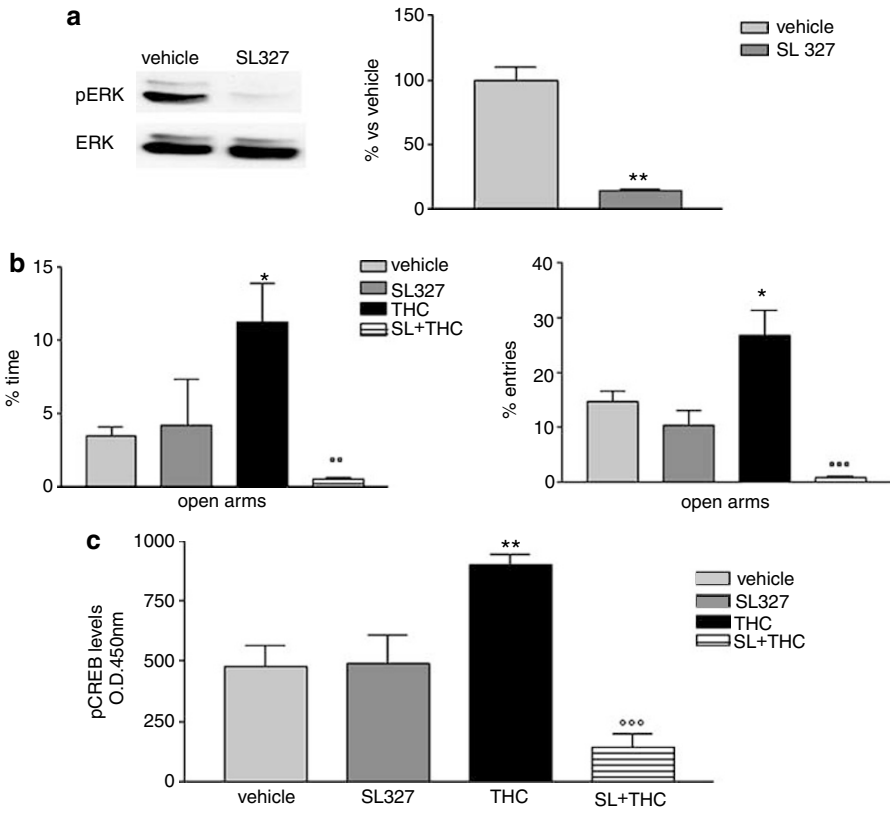

Figure 9 (a) Effect of SL327 microinjection (30 μg) in the prefrontal cortex on p-ERK immunoreactivity. $^{**}p<0.01$ *vs* vehicle (Student's *t*-test). (b) Effect of SL327 pretreatment (30 μg in the prefrontal cortex, 5 min before THC) on THC anxiolytic effect evaluated in the EPM. $^{*}p<0.05$ *vs* vehicle; $^{\circ\circ}p<0.01$, $^{\circ\circ\circ}p<0.001$ *vs* THC alone (one-way ANOVA followed by Tukey's test). (c) Effect of SL 327 pretreatment (30 μg in the prefrontal cortex, 5 min before THC) on THC-induced CREB phosphorylation in the prefrontal cortex. $^{**}p<0.01$ *vs* vehicle; $^{\circ\circ\circ}p<0.001$ *vs* THC alone (one-way ANOVA followed by Tukey's test).

such effects (for a review, see Phelps and LeDoux, 2005). Along similar lines, parts of the prefrontal cortex have been reported to influence the induction of anxiety and fear-related behavior and may play a role in autonomic reactions during stress and anxiety (Davidson, 2002). Interestingly, both the prefrontal cortex and the amygdala, where we found less cFos activation, can be activated by environmentally evoked anxiety (for a review, see Kovacs, 1998). Likewise, we found that vehicle-treated animals exposed to the EPM had higher levels of cFos in these brain regions than rats handled in the same manner but not subjected to the test (naïve). This is in agreement with Hinks *et al* (1996) and Duncan *et al* (1996) who reported similar increases in cFos expression, respectively in the amygdala and in the medial prefrontal areas after exposure to the EPM. In a similar stressful situation that induces anxiety in rats, that is exposure to cat odor, benzodiazepines had an anxiolytic effect that was paralleled by reduction in cFos expression in the medial prefrontal cortex and—though to a lesser extent—in the amygdala (McGregor *et al*, 2004).

Therefore, as already proposed in McGregor's paper for midazolam or more in general for benzodiazepines (McNaughton and Gray, 2000), we suggest that THC disinhibits the approach in situations of fear or uncertainty and promotes risk assessment behavior at the expense of flight. This shift in defensive behavior involves action on different brain regions: the prefrontal cortex may play a key role in coping with stress, and THC can deactivate this stress-related circuitry, whereas THC's effect in the amygdala might reflect specific action on the unconditioned fear response. Therefore, we can speculate that THC's anxiolytic effect may be at least partially due to prevention of the activation of an anxiety circuit involving the prefrontal cortex and amygdala.

The second result worth commenting is the association of THC's anxiolytic effect with increased CREB activation and related intracellular pathways in the prefrontal cortex and hippocampus. As for cFos experiments, we first checked the effect of EPM *per se* on these biochemical parameters. In the hippocampus neither phospho-CREB nor PKA, pCAMKII, and pERK levels were altered by EPM exposure. THC injection instead, significantly increased pCREB along with a decrease in the activity of CAMKII. As this kinase has an inhibitory effect on CREB activation, its reduction accounts for the increased pCREB levels triggered by THC through a CB1-dependent mechanism.

In the prefrontal cortex EPM exposure *per se* did not alter CREB activity, however it significantly reduced pCAMKII contents and provoked a trend to increase in pERK. The injection of THC instead increased pCREB level, did not induce further changes in pCAMKII other than those provoked by EPM exposure, and produced an increase in ERK activation that resulted more sustained and significantly different from that induced by EPM *per se*. On this

basis, we suggest that in the prefrontal cortex THC's anxiolytic effect is associated with CB1 receptor activation triggering CREB phosphorylation via ERK cascade. This picture is also supported by the experiments with SL327. The selective block of ERK activation in the prefrontal cortex fully prevented both THC-induced increase in CREB phosphorylation and THC's anxiolytic effect, highlighting the causative link between these events.

To our knowledge, this is the first study aimed at finding a neural correlate of THC's anxiolytic effect and it indicates that CREB is an important player. In line with our results, Barrot *et al* (2005) reported that in a model of protracted social isolation in adult rats, where they observed increased anxiety-like behavior, CREB activity was reduced in the nucleus accumbens. Likewise, Pandey *et al* (2005) found that lowered CREB function in the amygdala was associated with anxiety-like behavior in alcohol preferring rats, and increasing CREB phosphorylation in these animals relieved anxiety.

Taken together these results suggest that CREB is a key regulator of the reactivity of brain circuits and could therefore influence individual sensitivity to emotional stimuli. However, as a transcription factor, CREB itself is unlikely to be the molecule acutely affecting behavior. Rather, changes in CREB activity could alter the expression of specific gene products involved in the modulation of anxiety such as neuropeptide Y and brain-derived neurotrophic factor (Heilig, 2004; Branchi *et al*, 2006). This hypothesis requires further research.

Lastly, we did not find any alteration in CREB activation in the amygdala, where cFos levels were lowered, as they were in the prefrontal cortex. This lack of activation might be explained by some indirect effect of THC in this area rather than a direct one (ie not triggered by the stimulation of CB1 receptors in the amygdala). Consistent with this notion, there are extensive reciprocal connections between the amygdala and prefrontal cortex (Amaral *et al*, 1992) and the prefrontal cortex inputs excite neurons in the amygdala (Likhtik *et al*, 2005).

On this basis we might suggest that the THC-induced reduction in neural activation in the prefrontal cortex might also lead to neural deactivation in the amygdala. On the other hand, a direct effect of THC in the amygdala also cannot be ruled out, as suggested by the presence of CB1 receptors in this brain region. If this is the case, the intracellular pathway linked to CB1 receptor stimulation is still unclear but it does not involve CREB activation.

In conclusion, this study casts fresh light on the neurochemical and neuroanatomical substrates of THC's anxiolytic effect and highlights the importance of the cannabinoid system in these behaviors. The stimulation of CB1 receptors in the prefrontal cortex, amygdala and hippocampus, with the subsequent activation of different signaling pathways, might be the initial event underlying the effects of the cannabinoids on anxious states.

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