

Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling

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Cognitive impairment is one of the most important negative consequences associated with cannabis consumption. We found that CB1 cannabinoid receptor (CB1R) activation transiently modulated the mammalian target of rapamycin (mTOR)/p70S6K pathway and the protein synthesis machinery in the mouse hippocampus, which correlated with the amnesic properties of delta9-tetrahydrocannabinol (THC). In addition, non-amnesic doses of either the mTOR blocker rapamycin or the protein synthesis inhibitor anisomycin abrogated the amnesic-like effects of THC, pointing to a mechanism involving new protein synthesis. Moreover, using pharmacological and genetic tools, we found that THC long-term memory deficits were mediated by CB1Rs expressed on GABAergic interneurons through a glutamatergic mechanism, as both the amnesic-like effects and p70S6K phosphorylation were reduced in GABA-CB1R knockout mice and by NMDA blockade.

The endogenous cannabinoid system (ECS) is extensively expressed in the CNS and is important in synaptic plasticity¹. The predominant cannabinoid receptor in the CNS, CB1R, is expressed at presynaptic sites where, on activation by endocannabinoids or exogenous cannabinoid agonists, it regulates both inhibitory and excitatory neurotransmission¹. In the hippocampus, CB1R activation impairs memory formation and consolidation^{2,3}, and CB1Rs are mainly located in GABAergic terminals of basket cells⁴ to control GABA release⁵. In addition, CB1Rs are located, to a minor extent, in glutamatergic terminals^{6,7} where they are critical for neuroprotection⁸ through the modulation of glutamate release⁹.

THC-mediated CB1R stimulation *in vivo* triggers the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphoinositide-3 kinase (PI3K)/protein kinase B (also known as Akt) signaling cascades^{10–12}. mTOR is a serine/threonine kinase that represents an important downstream target of the PI3K/Akt pathway^{13,14}. mTOR regulates multiple cellular processes, including neuronal development and long-term modification of synaptic strength^{15,16}. Protein synthesis underlying activity-dependent synaptic plasticity is critically controlled at the level of translation, where mTOR is important as it regulates the translational rate and the initiation step^{17,18}. Thus, mTOR phosphorylates both the 70-kDa ribosomal protein S6 kinase (p70S6K) and the eukaryotic initiation factor 4E binding proteins (4E-BPs). The mTOR-dependent phosphorylation of p70S6K on T389 results in its activation¹⁹ and this activation also requires the phosphorylation of p70S6K on T421/S424 through an ERK-dependent mechanism²⁰. p70S6K is a direct modulator of ribosomal protein S6 (ref. 21), whose phosphorylation state correlates with

translation rates²². 4E-BPs, and particularly 4E-BP2, the main isoform found in the brain²³, are involved in controlling the binding of the eukaryotic initiation factor 4E (eIF4E) to the initiation complex eIF4F. This complex is integrated by eIF4E, the scaffold protein eIF4G and the RNA helicase eIF4A and promotes cap-dependent translation²⁴. After mTOR phosphorylation of 4E-BP2, eIF4E is liberated to allow its association with the other components in the eIF4F complex that initiates translation¹⁷. The involvement of mTOR in the regulation of the protein synthesis machinery could relate this intracellular signaling cascade to synaptic plasticity and memory processes^{17,18,25}.

We sought to evaluate the possible involvement of the mTOR pathway in the cognitive impairments produced by cannabinoid agonists, which represent an important negative consequence associated with cannabis consumption and a serious drawback for the therapeutic use of cannabinoid agents. We found that CB1R activation, mainly in GABAergic interneurons, by exogenous or endogenous cannabinoids can trigger the activation of the mTOR pathway and the protein synthesis machinery in the hippocampus through a glutamatergic mechanism, which underlies the characteristic long-term memory impairment.

RESULTS

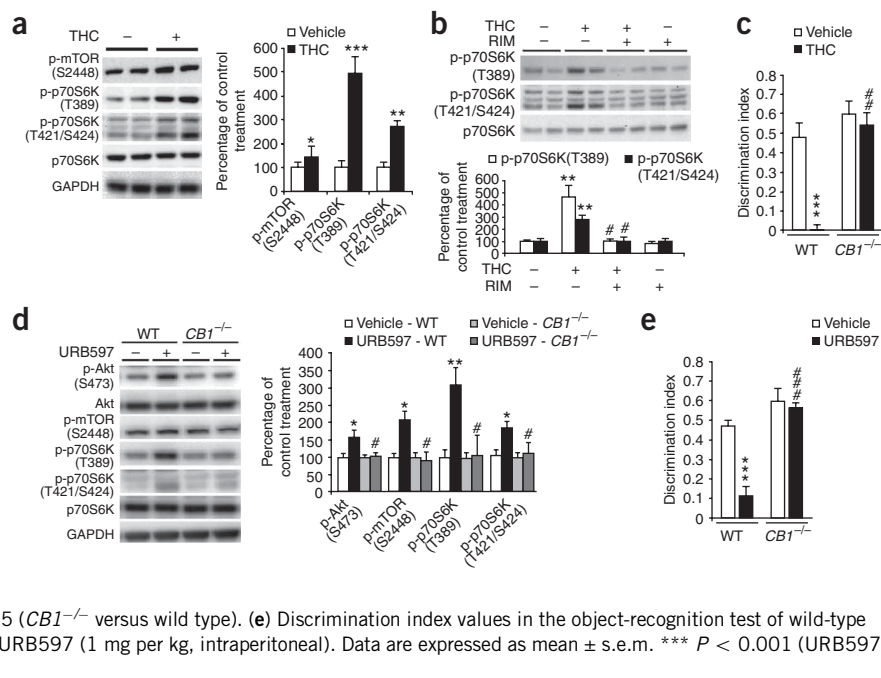
Cannabinoids modulate mTOR signaling and protein synthesis

We found that acute THC administration (3 or 10 mg per kg of body weight, intraperitoneal) modulated the mTOR/p70S6K signaling cascade in the mouse hippocampus (Fig. 1a,b and Supplementary Fig. 1). Notably, in the object-recognition test, a behavioral procedure involving hippocampal function²⁶, these doses of THC administered

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Figure 1 Modulation of the mTOR pathway by cannabinoids acting on CB1R. (a) Immunoblot (full-length blots are presented in **Supplementary Fig. 10**) of hippocampal samples and optical density quantification of mTOR and p70S6K phosphorylation 30 min after THC treatment (10 mg per kg, intraperitoneal, $n = 4$ per group). Data are expressed as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (THC versus vehicle). (b) p70S6K phosphorylation after mice were pretreated with rimonabant (RIM, 3 mg per kg, intraperitoneal) prior to THC (10 mg per kg, intraperitoneal) administration ($n = 4$ per group). Data are expressed as mean \pm s.e.m. # $P < 0.05$ (RIM versus THL). (c) Discrimination index values in the object-recognition test of wild-type (WT) and $CB1^{-/-}$ mice ($n = 6-8$ per group) after receiving THC (10 mg per kg, intraperitoneal). Data are expressed as mean \pm s.e.m. ## $P < 0.01$ (genotype effect). (d) Akt, mTOR and p70S6K phosphorylation in hippocampal samples of wild-type and $CB1^{-/-}$ mice treated with URB597 (1 mg per kg, intraperitoneal) ($n = 6$ per group). Data are expressed as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ (URB597 versus vehicle) and # $P < 0.05$ ($CB1^{-/-}$ versus wild type). (e) Discrimination index values in the object-recognition test of wild-type and $CB1^{-/-}$ mice ($n = 6-8$ per group) after receiving URB597 (1 mg per kg, intraperitoneal). Data are expressed as mean \pm s.e.m. *** $P < 0.001$ (URB597 versus vehicle) and ### $P < 0.001$ (genotype effect).



immediately after training produced a measurable deficit in long-term memory (assessed 24 h after training), resulting in a low discrimination index, which is an indicator of the discrimination between familiar and novel objects. Lower doses did not produce either p70S6K activation or memory deficits (**Supplementary Fig. 1**). The phosphorylation of Akt (S473), mTOR (S2448), p70S6K (T389) and p70S6K (T421/S424) was transient after THC administration (**Supplementary Fig. 1**). The changes in phosphorylation of p70S6K were mediated through CB1R activation, as they were sensitive to pretreatment with the CB1R antagonist rimonabant (3 mg per kg, intraperitoneal, 30 min before THC administration; **Fig. 1b**), whereas rimonabant alone did not show any effect on phosphorylation (**Supplementary Fig. 2**). Moreover, the amnesic-like effects produced by THC (10 mg per kg, intraperitoneal) were absent in CB1R constitutive knockout mice (**Fig. 1c**). The hippocampal activation of Akt/mTOR/p70S6K pathway was also observed after the administration of cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester (URB597, 1 mg per kg, intraperitoneal), a selective inhibitor of the fatty-acid amide hydrolase (FAAH; **Fig. 1d**). FAAH is involved in the degradation of the endocannabinoid anandamide and its blockade enhances ECS activity²⁷. The effect of URB597 on the Akt-mTOR-p70S6K pathway paralleled its amnesic-like effect (**Fig. 1d,e**) and both were dependent on CB1R, as they were absent in CB1R constitutive knockout mice (**Fig. 1d,e**), indicating that the ECS is physiologically relevant in the control of the activity of this signaling pathway in the hippocampus and on memory consolidation.

mTOR signaling modulates synaptic plasticity²⁸ through its major role in protein translation regulation. Therefore, we asked whether the effect of THC on mTOR would activate the protein translation machinery in the hippocampus and what was the time course of this modulation. We analyzed the phosphorylation of S6 (S235/236) and the translation initiation factors eIF4E (S209), eIF4G (S1108) and eIF4B (S422) by immunoblot, as translation initiation is the most rate-limiting step in protein translation^{21,29}. Phosphorylation of eIF4E (S209), eIF4G (S1108) and eIF4B (S422) showed a similar temporal pattern (**Fig. 2a**), with each being substantially enhanced 30 min after THC administration, peaking between 30 min and 2 h, and returning

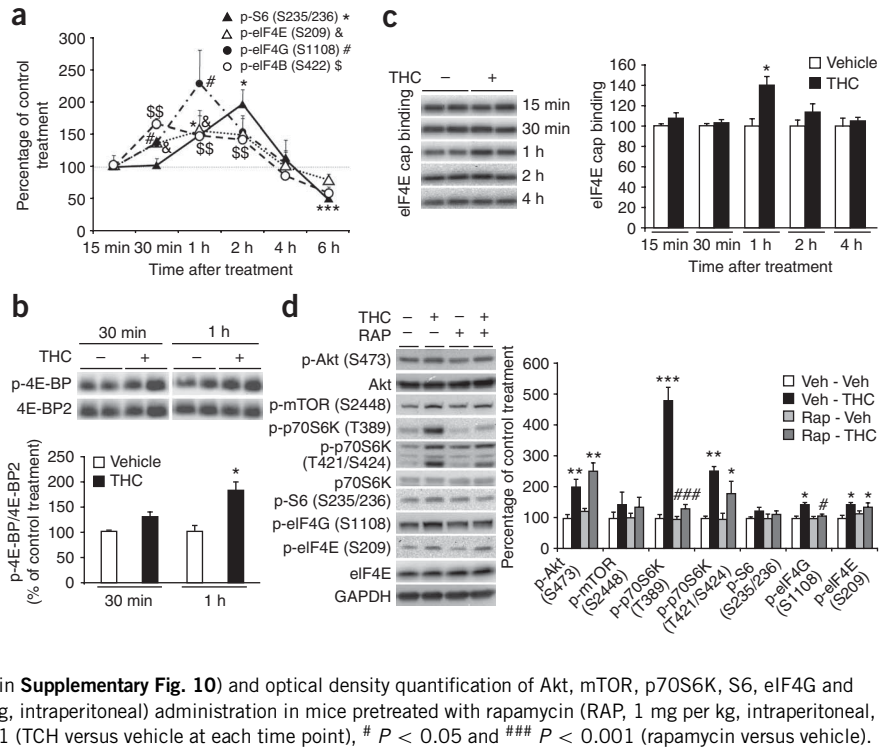
to basal levels at 4 h (**Fig. 2a**). We also observed an increase in the phosphorylation of 4E-BP2 after 1 h (**Fig. 2b**). Accordingly, we observed an increase in the binding of eIF4E to the cap analog 7mGTP affixed to Sepharose (cap resin) specifically in hippocampal homogenates at 1 h post THC administration (**Fig. 2c**).

These data indicate that protein translation initiation was transiently modulated in the hippocampus by acute THC administration. To clarify the role of the mTOR pathway activation in this process, we used the specific mTOR inhibitor rapamycin (1 mg per kg, intraperitoneal, 5 d) and an administration protocol that has been shown to have no effect on the cognitive performance measured in the object-recognition test (**Supplementary Fig. 3**), but is effective in the biochemical characterization of the pathway once it is activated (**Fig. 2d**). In this sense, others have also shown the efficacy of this treatment in modulating mTOR signaling in the hippocampus²⁵. Thus, the effect of THC after 30 min on p70S6K (T389), eIF4G (S1108), S6 (S235/236, inhibition visible after 2 h of THC, data not shown) and partially p70S6K (T421/S424) phosphorylation was dependent on mTOR (**Fig. 2d**), whereas Akt (S473), mTOR (S2448) and eIF4E (S209) phosphorylation were not modified by rapamycin (**Fig. 2d**). Instead, eIF4E (S209), and to some degree p70S6K (T421/S424), phosphorylation were dependent on MAPK/ERK signaling, as pretreatment with the inhibitor of this pathway, SL327, reduced the effects induced by THC in the phosphorylation of those residues (**Supplementary Fig. 4**).

mTOR and protein translation mediate THC-induced amnesia

Next, we studied whether the amnesic-like effects of THC (10 mg per kg, intraperitoneal, post-training) were dependent on mTOR signaling, as we hypothesized from previous findings in which a sustained increase in mTOR signaling or excess protein synthesis was associated with cognitive deficits^{25,30}. We used two behavioral procedures, the object-recognition and the context-recognition task, to evaluate the effects of THC on hippocampal-dependent long-term memory, a cognitive response that requires new protein synthesis³¹. These procedures have the advantage that robust hippocampal-dependent learning can be achieved by a single training episode. THC administration

Figure 2 Temporal modulation of protein translation machinery and mTOR involvement. **(a)** Optical density values for S6, eIF4E, eIF4G and eIF4B phosphorylation in the hippocampus expressed as a ratio between THC- and vehicle-treated mice for each time point. Data are expressed as mean \pm s.e.m. *, &, #, \$ $P < 0.05$ (THC versus vehicle at each time point), \$\$ $P < 0.01$ (THC versus vehicle at each time point) and *** $P < 0.001$ (THC versus vehicle at each time point). **(b)** Representative immunoblot and optical density quantification of 4E-BP phosphorylation after THC (10 mg per kg, intraperitoneal) administration. Hippocampal homogenates obtained at 30 min or 1 h after THC or vehicle administration were immunoprecipitated with antibody to 4E-BP2 and immunodetected with an antibody to phospho-4E-BP1 (Thr37/46) that is known to cross-react with 4E-BP2 and 4E-BP3 at equivalent phosphorylation sites. After stripping, 4E-BP2 was immunodetected in the same blot. Data are expressed as mean \pm s.e.m. **(c)** Immunoblot and optical density quantification of eIF4E on cap analog m7GTP-bound hippocampal proteins after THC (10 mg per kg, intraperitoneal) administration. Data are expressed as mean \pm s.e.m. **(d)** Immunoblot (full-length blots are presented in **Supplementary Fig. 10**) and optical density quantification of Akt, mTOR, p70S6K, S6, eIF4G and eIF4E phosphorylation 30 min after THC (10 mg per kg, intraperitoneal) administration in mice pretreated with rapamycin (RAP, 1 mg per kg, intraperitoneal, 5 d). Data are expressed as mean \pm s.e.m. ** $P < 0.01$ (THC versus vehicle at each time point), # $P < 0.05$ and ### $P < 0.001$ (rapamycin versus vehicle).



immediately after training had a marked amnesic-like effect in both procedures, with a reduction in the discrimination index in the object-recognition task (**Fig. 3a**) and a decline of the freezing time in the context-recognition task (**Fig. 3b**). The possible influence of the acute pharmacological effects of THC on locomotion, exploration or nociception during the training or the test session was disregarded, as the animals were drug free during these periods. Notably, a non-amnesic pretreatment with rapamycin (1 mg per kg, intraperitoneal, 5 d; **Supplementary Fig. 3**) abolished the amnesic-like effects of THC in both hippocampal-dependent tasks (**Fig. 3a,b**), suggesting a correlation between the effects of THC on mTOR-related signaling and the impaired cognitive responses of THC-treated mice.

The protein synthesis inhibitor anisomycin was used to confirm the role of protein translation in the amnesic-like effects of THC. Systemic administration of high doses (50 and 100 mg per kg, intraperitoneal) of anisomycin has been commonly used to affect memory consolidation (**Supplementary Fig. 5**). We used low doses of anisomycin (12 and 18 mg per kg, intraperitoneal, 25 min after training) that did not generate amnesic-like effects in the object-recognition test (**Supplementary Fig. 5**) to block the cognitive effects of THC (10 mg per kg, intraperitoneal, administered 45 min after training). A reduction of the amnesic-like effects of THC was observed after the administration of 12 mg per kg of anisomycin, whereas the highest non-amnesic dose of anisomycin (18 mg per kg) completely abolished this cognitive impairment (**Fig. 3c**).

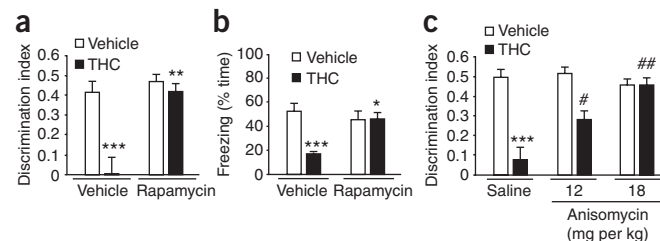


Figure 3 The amnesic-like effects of THC are blocked by the administration of rapamycin or anisomycin. **(a)** Discrimination index values in the object-recognition test of mice ($n = 6-8$ per group) treated with THC (10 mg per kg, intraperitoneal) after pretreatment with rapamycin (1 mg per kg, intraperitoneal, 5 d). Data are expressed as mean \pm s.e.m. *** $P < 0.001$ (THC versus vehicle), ** $P < 0.01$ (rapamycin versus vehicle). **(b)** Percentage of freezing time in the context recognition test of mice ($n = 6-8$ per group) treated with THC (10 mg per kg, intraperitoneal) after pretreatment with rapamycin (1 mg per kg, intraperitoneal, 5 d). Data are expressed as mean \pm s.e.m. * $P < 0.001$ (rapamycin versus vehicle). **(c)** Discrimination index values in the object recognition test of mice ($n = 4-6$ per group) treated with THC (10 mg per kg, intraperitoneal) after pretreatment with non-amnesic doses of anisomycin (**Supplementary Fig. 5**). Data are expressed as mean \pm s.e.m. # $P < 0.05$ (anisomycin versus saline) and ## $P < 0.001$ (anisomycin versus saline).

Postsynaptic modulation of mTOR involves NMDA receptors

We next determined the location of the subcellular sites in the hippocampus where mTOR was activated by immunostaining for phosphorylated p70S6K (T389) and S6 (S235/236) induced by THC (**Fig. 4a**). We found that both proteins were modulated in principal neurons that showed a somatodendritic pattern of distribution (**Fig. 4a**). These phosphorylated proteins did not colocalize with CB1R (**Supplementary Fig. 6**), but did colocalize with the dendritic marker microtubule-associated protein 2 (MAP2) (**Fig. 4b**). Moreover, phospho-p70S6K (T389) induced by THC was largely absent from GABAergic interneurons labeled with an antibody to glutamic acid decarboxylase (GAD) 65/67 (**Fig. 4c**).

CB1Rs in the hippocampus are mainly located in GABA nerve terminals⁴ and their stimulation reduces the inhibitory tone of GABA⁵, leading to an imbalance of the excitatory/inhibitory input. We pharmacologically tested whether either glutamatergic or GABAergic transmission could mediate the behavioral and biochemical effects of THC *in vivo* that have been previously shown for THC modulation of MAPK/ERK¹¹. For this purpose, we pretreated mice with the mGluR1/5 antagonist MPEP (10 mg per kg, intraperitoneal),

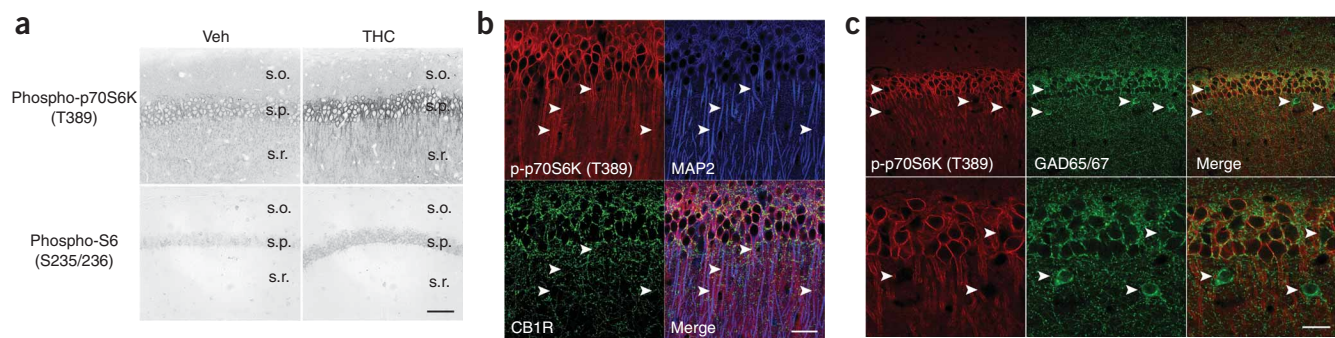


Figure 4 THC effects of p70S6K and S6 occur at the postsynaptic level and not in the GABAergic neurons. **(a)** Mice treated with THC (10 mg per kg, intraperitoneal) were processed for immunohistochemistry after 30 min (phospho-p70S6K) or 2 h (phospho-S6 detection). Confocal images (phospho-p70S6K) or bright-field images (phospho-S6) of stained coronal brain sections are presented in grayscale for comparative purposes (see Online Methods for image acquisition settings). SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar represents 80 μ m (p70S6K) and 140 μ m (S6). **(b)** Immunodetection of p-p70S6K (T389), MAP2 and CB1R in coronal brain sections from THC-treated mice (see Online Methods for image acquisition settings and **Supplementary Fig. 6** for CB1R antibody specificity). Arrowheads indicate phospho-p70S6K (T389) that did not colocalize with CB1R but instead colocalized with the dendritic marker MAP2. Scale bar represents 50 μ m. **(c)** Immunodetection of phospho-p70S6K (T389) and GAD65/67 in coronal brain sections from THC-treated mice (see Online Methods for image acquisition settings). Phosphorylated p70S6K did not colocalize with GAD65/67 (GABAergic neurons, arrowheads). Scale bars represent 45 μ m (upper panels) and 25 μ m (lower panels).

the AMPA antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]-quinoxaline-7-sulfonamide disodium salt (NBQX, 10 mg per kg, intraperitoneal), the NMDA antagonist MK801 (0.1 mg per kg, intraperitoneal), the GABA_A antagonist bicuculline (0.5 mg per kg, intraperitoneal) or the GABA_B antagonist CGP55845 (20 mg per kg, intraperitoneal) 20 min before administering THC (10 mg per kg, intraperitoneal). Only MK801 pretreatment substantially reduced the increase in phosphorylation of p70S6K (T389) induced by THC (**Fig. 5a**), whereas treatment with bicuculline or CGP55845 alone increased the phosphorylation of p70S6K (T389) (data not shown). In addition, MK801 abrogated the MAPK/ERK-dependent phosphorylation of eIF4E (S209) and p70S6K (T421/S424) (**Supplementary Fig. 7**). Notably, these biochemical changes correlated with the suppression of THC-induced cognitive impairment caused by pretreatment with non-amnesic doses of MK801 (0.01 and 0.1 mg per kg, intraperitoneal) (**Fig. 5b** and **Supplementary Fig. 8**) and with the lack of effect of bicuculline (0.5 mg per kg, intraperitoneal) and CGP55845 (20 mg per kg, intraperitoneal) pretreatment on THC-impaired cognitive responses (**Fig. 5c** and **Supplementary Fig. 8**).

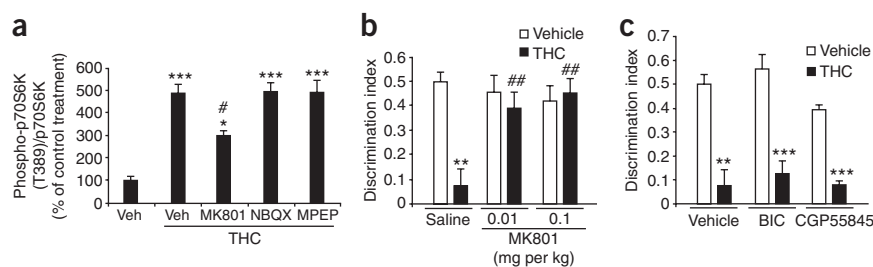
GABAergic CB1Rs are crucial for the effects of THC

To understand the specific role of CB1Rs in the different neuronal types in the hippocampus in relation to the behavioral and biochemical

responses to acute THC administration, we used two available lines of CB1R conditional knockout mice previously described⁸. One mouse mutant line had a deletion of the CB1R gene (*Cnr1*) in GABAergic interneurons⁸ (Cre recombinase driven from the *Dlx5/6* promoter excised the *loxP*-flanked *Cnr1* gene, hereafter referred to as *GABA-CB1^{-/-}*), whereas the other mutant line did not express CB1Rs in cortical glutamatergic principal neurons⁸ (Cre recombinase driven from the *Neurod6* promoter excised the *loxP*-flanked *Cnr1* gene, hereafter referred to as *Glu-CB1^{-/-}*). We found that 30 min after acute administration of THC (10 mg per kg, intraperitoneal) the phosphorylation of p70S6K (T389) was prevented in *GABA-CB1^{-/-}* mice, whereas it was increased in wild-type mice. The phosphorylation of p70S6K (T389) was also enhanced in *Glu-CB1^{-/-}* mice, although to a lower degree than in wild-type mice (**Fig. 6a**). Consistent with the results of our biochemical studies, the amnesic-like effects induced by THC in the object-recognition test were fully suppressed in *GABA-CB1^{-/-}* mice (**Fig. 6b**), whereas these cognitive responses were not modified in *Glu-CB1^{-/-}* mice (**Fig. 6b**).

These data suggest that CB1R in GABAergic terminals is an important element in the mTOR modulation by THC and in the amnesic-like effects induced by this cannabinoid agonist. Moreover, it represents, to the best of our knowledge, the first functional role directly attributed to CB1R in GABAergic neurons, which are the neuronal population in which CB1Rs are more profusely expressed.

Figure 5 The THC-mediated activation of mTOR and the memory impairment are sensitive to NMDA receptor blockade. **(a)** Optical density quantification of phospho-p70S6K (T389) after pretreatment with MK801, NBQX or MPEP 20 min before THC administration. Data are expressed as mean \pm s.e.m. * $P < 0.05$ (THC versus vehicle), *** $P < 0.001$ (THC versus vehicle), # $P < 0.05$ (MK801 versus saline). **(b)** Discrimination index values in the object-recognition test of mice ($n = 6-8$ per group) pretreated with MK801 before THC (10 mg per kg, intraperitoneal) administration. Data are expressed as mean \pm s.e.m. ## $P < 0.01$ (MK801 versus saline) and ** $P < 0.01$ (THC versus vehicle). **(c)** Discrimination index values in the object-recognition test of mice ($n = 4-6$ per group) treated with bicuculline (BIC, 0.5 mg per kg, intraperitoneal) or CGP55845 (20 mg per kg, intraperitoneal) before THC (10 mg per kg, intraperitoneal) administration. Data are expressed as mean \pm s.e.m. ** $P < 0.01$ (THC versus vehicle).



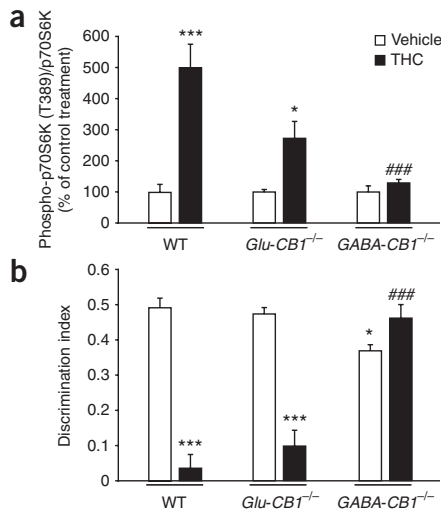


Figure 6 THC effects on mTOR signaling and cognitive function depend on CB1R localized in GABAergic neurons. **(a)** Conditional knockout mice lacking CB1R in GABAergic interneurons (*GABA-CB1^{-/-}*) or cortical glutamatergic neurons (*Glu-CB1^{-/-}*) or wild-type littermates (WT) were injected with THC (10 mg per kg, intraperitoneal) and hippocampal samples were obtained after 30 min ($n = 4-8$ per group). p70S6K (T389) phosphorylation was measured by immunoblot as a read-out of mTOR activity. Optical density analysis of the immunoreactive bands for phospho-p70S6K (T389) and p70S6K were quantified and represented as a proportion (phospho-p70S6K (T389)/p70S6K) referred to its control (vehicle values). Data are expressed as mean \pm s.e.m. * $P < 0.05$ (THC versus vehicle), *** $P < 0.001$ (THC versus vehicle), ### $P < 0.001$ (THC effects between genotypes). **(b)** Discrimination index values in the object-recognition test of conditional knockout mice lacking CB1R in GABAergic interneurons (*GABA-CB1^{-/-}*) or cortical glutamatergic neurons (*Glu-CB1^{-/-}*) or wild-type littermate mice ($n = 4-8$ per group) after receiving THC (10 mg per kg, intraperitoneal). Data are expressed as mean \pm s.e.m. * $P < 0.05$ (basal effect between genotypes).

the activity of PI3K and MAPK/ERK, resulting in the phosphorylation of eIF4E (S209)^{35,36}. The administration protocol for rapamycin that we used in this study did not produce apparent changes in the basal phosphorylation of mTOR in the hippocampus and did not result in memory impairments. Indeed, higher doses of subchronically (10 or 30 mg per kg) or acutely (150 mg per kg) administered rapamycin were required to affect long-term memory, which is consistent with previous studies³⁷. 4E-BP phosphorylation, another landmark of protein translation initiation¹⁷, was increased in the hippocampus 1 h after THC administration. At the same time, we also observed an increase in eIF4E binding to the cap analog, matching the increase in 4E-BP phosphorylation with the expected increase in available eIF4E and its enhanced phosphorylation.

Several studies have shown that both the mTOR and ERK pathways cooperate to stimulate protein translation³⁸⁻⁴⁰. ERK activation has been previously described in the hippocampus after systemic THC administration^{10,11}. Consistent with this cooperative role, THC-induced eIF4E phosphorylation was blocked by pretreatment with the ERK inhibitor SL327. In addition, ERK signaling inhibition also attenuated THC-induced p70S6K phosphorylation at T421/S424 sites. These results correlate with the partial reduction in the phosphorylation on p70S6K (T421/S424) caused by rapamycin that we observed, indicating that both the mTOR and ERK signaling pathways participate in the phosphorylation of p70S6K (T421/S424). In contrast, the phosphorylation of p70S6K at T389 by acute THC depended exclusively on mTOR activity, consistent with previous studies⁴¹. Altogether these findings suggest a role for mTOR and ERK in the modulation of protein translation rates in the hippocampus after THC administration.

We then investigated the possible relationship between the effects of THC on protein translation through mTOR and the well-known amnesic-like responses caused by THC. The cognitive impairment identified in hippocampal-dependent tasks induced by THC has been reported to be mediated by activation of the CB1R in this brain area^{2,3}. Pretreatment with a non-amnesic dose of rapamycin abolished the amnesic-like effects of THC in two hippocampal-dependent tasks, the object-recognition and the context-recognition test. This behavioral response was correlated with rapamycin blockade of the changes promoted by THC on mTOR-related signaling. To assess the role of the protein translation initiation detected in the hippocampus after THC in memory impairment, we used the protein synthesis inhibitor anisomycin. Non-amnesic doses of anisomycin blocked the memory deficits induced by THC, indicating that protein synthesis is involved in THC's effects on cognitive responses. Anisomycin has been reported to stimulate the stress-activated p38 MAPK, which could also participate

DISCUSSION

Our results demonstrate for the first time, to the best of our knowledge, that acute activation of CB1R *in vivo* modulates the mTOR pathway, increasing protein translation and regulating the memory deficits promoted by cannabinoids. Moreover, CB1Rs expressed on GABAergic terminals and NMDA receptor activation are necessary for the modulation of the mTOR pathway and the amnesic-like effects of cannabinoids.

The acute administration of THC or the FAAH inhibitor URB597 increased mTOR phosphorylation, which was accompanied by robust changes in phosphorylation of its downstream effector p70S6K (T389) and concomitant phosphorylation of ribosomal protein S6 (S235/236), causing a putative increase in translation rates^{21,22}. In addition, cannabinoid stimulation increased the phosphorylation of p70S6K at T421/S424, which are critical for its enzymatic activity, through an ERK-dependent pathway. Consistent with this, we found that this phosphorylation was sensitive to pretreatment with the specific ERK inhibitor SL327. All of these signaling events were selectively mediated by CB1R activation, as was shown by the blockade with the cannabinoid antagonist rimonabant or by the deletion of *Cnr1*. However, another explanation could be that THC or anandamide (by URB597 treatment), as a result of their lower intrinsic efficacy, would occlude the 2-araquidonoyl glycerol tone, as has been suggested by others³². This hypothesis is unlikely because endocannabinoid tone occlusion by pretreatment with rimonabant did not affect p70S6K phosphorylation (Supplementary Fig. 2).

Translation is a very energy-consuming event in the cell³³ and it is initiated when mRNA binds to the ribosome through the 5' mRNA cap structure by the cap-binding complex eIF4F³⁴. This multi-protein complex is formed by three translation initiation factors, eIF4E (a cap-binding protein), eIF4A (an RNA helicase) and eIF4G (a large scaffolding protein), to which a fourth initiation factor (eIF4B) can later bind to stimulate eIF4A activity. We found that THC administration transiently increased the phosphorylation of S6 and the translation initiation factors eIF4E (S209), eIF4G (S1108) and eIF4B (S422) in the hippocampus, all of which are related to translation increase¹⁷. Notably, THC-induced phosphorylation of eIF4E (S209) was not sensitive to low doses of rapamycin, which suggests that THC uses a mechanism that is independent of mTOR/4E-BP. Previous studies have described an alternative mechanism in the presence of rapamycin, suggesting that the lack of negative feedback by p70S6K could increase

in reducing THC-induced amnesia. However, this hypothesis seems unlikely because anisomycin did not alter the discrimination index in mice, although it produced an increase in the phosphorylation of p38 (data not shown). In addition, p38 phosphorylation induced by anisomycin was not modified by THC treatment (data not shown), whereas anisomycin blocked the amnesic-like effects of THC under similar experimental conditions. Furthermore, although p38 MAPK may be involved in various forms of synaptic plasticity, its role in memory consolidation is still controversial. Consistent with our hypothesis, an imbalance in protein synthesis was previously reported to cause memory deficits in an animal model of fragile X mental retardation³⁰ and to participate in the cognitive deficits occurring in several single-gene disorders⁴².

The results of our immunohistochemical experiments suggest that the signaling events described above occur postsynaptically. Indeed, the mTOR signaling cascade did not colocalize with GABAergic neurons and the phosphorylation of p70S6K and S6 was detectable on principal pyramidal cells of the hippocampus. In contrast, most of the CB1Rs in the hippocampus are located in GABA nerve terminals⁴, leading to a reduction of GABA inhibitory tone that enhances pyramidal glutamatergic inputs⁴³.

We next pharmacologically characterized the possible involvement of GABAergic and glutamatergic mechanisms in the biochemical and amnesic-like effects of THC. Pretreatment with antagonists for AMPA, mGluR1/5, GABA_A or GABA_B receptors did not affect the cognitive deficits associated with THC. Only NMDA blockade with non-amnesic doses of MK801 prevented the phosphorylation of p70S6K (T421/S424) and eIF4E (S209) and it reduced, but it did not abolish, the phosphorylation of p70S6K (T389) induced by THC. On the other hand, GABA receptor blockade by bicuculline or CGP55845 increased the phosphorylation of p70S6K, suggesting that a reduction in GABAergic inputs would enhance the glutamatergic tone, mimicking the THC-induced mTOR activation described here. Moreover, NMDA blockade avoided the amnesic-like effects of THC, suggesting that the stimulation of CB1Rs induced a postsynaptic activation of the signaling mechanisms mediated by NMDA receptors, leading to memory impairment. Consistent with this, recent data indicate that enhanced NMDA receptor-mediated synaptic plasticity is related to impaired learning and memory⁴⁴.

Using different lines of CB1R conditional knockout mice lacking CB1R in GABAergic or glutamatergic terminals⁸, we found that mTOR activation elicited by THC, as measured by p70S6K (T389) phosphorylation, was reduced in the hippocampus of *GABA-CB1*^{-/-} mice and, to a lesser extent, *Glu-CB1*^{-/-} mice. In addition, memory impairment induced by THC was abrogated in the *GABA-CB1*^{-/-} mice, whereas this response was not modified in *Glu-CB1*^{-/-} mice. CB1Rs are heavily expressed in GABAergic terminals in the hippocampus, but the role of this neuronal population in the intracellular signaling effects of cannabinoids and their functional relevance is unknown. In this study, we found that both THC-induced cognitive impairment and activation of the mTOR pathway are mainly mediated through the stimulation of CB1Rs in GABAergic terminals, whereas those in glutamatergic terminals did not produce such effects. Altogether, these findings suggest that the stimulation of CB1R, mainly expressed in GABAergic interneurons, would contribute to an imbalance between the excitatory and inhibitory inputs in the hippocampus. This is consistent with excitatory transmission being less sensitive to the effects of cannabinoids than inhibitory transmission⁴⁵. This imbalance would promote mTOR and MAPK/ERK signaling through the activation of NMDA receptors, resulting in the enhancement of protein synthesis that underlies the cognitive impairment produced by THC

(Supplementary Fig. 9). The ECS has a specific physiological role in the control of the cognitive processes of aversive memories, as revealed by genetic⁴⁶ and pharmacological tools⁴⁷. The mechanism involved in this would be exogenously overactivated by the administration of THC or by the increase in the endocannabinoid anandamide, leading to the amnesic-like effects that have been observed. Consistent with our results, recent reports propose that other long-term memory deficits can be associated with an overactivation of the mTOR signaling pathway and an imbalance in protein synthesis^{25,30}.

The elucidation of the mechanisms involved in the amnesic effects of cannabinoids allows for a better understanding of a serious drawback of cannabis consumption. The cognitive impairments are also important side-effects that can be associated with the therapeutic use of cannabinoids. Our results identify the specific target underlying these effects and would therefore be useful for facilitating the development of new therapeutic strategies leading to the prevention of these deleterious effects of cannabinoid compounds.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

E.P. conducted the biochemical, immunohistochemical and behavioral experiments and wrote the manuscript. G.M. provided CB1 conditional knockout mice. A.B.-G. conducted behavioral experiments and wrote the manuscript. B.L. provided CB1 conditional knockout mice. R.M. funded the project, participated in experimental design and wrote the manuscript. A.O. conceptualized, supervised and funded the project, participated in experimental design, and wrote the manuscript.

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ONLINE METHODS

Animals. Male CD-1 mice (Charles River), male CB1 constitutive knockout mice⁴⁸, conditional knockouts (*GABA-CB1*^{-/-}, *Glu-CB1*^{-/-})⁸ and their wild-type littermates, weighing 25–30 g were used. CB1 constitutive knockout mice were bred by backcrossing chimeric animals to the C57BL/6J background and crossing heterozygotes⁴⁸. Conditional CB1 knockout mice were in a mixed background with a predominance of C57BL/6-N and experimental animals were littermates bred as described previously⁸. Mice were housed in cages of five and maintained at a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%). Food and water were available *ad libitum*. Lighting was maintained at 12-h cycles (on at 8 a.m. and off at 8 p.m.). All of 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. Mice were generated and genotyped as described previously^{8,48}. 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-Institut Municipal d'Assistència Sanitària-Universitat Pompeu Fabra).

Drugs and treatments. THC was provided by THC-Pharm-GmbH, URB597 by Biomol-International, NBQX and CGP55845 by Tocris, and rapamycin by Calbiochem. SL327, Cremophor-EL, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate ((+)-MK801), 6-methyl-2-(phenylethynyl)pyridine (MPEP) and bicuculline were provided by Sigma. Rimonabant was kindly provided by Sanofi-Aventis Recherche. MK801 and NBQX were dissolved in 0.9% bacteriostatic sodium chloride (saline, vol/vol). THC and rimonabant were diluted in vehicle preparation (5% ethanol (vol/vol), 5% cremophor-EL (vol/vol), 90% saline (vol/vol)). SL327 was dissolved in 50% dimethyl sulfoxide (DMSO, vol/vol). Rapamycin, CGP55845, URB597 and bicuculline were dissolved in DMSO. MPEP was dissolved in 5% poly-ethylenglycol (vol/vol), 5% Tween-80 (vol/vol) and 90% saline (vol/vol). All drugs were administered intraperitoneally in a volume of 10 ml per kg of body weight, except for rapamycin, CGP55845, SL327, URB597 and bicuculline (2 ml per kg). Rimonabant (3 mg per kg) was administered either 30 min before THC or 30 min before sample extraction. MK801 (0.01, 0.1 or 0.3 mg per kg, intraperitoneal) and NBQX (10 mg per kg, intraperitoneal) were administered 20 min before THC treatment. MPEP (10 mg per kg, intraperitoneal) was administered 15 min before THC treatment. SL327 (50 mg per kg, intraperitoneal) was administered 1 h before THC. Rapamycin (1 mg per kg, 10 and 30 mg per kg, intraperitoneal) was administered once daily for 5 d and mice were tested 3 h after the last rapamycin injection. To examine the acute effect, we administered rapamycin as a single acute injection (150 mg per kg, intraperitoneal). Anisomycin (12, 18, 25, 50 or 100 mg per kg, intraperitoneal) was administered 20 min before THC. Bicuculline (0.5 or 2.5 mg per kg, intraperitoneal) and CGP55845 (20 mg per kg, intraperitoneal) were administered 20 min before THC. Mice were killed at the time points indicated after receiving THC or vehicle and their brains were rapidly removed. The hippocampus was dissected from the brain, frozen on dry ice and stored at –80 °C until used.

Immunoblot analysis. Frozen hippocampal tissues were processed as previously reported¹². For immunoblotting, we used antibodies to phospho-p70S6K (T389) (1:400), phospho-p70S6K (T421/S424) (1:200), p70S6K (1:500), phospho-S6 (S235/236) (1:1,200), phospho-mTOR (S2448) (1:500), phospho-Akt (S473) (1:200), Akt (1:2000), phospho-eIF4G (S1108) (1:500), phospho-eIF4E (S209) (1:75), eIF4E (1:500), phospho-eIF4B (S422) (1:400), phospho-4E-BP1 (Thr37/46) (1:100) and 4E-BP2 (1:500) from Cell Signaling, phospho-ERK1/2 (T202/Y204) (1:5,000) and ERK1/2 (1:10,000) from Sigma, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5,000) from Santa Cruz Biotechnology. Blots containing equal amounts of hippocampal protein samples to compare in each specific experiment were cut horizontally, producing strips at different molecular weights to be probed with different primary antibodies (see **Supplementary Fig. 10** for full-length blot detection with each primary antibody). Bound primary antibodies were detected with horseradish peroxidase-conjugated antibodies to rabbit or mouse antibodies (Pierce, diluted 1:5,000) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Pierce). When necessary, Immobilon-P

membranes (Millipore) were stripped in buffer containing 100 mM glycine (pH 2.5), 200 mM NaCl, 0.1% Tween 20 (vol/vol) and 0.1% beta-mercaptoethanol (vol/vol) for 45 min at 21 ± 1 °C, followed by extensive washing in 100 mM NaCl, 10 mM Tris and 0.1% Tween 20 (pH 7.4) before re-blocking and re-probing¹⁶. 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). Representative cropped immunoblots for display were processed with Adobe Photoshop 7.0. For quantitative purposes, the optical density values of active phospho-specific antibodies were normalized to the detection of nonphospho-specific antibodies or to GAPDH values in the same sample and expressed as a percentage of control treatment.

m7GTP-Sepharose pull-down. Proteins (500 µg) from hippocampal lysates obtained at different times (15 min, 30 min, 1 h, 2 h, 4 h and 6 h) after either vehicle or THC (10 mg per kg, intraperitoneal) administration were incubated for 2 h at 4 °C with m7GTP-Sepharose 4B (25 µl of 50:50 slurry) (GE Healthcare). Samples were then washed three times with lysis buffer before elution in 50 µl of Laemmli buffer at 95 °C for 5 min. The amount of eIF4E was analyzed by sodium dodecyl sulfate-PAGE and immunoblot as described previously with the antibody to eIF4E.

Immunoprecipitation. Brain homogenates containing 1 mg of total protein were incubated with antibody to 4E-BP2 (1:50) overnight at 4 °C. We then added 50 µl of 50% protein A Sepharose slurry (Sigma) and incubated them for 2 h at 4 °C. The protein A Sepharose beads were washed five times with 1 ml of lysis buffer. Bound proteins were eluted by 50 µl of Laemmli buffer at 95 °C for 5 min. Phospho-4E-BP2 content was analyzed by immunoblot with the antibody to phospho-4E-BP1 (Thr37/46), taking advantage of the cross-reactivity of this antibody for the equivalent phosphorylated sites in 4E-BP2.

Tissue preparation for immunohistochemistry and immunofluorescence. After pharmacological treatment, mice were deeply anesthetized by intraperitoneal injection (0.2 ml per 10 g body weight) of a mixture of ketamine (100 mg per kg) and xylazine (20 mg per kg) and killed via intracardiac perfusion of 4% paraformaldehyde in 0.1 M Na₂HPO₄/NaH₂PO₄ phosphate buffer (pH 7.5), delivered with a peristaltic pump. Brains were removed and post-fixed overnight at 4 °C in the same fixative solution. Brain sections (30 µm) were obtained with a vibratome (Leica, France) and kept in a solution containing 30% ethylene-glycol (vol/vol), 30% glycerol (vol/vol) and 0.1 M phosphate buffer at –20 °C until they were used for immunodetection.

Immunohistochemistry. The immunohistochemical procedure that we used was adapted from a previously described protocol⁴⁹. On day 1, free-floating slices were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 10 min in TBS containing 3% H₂O₂ (vol/vol) and 10% methanol (vol/vol), and then washed three times for 10 min each in TBS. After a 15-min incubation in 0.2% Triton X-100 (vol/vol) in TBS, slices were rinsed three times in TBS. Slices were incubated with polyclonal antibody to CB1R (1:500, rabbit, Frontier Science) or antibody to phospho-S6 (S235/236) (1:200), overnight at 4 °C. The next day, after three rinses in TBS, slices were incubated for 2 h at 21 ± 1 °C with secondary biotinylated antibody (1:400) (Vector Laboratories). After three washes, slices were incubated for 90 min in an avidin-biotin-peroxidase complex solution (1:100) (Vector Laboratories). After washing once in TBS and twice in Tris buffer (0.25 M Tris, pH 7.5) for 10 min each, slices were placed in a solution of Tris buffer containing 0.1% 3,3'-diaminobenzidine (30 mg per 100 ml) and developed by H₂O₂ addition (0.02%). Sections were mounted onto gelatin-coated slides and dehydrated through alcohol to xylene for light microscopic examination. Displayed images were converted to grayscale with Adobe Photoshop 7.0.

Immunofluorescence. Free-floating slices were rinsed in TBS, incubated for 15 min in 0.2% Triton X-100 in TBS and then incubated overnight at 4 °C with antibody to CB1R (1:500, rabbit), phospho-p70S6K (T389) (1:100, mouse), MAP2 (1:1,000, chicken, AbCam) or GAD65/67 (1:100, rabbit, Chemicon). The next day, after two rinses in TBS, sections were incubated for 2 h at 21 ± 1 °C with antibody to mouse Alexa Fluor 647 (1:500, Invitrogen), rabbit Cy3 (1:500) (Jackson ImmunoResearch Laboratories) or chicken biotinylated



antibody (1:400, Vector Laboratories). After a 10-min wash, the slices were incubated for 15 min with an antibody to streptavidin–Alexa Fluor 488 (Invitrogen). After two washes (10 min each), the tissue sections were mounted onto gelatin-coated slides with Mowiol mounting media. We included 0.1 mM NaF in all of the buffers and incubation solutions.

Confocal images were obtained using a Leica TCS SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Tissue sections were examined with a 40× 1.25-NA oil-immersion Leica Plan Apochromatic objective. Alexa 647, Cy3 and Alexa 488 were excited with a 633-nm helium neon laser, a 543-nm green neon laser and the 488-nm line of an argon laser. A triple dichroic 488/543/633 beam-splitter was used and spectral windows between 495 and 535 nm, 555 and 630 nm, and 645 and 750 nm were set to collect Alexa 488, Cy3 and Alexa 647 fluorescence, respectively. Images were 8 bit, 1024 × 1024 pixels. Grayscale digital images were imported to Adobe Photoshop 7.0 and pseudo-colored for display purposes.

Cognitive tasks. The cognitive tasks that we used depend on hippocampal function and provide a robust learning triggered by a single trial^{26,50}. All behavioral experiments were performed under blind conditions.

Object-recognition task. On day 1, mice were habituated for 10 min to the maze in which the task was performed. On the second day, mice were put back in the maze for 10 min, two identical objects were presented and the time that the mice spent exploring each object was recorded. The mice were again placed in the maze 24 h later for 10 min, one of the familiar objects was replaced with a novel object and the total time spent exploring each of the two objects (novel and familiar) was computed. Object exploration was defined as the orientation of the nose to the object at a distance of less than 2 cm. A discrimination index was calculated as the difference between the times spent exploring either the novel or familiar object divided by the total time exploring the two objects. A higher discrimination index is considered to reflect greater memory retention for the familiar object. Pharmacological treatment with URB597 was performed immediately after training. THC treatments were carried out either immediately after training in the case of rapamycin blockade or 45 min after training in

the case of anisomycin, MK801, bicuculline and CGP55845 pretreatment. Rapamycin was administered 3 h before any behavioral task in all cases.

Context-recognition task. In the context-recognition task, mice learn to fear a new environment because of its temporal association with an aversive stimulus (foot shock). When exposed to the same context, conditioned mice show freezing behavior⁵⁰. Mice were trained and tested in conditioning chambers (PanLab) that had a stainless-steel grid floor through which electric foot shocks could be delivered. On the training day, a mouse was placed in the conditioning chamber for 2 min before the onset of the unconditioned stimulus (0.4-mA foot shock for 2 s, 30 s between shocks, repeated six times) and then remained in the chamber for 30 s. Immediately after training, the mouse received an injection of THC (10 mg per kg, intraperitoneal) or its vehicle and was returned to its home cage. Testing for contextual fear conditioning was performed 24 h after training by measuring freezing behavior, which was defined as a complete lack of movement except for respiration, for 5 min in the same conditioning chamber. Results are expressed as a percentage of freezing time. Rapamycin was administered 3 h before any behavioral task in all cases.

Statistical analysis. The results of the immunoblot experiments were evaluated by one-way analysis of variance (ANOVA) with treatment as the between group factor. In the behavioral experiments, data were analyzed by two-way ANOVA with THC and pretreatment (rapamycin, anisomycin, bicuculline, CGP55845 or MK801) as the between factors, followed by one-way ANOVA when required. Comparisons were considered to be statistically significant when the level of significance was $P < 0.05$.

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