Dissociation of the Pharmacological Effects of THC by mTOR Blockade

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The potential therapeutic benefits of cannabinoid compounds have raised interest in understanding the molecular mechanisms that underlie cannabinoid-mediated effects. We previously showed that the acute amnesic-like effects of delta9-tetrahydrocannabinol (THC) were prevented by the subchronic inhibition of the mammalian target of rapamycin (mTOR) pathway. In the present study, we assess the relevance of the mTOR pathway in other acute and chronic pharmacological effects of THC. The rapamycin derivative temsirolimus, an inhibitor of the mTOR pathway approved by the Food and Drug Administration, prevents both the anxiogenic- and the amnesic-like effects produced by acute THC. In contrast, THC-induced anxiolysis, hypothermia, hypolocomotion, and antinociception are not sensitive to the mTOR inhibition. In addition, a clear tolerance to THC-induced anxiolysis, hypothermia, hypolocomotion, and antinociception was observed after chronic treatment, but not to its anxiogenic- and amnesic-like effects. Temsirolimus pre-treatment prevented the amnesic-like effects of chronic THC without affecting the downregulation of CB1 receptors (CB1R) induced by this chronic treatment. Instead, temsirolimus blockade after chronic THC cessation did not prevent the residual cognitive deficit produced by chronic THC. Using conditional knockout mice lacking CB1R in GABAergic or glutamatergic neurons, we found that GABAergic CB1Rs are mainly downregulated under chronic THC treatment conditions, and CB1–GABA–KO mice did not develop cognitive deficits after chronic THC exposure. Therefore, mTOR inhibition by temsirolimus allows the segregation of the potentially beneficial effects of cannabinoid agonists, such as the anxiolytic and antinociceptive effects, from the negative effects, such as anxiogenic- and amnesic-like responses. Altogether, these results provide new insights for targeting the endocannabinoid system in order to prevent possible side effects.
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INTRODUCTION

Cannabis is the most widely used illicit drug in the world. Apart from their widespread recreational use, marijuana-derived compounds present a variety of potential therapeutic applications acting on the endocannabinoid system (Pertwee, 2009). This neuromodulatory system regulates a variety of physiological processes, including memory (Marsicano and Lafenêtre, 2009), anxiety (Viveros et al, 2005), and nociception (Pertwee, 2001), and represents an emerging therapeutic target based on the well-demonstrated medicinal properties of compounds acting on this system (Piomelli, 2005; Pertwee, 2009). However, the administration of cannabinoid agonists may produce memory impairment (Castellano et al, 2003; Puighermanal et al, 2012), anxiety-like responses (Rubino et al, 2007), alter motor coordination and cerebellar learning (Skosnik et al, 2008), and have potential addictive properties (Maldonado et al, 2011), all representing important drawbacks for their therapeutic applications. Natural cannabinoids exert their actions by binding to at least two receptor types, named CB1 and CB2 cannabinoid receptors (CB1R and CB2R, respectively). CB1R are widely distributed in the brain where they are mainly localized at presynaptic neuronal terminals of the hippocampus, amygdala, basal ganglia, cortex, hypothalamus, and cerebellum (Wilson and Nicoll, 2002; Mackie, 2005). In forebrain regions, such as the neocortex, hippocampus, and amygdala, CB1R are much more abundantly expressed in GABAergic interneurons than in glutamatergic principal neurons (Marsicano and Lutz, 1999; Katona et al, 1999). The development of conditional CB1R knockout (CB1-KO) mice allowed to assess the role of CB1R in diverse cellular populations (Monory et al, 2006; Bellocchio et al, 2010), where they may

have potentially different functions. CB1R in forebrain GABAergic neurons were found critical for the amnesic-like effects of acute delta9-tetrahydrocannabinol (THC), as CB1-KO mice lacking CB1R in these neurons (GABA-CB1-KO) were not sensitive to the object-recognition memory deficits produced by THC (Puighermanal et al, 2009). These effects on memory seem to be the result of an imbalance between excitatory and inhibitory inputs produced by THC administration on the basis of the different levels of CB1R expression at GABAergic and glutamatergic terminals (Puighermanal et al, 2009; Kawamura et al, 2006). Instead, abolition of CB1R in glutamatergic neurons did not affect the memory deficits produced by THC (Puighermanal et al, 2009). This bimodal contribution of CB1R has also been recently reported in the anxiety-like responses. Indeed, the anxiolytic-like effects of a low dose of the cannabinoid agonist CP-55,940 were mediated by CB1R on glutamatergic terminals, whereas CB1Rs on GABAergic terminals were required to induce the anxiogenic-like effect of a high dose of this cannabinoid (Rey et al, 2012).

Several studies have reported that CB1R undergoes downregulation following chronic THC administration (reviewed in Sim-Selley, 2003). This adaptive mechanism is thought to contribute to tolerance to most of the cannabinoid-mediated behavioral effects, such as antinociception, hypolocomotion, hypothermia, and catalepsy (Hutcheson et al, 1998). In contrast, a lack of tolerance to the cognitive-impairing effects has been previously reported after chronic THC treatment (Boucher et al, 2009; Zanettini et al, 2011), pointing to a possible differential mechanism involved in these cannabinoid responses.

At the molecular level, the acute administration of THC triggers a variety of CB1R-dependent intracellular signaling mechanisms within the brain, including the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), the phosphoinositide-3 kinase (PI3K)/Akt/glycogen synthase kinase 3 (GSK-3), and the mammalian target of rapamycin (mTOR) signaling pathways (Derkinderen et al, 2003; Ozaita et al, 2007; Puighermanal et al, 2009). mTOR is a serine/threonine kinase sensitive to inhibition by the macrolide rapamycin (sirolimus), or its derivative temsirolimus (also known as CCI-779) (Guertin and Sabatini, 2009). mTOR is mainly associated with neural plasticity in the brain through the regulation of mRNA translation (Jaworski and Sheng, 2006; Hoeffer and Klann, 2010). Interestingly, mTOR pathway activation was involved in the memory impairment produced by endocannabinoids or acute THC administration, as pre-treatment with the mTOR inhibitor rapamycin blocked the amnesic-like effects promoted by both THC administration (Puighermanal et al, 2009) and inhibition of anandamide degradation (Busquets-Garcia et al, 2011). Moreover, enhanced levels of mTOR activity have been described in animal models presenting cognitive deficits such as the $Tsc2^{+/-}$ mice or $Fmr1$ knockout mice (Ehninger et al, 2008; Sharma et al, 2010).

We previously reported that CB1R activation, mainly in GABAergic interneurons, by acute exogenous or endogenous cannabinoids can trigger the activation of the mTOR pathway and the protein synthesis machinery in the hippocampus through a glutamatergic mechanism underlying the characteristic long-term memory impairment (Puighermanal et al, 2009; Busquets-Garcia et al, 2011). However, the role of the mTOR signaling in other central effects of acute THC as well as the role of mTOR signaling in memory performance after chronic THC exposure were unexplored. Thus, the aim of this study was to investigate the participation of the mTOR signaling pathway in several potential therapeutic and side effects of THC, such as the amnesic, anxiolytic, anxiogenic, hypothermic, and hypolocomotor responses, as well as the possible differential role of CB1R downregulation in chronic THC effects.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (Charles River, Lyon, France) aged between 9 and 11 weeks were used in pharmacological studies. CB1R conditional knockout mice lacking CB1R either in forebrain GABAergic neurons (GABA-CB1$^{-/-}$) or in cortical glutamatergic neurons (Glu-CB1$^{-/-}$) and wild-type littermates (CB1$^{+/+}$) were in C57BL/6N genetic background, and were obtained and genotyped as previously described (Monory et al, 2006; Bellocchio et al, 2010). Mice were housed in cages of four and maintained at controlled temperature ($21 \pm 1\,^{\circ}C$) and humidity ($55 \pm 10\%$). Food and water were available ad libitum. Lighting was maintained at 12-h cycles (on at 0800 hours and off at 2000hours). All the experiments were performed during the light phase of the dark/light cycle. Animals were habituated to the experimental room and handled for 1 week before starting the experiments. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal, CEEA-PRBB). Our institution has the Animal Welfare Assurance (no. A5388-01, IACUC Approval Date 6 August 2009) granted by the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health (USA). The observers in all the behavioral studies were blind to the experimental groups under analysis.

Drugs and Treatments

THC was obtained from THC Pharm GmbH (Frankfurt, Germany), temsirolimus from LC Laboratories (Woburn, MA), and rimonabant from Sanofi-Aventis (Sanofi-Aventis Recherche, Montpellier, France). THC and rimonabant were dissolved in 5% ethanol, 5% cremophor, and 90% saline. Temsirolimus was dissolved in 2% ethanol, 8% cremophor, and 90% saline, and administered in all instances 20 min before THC. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Rimonabant pre-treatment was performed 20 min before THC administration. For the chronic treatments, THC or its vehicle were administered once daily for 6 consecutive days. Temsirolimus pre-treatment in this chronic protocol was performed 20 min before THC (or its vehicle) administration. In another set of animals, mice received THC (or its vehicle) for 6 days and temsirolimus for the next 4 days, and object-recognition performance was tested daily during this chronic procedure.

Immunoblot Analysis

Brain tissues were dissected on ice and immediately frozen for 30 or 240 min after pharmacological treatment. Protein samples were prepared in lysis buffer supplemented with protease and phosphatase inhibitors (Puighermanal et al, 2009). Equal amounts of protein samples (40 μg/well) were separated in 10% polyacrylamide gels before electrophoretic transfer onto Immobilon-P membranes (Merk Millipore, Billerica, MA). After blocking, membranes were incubated for 120 min with primary antibodies: anti-phospho-p70S6K(T389) (1:500), anti-p70S6K (1:500) (Cell Signaling, Beverly, MA), anti-CB1R (1:1000) (Frontier Science, Ishikari, Japan), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA), and the corresponding secondary antibodies coupled to horseradish peroxidase. Immuno-chemiluminescence was produced by incubation of the membranes with West-femto ECL substrate (Thermo Fisher Scientific, Rockford, IL). The optical density of the relevant immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v4.6.3 (Bio-Rad). The detection obtained with the phospho-specific antibody against p70S6K was normalized to the detection of total p70S6K in the same sample and expressed as a percentage of the control/vehicle treatment. The values for CB1R were normalized to the detection of GAPDH in the same samples and expressed as a percentage of the control/vehicle treatment.

Body Temperature

Body temperature was measured with a thermo-coupled flexible probe (Panlab, Madrid, Spain) located in the rectum for 10 s. Temperature measurements were performed before (basal) and 120 min after THC, or vehicle, administration in mice that received or not the pre-treatment with temsirolimus or its vehicle. Data are represented as the difference in temperature from the basal measurement.

Locomotor Activity Test

Locomotor responses of THC were evaluated by using individual locomotor activity boxes (9 × 20 × 11 cm; Imetronic, Pessac, France) provided with two lines of six infrared beams to evaluate both horizontal and vertical activity under a dim light (20–25 lux). Mice were placed in the boxes during 20 min (240 min after THC administration) and the total locomotion score was analyzed.

Visceral Pain Test

The acetic acid test was performed after the locomotor activity test 260 min after THC administration. Mice received an i.p. administration of 0.8% acetic acid solution injected in a volume of 10 ml/kg of body weight. The injection produced the typical nociceptive reaction, characterized by contractions of the abdominal musculature followed by extension of the hind limbs (writhings). Mice were placed in individual transparent cylinders (35 cm high, 16 cm diameter) for observation, and the number of writhings was recorded during 15 min starting 5 min after the acetic acid injection.

Object-Recognition Task

Object-recognition memory was assayed in a V-maze. The acute test consisted of a single training and the test session was performed as previously described (Puighermanal et al, 2009). Drugs were always administered immediately after the training session, and the test session was performed 24 h later. The repeated assessment of object-recognition memory was carried out as previously described (Busquets-Garcia et al, 2011). Briefly, the first cognitive assessment (test 1) on the chronic object-recognition procedure was performed just before the second drug administration (day 2). Memory was tested again 24 h later using the novel object explored the day before (now the familiar object) and a brand new object (now the novel object, to be the familiar object the day after). In each test session, a discrimination index (DI) was calculated as the difference between the time spent exploring the novel (T_N) and the familiar object (T_F) divided by the total exploration time ($T_N + T_F$): DI $= [T_N - T_F]/[T_N + T_F]$. DI values above 0.3 were considered to reflect memory retention for the familiar object.

Elevated Plus-Maze

The elevated plus-maze was performed 240 min after THC administration in a different set of animals. At the beginning of the 5 min observation session, each mouse was placed in the central neutral zone facing one of the open arms. The cumulative time spent in open and closed arms was then recorded. An arm visit was counted when the mouse moved both front paws into the arm. Data are represented as percentage of time spent in open arms and the number of visits in the open arms.

Statistical Analysis

Statistical analyses were performed using t-tests when assessing two-group comparisons (immunoblot studies), and by means of ANOVAs with/without repeated factors for multiple-group comparisons (behavioral studies). When appropriate, post-hoc individual differences between groups were determined using the Dunnett's test. Differences were considered significant when $P < 0.05$. SPSS v19 software was used for statistical analyses.

Results

Acute THC Modulates mTOR Signaling in Different Brain Areas

Different brain areas expressing high levels of CB1R were analyzed 30 min after THC administration (10 mg/kg). Brain tissues were processed for immunoblot analysis to reveal the phosphorylation of the mTOR-dependent site (Thr389) on 70 kDa ribosomal protein S6 kinase (p70S6K(T389)) (Jefferies et al, 1997). All the brain areas analyzed, hippocampus, striatum, cerebellum, frontal cortex, and amygdala, showed an increase in the phosphorylation of p70S6K(T389) after acute THC administration (Figure 1a).
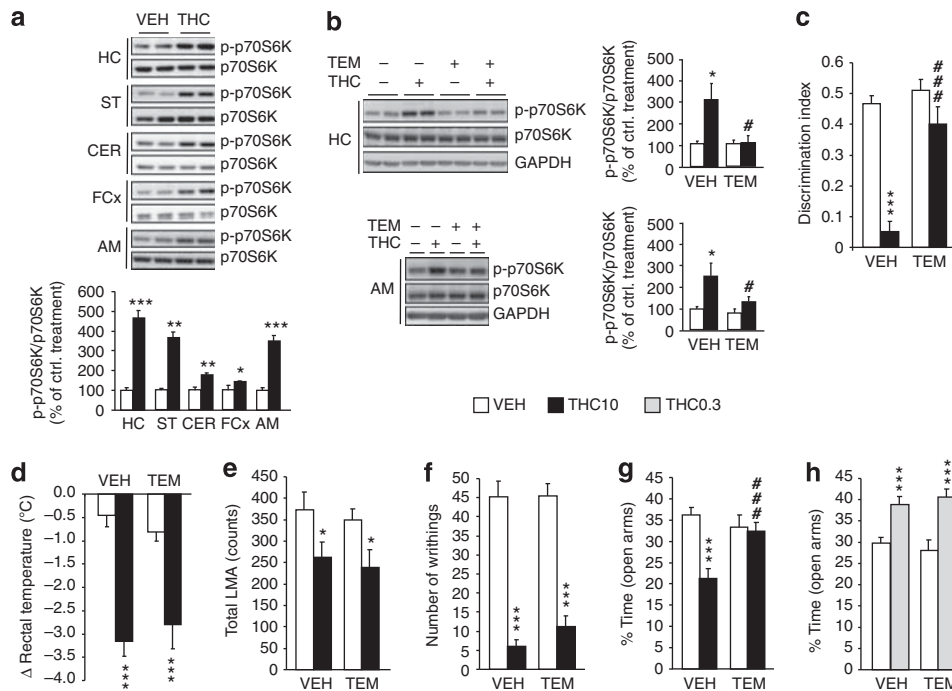
Figure 1 Phosphorylation of the downstream effector of mTOR p70S6K(T389) in selected brain areas and role of this pathway in the biochemical and behavioral effects after acute THC administration. (a) Representative immunoblot and optical density quantification of phospho-p70S6K(T389) and p70S6K on hippocampal samples obtained 30 min after THC (10 mg/kg) or vehicle administration in mice ($n = 5$ mice/group). (b) Representative immunoblot and optical density quantification of phospho-p70S6K(T389), p70S6K, and GAPDH in the hippocampus and amygdala after THC administration following a pre-treatment with temsirolimus (1 mg/kg, 20 min before THC or vehicle administration) or vehicle ($n = 5$ mice/group). (c) Temsirolimus pre-treatment (1 mg/kg, 20 min before THC or vehicle) prevents the deficit in memory consolidation promoted by THC ($n = 10$-11 mice/group). (d) Temsirolimus does not block the hypothermic effect measured 120 min after acute THC administration ($n = 9$–10 mice/group). (e) Temsirolimus does not affect the hypolocomotor effect detected 240 min after THC administration ($n = 9$–10 mice/group). (f) Temsirolimus does not affect the antinociceptive effect observed 260 min after THC administration in the model of visceral pain ($n = 12$–13 mice/group). (g) Temsirolimus pre-treatment prevents the anxiogenic-like effect observed 240 min after THC administration (10 mg/kg) ($n = 20$–21 mice/group). (h) Temsirolimus pre-treatment does not affect the anxiolytic-like effect of THC (0.3 mg/kg) in the elevated plus-maze represented by the time that the mice spent in the open arms ($n = 14$–15 mice/group). HC, hippocampus; ST, striatum; CER, cerebellum; FCx: frontal cortex; AM, amygdala; LMA, locomotor activity. $*P < 0.05$, $***P < 0.001$ effect of THC; $\#P < 0.05$, $\#\#\#P < 0.001$ effect of temsirolimus.

As previous studies showed that the subchronic (5 days) treatment with the mTOR inhibitor rapamycin before THC administration prevented the phosphorylation of p70S6K(T389) in hippocampus (Puighermanal et al, 2009), we evaluated the possibility to use a single systemic dose of temsirolimus, a rapamycin analog, to prevent specific effects of THC that could be mediated by the activation of the mTOR pathway. We found that a single administration of temsirolimus, at a dose that did not affect object-recognition memory consolidation on its own (1 mg/kg) ($F_{(5,42)} = 19.806$, n.s. compared to vehicle group; Supplementary Figure S1), was effective at preventing the increase in phosphorylation of p70S6K(T389) promoted by THC (10 mg/kg) administration in the hippocampus and in the amygdala, both regions involved in memory and anxiety-like responses (Figure 1b).

Role of mTOR Pathway in the Pharmacological Effects of THC

We analyzed the behavioral significance of mTOR signaling activation in the acute effects of THC using the blockade of mTOR activity by temsirolimus. We focused on object-recognition memory consolidation, body temperature, locomotor activity, nociception, and anxiety-like responses.

Mice were pre-treated with temsirolimus (1 mg/kg) or its vehicle 20 min before the cannabinoid agonist administration. THC (10 mg/kg) promoted a deficit in object-recognition memory consolidation measured 24 h after administration (Puighermanal et al, 2009), an effect readily prevented by temsirolimus pre-treatment ($F_{(3,22)} = 11.684$, $P < 0.01$ compared to THC group; Figure 1c). In contrast, the effects of THC (10 mg/kg) on body temperature ($F_{(3,39)} = 47.454$, $P < 0.001$ compared to vehicle group; Figure 1d), locomotion ($F_{(3,39)} = 3.318$, $P < 0.05$ compared to vehicle group; Figure 1e), and nociception ($F_{(3,49)} = 86.293$, $P < 0.001$ compared to vehicle group; Figure 1f) were not affected by temsirolimus.

Anxiety-like responses are modulated by cannabinoids in a bimodal manner. THC promotes anxiogenic-like responses in rodents at doses > 3 mg/kg that depend on CB1R activation (Supplementary Figure S2), whereas lower doses induce anxiolytic-like effects (Rubino et al, 2007). Under these conditions, temsirolimus pre-treatment blocked the anxiogenic-like responses induced by THC (10 mg/kg) ($F_{(3,83)} = 12.549$, $P < 0.001$ compared to vehicle group) in the elevated plus-maze ($F_{(3,83)} = 9.691$, $P < 0.01$ compared to THC group; Figure 1g), but did not affect the anxiolytic-like effects of THC (0.3 mg/kg) ($F_{(3,29)} = 27.798$, $P < 0.001$; Figure 1h), as shown by the percentage of time

spent in the open arms (Figure 1g and h) and the percentage of entries in the open arms (Supplementary Figure S3). Interestingly, these results correlated with the fact that the high dose of THC (10 mg/kg) increased the phosphorylation of p70S6K(T389) in the amygdala (Figure 1a and b), whereas the low dose of THC (0.3 mg/kg) that produced anxiolytic-like effects were not accompanied by mTOR signaling activation in any of the brain areas (Supplementary Figure S4).

Pharmacological Responses to Chronic THC Administration

Repeated exposure to cannabinoids produces tolerance to several pharmacological responses, an effect that correlates with the downregulation of CB1R in different brain structures (Sim-Selley, 2003; Martin et al, 2004). We evaluated the effect of the repeated exposure to THC on locomotor activity, nociception, anxiogenic- and anxiolytic-like behavior, and object-recognition memory. Mice were treated for 6 days once a day with vehicle (group VEH/VEH) or THC at 10 or 0.3 mg/kg (groups THC10/THC10 and THC0.3/THC0.3). To assess the effect of acute THC under similar experimental conditions, two groups of mice received vehicle for 5 days, and a challenge administration of THC (10 or 0.3 mg/kg) on the sixth day (groups VEH/THC10 and VEH/THC0.3). After chronic THC (10 mg/kg) treatment, tolerance to the hypolocomotor ($F_{(2,61)} = 4.824$, n.s. compared to vehicle group; Figure 2a) and antinociceptive effects of THC 10 mg/kg treatment ($F_{(2,42)} = 23.789$, n.s. compared to vehicle group; Figure 2b) was observed, but not to its anxiogenic-like (10 mg/kg) ($F_{(2,51)} = 3.308$, $P < 0.05$ compared to vehicle group; Figure 2c) or the anxiolytic-like response (0.3 mg/kg) ($F_{(2,59)} = 9.338$, $P < 0.01$ compared to vehicle group; (Figure 2d). Instead, the anxiolytic-like response obtained with THC at the dose of 0.3 mg/kg was obliterated in mice that previously received THC at the dose of 10 mg/kg during 5 days (Supplementary Figure S5).

In separate groups of animals, and using the same chronic administration schedule, object-recognition memory was assessed every day during chronic THC (10 mg/kg) or vehicle administration. We found that tolerance was not developed for the amnesic-like effect of THC ($F_{(1,72)} = 1197.004$, $P < 0.001$ compared to vehicle group; Figure 3a, test 1–6), and the cognitive performance in this test was strongly disrupted even 3 days after the last THC injection ($F_{(1,27)} = 197.1$, $P < 0.001$ compared to vehicle group; Figure 3a, test 7–9), with DIs below 0.2 values. This is in contrast to the hypothermic effects of THC that developed a rapid and complete tolerance in the same experimental group after 3 days of THC treatment ($F_{(1,17)} = 31.565$, n.s. on day 3; Figure 3b).

The activity of hippocampal mTOR signaling pathway was studied through the phosphorylation of p70S6K(T389) in a subset of mice analyzed for cognition. Three time points were considered to study the phosphorylation of p70S6K(T389) in relation with the cognitive performance observed in the object-recognition test: day 6 of chronic THC or vehicle treatment (test 5); 3 days after the last THC or vehicle administration (day 9, test 8), and 7 days after the last THC or vehicle administration (day 13, test 12)
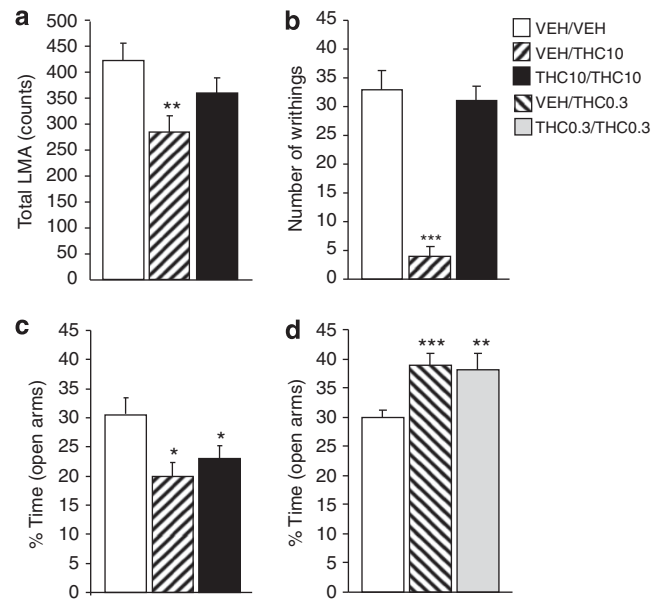
Figure 2 Tolerance to hypolocomotion and antinociception but not to the anxiogenic- and anxiolytic-like responses after chronic THC treatment. Tolerance to the hypolocomotor ($n = 20$–21 mice/group) (a), and antinociceptive effect ($n = 12$–14 mice/group) (b), were revealed after chronic THC administration (10 mg/kg, 6 days, once daily). No tolerance to the anxiogenic-like responses (c) was observed after chronic THC administration (10 mg/kg, 6 days, once daily) ($n = 16$–18 mice/group). No tolerance to the anxiolytic-like response appears after chronic THC administration (0.3 mg/kg, 6 days, once daily) ($n = 18$–19 mice/group) (d). LMA, locomotor activity. Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with vehicle treatment.

(Figure 3c). An additional group of mice received vehicle for 5 days and a challenge administration of THC (10 mg/kg) on the sixth day. Hippocampal samples were obtained 30 min after the last THC administration for samples collected after test 5, and immediately after the test for samples collected after test 8 and test 12. The response on p70S6K(T389) phosphorylation after acute THC administration was higher than that observed after chronic THC administration ($P < 0.01$ compared to vehicle group; Figure 3c). However, p70S6K phosphorylation was still significantly increased after chronic THC administration, correlating with the disruption of object-recognition performance ($P < 0.05$ compared to vehicle group; Figure 3c, after test 5). Interestingly, 3 days after chronic THC treatment cessation (Figure 3c, after test 8), p70S6K(T389) phosphorylation was slightly, although non-significantly (n.s. compared to vehicle group), increased in those mice that still showed a residual object-recognition memory impairment (Figure 3a). Finally, no differences in the phosphorylation of p70S6K were revealed 7 days after chronic THC treatment, which was associated to the total recovery of the THC-induced amnesic-like effects (n.s. compared to vehicle group; Figure 3c, after test 12).

Temsirolimus Prevents the Memory Deficit Promoted by Chronic THC Administration

To further characterize the involvement of the mTOR signaling pathway in the memory impairment produced by
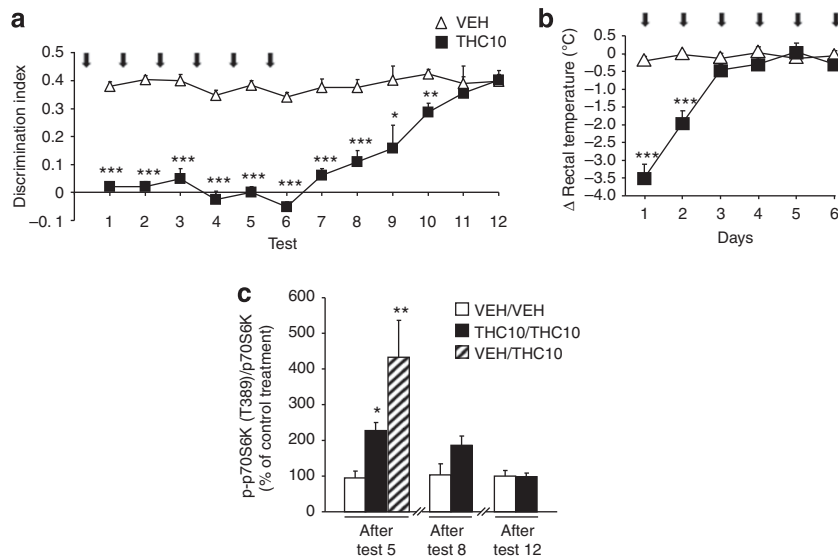
Figure 3 Absence of tolerance to the amnesic-like effect and tolerance to the hypothermic effects during chronic THC treatment. DI values for 12 consecutive tests on mice ($n = 30$–35 mice/group) treated chronically (black arrows for administration days) with THC 10 mg/kg, 6 days, once daily) (black square), or vehicle (white triangle) in the object-recognition memory test (a). Effects of THC (10 mg/kg) or vehicle ($n = 10$–15 mice/group) on body temperature during the 6 days of treatment (b). Quantitative analysis of p70S6K(T389) immunodetection in the hippocampus of mice ($n = 4$–5 mice/group) treated acutely or chronically with THC or vehicle, and sacrificed 30 min after test 5, or immediately after test 8 and test 12 of the experimental protocol (c). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle treatment.

chronic THC (10 mg/kg) exposure, mice were pre-treated with temsirolimus (1 mg/kg) or vehicle 20 min before each THC administration during the 6 days of chronic exposure. Chronic treatment with temsirolimus had no intrinsic effects in object-recognition memory consolidation, but it fully prevented the amnesic-like effects produced by chronic THC ($F_{(1,99)} = 3900.660$, $P < 0.001$ compared to THC group; Figure 4a), without affecting the exploration time (Supplementary Figure S6a). In contrast, when temsirolimus was administered starting the next day after chronic THC treatment cessation, the mTOR inhibition did not reverse the residual deficit in object-recognition performance ($F_{(1,100)} = 3032.196$, n.s. compared to THC group; Figure 4b). In addition, temsirolimus administration after THC cessation did not affect exploration time in the object-recognition test (Supplementary Figure S6b).

## Differential Downregulation of Hippocampal CB1R after Chronic THC Administration

After chronic THC treatment, a strong CB1R downregulation was observed in the hippocampus ($F_{(2,12)} = 111.76$, $P < 0.001$ compared to vehicle group; Supplementary Figure S7). Interestingly, hippocampal CB1R were similarly downregulated by THC administration in those mice pre-treated with temsirolimus ($F_{(2,12)} = 111.76$, n.s. compared to THC group; Supplementary Figure S7), suggesting that mTOR activity is not involved in the THC-induced CB1R downregulation. We investigated if this downregulation would affect similarly hippocampal CB1Rs located in glutamatergic and GABAergic neurons using the conditional CB1R knockout mice, which lack CB1R expression primarily from cortical glutamatergic neurons (including hippocampal pyramidal cells, Glu-CB1$^{-/-}$) (Monory et al, 2006; Bellocchio et al, 2010) or from forebrain GABAergic

neurons (including hippocampal interneurons, GABA-CB1$^{-/-}$) (Monory et al, 2006; Bellocchio et al, 2010). Thus, remaining CB1R in the hippocampus of GABA-CB1$^{-/-}$ mice will mainly represent receptors expressed in glutamatergic neurons, whereas hippocampal CB1R in Glu-CB1$^{-/-}$ mice will mainly correspond to those expressed in GABAergic interneurons. Chronic administration of THC (10 mg/kg) resulted in a strong downregulation of hippocampal CB1R, in Glu-CB1$^{-/-}$ mice ($F_{(1,10)} = 63.733$, $P < 0.001$ compared to vehicle group; Figure 5a), whereas this effect was less pronounced in GABA-CB1$^{-/-}$ mice ($F_{(1,11)} = 10.895$, $P < 0.05$ compared to vehicle group; Figure 5a). Although the downregulation was strongly detected in Glu-CB1$^{-/-}$ mice, the memory deficit associated to chronic THC administration did not undergo tolerance in these knockout mice ($F_{(5,43)} = 372.939$, $P < 0.001$ compared to vehicle group; Figure 5b). On the other hand, as previously shown under acute treatment conditions (Puighermanal et al, 2009), no amnesic-like effect of THC was observed in GABA-CB1$^{-/-}$ mice as long as the chronic treatment lasted ($F_{(5,43)} = 372.939$, n.s. compared to vehicle group; Figure 5b), supporting the role of GABAergic CB1R on the memory deficits produced by acute and chronic THC administration.

Discussion

This study describes the widespread activation of p70S6K(T389) by THC in the brain, one of the main downstream targets of the mTOR complex 1 signaling pathway. Preclusion of this process by temsirolimus allows to dissociate several therapeutic and side effects of THC. Indeed, temsirolimus prevents the amnesic- and anxiogenic-like effects of THC, leaving the anxiolytic,

antinociceptive, hypothermic, and hypolocomotor effects of this cannabinoid agonist unaffected. Tolerance to THC-induced anxiolysis, hypothermia, hypolocomotion,
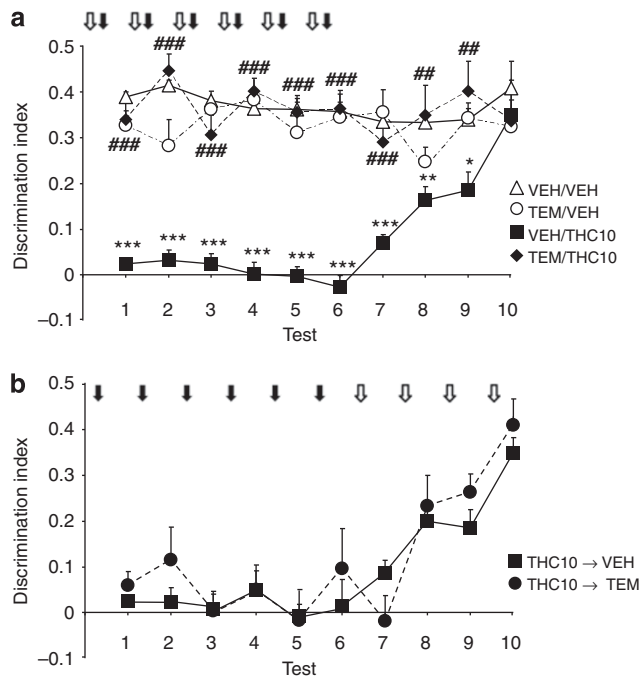

Figure 4 mTOR inhibition by temsirolimus prevents THC-induced long-lasting memory impairment, but does not resolve the residual cognitive deficit when administered after chronic THC treatment. DI values for 10 consecutive test sessions in the object-recognition memory test for mice chronically treated with THC or vehicle receiving a pre-treatment with temsirolimus or its vehicle. Black arrows indicate THC or vehicle administration and white arrows indicate temsirolimus or vehicle administration. Temsirolimus administration (1 mg/kg) 20 min prior to THC prevents the memory impairment produced by the cannabinoid agonist ($n = 20$–25 mice/group) (a). Temsirolimus administration (white arrows) after THC (black arrows) cessation did not resolve the residual cognitive deficit lasting beyond the end of THC exposure (b). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with VEH/VEH treatment; ##$P < 0.01$, ###$P < 0.001$ compared with VEH/THC10 treatment.

and antinociception was observed after chronic treatment, but not to its anxiogenic- and amnesic-like effects. In addition, a higher sensitivity to downregulation by chronic THC administration of CB1R expressed in hippocampal GABAergic neurons was observed in comparison to those expressed in glutamatergic terminals.

In the present study, THC activation of p70S6K(T389) was observed in homogenates of different brain areas where we previously described the increased phosphorylation in Akt(S473) and GSK-3$_\beta$(S9) in response to acute THC (Ozaita et al, 2007), both upstream kinases modulating mTOR activity (Navé et al, 1999; Inoki et al, 2006). Interestingly, the modulation of p70S6K(T389) by THC is readily sensitive to the systemic pre-administration of a low dose of the rapamycin analog, temsirolimus (Guertin and Sabatini, 2009; Dancey, 2005), which was recently approved by the Food and Drug Administration for cancer treatment (Hudes et al, 2007). Temsirolimus has similar potency and specificity for mTOR than rapamycin, but longer stability and increased solubility (Rini, 2008). We found that temsirolimus (1 mg/kg) showed a suitable inhibitory effect of mTOR-dependent p70S6K(T389) phosphorylation in hippocampus and amygdala after THC administration, without affecting basal levels of p70S6K(T389) phosphorylation. This low dose of temsirolimus did not affect object-recognition memory consolidation on its own after acute or chronic administration in agreement with the previous findings obtained with low doses of rapamycin (Puighermanal et al, 2009; Blundell et al, 2008). In addition, temsirolimus did not affect locomotor activity, nociception, body temperature, or anxiety-like responses when administered at this low dose.

The mTOR pathway regulates a plethora of functions in the cell by integrating different stimuli (growth factors and energy status among others) and giving rise to different outputs, such as transcription and translation control, cell growth, and inhibition of autophagy (Dobashi et al, 2011). mTOR has a crucial role in the translational control by phosphorylating p70S6K and eukaryotic initiation factor 4E-binding protein (4E-BP), and this property has been associated to the modulation of synaptic plasticity (Richter and Klann, 2009). The mTOR pathway has been recently
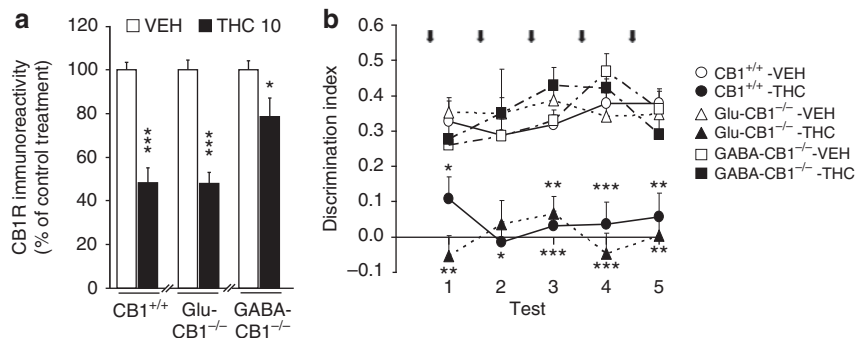
Figure 5 Chronic THC treatment differentially affects CB1R populations in the hippocampus. (a) Quantification of the immunoreactivity detected in wild-type (CB1$^{+/+}$), CB1R knockout mice in glutamatergic neurons (Glu-CB1$^{-/-}$), and CB1R knockout mice in GABAergic neurons (GABA-CB1$^{-/-}$) after chronic THC administration (10 mg/kg, 6 days, once daily). Note the strong downregulation of CB1R in the Glu-CB1$^{-/-}$ mice compared to that on GABA-CB1$^{-/-}$ mice ($n = 4$–6 mice/group). (b) Object-recognition memory performance of CB1$^{+/+}$, Glu-CB1$^{-/-}$, and GABA-CB1$^{-/-}$ mice during chronic exposure to THC (10 mg/kg) or its vehicle. Drug administration was performed after each memory test on 5 consecutive days ($n = 6$–8 mice/group). Data are expressed as mean ± SEM. *$P < 0.05$, ***$P < 0.001$ compared with vehicle treatment.

involved in the modulation of cognitive performance (Krab et al, 2008; Hoeffer and Klann, 2010), and mTOR signaling deregulation in the brain has been associated to intellectual disability due to aberrant synaptic plasticity (Troca-Marín et al, 2012). In agreement, low doses of rapamycin have shown to improve cognitive performance in an animal model for tuberous sclerosis, a rare disease producing intellectual disability, where mTOR activity is permanently increased (Ehninger et al, 2008). We previously described that the mTOR signaling pathway is critically involved in the acute amnesic-like effects of THC, as rapamycin pre-treatment blocked the phosphorylation of p70S6K(T389) by THC in the hippocampus, as well as the associated memory impairment (Puighermanal et al, 2009). The present findings reveal that temsirolimus blocked the anxiogenic-like effects promoted by THC administration, revealing a role for mTOR in anxiety modulation. Notably, only the anxiogenic-like responses triggered by THC were modulated by mTOR activity inhibition, without affecting THC anxiolytic-like effects, pointing to different molecular mechanisms involved in this bimodal effect of cannabinoids in anxiety-like responses (Viveros et al, 2005; Ruehle et al, 2012; Rey et al, 2012). Accordingly, we report that the high dose of THC (10 mg/kg) enhanced the phosphorylation of p70S6K(T389), whereas the low dose of THC (0.3 mg/kg) did not affect the activity of this component in the mTOR signaling.

In this study, THC administered chronically induced tolerance to its hypolocomotor, antinociceptive, and hypothermic effects, as previously described (Thorat and Bhargava, 1994; Rubino et al, 2006). In contrast, no tolerance to the anxiogenic- and amnesic-like effects of repeated THC (10 mg/kg) administration was detected, in agreement with previous behavioral (Boucher et al, 2009) and electrophysiological studies (Hoffman et al, 2007; Fan et al., 2010). Remarkably, hippocampal long-term potentiation was shown to be impaired by repeated THC administration under conditions close to those used in the present study, and this effect persisted for 3 days after the last THC injection (Hoffman et al, 2007), an observation that fits with the time course for the recovery of object-recognition memory performance reported herein.

Interestingly, temsirolimus pre-treatment blocked the object-recognition memory deficit when co-administered with chronic THC. The Akt/mTOR pathway has been associated with the modulation of structural plasticity in dendrites and dendritic spines (Jaworski and Sheng, 2006), and the activation of this signaling pathway by THC could alter synaptic plasticity mechanisms, impairing the object-recognition memory consolidation. In agreement, THC has been reported to inhibit activity-dependent synaptic loss in vitro (Kim et al, 2008), a relevant process for structural plasticity (Bruel-Jungerman et al, 2007), for which cannabinoids do not develop tolerance (Kim et al, 2008). The observation that temsirolimus did not resolve the residual memory deficit when administered after chronic THC exposure suggests that the resulting alterations after chronic THC administration are no longer sensitive to mTOR modulation. The acute effect of THC enhancing mTOR activity and the protein synthesis machinery to achieve its amnesic-like effects (Puighermanal et al, 2009) and the role of mTOR signaling in mRNA translation modulation

(Hoeffer and Klann, 2010) reinforce the idea that the mTOR-insensitive residual memory deficit could be the result of mTOR-dependent plastic changes that are temporarily stabilized. Together, these data suggest that adequate synaptic plasticity is necessary for the object-recognition memory task, and THC, by modulating mTOR signaling, could underlie a harmful effect on synaptic plasticity that promotes amnesic-like effects, a consequence that can be prevented by mTOR inhibition.

The downregulation of hippocampal CB1R induced after chronic THC exposure was mainly due to a decrease in CB1R located on hippocampal GABAergic neurons. These data using conditional KO mice are in agreement with previous electrophysiological results reporting the development of tolerance to GABA release inhibition, but not to glutamate release inhibition after chronic THC (Hoffman et al, 2007). Taking into account that the amnesic-like effects of acute (Puighermanal et al, 2009) and chronic THC administration (present data) in the object-recognition test depend on GABAergic CB1R, tolerance to this effect would be expected. However, GABAergic CB1R are 10–20 times more abundant in GABAergic than in glutamatergic hippocampal terminals (Marsicano and Lutz, 1999, Kawamura et al, 2006; Monory et al, 2006; Katona et al, 2006). We could then hypothesize that although the downregulation in GABAergic CB1R is more pronounced than in glutamatergic CB1R, this might not be sufficient to influence the unbalancing effect of THC on the excitatory/inhibitory presynaptic control (Puighermanal et al, 2009) even under these conditions of CB1R downregulation. Indeed, after chronic THC exposure, p70S6K(T389) phosphorylation was found to be increased in the hippocampus, pointing to the involvement of excitatory transmission, as was previously observed after acute THC exposure (Puighermanal et al, 2009). Consequently, further investigations will clarify possible differences in CB1R functionality in these neuronal populations after chronic THC administration and the potential downstream effectors of mTOR signaling that mediate the residual cognitive deficits resulting of THC treatment.

The present study suggests that the medicinal cannabis, such as Cesamet (nabilone), Marinol (THC), and Sativex (THC with cannabidiol) (Pertwee, 2012), with the combination of an mTOR signaling inhibitor like temsirolimus could represent an interesting therapeutic approach to minimize important side effects, such as the anxiogenic- and amnesic responses. According to our results and those previously reported (Monory et al, 2007; Puighermanal et al, 2009; Rey et al, 2012), different populations of CB1R mediate several therapeutic and side effects of cannabinoids. Indeed, CB1R in GABAergic neurons are involved in the amnesic- (Puighermanal et al, 2009) and anxiogenic-like effects of THC (Rey et al, 2012), whereas CB1R in principal forebrain glutamatergic neurons are necessary for the anxiolytic properties of low doses of the cannabinoids (Rey et al, 2012). This could be related to the different signaling complexes associated to CB1R in specific cellular environments (ie, GABAergic vs glutamatergic neurons). These CB1R signaling complexes may be different downstream of the receptor itself (Smith et al, 2010; Steindel et al, 2013), to the expression of different CB1R isoforms (Shire et al, 1995), or to the heterodimerization of CB1R with other

G-protein-coupled receptors (Wager-Miller et al, 2002; Milligan and Smith, 2007).

Overall, these results describe the specific role of mTOR in the amnesic, anxiolytic, anxiogenic, hypothermic, and hypolocomotor effects of THC, as well as the distinct downregulation of different populations of hippocampal CB1R after chronic THC exposure, showing a differential tolerance to THC pharmacological effects. This specific role of mTOR provides an interesting tool to dissociate cannabinoid responses related to their therapeutic applications from those related with their side effects.

DISCLOSURE

The authors declare no conflict of interest.

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