TESI DOCTORAL UPF / 2011

Emma Puighermanal Puigvert

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MOLECULAR MECHANISMS UNDERLYING THE EFFECTS OF CANNABINOIDS IN THE BRAIN

Emma Puighermanal Puigvert

DOCTORAL THESIS UPF / 2011

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A la meva mare

When everyone thinks the same,
nobody is thinking

Walter Lippmann

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Ser libre no es hacer lo que uno quiere, sino querer lo que se hace

Jean-Paul Sartre

Abstract

The endocannabinoid system is an endogenous neuromodulatory system that regulates a plethora of physiological functions, including the control of movement, memory, anxiety, and pain, among others. Cannabinoid compounds are mainly found in the *Cannabis sativa* plant and exert their effects by acting at the endocannabinoid system. Cannabinoids are potential therapeutic agents, mainly for multiple sclerosis, pain, and emesis, although an important caveat to their use is the possible adverse effects, such as memory impairment and anxiety. This thesis mainly addresses the molecular mechanisms underlying some of the physiological processes controlled by the endocannabinoid system as well as specific pharmacological effects triggered by Δ9-tetrahydrocannabinol, the main psychoactive compound of marijuana plant. The combination of biochemical, pharmacological, and behavioral approaches allowed the elucidation of certain signaling cascades responsible for particular effects induced by cannabinoids.

Resum

El sistema endocannabinoid és un sistema neuromodulador endogen que regula diverses funcions fisiològiques, incloent el control del moviment, la memòria, l'ansietat i el dolor, entre altres. Els compostos cannabinoids es troben principalment a la planta *Cannabis sativa* i exerceixen els seus efectes actuant al sistema endocannabinoid. Els cannabinoids tenen potencial terapèutic, principalment per l'esclerosi múltiple, el dolor i l'èmesi, tot i que una limitació important pel seu ús recau en els possibles efectes adversos, tal com l'alteració de la memòria i l'ansietat. Aquesta tesi exposa principalment els mecanismes moleculars responsables d'alguns processos fisiològics controlats pel sistema endocannabinoid així com efectes farmacològics desencadenats pel Δ9-tetrahidrocannabinol, el principal compost psicoactiu de la planta de marihuana. La combinació d'aproximacions bioquímiques, farmacològiques i comportamentals ha permès revelar algunes cascades de senyalització responsables de determinats efectes induïts pels cannabinoids.

Abbreviations

2-AG: 2-arachidonoylglycerol

4E-BP: eukaryotic initiation factor 4E binding protein

5'TOP: 5′ terminal oligopyrimidine

AEA: N-arachidonoylethanolamine (anandamide)

AMPK: AMP-activated protein kinase

BDNF: brain-derived neurotrophic factor

CB1R: CB1 cannabinoid receptor

CB2R: CB2 cannabinoid receptor

CCK: cholecystokinin

CNS: Central Nervous System

CREB: cAMP responsive element binding protein

DAGL: diacylglycerol lipase

eCB-LTD: endocannabinoid-mediated long-term depression

ECS: endocannabinoid system

eIF: eukaryotic initiation factor

ERK1/2: extracellular signal-regulated kinase 1 and 2

FAAH: fatty-acid amide hydrolase

FKBP12: FK506-binding protein 12

FMRP: fragil X mental retardation protein

GABA: γ-aminobutyric acid

GAP: GTPase-activating protein

GASP1: G-protein-coupled receptor-associated sorting protein 1

GSK-3: glycogen synthase kinase-3

HFS: high frequency stimulation

LTD: long-term depression

LTP: long-term potentiation

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

mGluR: metabotropic glutamate receptors

Mnk1/2: MAPK signal-integrating kinase/MAPK-interacting kinase 1 and 2

mTOR: mammalian target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

NAPE-PLD: N-acyltransferase and phospholipase D

NMDAR: N-methyl-D-aspartate receptor

p70S6K: p70 ribosomal S6 kinase

PDK1: PI3K-dependent kinase

PI3K: phosphatidylinositol-3 kinase

PKA: protein kinase A

PRAS40: 40 KDa Pro-rich Akt substrate

Rheb: Ras homolog enriched in brain protein

THC: Δ9-tetrahydrocannabinol

TSC: tuberous sclerosis complex

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Andrés Ozaita, Emma Puighermanal and Rafael Maldonado

J Neurochem **102**, 1105-1114 (2007)

Article 2 93

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

Emma Puighermanal, Giovanni Marsicano, Arnau Busquets-Garcia, Beat Lutz, Rafael Maldonado and Andrés Ozaita

Nat Neurosci **12**, 1152-1158 (2009)

Article 3 115

Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses

Arnau Busquets-Garcia*, Emma Puighermanal*, Antoni Pastor, Rafael de la Torre, Rafael Maldonado and Andrés Ozaita

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*Equal contribution

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Role of mTOR in the pharmacological effects of THC

Emma Puighermanal*, Arnau Busquets-Garcia*, Rafael Maldonado and Andrés Ozaita

*Equal contribution

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Introduction

1. The endocannabinoid system

1.1. Natural and synthetic cannabinoids

Marijuana and other derivates of the plant *Cannabis sativa* have been used for recreational purposes and as a medicinal herb for thousands of years. To date, more than 70 unique compounds derived from the hemp plant (phytocannabinoids) have been identified[1]. The pharmacological effects of cannabis in humans are well known and include mood-altering properties, sedation, impairments of memory and motor function, analgesia, antiemesis, and appetite stimulation, among others[2]. The search for the identification of the psychoactive ingredients of cannabis dates back to the 19th century, but the milestone discovery did not appear until 1964, when Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound of the plant, was isolated[3]. There is no doubt that phytocannabinoids have remarkably influenced in the research of the endocannabinoid system (ECS). Probably, the subsequent discoveries of the ECS would not have been made without the precedence of THC isolation. Since then, a number of biologically active analogs of THC have been synthesized. These compounds are collectively called cannabinoids for their cannabimimetic properties and have been used in laboratory animals to produce several behavioral effects analogous to those reported in humans[4]. The main effects of THC were characterized in a mouse behavioral assay, known as the tetrad test, consisting in catalepsy, reduced motility, analgesia, and reduction in body temperature[5].

Other cannabinoids also present in the plant are cannabidiol, Δ^8-tetrahydrocannabinol, cannabinol, cannabichromene, and

cannabigerol, among others[6] (Figure 1). THC and many of its metabolites are highly lipophilic and essentially water-insoluble hence they easily bind to tissues and in particular to fat[7].

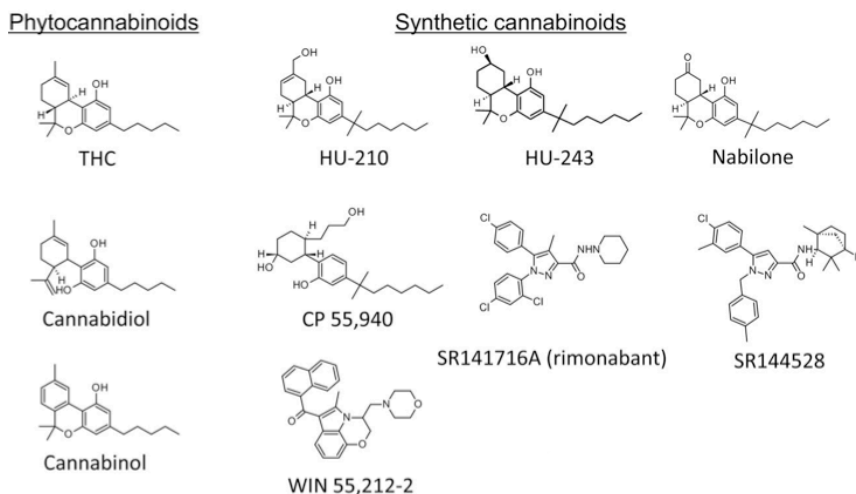

Figure 1. **Chemical structure of representative phytocannabinoids and synthetic cannabinoids** (modified from Maldonado *et al*, 2011[8]).

Studies that link the structure of phytocannabinoids with their pharmacological activity and the cloning of cannabinoid receptors allowed the development of new molecules displaying different intrinsic activity and selectivity for cannabinoid receptors. These synthetic cannabinoid agonists include HU-210, CP-55,940, nabilone and WIN 55,212-2 (Figure 1). On the other hand, the generation of selective antagonists for the different cannabinoid receptors, such as SR141716A (rimonabant)[9] and AM251[10] for the CB1 cannabinoid receptor subtype, and SR144528[11] and AM630[12] for the CB2 cannabinoid receptor subtype, represents an excellent tool to advance in the knowledge of the endocannabinoid system (Figure 1).

1.2. Components of the endocannabinoid system

1.2.1. Cannabinoid receptors: structure and distribution

The ECS is composed of the cannabinoid receptors, their endogenous ligands (endocannabinoids), and the enzymes involved in the synthesis and degradation of these endocannabinoids. Since the discovery of the first cannabinoid receptor in 1990, our understanding of the ECS has been strikingly expanded.

Cannabinoids exert their pharmacological actions through the activation of at least two distinct cannabinoid receptors: CB1 and CB2 cannabinoid receptors (CB1R and CB2R), although compelling evidences supports the existence of other receptors that bind cannabinoid ligands, such as GPR55[13,14]. CB1R was cloned in 1990[15] and three years later CB2R was also cloned[16]. Both receptors are G-protein-coupled receptors with seven-transmembrane domains, however there are considerable differences regarding their body distribution[17]. The abundance of CB1R and scarcity of CB2R in the Central Nervous System (CNS) entail that CB1R is the primary responsible for the psychoactive effects of exogenous and endogenous cannabinoids. Indeed, CB1R is the most abundant seven-transmembrane receptor in the brain and its distribution has been well characterized both in rodents[18,19] and humans[20] (Figure 2). CB1R is abundantly expressed in caudate-putamen, globus pallidus, hippocampus, and cerebellum[18,21], and is also found in multiple central areas including the amygdala, hypothalamus, thalamus, and the spinal cord, among other structures[19,22]. However, CB1R is also expressed in several peripheral organs, such as liver, smooth muscle, gastrointestinal tract, testis, eye, and vascular endothelium[17].

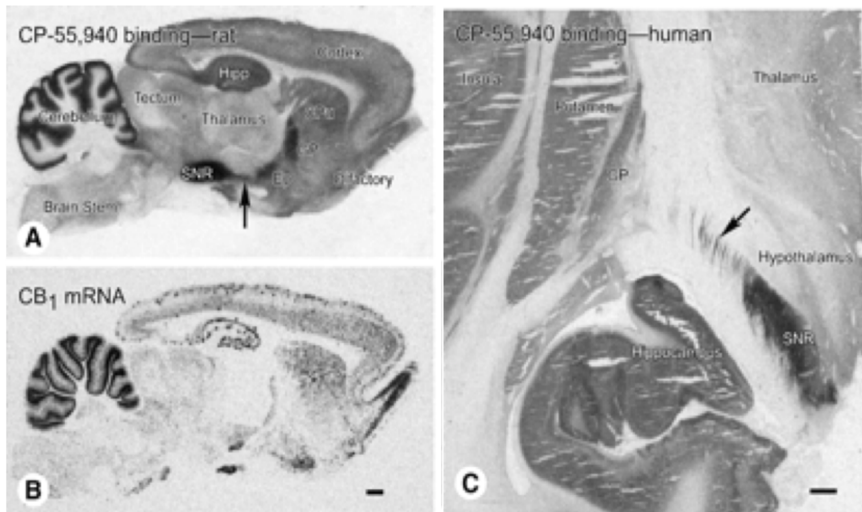

Figure 2. **Distribution of CB1R in the brain**.

Autoradiographic images show CB1R localization in rat (A) and human brain (C) marked by the tritiated ligand CP-55,940 as described by *in vitro* binding assay[18]. Sagittal section of rat brain hybridized with a CB1-specific oligonucleotide probe (B) shows locations of neurons that express the mRNA at this level. In both rat and human, high levels of CB1R are visible in the basal ganglia structures globus pallidus (GP), entopeduncular nucleus (Ep), substantia nigra pars reticulata (SNR), cerebellum, hippocampus (Hipp), cortex, and caudate putamen (CPu). Low binding is seen in the brain stem and thalamus (modified from Freund *et al*, 2003[23]).

CB1Rs are mainly confined at the presynaptic terminals of central and peripheral neurons, where they modulate the release of a number of different excitatory and inhibitory neurotransmitters that include glutamate, γ-aminobutyric acid (GABA), acetylcholine, noradrenaline, dopamine, serotonin, and cholecystokinin (CCK)[24-26]. Indeed, it is thought that it is the ability of CB1R agonists to inhibit neurotransmitter release that yields many of their effects when administered *in vivo*.

CB2Rs are predominantly located in the immune system, being found in cells (e.g., macrophages, B- and T-lymphocytes) and tissues (e.g., spleen, tonsils, lymph nodes) of this system[27]. However, the presence of CB2R has also been demonstrated at the central level in perivascular microglial cells[28], vascular endothelial cells[29], and in brainstem neurons[30]. The functional role of these central CB2Rs has not been yet clarified[30,31], although it has been shown to modulate neuroinflammatory responses upon microglial activation[31-34]. The possible lack of psychotropic effects mediated by CB2R agonists points this receptor as an interesting potential therapeutic target.

Cannabinoids differ in their affinity for binding cannabinoid receptors. There are several cannabinoid agonists that possess similar affinities for CB1R and CB2R, while others are more specific for each receptor. Some of the most studied cannabinoids and their affinity for CB1R and CB2R are summarized in Table 1.

The orphan G-protein coupled receptor GPR55 has been recently classified as another member of the cannabinoid family, potentially explaining some of the physiological effects that are non-CB1R/CB2R mediated. It has been linked to Gq, G_{12} and G_{13} coupling as well as the activation of RhoA and phospholipase C, although its stimulation triggered by endogenous or exogenous cannabinoids seems to be dependent on the cell type and tissue[35].

Cannabinoid Receptor Ligand	K_i	
	CB_1	CB_2
	nM	
Section II.C.1		
(−)-Δ^9-THC	5.05–80.3	3.13–75.3
HU-210	0.06–0.73	0.17–0.52
CP55940	0.5–5.0	0.69–2.8
R-(+)-WIN55212	1.89–123	0.28–16.2
Anandamide	61–543	279–1940
2-AG	58.3, 472	145, 1400
Section II.C.2		
Agonists with higher CB_1 than CB_2 affinity		
ACEA	1.4, 5.29	195, >2000
Arachidonylcyclopropylamide	2.2	715
R-(+)-methanandamide	17.9–28.3	815–868
Noladin ether	21.2	>3000
Agonists with higher CB_2 than CB_1 affinity		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
Section II.C.3		
Rimonabant (SR141716A)	1.8–12.3	514–13,200
AM251	7.49	2290
AM281	12	4200
LY320135	141	14,900
Taranabant	0.13, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
Section II.C.4		
SR144528	50.3–>10,000	0.28–5.6
AM630	5152	31.2
JTE-907	2370	35.9
Section II.C.5		
11-OH-Δ^8-THC	25.8	7.4
Ajulemic acid	5.7, 32.3	56.1, 170.5
Cannabinol	120–1130	96–301
Cannabigerol	81	2600
Cannabidiol	4350–>10,000	2399–>10,000
N-Arachidonoyl dopamine	250	12,000
Virodhamine	912	N.D.

Table 1. **Ki values of CB1R/CB2R ligands for the *in vitro* displacement of a tritiated compound from specific binding sites on rat, mouse, or human CB1 and CB2 receptors** (modified from Pertwee, 2010[17]).

1.2.2. Endocannabinoids: 2-arachidonoylglycerol and anandamide

The cloning of the CB1R prompted a search for an endogenous cannabinoid receptor agonist. This search led rapidly to the discovery

that mammalian tissues produce, not just one, but at least two endogenous cannabinoids (endocannabinoids). These are lipids present in different tissues and finely regulated by the balance between synthesis and inactivation. The most studied endocannabinoids are N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG)[36-38] (Figure 3), both are synthesized on demand in response to elevations of intracellular calcium[39]. Anandamide (AEA) was the first endocannabinoid isolated and its name is based on the Sanskrit word *ananda* that means "bliss". AEA, as THC, behaves as a partial agonist at both CB1Rs and CB2Rs (Table 1), and also as an endogenous ligand for the vanilloid receptor TRPV1. 2-AG is the most prevalent endocannabinoid in the brain, and acts as a full agonist for both cannabinoid receptors, indicating that 2-AG is a true natural ligand for the cannabinoid receptors (Table 1).

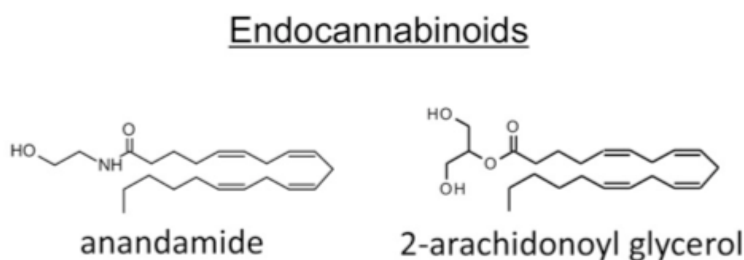

Figure 3. **Endocannabinoids structure** (modified from Maldonado *et al*, 2011[8]).

Other putative endocannabinoids include 2-arachidonoylglycerol ether (noladin ether)[40], N-arachidonoyldopamine[41], and O-arachidonoylethanolamine (virodhamine)[42] (Table 1), although their physiological relevance is under study.

Endocannabinoids are considered to act as retrograde messengers in the CNS[43] behaving as neuromodulators in a wide variety of physiological processes, thus preventing the presence of excessive neuronal activity in a manner that maintains homeostasis in physiological and pathological conditions. Neuronal activity is a potent stimulus for endocannabinoid synthesis and release. Once released by the postsynaptic neurons, endocannabinoids travel retrogradely across the synapse to bind presynaptic CB1Rs, suppressing neurotransmitter release at both excitatory and inhibitory synapses in a short- and long-term manner[23,44,45]. Activation of CB1R and subsequent long-term inhibition of transmitter release defines endocannabinoid-mediated long-term depression (eCB-LTD) (Figure 4). When eCB-LTD occurs at inhibitory terminals, it can facilitate the induction of long-term potentiation (LTP) at excitatory inputs[46]. Nevertheless, CB1R also mediates short-term plasticity, as in the case of depolarization-induced suppression of inhibition or excitation (DSI or DSE, respectively). In the same target cell, the difference between eCB-LTD and eCB-DSI/DSE relies on the duration of CB1R activity, which engages distinct signaling events in the neuron leading to a short or long suppression of neurotransmitter release[45].

1.2.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids

Both 2-AG and AEA are produced from ubiquitous lipids via several biosynthetic pathways. AEA is synthesized from the phosphatidylethanolamine present on the cell membrane by the activation of two enzymes: the N-acyltransferase and phospholipase D

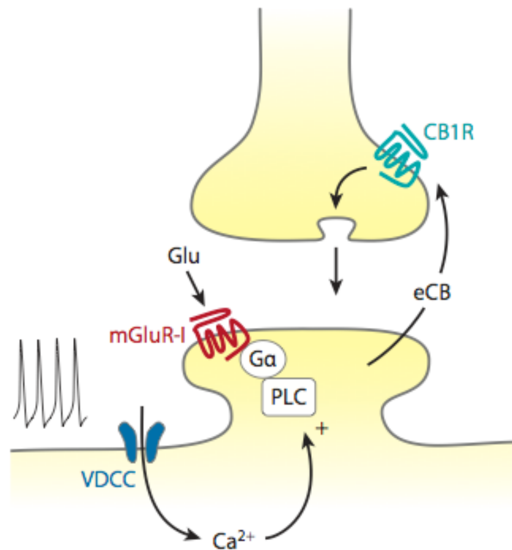

Figure 4. **Schematic illustration of the endocannabinoid-mediated long-term depression (eCB-LTD) in a synapse**.

The postsynaptic neuron can integrate action potential firing, which promotes Ca2+ levels elevation via voltage-dependent Ca2+ channels (VDCC), and synaptic release of glutamate, which activates the metabotropic receptors mGluR-I, to facilitate endocannabinoid mobilization and subsequent eCB-LTD. In this model, phospholipase C (PLC) participates as a coincidence detector (modified from Heifets and Castillo, 2009[45]).

(NAPE-PLD)[47] (Figure 5). 2-AG is generated when calcium stimulates phospholipase C, which transforms membrane phosphoinositides into a diacylglycerol, from which 2-AG is synthesized by diacylglycerol lipase (DAGL) (Figure 5). However, other pathways might also be involved in the synthesis of these endocannabinoids[48]. AEA is mainly degraded by fatty-acid amide hydrolase (FAAH)[49], while 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL)[50,51] (Figure 5). FAAH and MAGL are intracellular enzymes, although they are differently located. FAAH is expressed in the soma and dendrites of neurons[52], whereas MAGL is mainly distributed in presynaptic

terminals[53]. The identification of several enzymes involved in the synthesis and degradation of endocannabinoids prompted a search for inhibitory compounds that target these enzymes, and some of the most selective are shown in Figure 6.

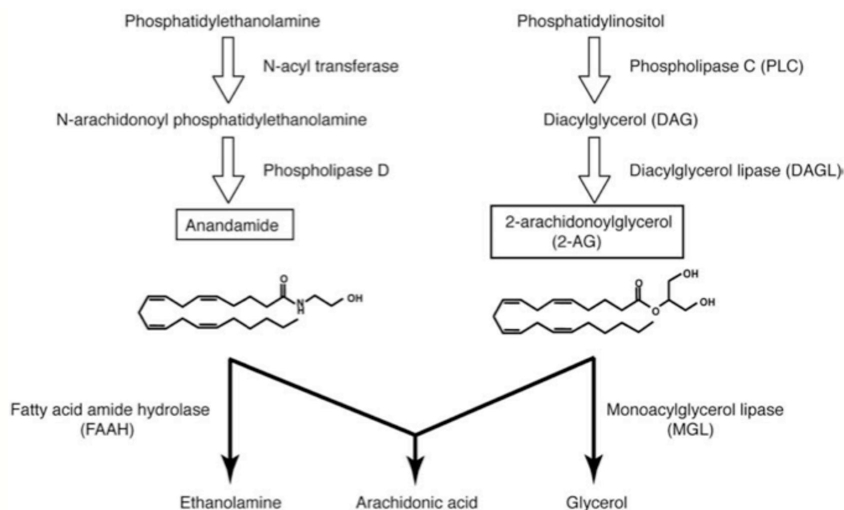

Figure 5. **Main pathways representing biosynthesis and degradation of endocannabinoids** (Hashimotodani *et al*, 2007[54]).

Although there is no doubt that both 2-AG and AEA are reuptaken from the synaptic cleft following their release, there is no convincing evidence that this uptake is mediated by any transporter protein[55-57].

1.3. Cannabinoid receptors signaling

Stimulation of cannabinoid receptors causes a great variety of effects by activating numerous signal transduction pathways. As members of the GPCR superfamily, both CB1R and CB2R were initially reported

to mediate the biological effects by activating heterotrimeric Gi/o type G proteins (α, β and γ), although they can also couple to other G proteins[58] (Figure 7). One of the most characterized CB1R-mediated effects through Gαi/o proteins is the inhibition of adenylyl cyclase activity and reduction in cyclic AMP production, accompanied by a subsequent decrease in protein kinase A (PKA) activity. Moreover, CB1R coupling to Gβγi/o can lead to the phosphorylation and activation of multiple members of the mitogen-activated protein kinases (MAPK) family, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase[58]. In addition, other proteins found to be modulated by CB1R stimulation are phosphatidylinositol 3-kinase (PI3K)[59], focal adhesion kinase[60], and some enzymes involved in energy metabolism[61].

Cannabinoids can activate the PI3K/Akt signaling pathway through CB1R in primary astrocytes[62] and oligodendrocytes[63,64], and also through CB2R[64,65]. Activated Akt promotes cell survival by inhibiting apoptosis through the phosphorylation of numerous substrates, including Bad, caspase-9, forkhead box protein O1 and O3, and glycogen synthase kinase-3 (GSK-3)[66]. Most of these signaling events triggered by CB1R stimulation are illustrated in Figure 7. However, cannabinoids can also promote Akt inhibition in transformed cells. Indeed, the antitumoral action of THC is mediated through ceramide accumulation and up-regulation of endoplasmic reticulum stress-related genes, which lead to the inhibition of Akt/mammalian target of rapamycin axis and the consequent induction of autophagy[67,68].

On the other hand, CB1R can also modulate various types of ion channels, including inhibition of N-type and P/Q-type calcium

currents and activation of A-type and inwardly rectifying potassium currents, a mechanism by which negatively regulates neurotransmitter release[69] (Figure 7).

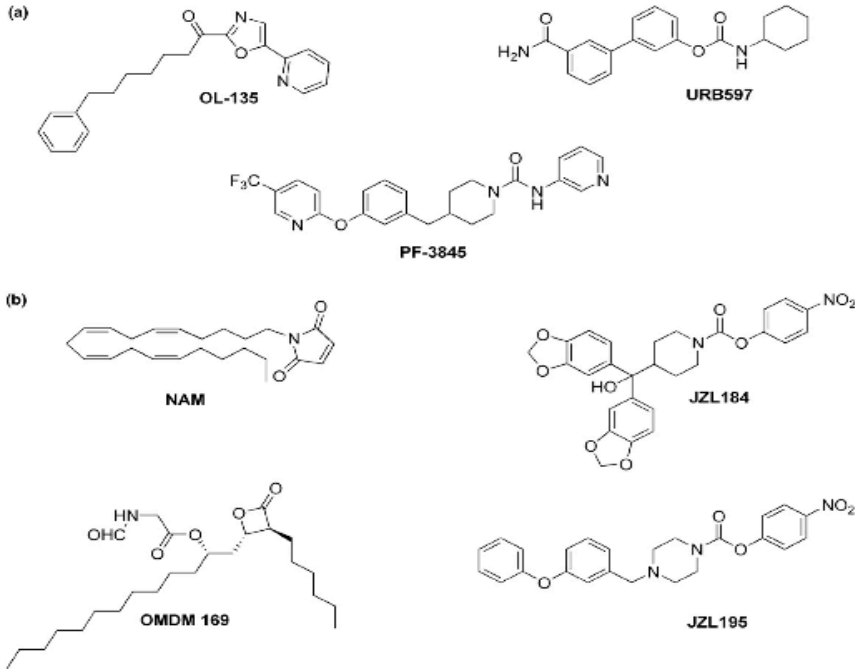

Figure 6. **Endocannabinoid hydrolysis inhibitors**.

(a) URB597, OL-135, and PF-3845 are selective FAAH inhibitors. (b) NAM, JZL184 and OMDM169 are MAGL inhibitors. JZL195 is a selective 'dual' MAGL–FAAH inhibitor increasing anandamide and 2-arachidonoylglycerol levels *in vivo* (adapted from Muccioli, 2010[48]).

During the last decade multiple proteins and signaling cascades activated by cannabinoids have been characterized. However, additional experiments need to be done to further clarify the complexity of the cannabinoid signal transduction in the cells.

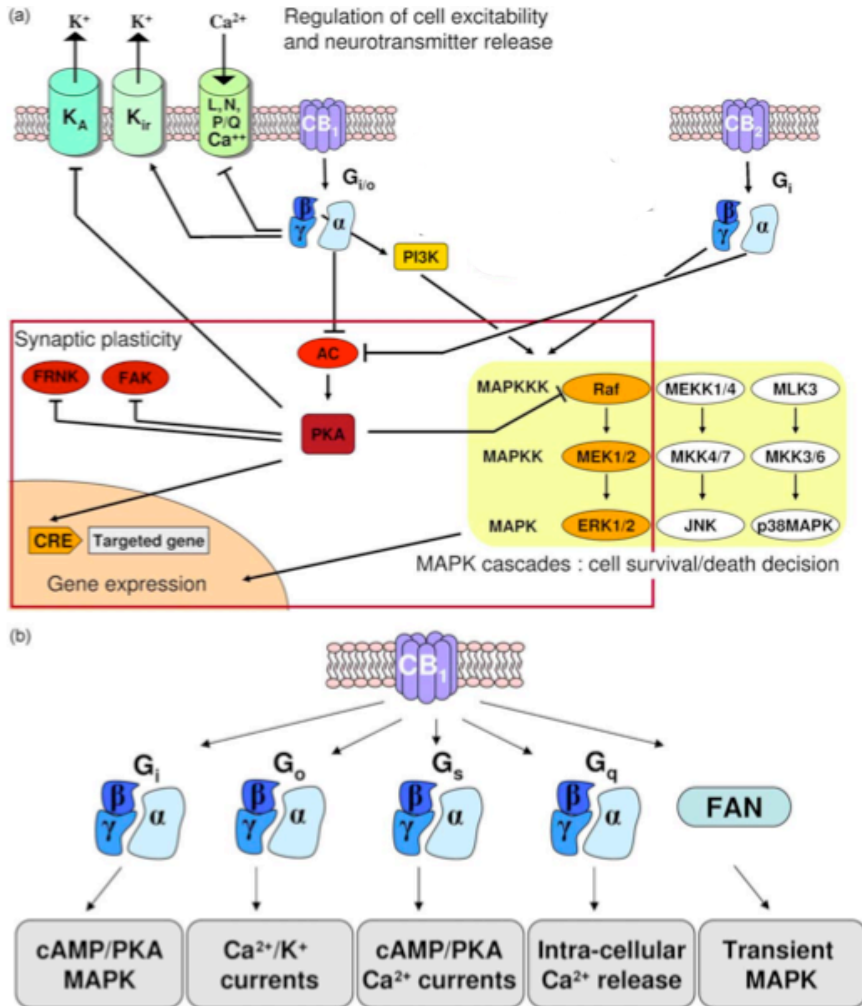

Figure 7. **Complexity of cannabinoid receptor signaling**.

(a) Both CB1R and CB2R are associated with protein Gαi-dependent inhibition of AC and Gβγ-dependent activation of different MAPK cascades. In addition, CB1R negatively regulates voltage-gated Ca2+ channels and positively regulates inwardly rectifying K+ channels, thereby inhibiting neurotransmitter release. Cross-talks between signaling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA. (b) CB1R also leads to activation of Gs and Gq proteins. Moreover, CB1R also signals trough non-G protein partners such as the adaptor protein FAN. Preferential activation of different intracellular effectors by each G protein contributes to diversity and selectivity of responses regulated by cannabinoid receptors (modified from Bosier *et al*, 2010[69]).

1.4. Physiological role of the endocannabinoid system and pharmacological effects of cannabinoids

The ubiquitous presence of the ECS in the CNS correlates with its role as a modulator of multiple physiological processes. Thus, its contribution to synaptic remodeling[60,70], neuronal differentiation[71] and neuronal survival[72,73] indicates that the ECS is a major homeostatic mechanism that guarantees a fine adjustment of information processed in the brain and provides counterregulatory mechanisms aimed at preserving the structure and function of brain circuits.

At the central level, the presence of CB1Rs in the basal ganglia and cerebellar circuits is responsible of the modulation of the fine control of movement and the implicit learning of motor routines[74]. Moreover, the ECS is also engaged in the homeostatic control of emotions and the regulation of motivated behavior among other central physiological functions. Thus, endocannabinoids control the motivation for appetite stimuli, including food and drugs, by acting on the reward circuits[8,75]. At the peripheral level, the ECS modulates the immune system, vascular beds, reproductive organs, gastrointestinal motility and metabolism, among others[76].

Numerous studies, mainly using rodents, have clearly demonstrated most of the pharmacological effects exerted by cannabinoid receptor agonists (Table 2). These effects include a decrease in motor and cognitive functions, reduced body temperature, catalepsy, antinociception, antiinflammatory effects, reduced emesis, stimulation of appetite, and neuroprotection, among others[77].

A comprehensive analysis of all physiological functions of endocannabinoids is beyond the scope of this thesis. Thus, this

introduction will mainly address the effects of cannabinoids in cognition, anxiety, and pain to facilitate the understanding of the results. The modulation of anxiety and pain by cannabinoids will be described below, while the cannabinoid effects on memory processes will be explained in the "Memory" section.

Body system	Effects
Psyche and perception	Fatigue, euphoria, enhanced well-being, dysphoria, anxiety, reduction of anxiety, depersonalisation, increased sensory perception, heightened sexual experience, hallucinations, alteration of time perception, aggravation of psychotic states, sleep
Cognition and psychomotor performance	Fragmented thinking, enhanced creativity, disturbed memory, unsteady gait, ataxia, slurred speech, weakness, deterioration or amelioration of motor coordination
Nervous system	Analgesia, muscle relaxation, appetite stimulation, vomiting, antiemetic effects, neuroprotection in ischaemia and hypoxia
Body temperature	Decrease of body temperature
Cardiovascular system	Tachycardia, enhanced heart activity, increased output, increase in oxygen demand, vasodilation, orthostatic hypotension, hypertension (in horizontal position), inhibition of platelet aggregation
Eye	Reddened conjunctivae, reduced tear flow, decrease of intraocular pressure
Respiratory system	Bronchodilation
Gastrointestinal tract	Hyposalivation and dry mouth, reduced bowel movements and delayed gastric emptying
Hormonal system	Influence on luteinising hormone, follicle-stimulating hormone, testosterone, prolactin, somatotropin, thyroid-stimulating hormone, glucose metabolism, reduced sperm count and sperm motility, disturbed menstrual cycle and suppressed ovulation
Immune system	Impairment of cell-mediated and humoral immunity, immune stimulation, anti-inflammatory and antiallergic effects
Fetal development	Malformations, growth retardation, impairment of fetal and postnatal cerebral development, impairment of cognitive functions
Genetic material and cancer	Antineoplastic activity, inhibition of synthesis of DNA, RNA and proteins

Table 2. **Physiological effects of cannabinoids**

Recompilation of dose-dependent effects observed *in vitro*, as well as in animal studies and clinical trials (Grotenhermen, 2003[77]).

1.4.1. Anxiety

Anxiety is an emotional response to dangerous situations, which enables an appropriate response (e.g., escape or fight) and is of paramount importance for survival. Anxiety is a complex mechanism

that involves numerous neurotransmitters, and compounds modulating GABAergic or serotonergic systems are currently used for treating anxiety-related disorders[78]. There are accumulating evidences showing the relationship between cannabinoids and anxiety-like behavior both in laboratory animals and humans[79]. The effects of cannabinoid agonists on emotional behavior have been studied in multiple paradigms in rodents, showing complex and sometimes contradictory results. Several factors might explain these results, including drug dose, genetic background, and environmental context[80,81]. In general, cannabinoid agonists display a biphasic effect, eliciting anxiolytic-like responses at low doses, whereas higher doses induce the opposite effect[79]. On the other hand, the context (e.g., stress) can also contribute to the modulation of anxiety by cannabinoids[82]. Prior exposure of animals to stress sensitizes them to the anxiogenic-like effects of CB1R agonists, and stress acts synergistically with CB1R agonists to activate certain brain regions, including central amygdala[83].

The physiological role of the ECS in the regulation of anxiety has been studied in animals using CB1R antagonists and CB1R knockout mice. Both pharmacological and genetic experimental approaches show an increase in the anxiogenic-like responses in different behavioral paradigms[84-87]. These results provide evidence for a basal endogenous anxiolytic cannabinoid tone. In this regard, anxiolytic drugs such as bromazepam and buspirone are ineffective in attenuating the anxiogenic-like response in the CB1R knockout mice[88].

Several neurochemical systems can modulate the effects of cannabinoids on anxiety. Thus, an interaction between THC and

nicotine was observed by co-administering sub-threshold doses of both drugs, which produce an anxiolytic-like response[89]. On the other hand, the possible involvement of μ and δ opioid receptors in CB1R-mediated anxiolytic-like effects has been described[90]. In contrast, κ opioid receptors are involved in the anxiogenic-like response produced by CP-55,940[91]. Thus, distinct mechanisms seem to mediate the anxiolytic and anxiogenic responses obtained upon CB1R activation.

Although several evidences suggest that cannabinoid compounds could have therapeutic potential in the treatment of anxiety-related neuropsychiatric disorders, the biphasic effects of CB1R agonists and the influence of other parameters, such as the context, limit their therapeutic use. This limitation prompted new studies in the field using pharmacological agents that enhance the endocannabinoid tone. Thus, several studies suggested that endocannabinoids, mainly AEA, might be synthesized in the amygdala during anxiety to bring back the normal homeostasis[92]. This hypothesis is supported by the increase in the endocannabinoid tone in the basolateral amygdala in response to anxiogenic situations[93]. In this regard, pharmacological or genetic disruption of FAAH produces anxiolytic-like effects through CB1R[94,95]. However, the role of 2-AG in anxiety processes has not been clearly elucidated. Only one recent study associates 2-AG with anxiolytic-like responses by using the MAGL inhibitor JZL184 in the marble buring test[96], although this paradigm is not a classical behavioral model to measure anxiety-related responses.

1.4.2. Pain

Analgesic properties have been ascribed to *Cannabis sativa* plant since ancient times, and recently, endocannabinoids have been proposed to participate in the endogenous control of natural nociception. Cannabinoids exert antinociceptive effects by acting at three different levels. First, electrophysiological and neurochemical studies have provided evidences that cannabinoids inhibit the ascending pathways involved in the transmission of nociceptive stimuli *in vivo*[97,98]. Second, cannabinoids play an outstanding role in the activation of the descending inhibitory pathway from the brainstem to spinal nociceptive neurons. This circuit comprises the midbrain periaqueductal gray and the rostral ventromedial medulla. This activation seems to be induced by the inhibition of GABA release in the axon terminal of presynaptic interneurons located in these structures, a mechanism similar to the one described for the analgesic effects of opioids[99]. In agreement, microinjection of CB1R agonists into these structures produces antinociception[100,101]. Finally, at the supraspinal level, cannabinoids also modify the subjective interpretation of pain by modulating the neuronal activity mainly at the level of the limbic structures, such as the amygdala[102,103].

Thus, the ECS controls pain circuits acting at peripheral, spinal and supraspinal levels. Indeed, administration of rimonabant causes hyperalgesia, indicating that endocannabinoids tonically regulate nociception[104-106]. Moreover, an enhancement of endocannabinoid levels after noxious stimuli is observed by *in vivo* microdialysis[107]. The different antinociceptive effects produced by cannabinoids are consistent with the anatomical location of CB1R in areas involved in

pain sensation in the brain, spinal dorsal horn, dorsal root ganglion, and peripheral afferent neurons[108,109]. However, apart from CB1R, CB2R plays a crucial role in the regulation of pain, especially in the central immune responses leading to neuropathic pain[33,34].

Different nociceptive models in laboratory animals have been widely used to demonstrated that cannabinoids suppress behavioral reactions to acute noxious stimuli, inflammatory pain, and nerve injury-induced pain. Thus, exogenous cannabinoids elicit antinociceptive effects in the hot plate test, which mainly involves supraspinal pathways[110-112], and in the tail flick paradigm, which mainly requires spinal mechanisms[110]. In addition, cannabinoid-induced pain relief is also observed in mechanical, chemical, nerve injury, inflammatory models, and visceral pain models, among others[113]. On the other hand, enhanced endocannabinoid levels also elicit antinociceptive responses. Thus, genetic or pharmacological disruption of FAAH produces CB1R-dependent analgesia in multiple pain assays[114-117]. A similar outcome is observed following acute blockade of MAGL, which reduces pain manifestations[118,119].

1.4.3. Therapeutic perspectives of cannabinoids

During the last decades, cannabinoids are gaining more weight in modern medicine due to their multiple pharmacological effects that can provide new interesting therapeutic agents. Some of the properties that might be of therapeutic use include analgesia, stimulation of appetite, antiemesis, muscle relaxation, immunosuppression, antiinflammation, sedation, improvement of mood, lowering of intraocular pressure, bronchodilation, neuroprotection, and

antineoplastic effects. Thus, the THC oral preparation dronabinol (Marinol) and the synthetic analogue nabilone (Cesamet) have been licensed for use in several countries for the suppression of nausea and vomiting produced by cancer chemotherapy and for appetite stimulation in the anorexia associated with AIDS. Recently, another cannabinoid compound, Sativex, was approved for the treatment of spasticity associated with multiple sclerosis and a large clinical trial to evaluate its efficacy against cancer pain has been now achieved. Sativex consists in an oromucosal spray containing THC and CBD in a proportion 1:1. The latter compound does not display the unwanted psychotropic effects of THC due to the lack of binding to CB1R. CBD exerts a plethora of actions including anticonvulsive, sedative, hypnotic, antipsychotic, antiinflammatory and neuroprotective properties without binding to cannabinoid receptors[120].

1.5. Tolerance and physical dependence of cannabinoids

Mechanisms involved in CB1R desensitization and down-regulation are essential to limit signal duration as well as in determining the intensity of the signal. In this regard, chronic exposure to THC and other cannabinoid agonists leads to biological adaptive mechanisms that may develop tolerance and physical dependence. After prolonged cannabinoid exposure, tolerance develops to most of the pharmacological effects including antinociception, hypothermia, hypolocomotion, catalepsy, ataxia, cardiovascular actions, and corticosterone release[121]. This tolerance has been reported to occur in rodents, pigeons, dogs, monkeys[122] and humans[123-125]. In laboratory animals, the degree and the time-course of tolerance are dependent on

the species used, the type of ligand, the dosage, the duration of the treatment, and the pharmacological responses evaluated. Although the pharmacodynamic events (e.g., decrease in total number and sensitivity of CB1Rs) are the main factors mediating the development of tolerance, the pharmacokinetic properties of cannabinoids (changes in drug absorption, distribution, biotransformation, and excretion) also influence the degree of tolerance, but to a lesser extend[126].

In general, tolerance occurs quite rapidly, and a decrease in the acute response has been observed after the second administration of cannabinoids[122]. However, tolerance can differentially develop depending on the physiological and behavioral response. For example, it has been shown that tolerance develops more rapidly to cannabinoid-induced hypothermia than hypolocomotion[127,128]. In this line, tolerance to the analgesic effect of WIN 55,212-2 develops more rapidly than other effects including hypothermia and catalepsy[129,130]. The different tolerance development seems to be due to distinct brain areas involved in the different pharmacological responses of cannabinoids. Apart from this region-dependency of tolerance, there is also time-dependency. While tolerance to certain pharmacological effects of cannabinoids, such as decrease in motor activity, hypothermia, and antinociception develops within a range of 3-7 days, other effects, such as memory alterations or certain neuroendocrine actions, need weeks or even months to develop tolerance[128]. This different functional tolerance suggests that the magnitude and rate of the neuroadaptive processes in the brain might differ under chronic cannabinoid exposure.

In agreement with the differential development of tolerance to the distinct pharmacological responses of cannabinoids, an important characteristic of cannabinoid signaling adaptation is the variation in the magnitude and rate of CB1R desensitization and down-regulation reported in different brain regions after chronic cannabinoid treatment[131]. Thus, chronic exposure to THC produced time-dependent and region-specific down-regulation and desensitization of CB1R[132]. Chronic THC administration activated certain proteins involved in receptor internalization such as G protein-coupled receptor kinases and β-arrestins in multiple brain areas. These effects were ERK1/2-dependent in the striatum and cerebellum, but ERK1/2-independent in the hippocampus and prefrontal cortex, suggesting that regional differences in the intracellular signaling events might account for distinct CB1R adaptive responses[133]. Recently, G-protein-coupled receptor-associated sorting protein 1 (GASP1), which targets CB1R for degradation after their agonist-mediated endocytosis, has been also implicated in tolerance phenomenon. Thus, repeated administration of WIN 55,212-2 promoted CB1R downregulation and tolerance to hypomotility, antinociception, and motor incoordination in wildtype mice, whereas no tolerance or CB1R downregulation was observed in the GASP1 knockout mice[134], indicating the critical role of this protein in CB1R dynamics.

Several mechanisms have been proposed to contribute to CB1R desensitization. Thus, phosphorylation of Ser residues on the intracellular loop 3 and C-terminal of the CB1R is important for regulation of coupling to G proteins and subsequent signal[135-137]. Tolerance to THC-induced antinociception was reversed by

pretreatment with a PKA inhibitor and a Src family tyrosine kinase inhibitor, indicating that these kinases might play a role in maintaining the tolerant state[138].

It is not totally clear whether chronic exposure to endocannabinoids regulates CB1R in a similar manner than THC and synthetic cannabinoids. The genetic studies using FAAH and MAGL knockout mice revealed some differences regarding tolerance and CB1R downregulation. Prolonged pharmacological or genetic inactivation of MAGL causes loss of analgesic responses to a MAGL inhibitor, cross-tolerance to exogenous cannabinoid agonists, and CB1R downregulation and desensitization in certain brain regions[139]. Conversely, none of these effects are observed in FAAH knockout mice or after sustained pharmacological disruption of FAAH, which instead maintained an analgesic phenotype and intact CB1R expression and function[114,117,140]. Thus, brain CB1R notably undergoes different adaptations in response to sustained elevations of the two main endocannabinoids, 2-AG and AEA. The fact that CB1R adaptations occurred only in mice with chronically inactivated MAGL, but not FAAH, suggests that constant elevations in 2-AG promote more severe adaptive changes in the ECS than AEA.

Several studies have reported the absence of somatic signs of spontaneous withdrawal after a chronic THC treatment in rodents, pigeons, dogs and monkeys, even after the administration of extremely high doses of THC[141]. Similarly, no physical manifestations of withdrawal have been reported after chronic treatment with other cannabinoid agonists[142], although somatic signs of spontaneous abstinence have been observed after the abrupt interruption of

chronic WIN 55,212-2 treatment, which has a shorter half-life than THC[143]. In agreement, rimonabant administration has been reported to precipitate somatic manifestations of withdrawal in THC-dependent rodents and dogs[141]. In rodents, this cannabinoid withdrawal syndrome is characterized by the presence of several somatic signs, such as wet dog shakes, front paw tremor, ataxia, hunched posture, ptosis, and piloerection, among others, with the absence of vegetative manifestations[141].

Several neurochemical changes observed during cannabinoid withdrawal syndrome are similar to those reported during withdrawal to other prototypical drugs of abuse. Thus, a compensatory up-regulation of cAMP pathway, mainly in the cerebellum, as well as decreased mesolimbic dopaminergic activity and increased corticotropin-releasing factor release, have been reported during cannabinoid withdrawal[141].

2. mTOR: a master switch in the maintenance of cellular homeostasis

One of the intracellular signaling systems that can be involved in the pharmacological responses produced by cannabinoids is the mammalian target of rapamycin (mTOR) signaling cascade. Indeed, the wide variety of effects triggered by cannabinoid agonists and the plethora of functions that mTOR orchestrates in the body points this protein as an interesting effector that could underlie some of the cannabinoid-mediated responses. mTOR, also known as FRAP or RAFT, is a large ubiquitously expressed multi-effector serine/threonine kinase highly conserved among species. Its kinase activity is modulated in response to a wide variety of stimuli, such as trophic factors (e.g., BDNF), hormones (e.g., insulin), mitogens, amino acids, cell energy status, and cellular stress[144,145] (Figure 8). mTOR serves as a key player in the integration of all these inputs, balancing between the anabolic and the catabolic states in the cell, and finally leading to numerous cellular processes, such as transcription, translation, and autophagy. When there is availability of nutrients and energy, mTOR promotes cell growth under favorable environmental conditions, being a sensor between growth and starvation. The dysregulation of mTOR activity is a critical component of several disease states where cell growth and homeostasis are compromised, such as cancer, metabolic alterations, neurodegenerative disorders, and aging. The mTOR signaling pathway is frequently dysregulated in a wide range of cancers and the inhibition of this kinase has emerged as a key avenue for the treatment of cancer. On the other hand, over-activation of the mTOR signaling cascade by over-feeding has gained interest as a new therapeutic approach for metabolic disorders such as

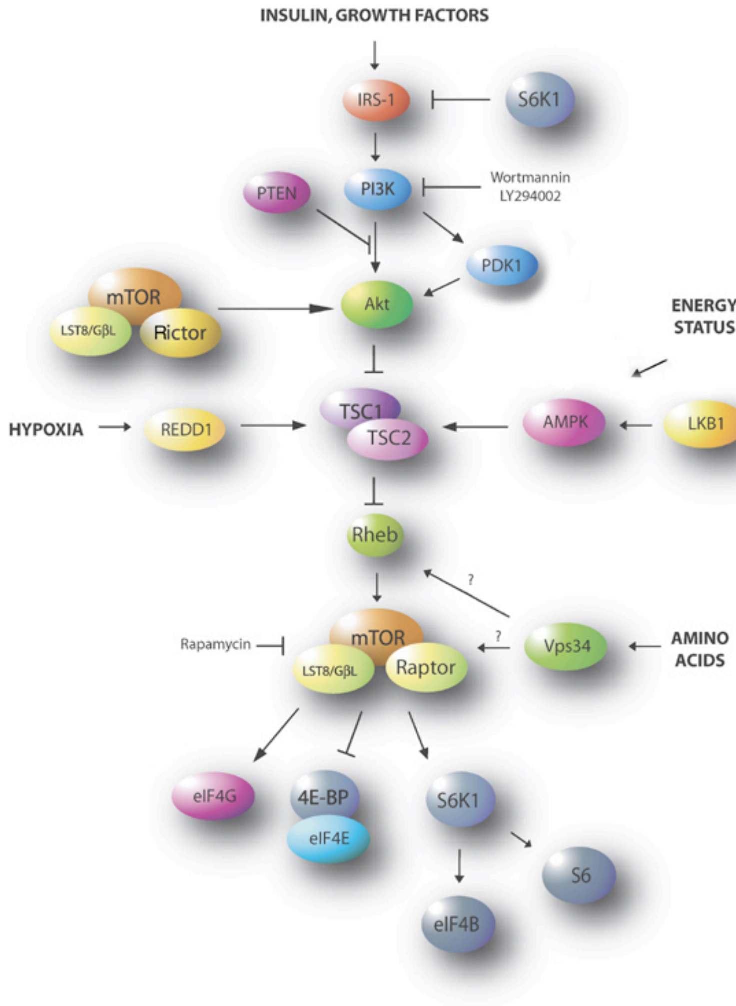

Figure 8. **Schematic diagram of mTOR control activity.**

mTOR is a signal integrator of different stimuli. Growth factors, hormones, and mitogens activate the class I PI3K/Akt signaling cascade. Akt is phosphorylated by an mTOR complex containing Rictor and LST8 (mTORC2), and followed by PDK1. Once Akt is activated, it phosphorylates and inhibits TSC2, which allows Rheb to activate mTOR. Amino acids can also promote the association of Rheb and mTOR via Vps34 (a class III PI3K). Energy status is also sensed by mTOR, as low ATP levels can activate AMPK, which activates TSC2, and finally leads to mTOR inhibition. Under hypoxia conditions, REDD1 gets activated and triggers mTOR inhibition through TSC2. Ultimately, mTOR forms a complex with Raptor and LST8, and mediates the phosphorylation of p70S6K (S6K1), 4E-BP, and eIF4G. The activation of p70S6K by mTOR triggers the phosphorylation of the ribosomal protein S6 and eIF4B (modified from Mamane *et al*, 2006[146]).

diabetes type II. Recently, several studies postulated mTOR as a controller of cell and tissue aging, pointing to mTOR inhibitors as promising tools to enhance longevity[147].

2.1. Regulation of mTOR by upstream signals

Nutrients, growth factors, and neurotransmitters can lead to mTOR activation through the canonical pathway, which involves the activation of receptors tyrosine kinase that trigger the activation of the class I PI3K signaling pathway. PI3K phosphorylates the membrane-bound phospholipid, phosphatidylinositol-4,5-biphosphate, converting it to phosphatidylinositol-3,4,5-triphosphate, which then recruits Akt to the cell membrane, where it is phosphorylated and activated by PI3K-dependent kinase 1 (PDK1) and mTORC2[148] (see below). mTORC2 is one of the two distinct protein complexes formed by mTOR (mTORC1 and mTORC2). Once Akt is activated, it can directly phosphorylate mTOR at Ser2448, which has been correlated with higher levels of mTOR activity[149-151]. However, Akt can also activate mTOR through two indirect pathways. On one hand, recent studies described PRAS40 (40 KDa Pro-rich Akt substrate), a direct inhibitor of mTORC1 (see below), as a novel Akt target. Activated Akt phosphorylates PRAS40, resulting in its dissociation from mTOR, which activates its effectors[152-154]. On the other hand, Akt can activate mTOR via phosphorylation and inhibition of the TSC2 subunit of the tuberous sclerosis complex (TSC). TSC consists in a complex formed by TSC1 (hamartin) and TSC2 (tuberin). TSC2 is a GTPase-activating protein (GAP) that hydrolyses the GTP bound to Rheb (Ras homolog enriched in brain protein). Inactivation of TSC2,

caused by Akt-mediated phosphorylation, results in a reduction of its GAP activity, leading to increased Rheb-GTP levels in the cell and thus stimulating the mTOR catalytic activity[155-157], although Rheb-GTP may also promote substrate recognition by mTOR[154,158]. In summary, Akt might activate mTOR through two distinct mechanisms: by inhibiting PRAS40 and/or by activating Rheb. A diagram depicting signaling events known to activate mTOR is shown in Figure 9.

In addition to the canonical PI3K signaling, the MAPK ERK1/2 can also activate mTOR under certain conditions. ERK1/2 activation promotes the phosphorylation and concomitant inhibition of TSC2, thereby promoting TSC1-TSC2 dissociation, which in turn leads to mTOR activation[159] (Figure 10). Moreover, ERK1/2 can also inhibit TSC2 indirectly via the activation of p90 ribosomal S6 kinase, which phosphorylates TSC2 in addition to Akt-mediated inhibitory modifications of TSC2[159,160]. The participation of the ERK signaling pathway in mTOR signaling activation is remarkably important in the hippocampus, as the phosphorylation of its downstream effectors driven by different stimuli, such as metabotropic glutamate receptor (mGluR) agonists and high-frequency stimulation (HFS), are attenuated by inhibitors of ERK1/2 and PI3K[161-163].

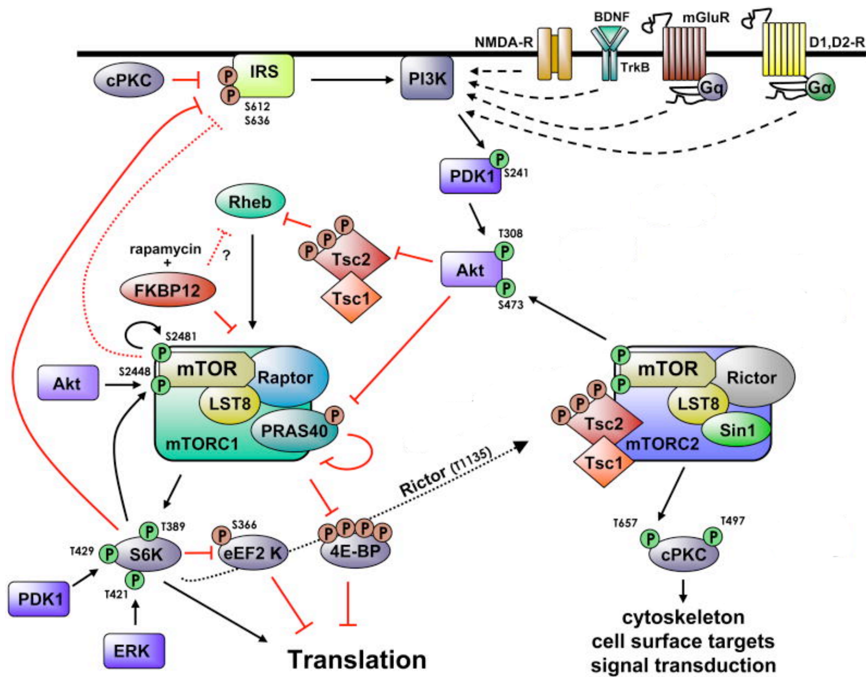

Figure 9. **Signal upstream and downstream regulators of mTOR complexes.**

Several surface receptors and channels, such as Trk-B, NMDAR, dopaminergic (D1R and D2R), and metabotropic glutamate receptors (mGluR) can converge to mTORC1 activation in the neuron via the activation of the canonical pathway (through PI3K). However, the upstream modulators of mTORC2 in the brain remain to be characterized. The activation of PI3K triggers the activation of Akt through their phosphorylations driven by mTORC2 and PDK1. Then, Akt can activate mTOR directly through the phosphorylation at Ser2448 site or indirectly via the inhibition of TSC2, which allows Rheb to activate mTOR, and via the inhibition of PRAS40, a negative regulator of mTORC1. mTORC1 positively regulates protein translation through the phosphorylation of its two main effectors p70S6K (S6K) and 4E-BP. Additionally, p70S6K can also control the activity of mTORC1 by the phosphorylation at Ser2448 site as well as the activity of mTORC2 (through Rictor). Moreover, p70S6K regulates eEF2K, a kinase that participates in the protein elongation stage. On the other hand, besides mTOR, other kinases can phosphorylate p70S6K, including PDK1 and ERK. mTORC1 exerts a negative feedback loop to control its activation. One of the best characterized mechanisms is the inhibitory effect that p70S6K exerts on IRS-1 activity, which interferes with its binding to the insulin receptor and promotes its degradation. In another mechanism, mTORC1 can phosphorylate IRS-1 (via Raptor) hence interfering with its association with PI3K. Rapamycin binds FKBP12 and prevents mTOR-Raptor association, thereby leading to an inhibition of the mTORC1 effectors phosphorylation (modified from Hoeffer and Klann, 2010[164]).

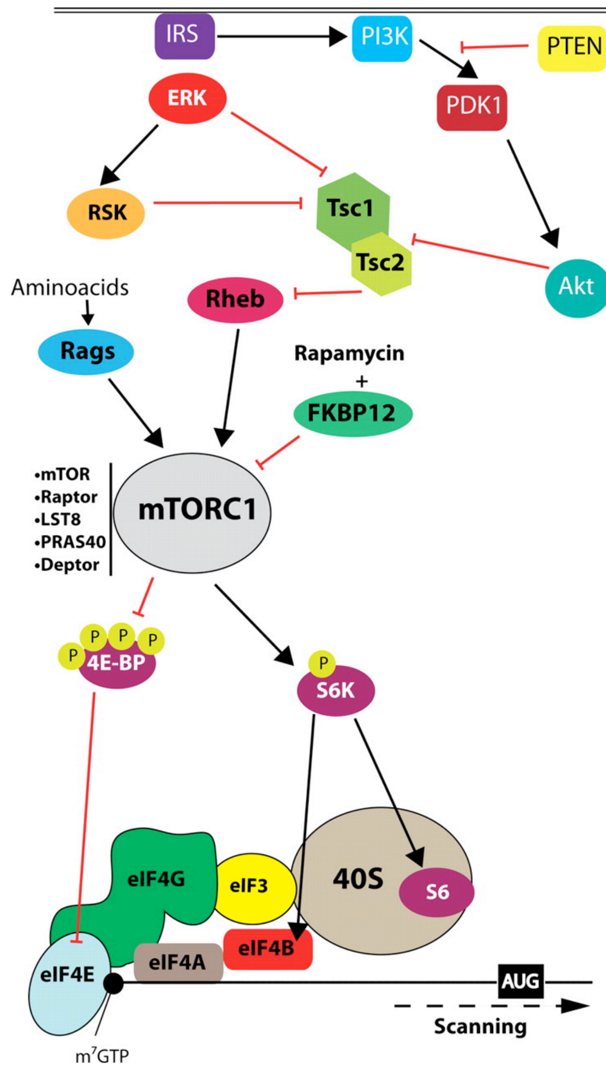

Figure 10. **mTORC1 signaling pathway.**

mTOR is a crucial downstream effector of the PI3K signaling pathway, where Akt and ERK are also involved. These two kinases can converge at TSC1/TSC2 complex by promoting its inhibition, thereby enhancing mTORC1 activity. mTORC1 promotes protein translation by phosphorylating two main substrates: p70S6K (S6K) and 4E-BP. p70S6K increases the translational rate by phosphorylating the ribosomal protein S6, and the translation initiation factor eIF4B. On the other hand, hyperphosphorylated 4E-BP releases eIF4E and allows it to bind to the cap structure of the mRNA. Then, other translational factors are recruited to the cap structure (m^7GTP), such as eIF4G, eIF4A, eIF3, and yield the ribosome binding and scanning to start translation (Gkogkas *et al*, 2010[165]).

Apart from growth factors and neurotransmitters, there are other upstream regulators of mTOR, such as different stress stimuli (hypoxia, DNA damage, etc.) and energy status[166]. Upon nutrient deprivation, mTOR senses low ATP levels by a mechanism involving the AMP-activated protein kinase (AMPK). This kinase phosphorylates and activates TSC2, possibly by stimulating its GAP activity towards Rheb, thereby inhibiting mTOR signaling[167] (Figure 8). In response of AMPK-dependent priming phosphorylation of TSC2, GSK-3 can further phosphorylate and activate TSC2[168]. Thus, both AMPK and GSK-3 act as negative regulators of mTOR by activating TSC2, whereas Akt and ERK1/2 activate mTOR via TSC2 inhibition.

mTOR senses the availability of amino acids through the involvement of class III PI3K. However, the recruitment of class I PI3K is required for mTOR activation triggered by growth factors, mitogens, and neurotransmitters[169].

2.2. mTOR protein complexes: mTORC1 and mTORC2

In mammalian cells, mTOR can form two distinct protein complexes called complex 1 (mTORC1) and complex 2 (mTORC2), which can be distinguished from each other based on their unique components and substrates[170] (Figures 9 & 11). The subunit composition of each mTOR complex dictates its substrate specificity. mTORC1 is referred when mTOR interacts with the adaptor protein Raptor and LST8 (also known as GβL), and is sensitive to rapamycin, a macrolide produced by a soil bacterium found on Easter Island[171,172]. Rapamycin, which binds to FK506-binding protein 12 (FKBP12) and prevents

mTOR from binding Raptor, inhibits mTORC1-induced phosphorylation of their main substrates: the 70-KDa ribosomal S6 kinase 1 and 2 (p70S6K), and the eukaryotic initiation factor 4E binding proteins (4E-BP)[173,174]. Increased mTORC1 formation promotes p70S6K activation and repression of 4E-BP[164] in a phosphorylation-dependent fashion. mTORC1 acts as a signal integrator for four major regulatory stimuli: nutrients, growth factors, energy and stress (Figure 8) and it regulates a wide range of processes in the cell, including cell growth and proliferation by controlling protein translation. Moreover, mTORC1 inhibits autophagy, a catabolic process triggered by nutrient deprivation in which long-lived proteins and/or cellular organelles are degradated by lysosomal machinery to obtain intracellular nutrient resources[175,176].

The other mTOR complex, mTORC2, contains Rictor instead of Raptor, and phosphorylates the prosurvival kinase Akt and several protein kinase C isoforms[177-181]. The function of Raptor and Rictor is to increase the substrate specificity of mTOR towards mTORC1 and mTORC2 targets, respectively[173,182]. Although rapamycin has been reported to selectively inhibit mTORC1, it can also inhibit mTORC2 after prolonged treatment in certain cell types[183]. mTORC2 elicits different biological functions from mTORC1, being mainly involved in the regulation of cytoskeletal organization and cell survival[184].

Although recent studies have described these two heteromeric and functionally distinct complexes, little is known about their possible mutual influences in their activity control.

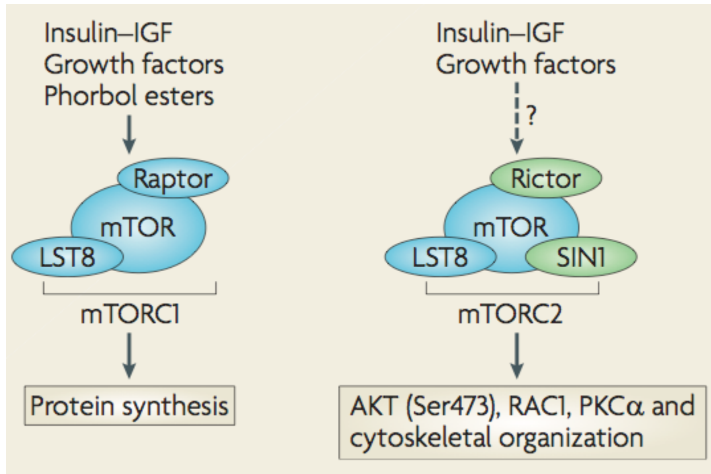

Figure 11. **mTOR complexes.**

mTOR can form two distinct multiprotein complexes, mTORC1 and mTORC2. mTORC1, the rapamycin sensitive complex, is formed by mTOR, Raptor and LST8 (also known as GβL), and mediates efficient cap-dependent translation initiation upon the stimulation of hormones (e.g., insulin), growth factors, mitogens, etc. mTORC2, the rapamycin insensitive complex (although long-term rapamycin treatment disrupts mTORC2 assembly), contains mTOR, Rictor, LST8, and SIN1, and is involved in the regulation of cytoskeletal organization. Akt, RAC1, and PKCα are the mTORC2 effectors described. Although the activity of mTORC2 responds to growth factors, the regulatory mechanisms of its activation remain unknown (Ma and Blenis, 2009[185]).

2.3. mTOR functions in the Central Nervous System

The development of selective mTOR inhibitors, the genetic modifications of specific proteins belonging to the mTOR pathway, and an increasing interest of the scientific community on its functions have dramatically accelerated the understanding about the role of mTOR in the CNS. mTOR is mainly known for its involvement in cell growth and proliferation because it acts as a sensor of the cellular metabolic resources and consequently controls the anabolic/catabolic processes. However, although neurons are post-mitotic cells, mTOR

plays a crucial role in the control of the CNS functions. Several studies have reported that mTOR controls the size of the neuronal soma[186], the morphogenesis of the dendritic spines[187-189], and the axon guidance[190], among others. Prolonged inhibition of mTOR by rapamycin chronic treatment diminished the total number of dendritic branches in *in vitro* hippocampal neuronal cultures, an effect that is mimicked by the inhibition of its upstream activators PI3K and Akt[187,188]. It is thought that mTOR promotes dendritic tree formation through its control of local protein translation and microtubule dynamics.

In the same line of these *in vitro* evidences, some animal models that show an over-activation of mTOR present brain hypertrophy with alterations in cell morphology. The conditional *Pten* knockout mouse line, which shows a constitutive activation of the PI3K/Akt/mTOR signaling pathway in the hippocampus and cortex, exhibits macrocephaly and neuronal hypertrophy, accompanied by behavioral abnormalities[191]. This phenotype is ameliorated by a chronic treatment with rapamycin[192].

Due to the crucial role that mTOR plays in neuronal physiology, it is expected that mTOR signaling is deregulated in a variety of neuropathological conditions, including brain tumors and neurodegenerative diseases (see 2.3.5. section).

2.3.1. mTOR and protein synthesis: the downstream effectors

Although mTOR complexes control a myriad of cellular processes, the most studied function of mTORC1 is its role in regulating protein translation[193,194]. The proper control of mRNA translation is

remarkably important due to the fact that alterations in this process give rise to a wide number of disease states, such as cancer, tissue hypertrophy and neurodegeneration. Translation is an energetically costly process divided into three steps: initiation, elongation, and termination. Initiation is the step at which the ribosome is recruited to the mRNA and is the major rate-limiting step and hence tightly regulated[195,196]. mTOR positively controls translation by regulating two crucial core components of the translation initiation machinery: p70S6K and 4E-BP[197]. On the other hand, mTOR also regulates several phosphatases, such as the protein phosphatase 2A, which likewise controls the mTOR effectors, thereby generating feedback and controlling translational rates.

2.3.1.1. 4E-BP, eIFs and cap-dependent translation

In eukaryotes, the majority of the mature mRNA transcripts posses a 5'-cap structure (m7-GTP)[198] that is recognized by the eIF4F complex during translation initiation. This complex is formed by eIF4E, eIF4G, eIF4A, and eIF4B[199]. Under basal conditions, the hypophosphorylated eIF4E remains bound tightly to 4E-BP, preventing the formation of the multimeric eIF4F. mTORC1 phosphorylates 4E-BP and triggers the release of eIF4E, allowing the binding to the cap structure of mRNAs and the initiation of translation. eIF4E is phosphorylated at a single residue (Ser209 in mammals) by the ERK1/2 substrate MAPK signal-integrating kinase/MAPK-interacting kinase 1 and 2 (Mnk1/2)[200-202]. When eIF4E binds the cap structure of mRNAs, it associates with eIF4G, which serves as a scaffolding for Mnk1/2, allowing the phosphorylation of

eIF4E (Figure 12). eIF4G is phosphorylated by mTORC1 and, although the precise role of this phosphorylation is not clear, it could increase eIF4F formation[193]. In this scenario, the recruitment of eIF4B, which potentiates the helicase activity of eIF4A by binding the eIF4F complex, is also necessary and mTOR-dependent through the activation of its substrate p70S6K[203,204]. Finally, eIF3, the small ribosomal subunit and a ternary complex (formed by eIF2, Met-tRNA and GTP) are recruited to the cap, allowing the assembly of the ribosome and the concomitant scanning which leads to the initiation of translation[144]. All these signaling events described above are illustrated in Figure 10.

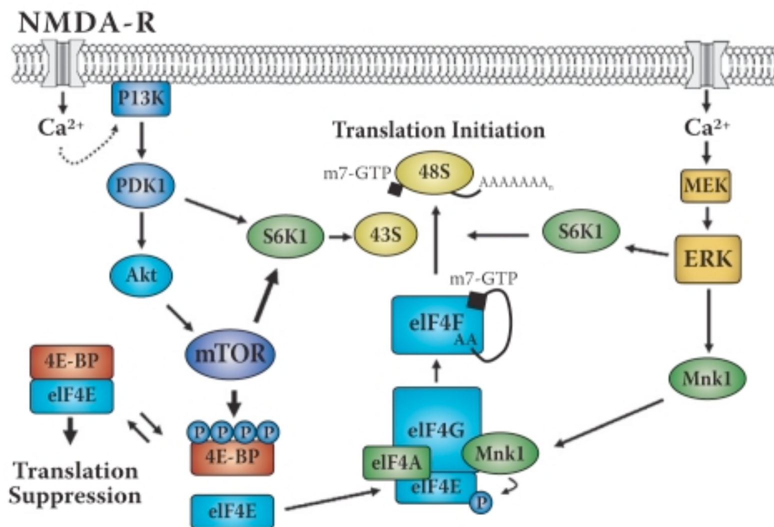

Figure 12. **mTOR and ERK signaling pathways converging on eIF4E.**

The translation initiation factor eIF4E is sequestered by 4E-BP2, which is the major 4E-BP isoform in the brain. When mTOR is activated by Akt, it phosphorylates and inhibits 4E-PB2, allowing eIF4E to bind to the cap structure (m7-GTP) of the mRNA. In parallel, MEK activates ERK1/2, which in turn, phosphorylates Mnk1/2. When eIF4G binds to the cap structure, it serves as a scaffolding protein for Mnk1/2 to phosphorylate eIF4E, which is associated with high translational rates. When the eIF4F complex is formed, the ribosome binds and starts scanning (Hoeffer and Klann, 2009[205]).

2.3.1.2. p70S6K and 5'TOP mRNAs

Apart from the regulation of cap-dependent mRNAs, mTORC1 also controls the translation of a subset class of mRNAs containing a 5′ terminal oligopyrimidine (5'TOP) tract[206] and encoding for components of the translational machinery, such as ribosomal proteins and elongation factors. This regulation is mediated by the other mTOR major substrate p70S6K[207]. The activation of p70S6K by mTORC1 induces mRNA translation through several effectors[185]. S6, a component of the 40S ribosomal subunit, was the first substrate of p70S6K identified (Figures 8 & 11). Although its role in promoting translation remains unclear, an increase in its phosphorylation correlates with high rates of translation under growth-promoting conditions. The activation of p70S6K is initiated by mTORC1-mediated phosphorylation of Thr389, allowing the subsequent phosphorylation of Thr229 by PDK1. Moreover, some studies reported that ERK1/2 could also mediate p70S6K activity by phosphorylating the residues Thr421/Ser424[208,209] (Figure 9). During the HFS-induced LTP, ERK plays an executive role in regulating mTOR signaling distinct from the canonical mechanism involving PI3K signaling. In the CA1 region of the hippocampus, p70S6K phosphorylation and 5'TOP mRNA-encoded proteins increased after the delivery of HFS. Rapamycin pretreatment only prevented the HFS-induced p70S6K phosphorylation at Thr389 site, but not in Thr421/Ser424 sites, whereas an ERK activity inhibitor blocked all phosphorylations[163]. In agreement, the activation of ERK1/2-p70S6K (Thr421/Ser424) was also observed in the mGluR-dependent LTD[210]. Moreover, mTOR and ERK signaling pathways also converge at

regulation of eIF4E during β-adrenergic receptor-induced LTP[211], which is also observed during mGluR-LTD[212].

Apart from the phosphorylation of the ribosomal protein S6 upon p70S6K activation, some studies demonstrated that p70S6K can also phosphorylate mTOR at the same residue targeted by Akt (Ser2448), as part of a feedback loop with unknown functions[213,214] (Figure 9). There are several negative feedback regulatory mechanisms for mTOR activity. Upon growth factor stimulation, the activation of p70S6K produced by mTORC1 induces inhibitory effect on tyrosine kinase receptors, via phosphorylation of the insulin response element-1, and finally suppresses PI3K-induced Akt activation[215,216] (Figure 9).

Besides the role of p70S6K promoting the translation of 5'TOP mRNAs, its activation also contributes to the cap-dependent translation through the phosphorylation of the translation initiation factor eIF4B, which in turn, enhances the helicase activity of eIF4A, facilitating the assembly of the ribosome into the cap structure[203,204] (Figures 8 & 11).

In addition to the regulation of translation initiation, mTORC1 is also involved in the control of translation elongation via the eukaryotic elongation factor 2 kinase, which is phosphorylated an inhibited by p70S6K, allowing the increase of the translation elongation rate[217] (Figure 9).

2.3.2. mTOR and synaptic plasticity

Synaptic plasticity is referred to the ability of the synapse to strengthen or weaken in response to experience. The most studied

forms of long-lasting synaptic plasticity are LTP and LTD, which are perdurable increases or decreases, respectively, in synaptic strength[218]. LTP is divided into two distinct temporal phases: early-phase LTP, which does not require new gene expression, is usually induced by one tetanic train stimulation and lasts 1-2h, and late-phase LTP, which requires new protein synthesis, is induced by repetitive tetanic trains and lasts for several hours[219]. While throughout the 1990s studies were focused entirely on transcription, mainly in the transcription factor cAMP responsive element binding protein (CREB)[220], in the last years emerging studies have evidenced the role of translation, especially the local protein synthesis at dendrites underlying long-lasting forms of synaptic plasticity[221]. The important role of newly synthesized proteins in synaptic plasticity has been postulated in several studies using a variety of pharmacological and genetic approaches[222].

The presence of ribosomes, translational factors, and mRNA has been reported in dendrites and dendritic spines, and not only in the neuronal soma[223,224], indicating that local protein synthesis in the proximity of synapses could elicit long-lasting synaptic plasticity without requiring transcription in the soma. Several lines of evidence suggest that mTOR is one of the critical regulators of this phenomenon. In specific protocols where LTP is induced in preparations where dendrites are separated from the soma, rapamycin or protein synthesis inhibitors prevent LTP induction, demonstrating that mTORC1-induced protein translation in active dendrites is sufficient to mediate persistent synaptic changes[225,226].

Numerous upstream regulators of mTOR are necessary for the induction and maintenance of LTP and LTD, including BDNF and

several surface receptors such as N-methyl-D-aspartate receptors (NMDAR), mGluRs, and dopaminergic receptors[227-230] (Figure 9). Most of the evidences linking mTORC1 signaling to long-lasting synaptic plasticity are based on the studies showing the blockade of changes in synaptic strength by rapamycin. The application of rapamycin demonstrated the first involvement of mTOR in NMDAR-dependent hippocampal L-LTP and its role in the regulation of translational machinery[231]. This effect is time-restricted and occurs in the dendrites of the CA1 hippocampal region[232].

On the other hand, it was also shown that LTP-induced p70S6K phosphorylation requires both mTOR and ERK1/2, and is correlated with enhanced S6 phosphorylation[163,233,234]. During late-phase LTP, mTOR activation promotes the local translation of the elongation factor eEF1A, a 5'TOP mRNA involved in elongation phase of the protein synthesis[233]. β-adrenergic receptor-induced LTP is another phenomenon requiring cooperation between mTOR and ERK1/2 to regulate *de novo* protein synthesis, required for long-lasting forms of synaptic plasticity. Treatment of mouse hippocampal slices with a β-adrenergic receptor agonist results in phosphorylation of the repressor 4E-BP as well as the phosphorylation of the ERK-target Mnk1/2, and the translation initiation factor eIF4E[211]. On the other hand, mTOR is also crucial in the establishment of another form of synaptic plasticity, the mGluR-induced LTD, which is altered in the fragil X syndrome. The mGluR1/5 agonist dihydroxyphenylglycine, which promotes mGluR-LTD, induces the phosphorylation of the mTOR downstream effectors, such as the ribosomal protein S6, and the synthesis of eEF1A in dendrites[212,229,235,236]. Thus, the intact mTORC1 function in

dendritic up-regulation of translational apparatus is essential for the proper persistent forms of translation-dependent synaptic plasticity. Several mRNAs translated in response to mTORC1 activation have been described, such as NR1, CaMKIIα, PSD95, Arc, and PKMζ[237-240], being all involved in synaptic plasticity.

Genetic studies also confirm the role of mTOR in plasticity. Unfortunately, the constitutive deletion of several mTOR signaling components including mTOR, Raptor, Rictor, and mLST8 is lethal[181]. This is not surprising due to the important role of mTOR in developmental processes[241]. However, there are mutant mice lacking upstream or downstream members of mTOR signaling that have contributed to highlight the relevance of mTOR in perdurable synaptic changes. Some transgenic lines for gene mutations that yield to alterations in synaptic plasticity are summarized in Table 3.

Mutation	FKBP-12 KO	TSC1+/-	TSC2+/-	4E-BP2 KO	S6K1 KO	S6K2 KO
E-LTP	=	ND	↓ | ↑	↑	↓	=
L-LTP	↑	ND	ND	↓	=	=
LTD	ND	ND	↓	↑	↑	↑

Table 3. **Long-lasting synaptic plasticity changes in mutant mice of translational regulatory components**

Normal (=), enhanced (↑), or impaired (↓) long-lasting synaptic plasticity in distinct knockout mice (KO) compared to wildtype control mice (modified from Gkogkas *et al*, 2010[165]). ND, no data; E-LTP, early long-term potentiation; L-LTP, late-long-term potentiation; LTD, long-term depression.

Conditional mutant mice lacking mTOR negative regulators, such as TSC proteins ($TSC1^{+/-}$ and $TSC2^{+/-}$) or $FKBP12^{-/-}$ (an immunophilin

to which rapamycin binds), exhibit altered synaptic plasticity and memory processes[234,242,243]. The deletion of the mTOR effectors, such as 4E-BP2$^{-/-}$, p70S6K1$^{-/-}$, and p70S6K2$^{-/-}$, also results in disparate phenotypes regarding long-lasting plasticity and memory[212,244-246] (see 3.3 section).

2.3.3. mTOR and NMDAR

Several studies have revealed the involvement of NMDARs in synaptic plasticity through the modulation of mTOR signaling cascade and neuronal protein translation. Calcium entry via NMDAR channels is crucial for the NMDAR-driven activation of intracellular signaling pathways[247,248]. NMDAR stimulation promotes the activation of several signaling kinases such as ERK1/2[249], which in turn leads to phosphorylation of Mnk1/2 and the subsequent phosphorylation of eIF4E[250], localized in postsynaptic densities and dendritic lipid rafts (Figure 12). An increase in eIF4E phosphorylation is commonly related to enhanced general translation[251], although the physiological function of this phosphorylation has not yet been completely clarified. Nevertheless, a direct connection of NMDAR activation and ERK/Mnk/eIF4E signaling cascade phosphorylation has been demonstrated in the mouse hippocampus[162]. In addition, it has been shown that NMDAR-dependent late-phase LTP is associated with an ERK-dependent increase in eIF4E phosphorylation[161]. These findings demonstrate a critical role for NMDAR activation in promoting the initiation of translation through ERK1/2 signaling.

In parallel with these responses involving the ERK1/2 signaling cascade, NMDAR activation can regulate translation through mTOR

signaling. Several studies demonstrated that upon NMDAR activation, several kinases highly involved in the modulation of mTOR are activated, including PI3K, PDK1, and Akt[252-255] (Figure 12). More direct evidence of the link between NMDAR activation and mTOR-dependent regulation of translation arise from studies in hippocampal neurons demonstrating that stimulation of NMDAR results in dendritic protein synthesis that is sensitive to rapamycin[256]. NMDAR-dependent late-phase LTP leads to the enhancement of p70S6K phosphorylation at the mTOR-dependent site, an effect that is blocked by rapamycin[232].

Although mTOR activation is currently associated with an increase in protein synthesis, an activation of mTOR can occasionally lead, on the contrary, to a translation suppression of certain mRNAs. Interestingly, it has been reported that NMDAR-mediated activation of mTOR gives rise to a reduction of mRNA translation of the potassium channel Kv1.1 in the hippocampus[257]. Altogether, these results support the evidence of the strong link between NMDAR activation and signal transduction pathways (ERK1/2 and mTOR) involved in the control of translation in neuronal dendritic compartments.

2.3.4. mTOR signaling dysregulation and neuronal disorders

mTOR signaling is disturbed in a large variety of human diseases, mainly particular types of cancer, which usually exhibit mutations in genes encoding upstream regulators of mTOR activity[258-261]. However, there are accumulating evidences tightly linking mTOR deregulation, abnormal translation and human neurological disorders[262].

One of the most studied neural diseases linked to mTOR is the

dominantly inherited tuberous sclerosis. The causes of this disease are mutations in genes encoding TSC1 and TSC2, which yield to an up-regulation of mTOR activity[263]. Tuberous sclerosis is clinically defined by the appearance and growth of benign hamartomas throughout the body and brain. Around 90% of the patients have seizures and 60% are mentally retarded, probably due to the lesions caused by hamartomas and cellular hypertrophy. Consequently, inhibition of mTOR activity counteracts this phenotype[264,265]. Another multiorgan disease exhibiting an increase in mTOR activity is neurofibromatosis type 1, a familial cancer syndrome hallmarked by tumors of the nerve tissue and memory impairment. In this disease, a mutation disrupting neurofibromin, a regulator of Ras signaling, yields to an up-regulation of the Ras/PI3K/Akt/mTOR signaling pathway activity[262]. On the other hand, Fragil X syndrome is a disease caused by transcriptional silencing of the *Fmr1* gene, which encodes for the fragil X mental retardation protein (FMRP), an RNA-binding protein capable of binding and sequestering numerous mRNAs, thus preventing or limiting their translation[266]. FMRP knockout mice display behavioral deficits mimicking those observed in human patients[267]. Several studies indicate that mTOR activity is enhanced in these mice[268] and p70S6K could phosphorylate and regulate FMRP[269].

Recent studies have suggested that mTOR signaling is also altered in neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease[241]. There are several hypotheses about the putative therapeutic effects of rapamycin, or its analogs, in age-related neurodegenerative disorders. First, it has been recently described that rapamycin treatment extends life span in yeast[270], worms[271], flies[272], and mice[273]. If mTOR inhibition can delay the aging

process, it is easy to speculate that it can also delay the appearance of neurodegenerative disorders by preserving a "younger" phenotype in the brain. In addition, neurodegenerative disorders are usually associated with neurotoxic insults, which can alter cell metabolism or induce stress response pathways, an effect that can be counteracted by mTOR inhibition. Rapamycin induces autophagy and decreases the translational rate, which facilitates the clearance and removing of misfolded or damaged proteins in the cell[176].

Alzheimer's disease is a progressive neurodegenerative disorder mainly characterized by the accumulation of β-amyloid containing plaques and the formation of neurofibrillary tangles formed by hyperphosphorylated tau, accompanied by gradual memory loss of the patients. One biochemical feature is the dysregulation of PTEN, Akt, p70S6K, and mTOR in postmortem brain samples from Alzheimer's disease patients[262,274,275]. Although some studies suggest that overall translation is decreased, certain proteins can accumulate, including tau, which can be translated upon mTORC1 activation[274,276]. Indeed, rapamycin feeding regimen extended life-span in mouse models of Alzheimer's disease[273,277,278]. Moreover, mTOR plays a crucial role in the inhibition of autophagy in Alzheimer's disease and Huntington's disease, since rapamycin treatment promotes clearance of huntingtin aggregates in mouse models of Huntington's disease[277-280].

The relationship between mTOR dysregulation and Parkinson's disease is more difficult to understand at the present moment. Parkinson's disease is the second most common neurodegenerative disorder characterized by the loss of dopamine-containing neurons in the substantia nigra. The main symptoms are rigidity, bradykinesia,

and tremor. Enhanced levels of REDD1 (regulated in development and DNA damage responses 1), a protein that inhibits mTOR signaling via TSC2 activation (Figure 8), are found in human Parkinsonian brains as well as in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a selective toxic of dopaminergic neurons[281]. Furthermore, the knockdown of REDD1 or TSC2 reduces the toxicity of 6-OHDA in a catecholaminergic cell line, suggesting that the increase of mTOR activity could be protective[281]. However, rapamycin treatment increases survival in the substantia nigra of mice treated with MPTP[282]. On the other hand, rapamycin facilitates clearance of α-synuclein, which is observed in Lewy bodies of Parkinson's disease, probably by promoting autophagy[283]. Interestingly, rapamycin might be also useful to reduce dyskinesia in mice treated with L-DOPA, which is a caveat of its clinical use for the treatment of Parkinson's disease[284].

3. Memory

3.1. Neuroanatomical substrates of memory

According to Eric Kandel, memory is the process by which the acquired knowledge of the world is encoded, stored, and later retrieved[219]. Thus, memory is a brain function that classifies, encodes, stores, and recovers distinct information relevant for the subject[285]. In general, memory can be divided in two major groups: declarative and non-declarative. Declarative (or explicit) memory is defined as the conscious memory for facts and events and is often further divided into episodic memory (memory for personal events) and semantic memory (memory for general facts). Moreover, it is thought to be acquired with relatively few exposures to the material to be learned[286,287]. On the other hand, non-declarative (or implicit) memory consists in procedural memory for habits or skills and usually requires an extensive acquisition phase[288].

Since the first explorations of the parahippocampal-hippocampal network by Ramón y Cajal in the 19th century, multiple anatomical analyses related with learning and memory processes have been published. The medial temporal lobe, including the hippocampus as well as the anatomically related entorhinal, perirhinal, and parahippocampal cortices, are crucial for the ability to learn and retain new declarative memories, however, it is not required for non-declarative memories[289]. The flow of information into the hippocampal formation starts in the entorhinal cortex, which is the main input to the hippocampus, and receives information from associational areas including the parahippocampal and perirhinal cortices[290-292]. The hippocampus is mainly composed by the dentate

gyrus and areas CA3 and CA1. The entorhinal cortex projects to the dentate gyrus and CA3 via the perforant pathway (layer II), whereas it projects directly to CA1 and subiculum through layer III. CA3 also projects to the CA1 through the Schaffer-collaterals[293]. Thus, hippocampal CA1 pyramidal neurons receive two anatomically segregated excitatory glutamatergic inputs: their distal portions of the apical dendrites are innervated by the perforant path (from entorhinal cortex), while their proximal regions are innervated by the Schaffer-collaterals, originating from CA3 excitatory neurons[291,294]. A diagram illustrating the neural circuitry in the rodent hippocampus is depicted in Figure 13. Remarkably, the dual sensory inputs that pyramidal CA1 neurons receive have been suggested to be essential for information processing, consolidation, storage, and retrieval in the hippocampus[295-297].

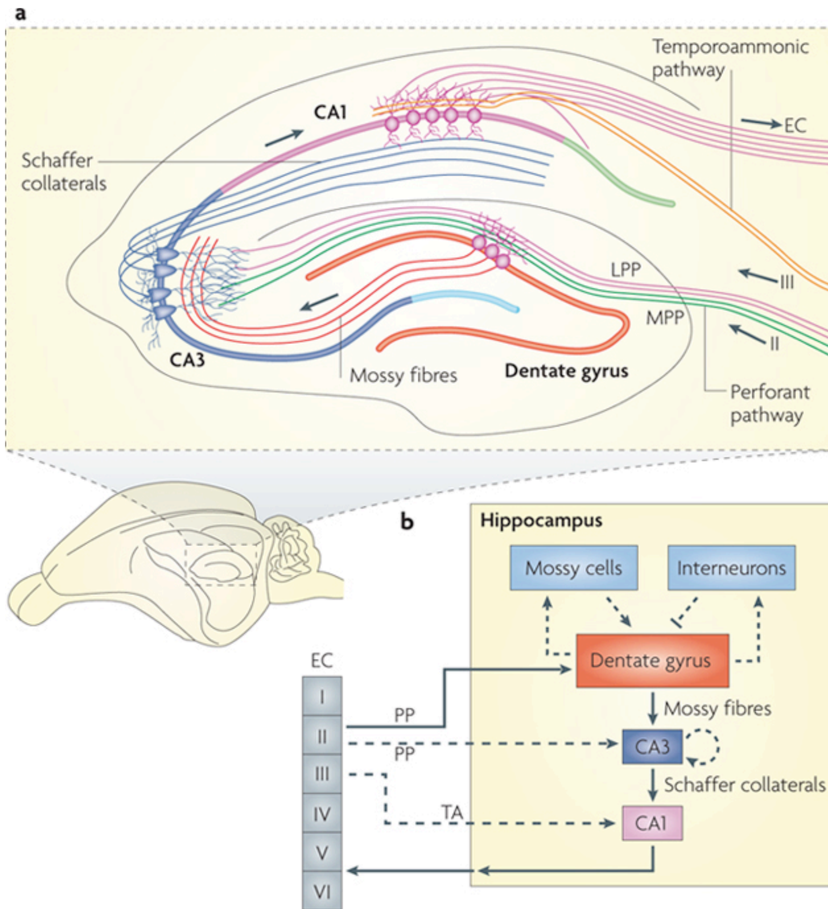

Figure 13. **The hippocampal network.**

Illustration of the hippocampal circuitry (a) and diagram of the hippocampal neural network (b). The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway (PP). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells (Deng *et al*, 2010[298]).

3.2. Behavioral models in animals to study learning and memory

Different behavioral paradigms have been used to study learning and memory in mice. Multiple mazes have been designed to evaluate memory based on different responses, from spontaneous exploratory behavior to complex action sequences. Positive reinforcers, such as food, sweetened water, refuge, or the opportunity to explore new objects, as well as negative reinforcers, such as water immersion, intense light, or a loud noise, have been applied[299]. Despite the diversity of mazes developed to evaluate spatial memory, the Morris water maze and radial arm maze are among the most frequently used[300]. In addition, multiple tests have been developed that are believed to mimick the natural behavior of mice, including T- and Y-maze alternation tasks[301], object recognition task[302], and the social recognition task[303], among others. Other tests require an aversive component (e.g., shock), including passive avoidance task, fear conditioning, and conditioned taste aversion, among others[304-306]. Some of these tasks are summarized in Box 1. Two memory paradigms, the object recognition task and the context recognition task, will be described in details because these are the paradigms used in the cognitive experimental procedures of this thesis.

Morris water maze
It is one of the most used spatial learning and memory tasks known to depend on the hippocampus. Animals swim in a murky pool of water to find the location of a submerged platform just beneath the surface of the water. To escape the water, mice use a variety of cues and strategies, including spatial cues around the pool in the room. Animals are trained for several days and the time/path length they take to find the platform is usually measured as a learning index. A more sensitive measure of spatial learning is performance in probe trials in which the platform is removed from the pool and the mice are allowed to search for it for a short period of time (for example, 60 sec). A common learning index for this test is the percentage of time that the mice spend looking for the platform in the quadrant where the platform was during training.

Novel object recognition task
It is a non-aversive, non-spatial test that requires hippocampal function. In this test, animals are allowed to freely explore two objects in an open field during training sessions. In the test sessions, one of the objects is replaced by a novel object. Short (e.g., 1 h) or long-term memory (e.g., 24 h) is measured as a ratio of time spent exploring the novel object versus the familiar object. Variants of this task use other stimuli, such as smells and even conspecifics (social recognition).

Radial arm maze
It is a spatial learning task with various versions. The apparatus has several arms (most commonly eight) that can be baited with food pellets at the end. Food-deprived animals are allowed to enter the arms and search for the hidden food. In a common version of this task, which is sensitive to both hippocampus and prefrontal cortex lesions, food-deprived animals are first (phase A) allowed to retrieve food pellets from 4 accessible arms of an 8-arm maze (the remaining 4 arms are blocked). After a retention interval of (e.g., 2 min) animals are brought back to the maze (phase B) and are given access to all 8 arms, but only the 4 previously blocked arms are now baited. Within-phase errors are committed when mice enter an arm previously visited in the same trial; across-phase errors are committed when mice enter an arm in phase B that they had already visited in phase A.

Fear conditioning
It is a Pavlovian aversive learning task in which animals associate a non-aversive conditioned stimuli (CS), such as a tone or context, with an aversive unconditioned stimulus (US; e.g., footshock). Conditioned responses, usually freezing (cessation of all but respiratory movement) are used as measures of memory. There are two common versions of this test: in tone conditioning the CS is a tone that precedes and co-terminates with the US; in context conditioning the CS is the context in which the animals are conditioned (that is, a chamber). Fear memories can last a lifetime but they can also be extinguished by repeated exposures to the CS without the US.

Conditioned taste aversion
It is an aversive learning task in which animals associate a food source (for example, saccharine flavoured water; CS) with malaise usually induced by LiCl injection (US). Avoidance of the food previously associated with malaise is used as a memory index.

Inhibitory avoidance
In this task the training apparatus has metal grids on the floor which can deliver a footshock. One part of the grid is covered to provide a safe platform for animals. During training, animals are placed on the safe platform and once they voluntarily step down to the grids they automatically receive a shock. Memory is assessed by measuring the time the animals spend on the platform before stepping down.

Passive avoidance
Here the animal learns to inhibit a natural tendency, namely to step into an apparently safer, dark compartment that has previously been associated with footshock.

Box 1. **Behavioral tests for learning and memory** (modified from Lee and Silva, 2009[304]).

3.2.1. Novel object recognition task

Recognition of objects, a judgement of the prior occurrence, is thought to be a critical component of human declarative memory. Due to the fact that object recognition is commonly impaired in human patients affected by neurodegenerative diseases or who have suffered brain injury[307-312], several behavioral tasks have been developed to study mnemonic phenomena in animal models based on recognition processes. However, declarative memory consists of a variety of cognitive processes related to familiarity and recollection with dissociable neural substrates[313,314], and in the object recognition paradigms only a subset of these processes implicated in declarative memory are involved. The test is based in the fact that rodents naturally tend to approach and explore novel objects, which are

assumed to have no natural significance to the animal, and which are not associated with a reinforcing stimulus. Rodents investigate them physically by touching and sniffing the objects, rearing upon and trying to manipulate them with their forepaws. They also show an innate preference for novel over familiar objects[315].

The standard one-trial object recognition task measures spontaneous behavior and is especially suited to test the effects of pharmacological and genetic interventions on declarative memory. A large advantage over food-rewarded maze learning tasks and classical delayed matching- or non-matching to sample tasks is that it does not require food or water deprivation, the application of reinforcing/aversive stimuli (food or electric shock delivery), the learning, retention and application of rules, or the learning of response–reward associations. On the contrary, it only requires a single training and is less stressful than tasks based on negative reinforcement of behavior (e.g., the hidden platform version of the water maze, the inhibitory and active avoidance, or fear conditioning tasks)[316,317].

The object recognition task typically consists of a training trial during which rodents explore two identical objects in a familiar arena, followed by a delayed test trial, in which a novel object is presented together with one familiar object already explored during the training trial. Usually, untreated animals spend more time exploring the novel object, suggesting that the familiar object was recognized[315,318]. It is important to note that this task evaluates memory for unique episodes or events (one-trial learning), which makes it more sensitive to amnesic experimental interventions, compared to other tasks, in which

incremental learning across multiple trials is induced, such as in the water-maze or radial-maze paradigms. Compared to tasks that require multiple learning trials, the one-trial object recognition task allows studying the effects of a drug on different stages of memory. Thus, the administration of a drug prior to the training trial allows the study of its effects in the acquisition (encoding) period. If it is administered after the training, in the consolidation (storage) stage, and if it is administered before the test, it influences the retrieval period.

Some of the disruptive effects on memory after drug delivery, lesions in certain brain structures or genetic manipulations have been studied in the object recognition task. However, several factors might influence the results obtained in this paradigm, including mouse strains, age and sex of the animals, the dimensions, shape, and the illumination of the apparatus in which object recognition is performed, whether rich spatial and contextual cues are present during the test, the type of objects, the duration of the trials, and the interval period between the training and the test, among others[319].

In our laboratory, and with the particular contribution of Arnau Busquets, we validated a new experimental procedure for the object recognition task by using a V-shape maze instead of the classical open field that is currently used. In this new procedure, both objects are placed at the end of each arm of the maze, which enables a higher exploration of the objects by mice and hence higher discrimination index values in control animals than when using the open field. We validated this task to study both acute and chronic pharmacological treatments. Briefly, mice were habituated to the V-maze on day one

for 9 min, and they were then returned to the homecage. On day two, two equal objects were placed at the end of the arms, and mice were left in the maze to freely explore the objects for 9 min. After 3 h (for short-term memory) or 24 h (for long-term memory), one familiar object was replaced by a novel object and the animals were put back into the maze. The time of exploration of each object was recorded during 9 min. Finally, the discrimination index (DI) value was calculated as follows:

$$\textbf{DI} = \frac{\textbf{time spent in novel object} - \textbf{time spent in familiar object}}{\textbf{total exploration time}}$$

DI values around 0.4 were considered as an indication of good memory. On the contrary, DI values < 0.2 were considered as an indication of memory disturbance.

For the assessment of object recognition memory during a chronic treatment, the experimental protocol was quite similar to the previously described above with the exception that two distinct, instead of two identical objects, were placed in the training trial. During each testing day, one familiar object was replaced by a novel one, and each test trial served as the training trial for the following day. Both the acute and the chronic protocols are illustrated in Figure 14.

It has been proposed that the medial temporal lobe system serves as a declarative memory system[320]. Lesions to the medial temporal lobe cause retrograde and anterograde amnesia in humans and primates[321]. The role of the medial temporal lobe, specially the hippocampus, in

the one-trial object recognition task has been investigated in rodents by excitotoxic damage, ablation lesions, or pharmacologically (infusion of lidocaine, muscimol, NMDAR antagonists, among others)[302,322].

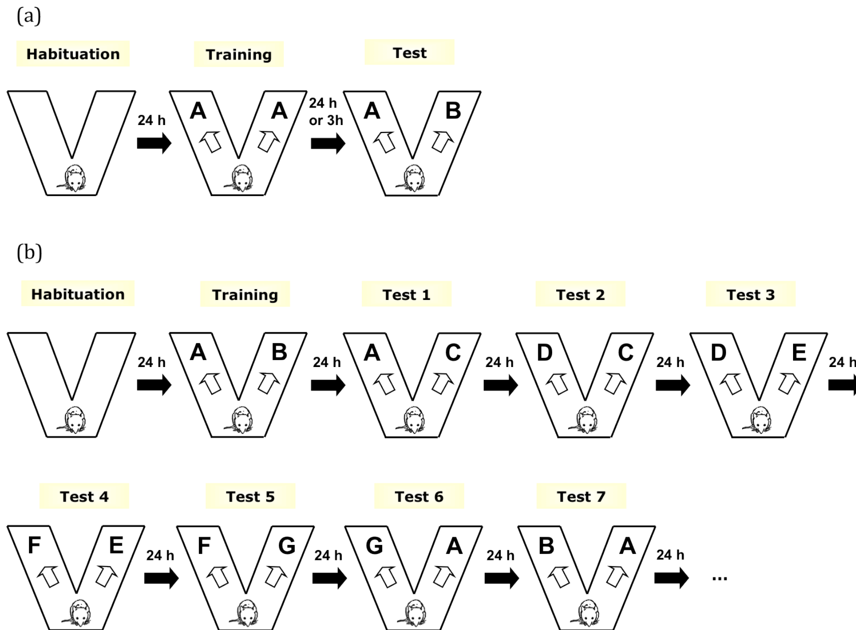

Figure 14. **Object recognition memory task**

(a) Protocol to study the effects of pharmacological acute administrations on short-term memory (3 h of delay between training and test) or long-term-memory (24 h of delay). (b) Protocol to study the effects of pharmacological chronic administrations on long-term memory.

It has been currently suggested that the hippocampus is not required for the encoding of object information after short intervals between the training and the test (few minutes), but it becomes important when object information has to be maintained over longer delays[323-325]. However, some controversial results have been reported in the studies analyzing this role of the hippocampus. Thus, temporary inactivation

of neuronal activity in the dorsal hippocampus by lidocaine given prior to the training impaired one-trial object recognition after 24 h, but not after 5 min of delay in mice[324]. In another study, muscimol was bilaterally infused into the dorsal hippocampus 3 or 6 h after novel object recognition training and the test was performed one day later. The results showed that hippocampus is essential for consolidation memory up to 3 h, but not 6 h post-training[325]. Moreover, compelling evidences suggest that hippocampal NMDAR has been implicated in certain types of long-term synaptic plasticity and memory consolidation[326].

On the other hand, the perirhinal cortex is part of the parahippocampal region and is located dorsally to the hippocampal formation. The perirhinal cortex has both direct and indirect connections with the hippocampus via the entorhinal cortex[290]. Lesions of the perirhinal cortex or locally drug infusions have demonstrated that perirhinal cortex also plays a crucial role in the object recognition memory in rodents and primates[327,328]. Thus, lidocaine infusion into the rat perirhinal cortex has been addressed in different memory stages, indicating that this brain structure is critical for the encoding of object information, the maintenance of the object memory trace during the consolidation period and the retrieval of object information during the test trial[329]. Deficits in object recognition in rats have also been observed after combined peri-postrhinal cortex lesions at delays of 15 min[330], 1 and 24 h[331]. Notably, even in the cases where hippocampal damage disrupts object recognition, this impairment is usually much less severe than the deficit caused by perirhinal lesions[332,333].

3.2.2. Context recognition task

Fear conditioning paradigms measure the ability of animals to associate and then remember between an auditory tone and footshock (cued fear conditioning), or between an environment and footshock (contextual fear conditioning). The auditory tone and the environmental context are considered the conditioned stimulus, while the footshock is the unconditioned stimulus of this associative learning. Both tasks are based on the natural tendency of mice to freeze in response to fearful stimuli. Freezing behavior is considered as a cessation of all body movement aside from respiration. Although, both tasks are sensitive to lesions of the amygdala, it is generally accepted that auditory fear conditioning requires the basolateral amygdala, while contextual fear conditioning depends on the hippocampus[334-337].

The context recognition task was also used in our cognitive studies to evaluate the responses in a second behavioral paradigm where the hippocampus also plays a crucial role. The protocol was performed in two consecutive days: day 1, training session and day 2, test session. During the training, mice acclimate to the electrifiable chamber around 2 min before the onset of the unconditioned stimulus (2 sec footshock of 0.35-1.5 mA intensity) and then they remained in the chamber for 30 additional sec. A single pairing of conditioned stimulus and unconditioned stimulus is adequate to induce strong learning of an association between both stimuli. 24 h later, mice are placed back in the chamber for 5 min with no shock presentation, and the freezing behavior is counted (Figure 15).

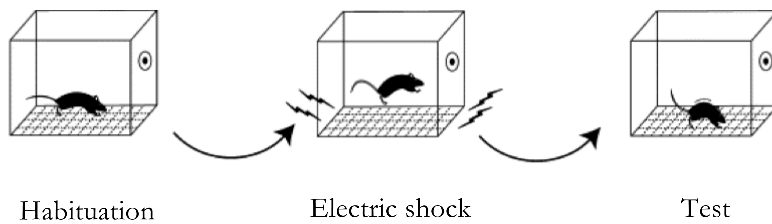

Figure 15. **Context recognition memory test** (modified from Schimanski *et al*, 2004[319]).

3.3. Neurobiological substrates of memory: role of mTOR and protein synthesis

Several neurobiological substrates have been characterized to underlie memory phenomenon. Excitatory transmission and LTP seem to play a major role in this process. Thus, calcium influx through NMDARs into the postsynaptic spines triggers biochemical processes associated to long-lasting synaptic plasticity and memory formation. Calcium/calmodulin-dependent protein kinase II is activated upon calcium entry, which participates in the enduring modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Moreover, during the late-phase of LTP, long-term memory storage requires the activation of PKA, MAPK and CREB, which are related to the growth of new synaptic connections[219]. The introduction of this thesis will mainly address the role of the mTOR signaling cascade and protein translation on memory processes.

Memories are usually divided into short-term memory, which lasts 1-3 h, and long-term memory, which lasts for days, months or even a

lifetime[338,339]. It is widely accepted that changes in gene expression contribute to enduring modifications of synaptic strength and are required for long-term memory. However, the regulation of transcription is only the first-step of the final gene expression. In this line, there is a growing research regarding the regulation of mRNA translation as the final step of gene expression, which does not imply mRNA synthesis or transport in the cells[340,341]. One of the advantages over the nuclear transcriptional control relies on the local translation, which allows the rapid synthesis of certain proteins in specific sites of the cell, without requiring the nucleus. This mechanism seems very important in neurons due to their asymmetry and their complex synapto-dendritic architecture. Thus, the asymmetric distribution of mRNAs within a neuron and the tightly control of translation could be transduced into certain forms of memory storage[341,342]. Nevertheless, the high complexity of the signaling network governing the control of translation indicates that numerous converging and diverging pathways could be involved in the control of protein synthesis during each type of memory.

It is thought that brain encodes and stores information by adjusting certain connections and thereby by modulating particular synapses while leaving others unaltered. To further understand where the long-term synaptic adjustment may occur, a putative mechanism termed “synaptic tagging” has been proposed, which consists in a local and persistent protein modification that serves as a marker for the synapse that will be modified[343,344]. Thus, the products of gene expression are delivered throughout the neuron but are only functionally incorporated in those synapses that have been tagged by previous

synaptic activity. Polarized mammalian cells transport mRNA-protein complexes to specific subcellular compartments, where mRNA translation can be promoted by physiological stimuli. Thus, local translation in neuronal dendrites is notably important for understanding the persistent changes in synaptic connectivity that might occur during memory storage[345].

mTORC1 is thought to be one of the major regulators of translational control. Additionally, both pharmacological and genetic approaches indicate that mTORC1 plays a crucial role in memory processes. Several studies show that mTORC1 signaling inhibition by rapamycin blocks long-term memory formation in mammals[346-348]. On the other hand, training of several memory tasks coincides with enhancement in the phosphorylation of the mTOR targets 4E-BP and p70S6K in the hippocampus and amygdala[234,347,349]. Strikingly, mutant mice lacking the translational control molecules downstream from mTORC1 exhibit altered memory (Table 4). Thus, 4E-BP2 knockout mice display impaired hippocampus–dependent and –independent forms of memory[244,245]. p70S6K (S6K 1 and 2) knockout mice also exhibit altered memory in contextual fear conditioning, conditioned taste aversion, and Morris water maze, although some differences are found between both knockouts[246]. On the other hand, mutant mice bearing genetic deletions of the upstream molecules that regulate mTORC1 signaling also display an altered memory phenotype (Table 4). Both TSC1 and TSC2 heterozygous knockout mice show hippocampus-dependent memory disruption[242,243] and rapamycin treatment rescues the memory deficit in the TSC2 mutant mice[242]. These results might underlie the mental retardation and autism symptoms of the tuberous

sclerosis patients[350]. Interestingly, the conditional knockout of FKBP12, another negative regulator of mTORC1, which shows increased levels of mTOR and p70S6K phosphorylation, exhibits enhanced contextual fear memory apart from perseveration and repetitive behaviors that are consistent with autistic and obsessive-compulsive disorders[234]. Taken together these results indicate that intact mTOR function is required for proper long-term memory storage.

Mutation	FKBP-12 KO	TSC1+/-	TSC2+/-	4E-BP2 KO	S6K1 KO	S6K2 KO
MWM	=	↓	↓ ↑	↓	=	=
CFC	↑	↓	↓	↓	↓	=

Table 4. **Memory phenotypes in mutant mice of translational regulatory components.**

Normal (=), enhanced (↑), or impaired (↓) memory in distinct knockout mice (KO) compared to wildtype control mice (modified from Gkogkas *et al*, 2010[165]). MWM, Morris water maze; CFC, contextual fear conditioning.

3.4. Cannabis and memory

It has been widely demonstrated that cannabis intake causes memory impairment in laboratory animals and humans. Impairments in cognition and learning in humans following marijuana consumption have been known since decades. Both acute and chronic exposures to cannabis are associated with dose-related cognitive impairments, most consistently in attention, working memory, verbal learning, and memory functions. In addition to reduced learning, heavy cannabis use is also associated with a decreased mental flexibility, increased

perseveration and reduced ability to sustain attention[351]. Long-term heavy cannabis users show impairments in memory and attention that persist beyond the period of intoxication and getting worse with increasing years of regular cannabis use[352].

In laboratory animals, the effects of exogenous cannabinoids administration on memory have been extensively studied by using multiple behavioral paradigms[353]. In general, these studies revealed that cannabinoid administration disrupts certain forms of memory (e.g., short-term memory or working memory), while other forms such as retrieval of previously learned information seem to be more resistant to cannabinoids.

3.4.1. Neuroanatomical basis of cannabinoid effects

In rodents, activation of cannabinoid receptors by endogenous or exogenous cannabinoid agonists impaired learning and memory by a mechanism that is postulated to involve the hippocampus[354]. Indeed, multiple studies showing memory impairment produced by cannabinoids have been performed in paradigms involving spatial tasks known to be hippocampus-dependent, including the 8-arm radial maze, spatial alternation in a T-maze, and the open-field water maze, among others[355]. However, in the majority of these studies, cannabinoid receptor agonists were administered systemically and the contribution of hippocampus was not directly confirmed. In contrast, CP-55,940 was infused intrahippocampally in another study that obtained similar working memory deficits to those found after systemic cannabinoid administration[356]. In a more recent study, intrahippocampal infusion of rimonabant completely blocked the

memory impairment produced by THC or CP-55,940 systemic administration in the radial arm maze task, without affecting other pharmacological properties of cannabinoids, as assessed in the tetrad assay[354]. Moreover, intrahippocampal administration of WIN 55,212-2 disrupted memory in the radial and T-maze delayed alternation tasks[357] and in the spontaneous object and place recognition tasks[358].

Electrophysiological evidences also suggest that the hippocampus plays a predominant role in the memory disruptive effects of cannabinoids. Thus, systemic administration of THC or WIN 55,212-2 disrupted memory in a delayed non-match-to-sample operant task that was related to depressed hippocampal cell firing[359]. In addition, exogenous cannabinoid agonists[360] and endocannabinoids decrease LTP in the hippocampus[361], which is related to memory perturbation in various behavioral paradigms. Interestingly, both THC and CP-55,940 decreased the power of θ, γ, and ripple oscillations in the hippocampus of rats, which correlated with memory impairment on the delayed alternation memory paradigm, a hippocampus-dependent task[362]. Both the electrophysiological and cognitive effects of cannabinoid agonists were attenuated by the administration of rimonabant[363-365]. On the other hand, rimonabant facilitates olfactory memory in the social recognition test[366] and working memory in the delayed-non-match-to-sample behavioral task[367]. In agreement with these pharmacological data, mice lacking CB1R showed an increase of LTP in the hippocampus[368], an improvement in memory retention in the object recognition paradigm[369,370] and an increased number of conditional changes in the active avoidance task[87].

Taken together, these findings are consistent with the notion that CB1Rs located in the hippocampus contribute to the amnesic-like effects produced by cannabinoid agonists. However, the involvement of CB1R in other brain regions cannot be excluded. As an example, THC infusion into the prefrontal cortex disrupted memory on a radial arm maze procedure of 1 h of delay[371], but not on the standard radial arm task[372]. Therefore, the type of cognitive task is likely to determine the neural substrates underlying the memory impairment produced by cannabinoids.

On the other hand, the endocannabinoid system has a specific role in facilitating extinction and/or forgetting processes[93,373]. In this sense, CB1R knockout mice showed strongly impaired short-term and long-term extinction in auditory fear conditioning tests, with unaffected memory acquisition and consolidation. Treatment of wildtype mice with rimonabant mimicked the phenotype of CB1R deficient mice, revealing that CB1R is required at the moment of memory extinction. Consistently, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, a region known to control extinction of aversive memories[93].

The widespread anatomical localization of CB1Rs in the brain may explain its involvement in multiple memory stages that might require different neural substrates. In the hippocampus, CB1R is highly expressed in interneurons, mainly in CCK-positive basket cells, which surround pyramidal neurons[374,375] (Figure 16). However, CB1R is not detected in parvalbumin-positive basket cells. In 2006, the development of a high-titer CB1R antibody allowed the localization of

CB1R in the terminals of glutamatergic neurons[376,377]. However, the density of immunogold labeling on excitatory terminals is 1/30 of that on inhibitory terminals (Figure 17). On the other hand, CB1R is also expressed in the cortex, which participates in certain types of memory, as well as in the amygdala, a structure involved in emotional memory processes (Figure 16).

3.4.3. Effects of chronic cannabinoid exposure

The long-term effects of cannabinoids on learning and memory have been less investigated than their acute effects. Several parameters including the type of CB1R agonist, the dose, the duration of the treatment, and the behavioral task have to be taken into account for discrepancies in the results[378]. Although contradictory data is found with regard to the effects of chronic cannabis exposure, there are more evidences pointing to reversible memory impairment after cannabis cessation[379-382]. However, the age of onset of cannabis consumption is critical and compelling studies reported that adolescents are more vulnerable than adults to the cognitive effects associated with chronic heavy marijuana consumption, probably due to the involvement of the ECS in the brain maturation during youth[383].

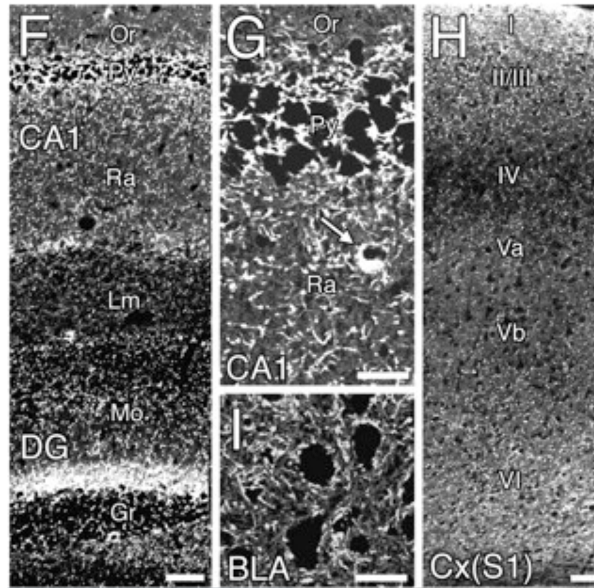

Figure 16. **Distribution of CB1R in the hippocampus, amygdala, and cortex.**

High-power views in the hippocampal dentate gyrus (F) and CA1 (F and G), primary somatosensory cortex (H), and basolateral amygdaloid nucleus (I). CB1 immunoreactivity shows a punctate or meshwork pattern in all of these regions. CB1-labeled perikarya are occasionally found in particular interneurons in cortical areas (arrow, G). In addition, CB1 immunoreactivity also shows laminar patterns in the hippocampus and cortex, reflecting different amounts of CB1 among afferents. Or, stratum oriens; Py, pyramidal cell layer; Ra, stratum radiatum; Lm, lacunosum moleculare layer; Mo, dentate molecular layer; Gr, dentate granular layer (modified from Kano *et al*, 2009[384]).

Neurocognitive abnormalities associated to chronic cannabis exposure in adults are apparent for several days after consumption, but might disappear one month after cessation. Nevertheless, adolescent heavy users show impairments in learning and working memory up to six weeks after cessation[383]. In agreement, adolescent rodents display greater memory impairments than adults. Early studies indicate that rats receiving a daily oral preparation of marijuana extract for 3-6 months, beginning the treatment when rats were immature, exhibit

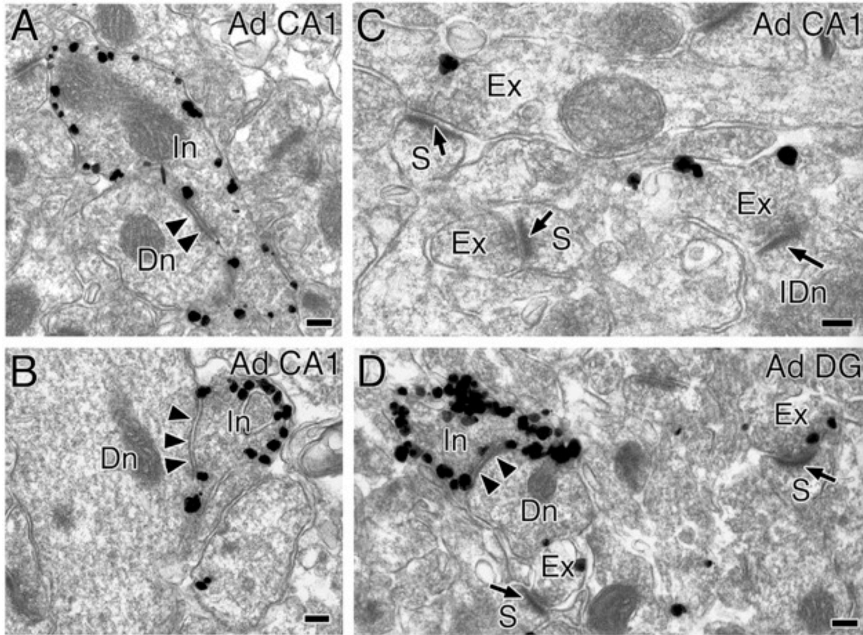

Figure 17. **Immunoelectron microscopy of CB1R in the hippocampus.**

Immunogold staining of presynaptic CB1R in the CA1 region (A-C) and the dentate gyrus (D) of the mouse hippocampus. Arrowheads and arrows indicate symmetrical and asymmetrical synapses, respectively. Dn, dendrite; Ex, excitatory terminal; IDn, interneuronal dendrite; In, inhibitory terminal; S, dendritic spine (modified from Kano *et al*, 2009[384]).

learning impairment on the Hebb-Williams maze[385], radial arm maze[386], and a differential reinforcement of low-rate responding[387]. These effects are observed 1-6 months after the last drug administration. Similar results are observed by using an equivalent dose of 20 mg/kg of THC chronically administered for 3 months followed by a drug-free period of 1 month[388]. Altogether, these studies show behavioral changes that persist even months after the last drug administration. However, in contrast to previous findings among rats exposed to cannabis when immature, adult exposed rats did not show impaired memory in the same tasks[389]. More recently, it has been

shown that chronic pubertal, but not adult chronic WIN 55,212-2 administration produced dysfunctional prepulse inhibition and impaired object recognition memory in rats[390]. In agreement, only THC-exposed adolescent rats, but not adults, display impaired object recognition and alterations in hippocampal protein expression[391]. Altogether, these studies indicate that adolescents are more vulnerable than adults to the memory impairment induced by cannabinoids.

Tolerance development to a spatial learning task has been shown to occur only in adult rats, but not in adolescents, after chronic THC treatment[392]. The authors relate these findings with the fact that CB1Rs in adolescent hippocampus are less functionally coupled to G proteins and desensitize more slowly in response to THC than those of adults. In another recent study, chronic THC treatment does not produce tolerance to the spatial memory impairment in mice, while it does for the locomotor suppressant effects[393]. Thus, the degree of tolerance development and long-lasting memory impairment might occur mainly depending on the age of onset, dose, and duration of the treatment.

3.4.4. Possible mechanisms underlying memory impairment by cannabinoids

The mechanisms involved in the modulation of learning and memory processes induced by cannabinoids have not been still fully clarified. The modulation of CB1R on the activity of other neurotransmitters seems to be involved in these cognitive effects. Memory impairment produced by cannabinoids has been related to an inhibition of

cholinergic activity in the CNS[394]. In agreement, both *in vitro*[395] and *in vivo*[396] studies have shown that cannabinoid agonists induce an inhibition of acetylcholine release in rat hippocampus. Another possible mechanism is the inhibition of CCK release, as blockade of CCK receptors impairs learning in a radial maze[397].

Since CB1Rs are much more densely expressed on GABAergic versus glutamatergic axon terminals in the hippocampus[377], another mechanism suggested is the activation of CB1R located in GABAergic terminals, leading to a suppression of GABA release[374] and a concomitant unspecific increase in excitatory firing. In this regard, a selective GABA reuptake blocker enhanced spatial learning[398]. In agreement, THC has been shown to act as a full agonist at CB1R located on GABAergic terminals in the hippocampus, while it acts as a partial agonist at CB1R present on glutamatergic terminals[399]. Thus, the predominant inhibitory action on GABA release triggered by cannabinoids could thereby modify the glutamatergic system, which is crucial in memory processes. In this regard, it has been shown that THC administration decreases GABA levels and increases glutamate levels in the rat prefrontal cortex, a brain structure also involved in memory[400]. Indeed, it has been suggested that CB1R activation during HFS of the medial perforant path increases glutamate release from perforant path synapses, but inhibits release of GABA from local circuit interneurons[401]. In addition, endocannabinoids released from pyramidal neurons can act on astrocytic CB1Rs and trigger glutamate release[402]. Moreover, an interaction between endocannabinoid and glutamatergic systems has been shown since THC-induced ERK1/2 phosphorylation *in vivo* in the CA1 and CA3 regions of hippocampus

is mediated by NMDARs[403]. The possible involvement of these mechanisms, or new mechanisms still unidentified, in the memory impairment produced by cannabinoids needs further clarification.

Objectives

Objective 1

To elucidate the intracellular signaling cascades activated by THC *in vivo* that could underlie several central effects attributed to cannabinoids, with special emphasis on the signaling pathways related to neuroprotection.

Article #1

Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain

Andrés Ozaita, Emma Puighermanal and Rafael Maldonado.

J Neurochem **102**, 1105-1114 (2007).

Objective 2

To study the molecular signaling pathways responsible of THC-induced memory impairment by using genetic and pharmacological approaches in two different behavioral models, the context and object recognition memory tasks.

Article #2

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

Emma Puighermanal, Giovanni Marsicano, Arnau Busquets-Garcia, Beat Lutz, Rafael Maldonado and Andrés Ozaita.

Nat Neurosci **12**, 1152-1158 (2009).

Objective 3

To dissect the functional role of the two main endocannabinoids, AEA and 2-AG, in memory, anxiety, and nociception by using acute and chronic pharmacological approaches in different behavioral tasks.

Article #3

Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses

Arnau Busquets-Garcia*, Emma Puighermanal*, Antoni Pastor, Rafael de la Torre, Rafael Maldonado and Andrés Ozaita.

*Equal contribution

Objective 4

To study the behavioral relevance of the mTOR signaling pathway in the effects triggered by acute and chronic THC exposures.

Supplementary information

Role of mTOR in the pharmacological effects of THC.

Emma Puighermanal*, Arnau Busquets-Garcia*, Rafael Maldonado and Andrés Ozaita.

*Equal contribution

Results

ARTICLE 1

Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain

Andrés Ozaita, Emma Puighermanal and Rafael Maldonado

J Neurochem **102**, 1105-1114 (2007)

ARTICLE 2

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

Emma Puighermanal, Giovanni Marsicano, Arnau Busquets-Garcia, Beat Lutz, Rafael Maldonado and Andrés Ozaita

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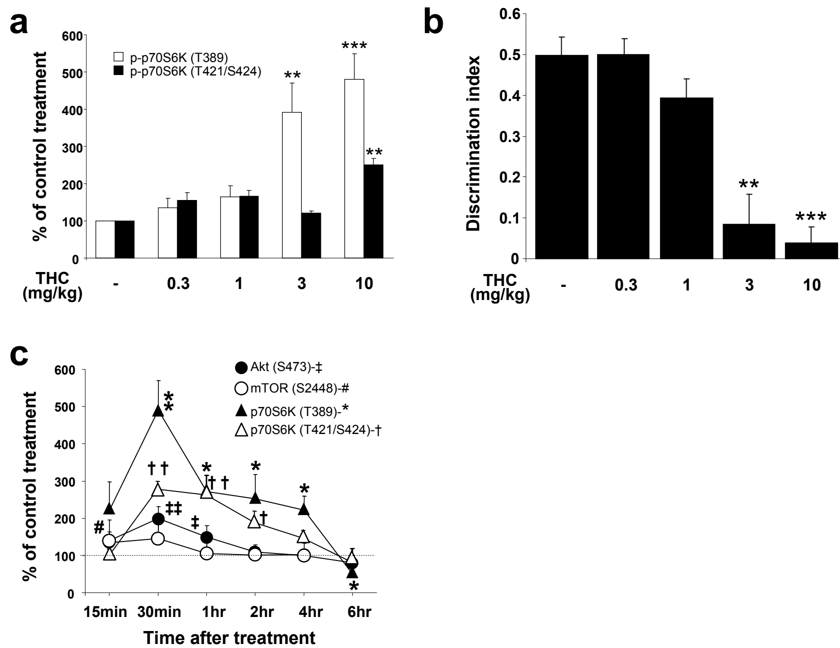
Supplementary figures

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

E. Puighermanal, G. Marsicano, A. Busquets-Garcia, B. Lutz, R. Maldonado, A. Ozaita



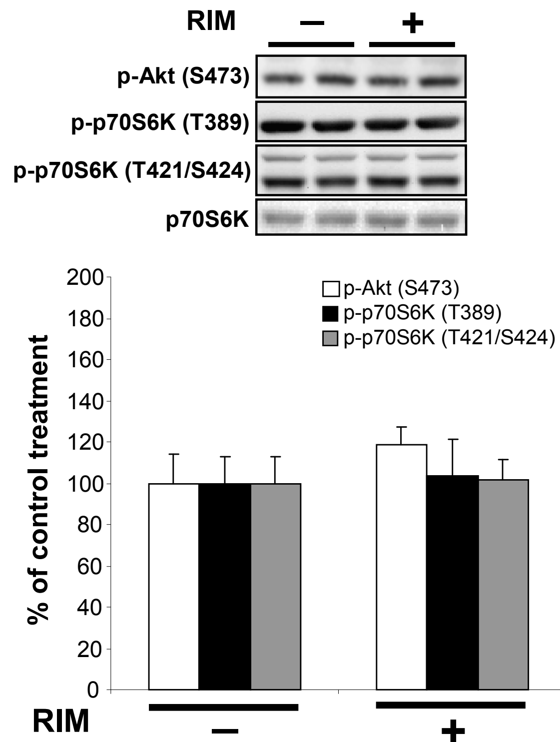
Supplementary figure 1. Effects of THC on mTOR signaling pathway in the hippocampus.

a) THC dose-dependent effect on mTOR signaling. Mice (n=4-6 per group) were treated with different doses of THC (0.3, 1, 3 and 10 mg/kg, i.p.) or its vehicle. 30 min later mice were sacrificed and hippocampal tissues were processed for immunoblotting. Optical density quantification is presented for p70S6K phosphorylation on residues (T389) and (T421/S424). Only the doses of 3 or 10 mg/kg induce the phosphorylation of these

residues. Data are expressed as mean ± s.e.m. **p<0.01, ***p<0.001 (THC vs. vehicle).

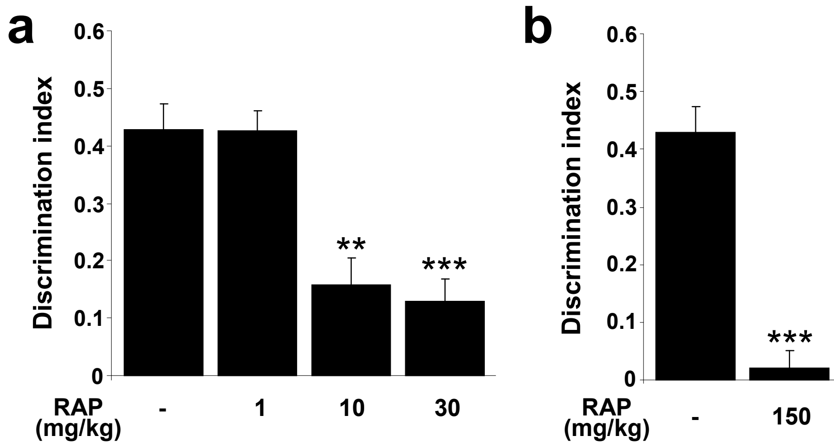
b) THC dose-dependent effect on memory impairment. Mice (n=4-6 per group) were treated with vehicle or different doses of THC (0.3, 1, 3 and 10 mg/kg, i.p.) immediately after the training session in the object recognition test. Only the doses of 3 or 10 mg/kg induced a significant amnesic-like effect tested 24 h later. Data are expressed as mean ± s.e.m. **p<0.01; ***p<0.001 (THC vs. vehicle).

c) Time course of mTOR activation in the hippocampus after THC administration. Mice were treated with either vehicle or THC (10 mg/kg, i.p.) and hippocampal tissues were processed at the indicated time points. The levels of phosphorylated Akt (S473), p70S6K (T389) and (T421/S424) were normalized to those of Akt or p70S6K, respectively, whereas the level of phosphorylated mTOR (S2448) were normalized to the housekeeping control GAPDH. Optical density values are expressed as a ratio between the THC and vehicle-treated mice (n=4-6 per group). Data are expressed as mean ± s.e.m. *,†,‡,#p<0.05,**; ††,‡‡p<0.01 (THC vs. vehicle for each time point).



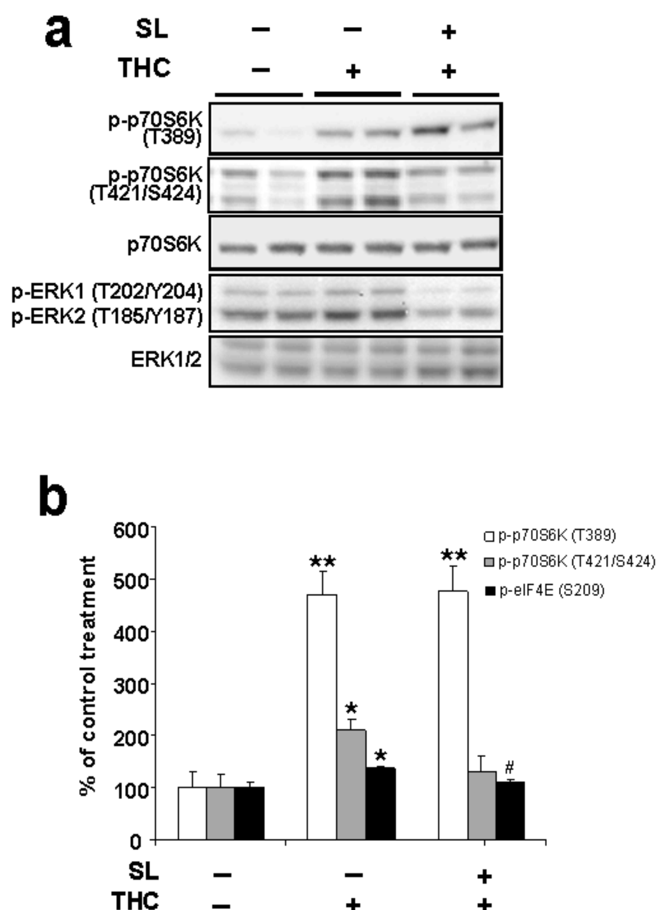
Supplementary figure 2. Endocannabinoid tone occlusion by pharmacological blockade of CB1R does not modify Akt or mTOR signaling.

Mice were pre-treated with rimonabant (RIM, 3 mg/kg, i.p.) or vehicle, 30 min before hippocampal tissue extraction (n=4 mice per group). Upper panel corresponds to a representative immunoblot showing the effects of CB1R blockade on the Akt and p70S6K phosphorylations. Each lane corresponds to an individual mouse of a representative experiment. **b)** Optical density quantification of immunoreactive bands for phosphorylated Akt (S374), p70S6K (T389) and (T421/S424). Note that no differences in mTOR signaling pathway activity were observed after endocannabinoid tone blockade. Data are expressed as mean ± s.e.m.



Supplementary figure 3. Dose-dependent effects of rapamycin on the object recognition test.

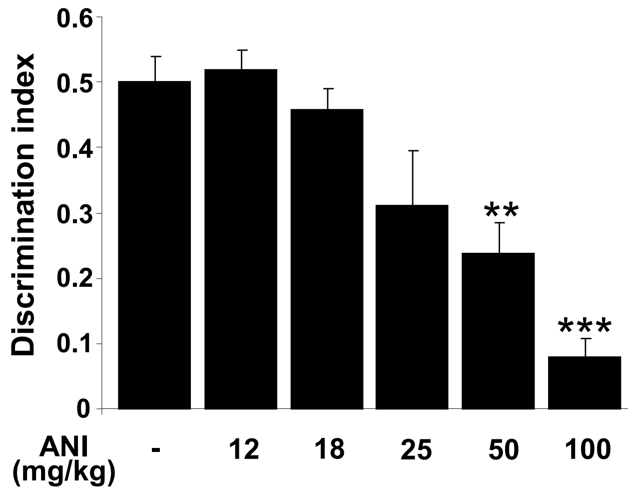
a) Mice (n=4-6 per group) were treated either with DMSO or a sub-chronic treatment of rapamycin (5 days) at different doses (1, 10, 30 mg/kg, i.p) with the last administration 3 h before the training phase in the object recognition test. The doses of 10 and 30 mg/kg produced long-term memory deficits when tested 24 h after training, while de dose of 1 mg/kg did not have effects on long-term memory *per se.* **b)** Amnesic-like effects of an acute dose of rapamycin (150 mg/kg, i.p.) administered 3 h before training in the object recognition test. Data are expressed as mean ± s.e.m. $^{**}p < 0.01$, $^{***}p < 0.001$ (rapamycin vs. vehicle).



Supplementary figure 4. Pharmacological blockade of ERK signaling attenuates the THC-induced p70S6K phosphorylation at T421/S424 sites and totally blocks eIF4E phosphorylation.

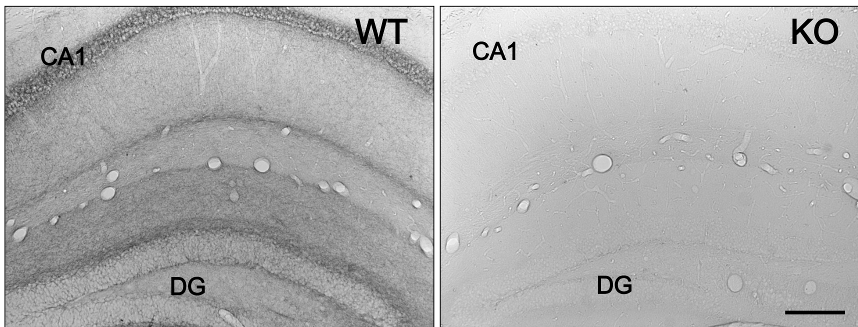
Mice were pre-treated with an inhibitor of MEK (the upstream kinase of ERK) SL327 (SL, 50 mg/kg, i.p.) or vehicle, 1 h before THC (10 mg/kg, i.p.) or vehicle administration (n = 4 mice per group). Mice were sacrificed 30 min later and hippocampal tissues were processed for immunoblotting. **a)** Representative immunoblot showing the effects of MAPK/ERK blockade on THC-mediated effects. Each lane corresponds to an individual mouse of a representative experiment. **b)** Densitometric values for phosphorylated p70S6K were normalized by those of p70S6K detection. Levels of

phosphorylated ERK1/2 bluntly diminished in the SL327 group, demonstrating that the inhibitor worked appropriately. SL327 markedly reduced the THC-induced phosphorylation of p70S6K at T421/S424 sites. Nevertheless, the levels of p70S6K phosphorylation at T389 site remained intact. eIF4E phosphorylation induced by THC was dependent on MAPK/ERK activation. Data are expressed as mean ± s.e.m. *p < 0.05, **p < 0.01 (THC vs. vehicle); #p < 0.05 (SL327 vs. vehicle).



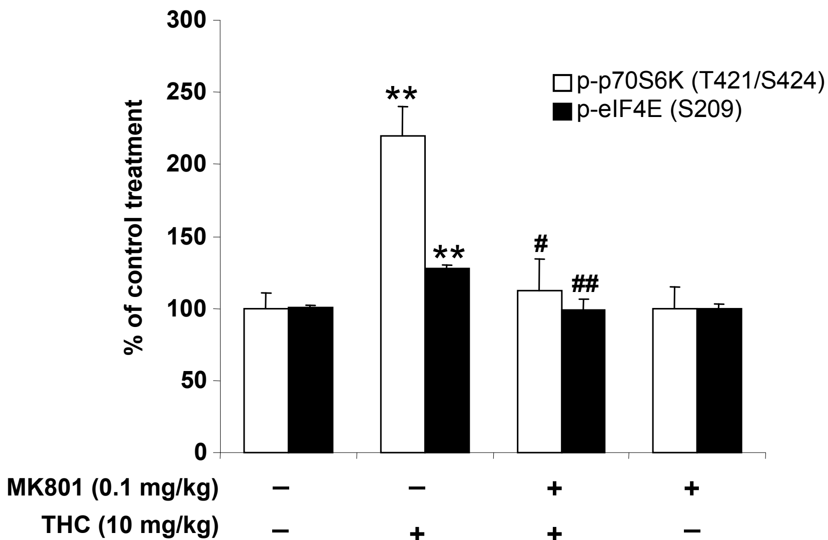
Supplementary figure 5. Dose-dependency of anisomycin effects on the object recognition test.

Mice (n = 4-6 per group) were treated with saline or different doses of anisomycin (12, 18, 25, 50, 100 mg/kg, i.p.) 25 min after training. Note that the higher doses of anisomycin produced long-term memory deficits detected 24 h later, while lower doses (12 and 18 mg/kg) did not. Data are expressed as mean ± s.e.m. **p < 0.01; ***p < 0.001 (anisomycin vs. saline).



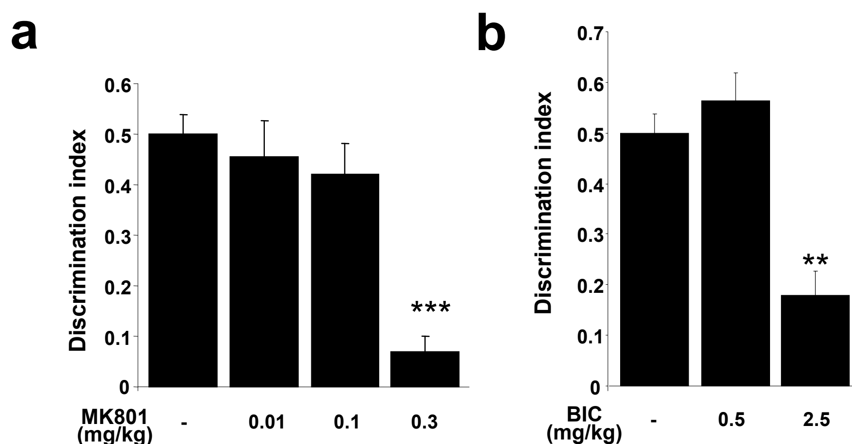
Supplementary figure 6. Anti-CB1R antibody specificity.

CB1R immuno-detection in wild-type mouse (WT) hippocampus was compared to that of CB1R knock-out mice (KO), to ascertain the authenticity of the staining. CB1R staining matched that reported previously[1,2] and was completely absent in the CB1R knock-out mouse. Abbreviations: DG, dentate gyrus; CA1, CA1 field of Ammon´s horn. Scale bar, 200 μm.



Supplementary figure 7. Glutamatergic mediation in the biochemical effects of THC.

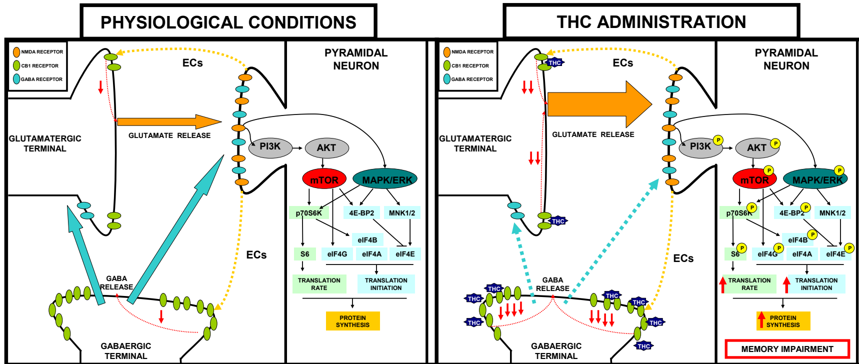
Mice (n = 4-6) were pre-treated with MK801 (0.1 mg/kg, i.p.) or saline 20 min before receiving either THC (10 mg/kg, i.p.) or its vehicle. Bars diagram represents the optical density analysis of the immuno-detection for p70S6K (T421/S424) and eIF4E (S209) phosphorylation in hippocampal lysates. Note that MK801 blocked the increase in phosphorylation of p70S6K and eIF4E promoted by THC. Data are expressed as mean ± s.e.m. $^{**}p < 0.01$ (THC vs. vehicle); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (MK801 vs. saline).



Supplementary figure 8. Dose-dependent effects of MK801 and bicuculline on the object recognition test.

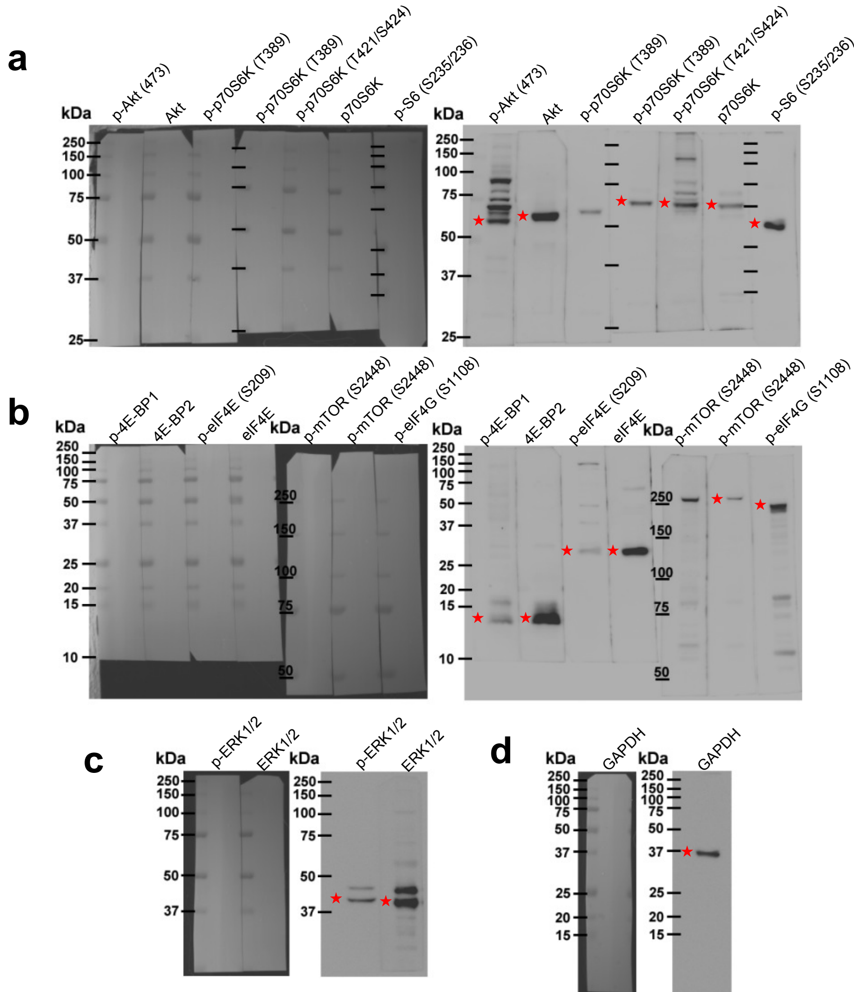
a) Mice (n = 4-6 per group) received either saline or MK801 at different doses (0.01, 0.1 and 0.3 mg/kg, i.p) 25 min after training. Note that only the dose of 0.3 mg/kg showed amnesic-like effects. Data are expressed as mean ± s.e.m. $^{***}p < 0.001$ (MK801 vs. saline).

b) Mice (n = 4-6 per group) received vehicle or bicuculline (BIC) at two doses (0.5 and 2.5 mg/kg, i.p). Only the dose of 2.5 mg/kg had amnesic-like effects. Data are expressed as mean ± s.e.m. $^{**}p < 0.01$ (bicuculline vs. vehicle).



Supplementary figure 9. Schematic diagram showing a possible mechanism involved in THC amnesic-like effects.

On the left, the physiological conditions are represented. mTOR pathway is localized in the somatodendritic compartment of the pyramidal neurons whereas CB1R are mainly localized in GABAergic neurons and to a minor extent in glutamatergic neurons. Endocannabinoid system (ECS), through CB1R, modulates the neurotransmitter release in GABAergic and glutamatergic terminals. At the post-synaptic level, mTOR and MAPK/ERK pathways are activated by glutamate receptors and modulate protein synthesis regulating the translation rate and translation initiation. On the right, a possible mechanism to explain the amnesic-like effects caused by THC. An unbalance between GABAergic and glutamatergic neurotransmission is produced by the THC mainly acting on CB1R located in GABAergic neurons. This unbalance leads to a glutamatergic activation of the mTOR pathway resulting in the phosphorylation of different downstream targets such as p70S6K, S6, 4E-BP2, eIF4E, eIF4B and eIF4G. This activation promotes an increase of the translation rate and translation initiation leading to an enhancement of the protein synthesis and the consequent amnesic-like effects promoted by THC.



Supplementary figure 10. Immunoblot detection on hippocampal homogenates. Antibodies used in this study were first tested for immunodetection in mouse hippocampal homogenates (50 mg of protein per lane). Digitalized images of the blots (lefts panels) as well as digitalized images of the chemiluminescence detection (right panels) were captured as described in the methods section. Detection of Akt, p70S6K and ERK1/2 was performed after electrophoretic separation of proteins in 10 % acrylamide gels, while S6, 4E-BP, eIF4E and GAPDH were detected after electrophoretic separation in 14 % acrylamide gels. mTOR and eIF4G were immunodetected after protein samples were separated on 7 % acrylamide

gels. Pre-stained molecular weight standards were run in parallel to accurately estimate the appropriate molecular weight of the immunoreactive bands (star): Akt (~60 kDa), p70S6K (~70 kDa), S6 (~32 kDa), 4E-BP (~15 kDa), eIF4E (~30 kDa), mTOR (~290 kDa), eIF4G (~220 kDa), ERK1/2 (~44 and 42 kDa), GAPDH (~37 kDa). All the subsequent experiments shown on Figures 1 and 2, and Supplementary figures 2 and 4 were performed by incubating the piece of the blot corresponding to the molecular weight of the target protein with its specific antibody. Therefore, blots were cut before immunodetection in order to perform simultaneously the exposure of several target proteins of different molecular weight ranges.

References

(1) Hájos, N. *et al.* Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur J Neurosci.* **12**, 3239-3249 (2000).

(2) Fukudome, Y. *et al.* Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M3-mediated indirect suppression through endocannabinoid signalling. *Eur J Neurosci.* **19**, 2682-2692 (2004).

ARTICLE 3

Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses

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Biol Psychiatry (2011)

*Equal contribution

This article will be also presented in the thesis of Arnau Busquets-Garcia

Busquets-Garcia A, Puighermanal E, Pastor A, de la Torre R, Maldonado R, Ozaita A. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. Biol Psychiatry. 2011;70 (5):479-486.

Supplemental Methods & Materials

Endocannabinoid quantification

Chemicals. Acetonitrile, formic acid, acetic acid, ammonia, trifluoroacetic acid (TFA) and tert-butyl methyl ether (TBME) were obtained from Merck (Darmstadt, Germany). Arachidonoylethanolamide (AEA), deuterated AEA (AEA-d8), 2-AG and deuterated 2-AG (2-AG-d8) were from Cayman Chemical (Ann Arbor, MI). Stock and working standard solutions were prepared in acetonitrile.

Sample extraction. A half right or left brain (cerebrum, cerebellum and brain stem, weighing 230 mg approximately) (AEA and 2-AG detection) or hippocampal tissue (2-AG detection) were homogenized on ice with a glass homogenizer in 1 mL 0.02% TFA (pH 3.0). Aliquots of 150 µl of brain homogenate were used for AEA analysis, while 20 µl aliquots of brain and hippocampal homogenates were used for 2-AG analysis. Aliquots were transferred to 12 ml glass tubes and diluted up to 1 ml in 0.02% TFA, spiked with internal standards (2.5 ng AEA-d8 or 125 ng 2-AG-d8), extracted with TBME (10 ml) and centrifuged (3,500 rpm, 5 min, room temperature). The organic phase was evaporated (40ºC, 20 min) under a stream of nitrogen, and extracts were reconstituted in 100 µl of (50:50, v/v) mobile phase A and B. Twenty µl were injected into the liquid chromatography–mass spectrometry (LC-MS)/MS system.

LC-MS/MS analysis. An Agilent 6410 triple quadrupole (Agilent Technologies, Wilmington, DE) equipped with a 1200 series

binary pump, a column oven and a cooled autosampler (4ºC) were used. The chromatographic separation was carried out with a Zorbax 80Å StableBond C8 column (2.1 x 50 mm, 1.8 μm particle size) maintained at 40ºC with a mobile phase flow rate of 0.4 ml/min. The composition of the mobile phase was: A: 5 mM ammonium acetate, 0.1% (v/v) formic acid in water (pH = 3.15), B: 5 mM ammonium acetate, 0.1% (v/v) formic acid in (95:5, v/v) acetonitrile:water. The initial conditions were 40% B. The gradient was initiated after 2 minutes, increasing linearly to 100% B for 6 minutes, maintained at 100% B for 2 minutes, to return to initial conditions for a further 5 minutes with a total run time of 15 minutes. The tandem quadrupole mass spectrometer operated on the positive electrospray mode. Desolvation gas temperature of 350ºC and a gas flow rate of 9 L/min were used. The pressure of the nebulizer was set at 40 psi and the capillary voltage at 4,000 V. The fragmentor was set at 135 V and the dwell time at 100 msec for all analytes. The collision energies were optimized at 12 V for AEA and AEA-d8, and 15 V for 2-AG and 2-AG-d8. The multiple reaction monitoring mode was used for the analysis with the following precursor to product ion transitions: m/z 348.2→62 for AEA, m/z 356.2→62 for AEA-d8, m/z 379.2→287 for 2-AG and m/z 387.2→295 for 2-AG-d8.

The response of the instrument was proven linear over the range of 20 pg to 500 pg for AEA and 2 ng to 30 ng for 2-AG (on column). The dynamic range was suitable for the quantification since the endogenous concentrations of AEA and 2-AG found in the samples were all within this range. The limit of detection on column was 8 pg for AEA and 200 pg for 2-AG. The average recovery of the

deuterated analogues in the extracted samples was estimated to be 95.7% for AEA-d8 and 78.8% for 2-AG-d8. Ion suppression was estimated to be 13.6% for AEA and absent for 2-AG.

The endogenous concentrations of AEA and 2-AG were calculated based on the response of the deuterated analogues. Variations in accuracy were < 10% for the individual sample replicates.

Chronic assessment of object-recognition memory

The effect of the chronic administration of URB597 and JZL184 on object-recognition memory was evaluated in the V-maze during 6 days (see Figure S1). A discrimination index was calculated as previously described (1) for each test session (see below). All the drugs were injected after the training/test sessions.

Objects were made out of different materials (marble, wood or plastic) and shapes, and had been tested in previous experiments to show no preference or dislike for control mice.

On the first day, mice were placed in the V-maze for habituation. On the second day, mice were placed back in the V-maze where two objects (X and A, Figure S1) were positioned at the end of the corridors. On the next day (Test 1), mice were placed in the V-maze where object X was replaced by object B. The time spent exploring object A (familiar object, Tf) and object B (novel object, Tn) was counted and the discrimination index (D.I.) was calculated as follows: D.I. = [Tn–Tf]/[Tn+Tf]. Thus, this session is the test session

#1, but at the same time is the training session for the test session #2, to take place the next day. After each test/training session, familiar objects were replaced by novel objects (B, C, D, E, F, G), following the alternation described in the drawing.

Supplemental Figures

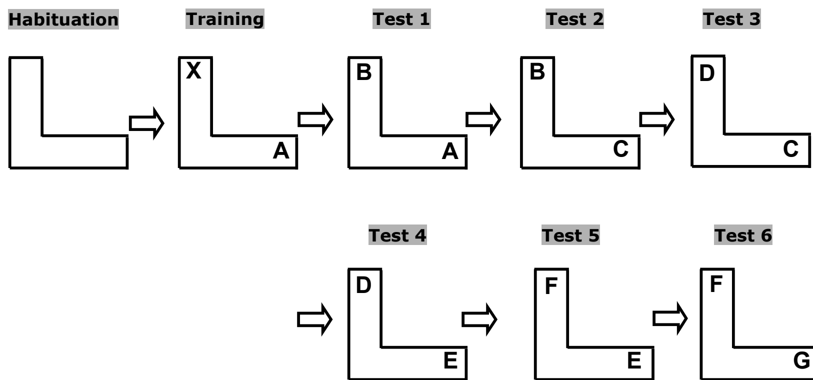

Figure S1. Schematic drawing of the object-recognition memory test used to study the effects of the chronic administration of URB597 and JZL184.

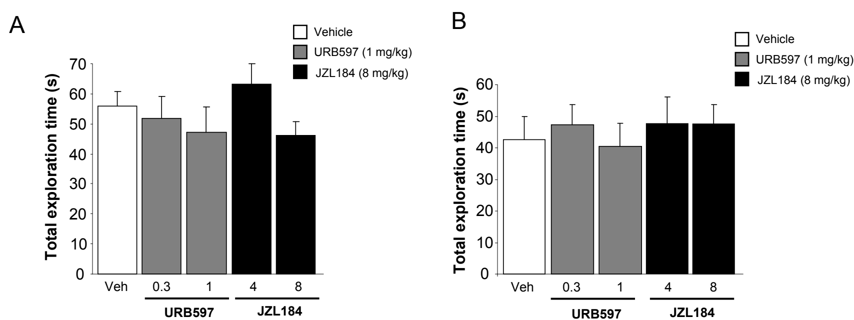

Figure S2. Total exploration time during the test session in the object-recognition memory test. (**A**) The exploration time for the novel and familiar objects was added and represented for each treatment in the short-term memory task (*n* = 8-10). (**B**) The exploration time for the novel and familiar objects was added and represented for each treatment in the long-term memory task (*n* = 8-10). Data are expressed as mean ± SEM. There were no significant differences between treatments in both tasks discarding changes in mice total activity or exploratory behavior as a consequence of the pharmacological treatment.

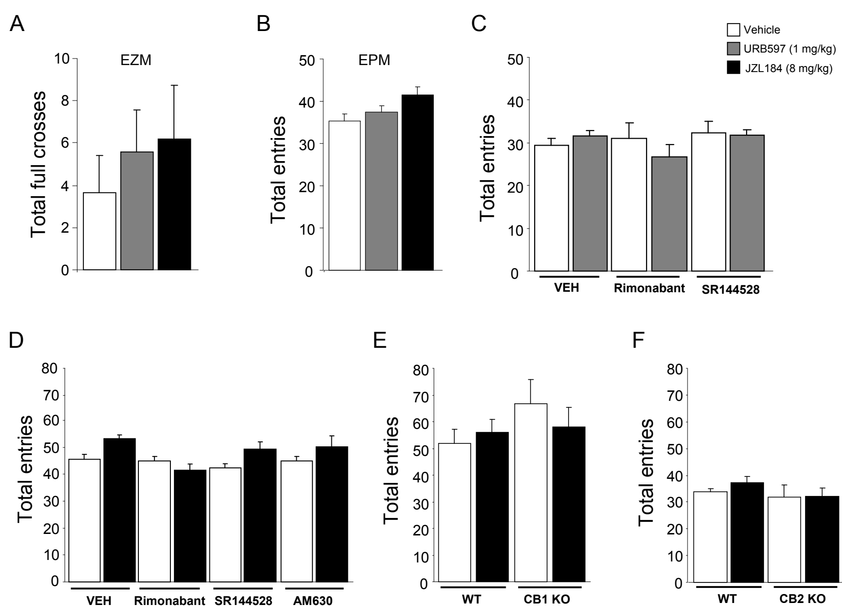

Figure S3. Total activity in the elevated zero maze (EZM) and the elevated plus maze (EPM) does not depend on the pharmacological treatment or the genotype. (**A**) Total full crosses in the EZM after the acute administration (2 h) of URB597, JZL184 or their vehicle (*n* = 10). No significant differences were observed between treatments. (**B**) Total number of entries in the EPM after the acute administration (2 h) of URB597, JZL184 or their vehicle (*n* = 10). (**C**) Total number of entries in the elevated plus maze after the acute administration (2 h) of URB597 or its vehicle in animals (*n* = 10) pre-treated (30 min) with rimonabant, SR144528 or its vehicle. (**D**) Total number of entries in the elevated plus maze after the acute administration (2 h) of JZL184 or its vehicle in animals (*n* = 10) pre-treated (30 min) with rimonabant, SR144528, AM630 or their vehicle. No differences were observed between treatments. **(E**) Total number of entries in the elevated plus maze after the acute administration (2 h) of JZL184 or its vehicle in WT and CB1 KO mice (*n* = 10). No significant differences were observed between treatments or genotypes. (**F**) Total number of entries in the elevated plus maze after the acute administration (2 h) of JZL184 or its vehicle in WT and CB2 KO mice (*n* = 10). Data are expressed as mean ± SEM. No significant differences were observed between treatments or genotypes

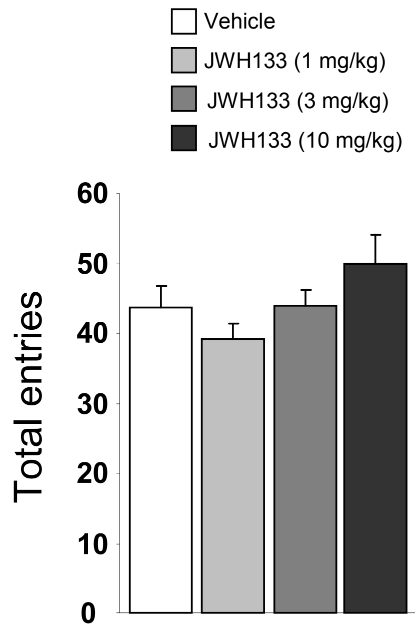

Figure S4. Total number of entries in the elevated plus maze after the acute administration (2 h) of JWH133 (1, 3, 10 mg/kg) or its vehicle (n = 10). Data are expressed as mean ± SEM. No significant differences were observed between treatments.

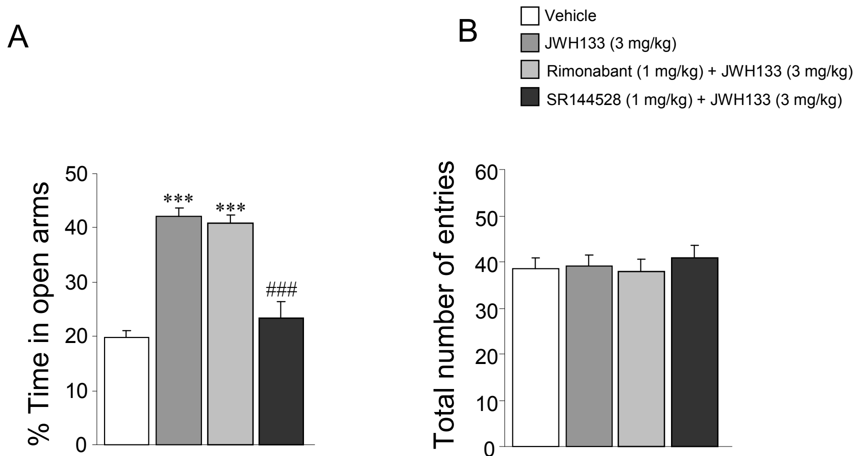

Figure S5. The anxiolytic-like effects of JWH133 are mediated by CB2 cannabinoid receptors. (**A**) The anxiolytic-like effects in the elevated plus maze (EPM) after a single administration of JWH133 (3 mg/kg) were blocked after pre-treatment with the CB2 receptor antagonist SR144258 (1 mg/kg) but were not affected by pre-treatment with the CB1 receptor antagonist rimonabant (1 mg/kg, i.p.) (*n* = 5-6 mice per group). (**B**) The total number of entries in the EPM after the acute administration of the different drugs was not modified in the same animals analyzed in **A**. Data are expressed as mean ± SEM. ***$p < 0.001$ compared to vehicle treatment; ###$p<0.001$ compared to acute JWH133, ANOVA followed by Dunnett's test.

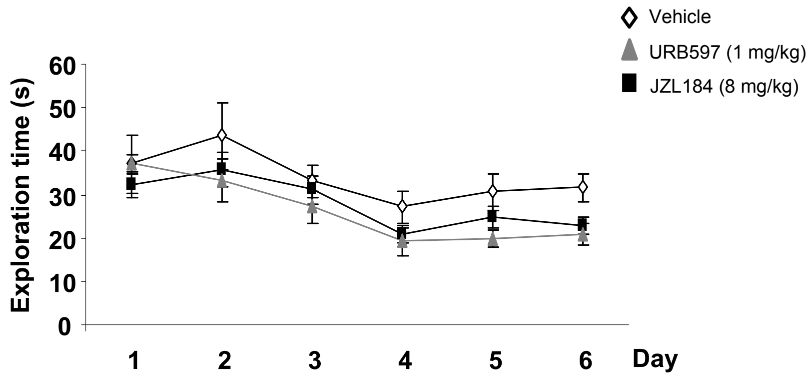

Figure S6. Total exploration time during the chronic treatment in the object-recognition memory test is represented (*n* = 10-12 mice per group). The pharmacological treatments did not modify exploratory behavior in the object-recognition task. Data are expressed as mean ± SEM.

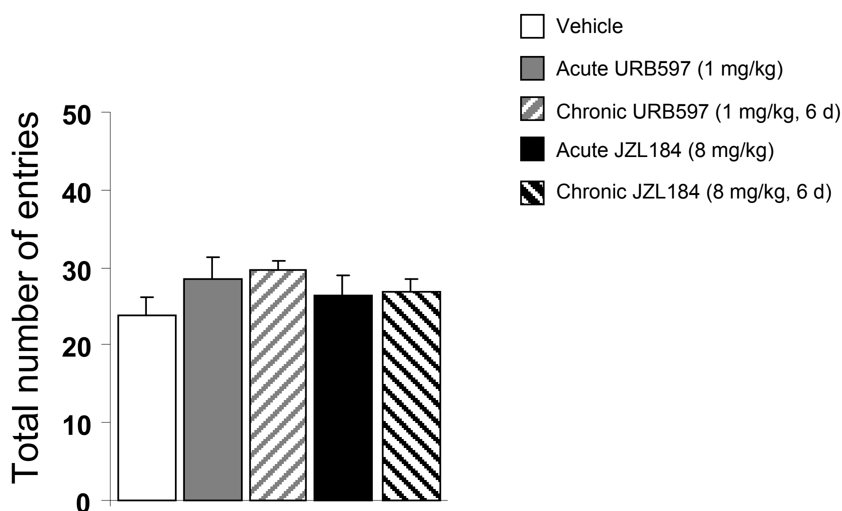

Figure S7. Total activity in the EPM does not depend on the pharmacological treatment. Total number of entries in the elevated plus maze after chronic administration of URB597 or JZL184. Mice (*n* = 15) were treated with URB597 or JZL184 for 6 days (dashed bars). Control groups were treated with vehicle (5 days) and on the sixth day they received URB597, JZL184 or vehicle (solid bars). Data are expressed as mean ± SEM. No significant differences were observed between treatments.

References

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Supplementary results

Role of mTOR in the pharmacological effects of THC

Emma Puighermanal*, Arnau Busquets-Garcia*, Rafael Maldonado and Andrés Ozaita

*Equal contribution

Role of mTOR in the pharmacological effects of THC

THC activates the mTOR signaling pathway in different brain areas

We previously reported that an acute THC treatment activates the mTORC1 signaling cascade in the hippocampus in a CB1R-dependent manner[404]. In the present study, we focused in the phosphorylation of the mTORC1 target p70S6K (Thr389), as a readout of mTORC1 activation. We treated mice with THC (10 mg/kg) or vehicle and we analyzed the levels of phospho-p70S6K 30 min after THC administration in different brain areas that express high levels of CB1R. We found that THC enhances the phosphorylation of p70S6K in the hippocampus, striatum, frontal cortex and amygdala (Figure 18).

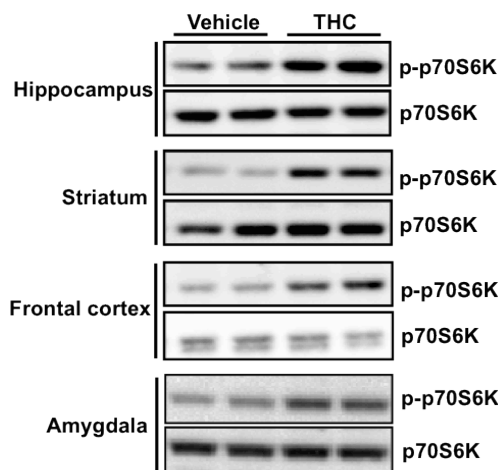


Figure 18. **Activation of mTOR by THC in different brain areas.**

Representative immunoblot showing the phosphorylation of p70S6K (Thr389) induced 30 min after THC administration (10 mg/kg) in the hippocampus, striatum, frontal cortex and amygdala (n=4-6 mice/group).

mTORC1 inhibitors, such as rapamycin and its analogues, are useful tools to elucidate the role of mTORC1. We previously used rapamycin

to abolish the memory impairment produced by THC[404]. However, the half-life of rapamycin is very long (around 60 h) and does not easily cross the blood-brain-barrier[405]. Thus, the use of local brain infusions, rapamycin-containing food, or systemic subchronic treatments (5 days) are usually required to properly inhibit mTORC1 *in vivo*[242,273,404]. Several rapamycin derivatives have been recently developed[170], such as temsirolimus (also known as CCI-779), which was the first mTORC1 inhibitor approved by the Food and Drug Administration for cancer treatment[406]. It has a comparable potency and specificity for mTORC1, but longer stability and increased solubility[407]. Therefore, we used temsirolimus to study the role of mTORC1 in the behavioral effects of THC. As mTORC1 is involved in the memory processes and its inhibition can lead to amnesic-like effects[205], we performed a dose-response experiment in the object recognition memory task to determine a dose of temsirolimus without intrinsic amnesic-like effects. For this purpose, we treated different groups of mice with the following doses of temsirolimus: 1, 5, 10, 30, or 100 mg/kg. Temsirolimus administration was performed immediately after training in the object recognition test, and the memory test was performed 24 h later. We chose the dose of 1 mg/kg, which did not show any intrinsic effect on memory (Figure 19B).

To further demonstrate that the dose of 1 mg/kg of temsirolimus was effective at inhibiting mTORC1, we pretreated mice with one single administration of temsirolimus (1 mg/kg) or vehicle 20 min prior to THC (10 mg/kg) or vehicle injection and the phosphorylation of p70S6K in the hippocampus and amygdala was analyzed 4 h later. At

this time, THC administration induces a significant mTOR-dependent increase in p70S6K (Thr389) phosphorylation[404]. We found that the non-amnesic dose of temsirolimus inhibits the increase in p70S6K phosphorylation produced by THC in the hippocampus (Figure 19A) and amygdala (Figure 21A).

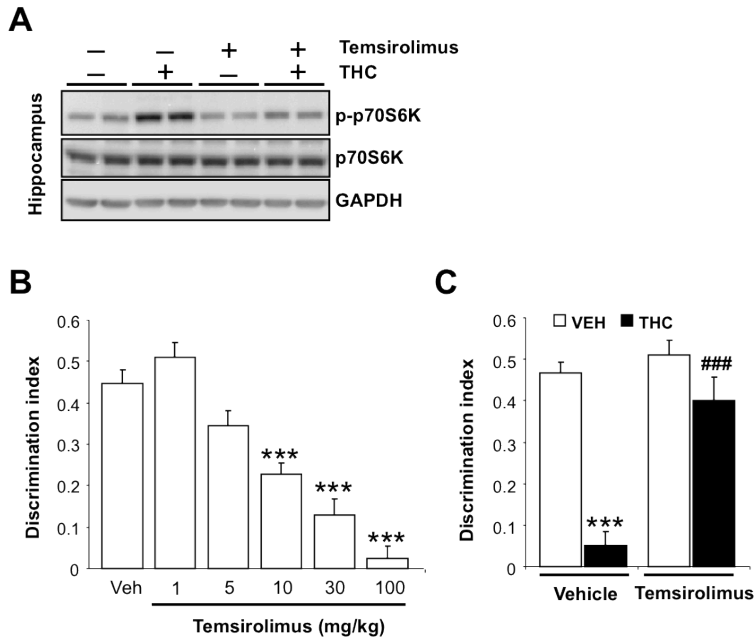


Figure 19. **mTOR inhibition by temsirolimus prevents the memory impairment produced by THC.**

A, Representative immunoblot of phosphorylation of p70S6K (Thr389) induced 4 h after THC administration (10 mg/kg) in the hippocampus and blockade of pretreatment of temsirolimus (1 mg/kg) 20 min before THC (n=5 mice/group). B, Discrimination index values of the dose-dependency effect of posttraining administration of temsirolimus in the object recognition memory test. C, Prevention of the memory impairment produced by THC (10 mg/kg) in the object recognition memory test by pretreatment of temsirolimus (1 mg/kg) 20 min before THC (n=8 mice/group).

mTOR participates in both amnesic-like and anxiogenic-like effects produced by THC

We used different behavioral paradigms to evaluate the possible involvement of the mTORC1 signaling pathway in some of the pharmacological effects of THC. On one hand, we used the object recognition memory task to determine if a single administration of temsirolimus could also prevent the memory impairment produced by THC, as we previously described by using the subchronic pretreatment with rapamycin[404]. For this purpose, mice were pretreated with temsirolimus (1 mg/kg) or vehicle after training and THC (10 mg/kg) or vehicle was administered 20 min later. We observed that mTORC1 inhibition fully prevented THC-induced amnesic-like effects (Figure 19C).

Due to the regional widespread activation of mTORC1 in the brain after THC administration, we also investigated if this signaling pathway participates in other behavioral effects of THC. For this purpose, we pretreated mice with temsirolimus (1 mg/kg) or vehicle and THC (10 mg/kg) or vehicle was administered 20 min later. Mice were analyzed afterwards for body temperature, locomotion and antinociception. Rectal temperature was measured in (i) basal conditions, (ii) before and (iii) 2 h after THC administration. Locomotor activity was measured 4 h after THC administration during 20 min, and the antinociceptive effects were assessed immediately after the locomotor test using the acetic acid test (number of writhings produced by an intraperitoneal injection of 0.8% acetic acid solution) during 15 min. We found that mTOR is not involved either in the hypothermic (Figure 20A) or the hypolocomotor effects

(Figure 20B) of THC since the pretreatment with temsirolimus did not block these responses. In addition, the antinociceptive effects of THC in the writhing test were not modulated by the pretreatment with temsirolimus, indicating that mTORC1 does not participate in these responses (Figure 20C).

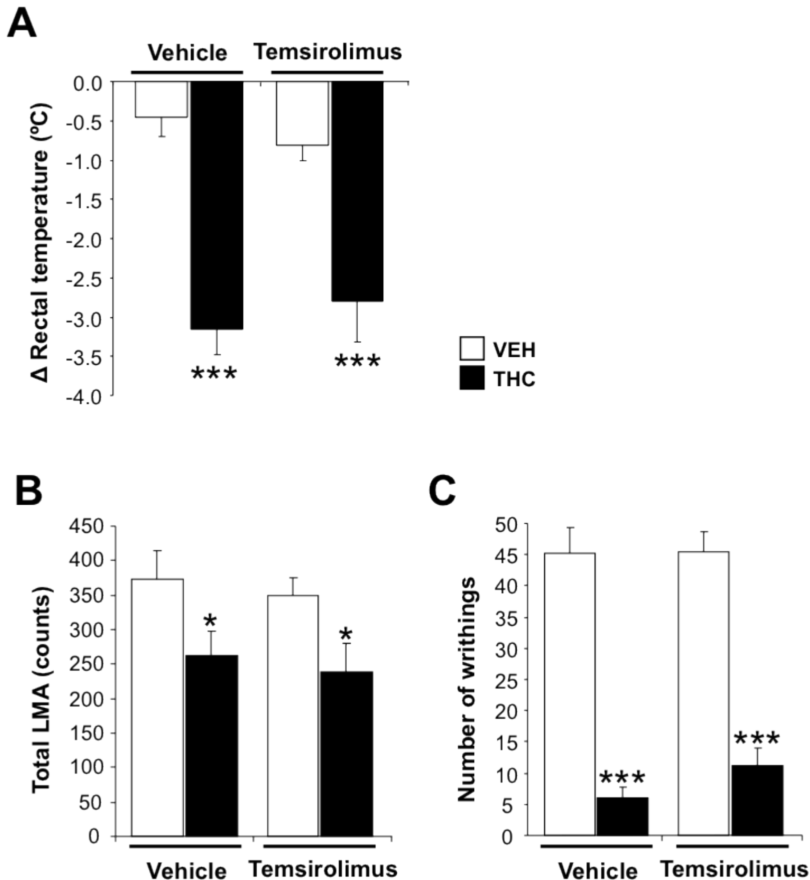


Figure 20. mTOR is not involved in the hypothermic, hypolocomotor, or antinociceptive effects of THC.

Temsirolimus administration (1 mg/kg) 20 min before THC (10 mg/kg) does not block the hypothermic effect produced by THC 2 h later (A), the hypolomotor effect induced by THC 4 h later (B), or the antinociceptive effect observed as a reduction in the number of writhings in the acetic acid test 4 h after THC administration (C) (n=10 mice/group). LMA, Locomotor activity.

Finally, we addressed whether mTOR was involved in the anxiety-related responses produced by THC. As mentioned in the introduction of this thesis, cannabinoids elicit biphasic effects on anxiety depending on the experimental conditions. Thus, low doses of THC usually produce an anxiolytic-like effect, whereas high doses induce the opposite response[89,90,408,409]. However, the mechanisms underlying the anxiety-related effects of cannabinoids remain to be characterized. To test the involvement of the mTORC1 signaling pathway, two distinct doses of THC were used: the dose of 10 mg/kg, which is known to produce anxiogenic-like effects, and the dose of 0.3 mg/kg, which produces anxiolytic-like responses. We pretreated mice with temsirolimus (1 mg/kg) or vehicle 20 min before THC (0.3 or 10 mg/kg) administration. Anxiety-like responses were evaluated in the elevated plus maze 4 h after THC administration. The dose of 10 mg/kg of THC produced a clear anxiogenic-like effect since both the percentage of time spent in the open arms (Figure 21B) and the percentage of visits in the open arms (Figure 21C) were lower in the THC-treated animals compared with the vehicle group. Interestingly, temsirolimus attenuated the anxiogenic-like effect produced by THC, indicating that mTORC1 participates in this response (Figures 21B & 21C). However, mTOR was not involved in the anxiolytic-like response induced by a low dose of THC (0.3 mg/kg), which produced an increase in the percentage of time spent in the open arms (Figure 21D) and in the percentage of visits to the open arms (Figure 21E). In agreement, only the administration of THC at the dose of 10 mg/kg (Figure 21A), but not at 0.3 mg/kg (data not shown), activated the mTORC1 effector p70S6K in the amygdala.

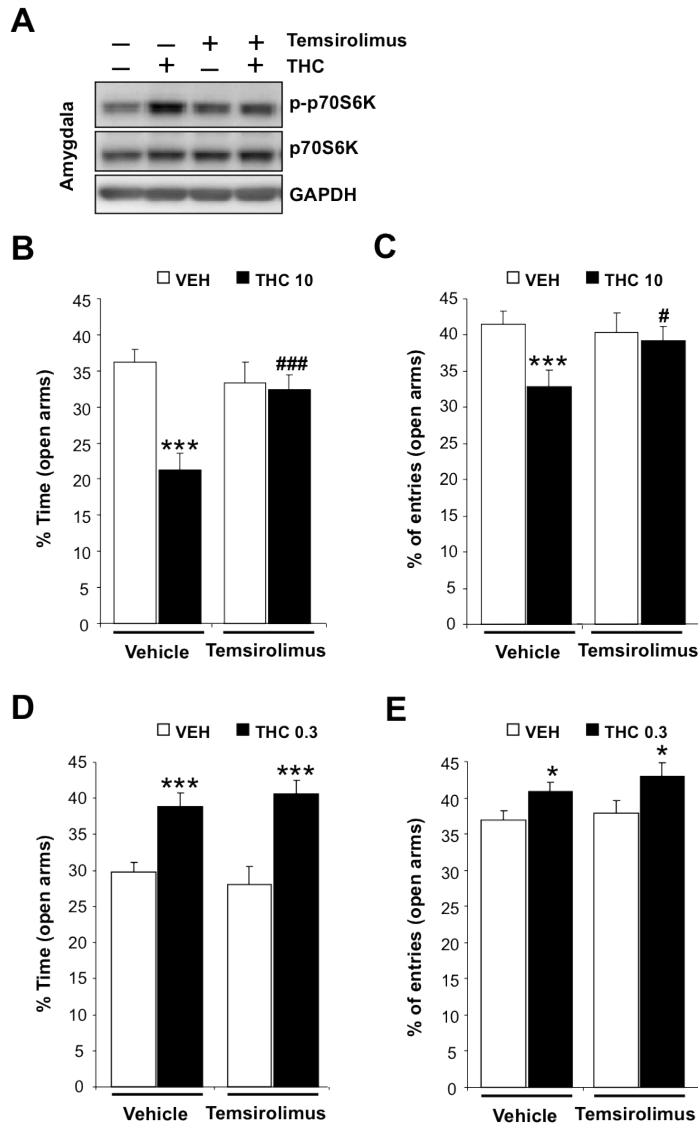


Figure 21. **mTOR participates in the anxiogenic-, but not in the anxiolytic-like effects, produced by THC.**

A, Representative immunoblot of phosphorylation of p70S6K (Thr389) induced 4 h after THC administration (10 mg/kg) in the amygdala and blockade of pretreatment of temsirolimus (1 mg/kg) 20 min before THC (n=5 mice/group). Temsirolimus administration (1 mg/kg) 20 min prior to THC prevents the anxiogenic-like effect produced by a high dose of THC (10 mg/kg) (B and C), but not the anxiolytic-like effect of a low dose (0.3 mg/kg) (D and E), 4 h after THC treatment in the elevated plus maze (n=10 mice/group).

Lack of tolerance to the memory impairment and anxiogenic-like responses after THC chronic treatment

Repeated exposure to cannabinoids produces profound tolerance that correlates with desensitization and downregulation of CB1R in different brain structures[130,410]. To test if chronic THC administration induces tolerance to the mTORC1 signaling pathway activation and to the disruptive effects in memory consolidation, mice received THC (10 mg/kg) once daily during 6 days and the object recognition memory test was performed as described previously in the introduction (see 3.2.1 section and Figure 14). After the 6-days chronic THC treatment, mice performance in the object recognition test was measured for seven additional days, where mice received vehicle instead of THC. The discrimination index of each test session was calculated. Rectal temperature was also evaluated before and 30 min after each THC administration. A clear tolerance to the hypothermic effect appeared after the third day of THC administration (Figure 22C). Interestingly, chronic exposure to THC did not induce tolerance to the memory impairment in the object recognition test (Figure 22A). The THC-treated mice showed low discrimination index values along the 6 days of THC administration, compared to the vehicle-treated animals. Surprisingly, mice required around 4 or 5 days after the cessation of THC to totally recover from the cognitive impairment (Figure 22A).

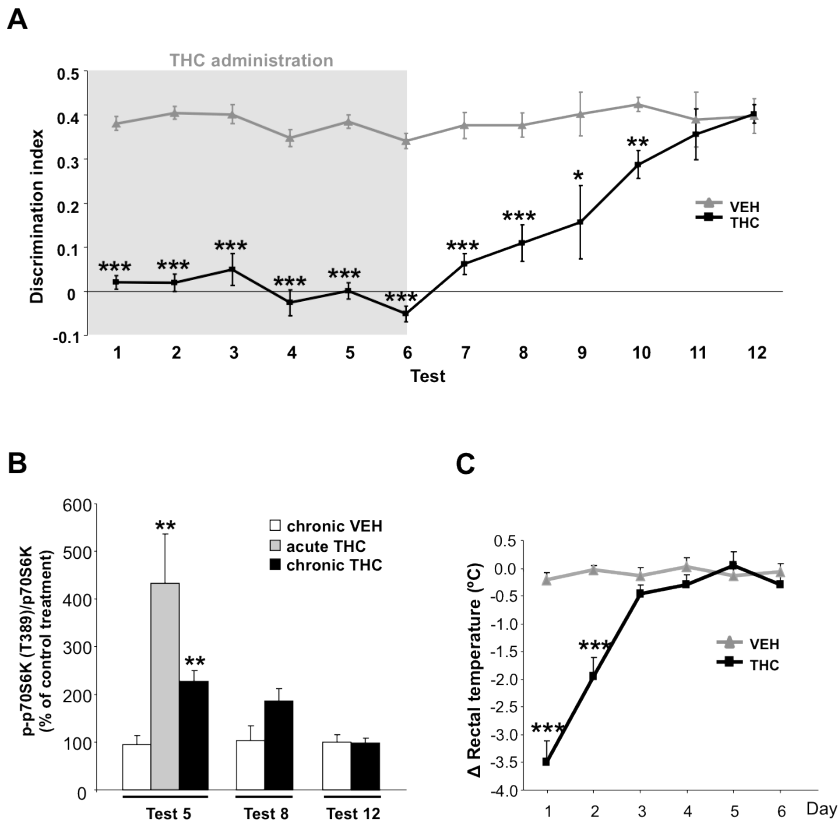


Figure 22. **Chronic THC treatment does not produce tolerance to the memory impairment and mTORC1 activation, while it develops tolerance to hypothermia.**

A, Discrimination index values of each test session of mice treated chronically with THC (10 mg/kg, 6 days, once daily) (black line) or vehicle (gray line) in the object recognition memory test. B, Quantitative analysis of immunoblots of p70S6K (Thr389) phosphorylation in the hippocampus of mice treated acutely or chronically with THC and sacrificed 30 min after test 5, 8 or 12 of the object recognition memory test. C, Changes in rectal temperature 2 h after each THC administration compared to basal along the 6 days chronic treatment (n=10-20 mice/group).

The phosphorylation levels of p70S6K were analyzed in the hippocampus of mice treated with THC or vehicle at 3 different stages: one group of animals was sacrificed 30 min after the last THC administration immediately after the performance of the test 5;

another group was sacrificed immediately after the test 8, when THC-treated mice still showed low discrimination index values even after the cessation of the drug administration; the third group was analyzed after they completely recovered from the memory impairment, immediately after the performance of the test 12. A slight tolerance to THC-induced p70S6K activation was revealed after the chronic treatment (Figure 22B), although p70S6K was still activated at the 6th day of THC administration, which correlates with the disruptive effects in memory. However, this activation was less pronounced than after an acute THC treatment. Interestingly, 3 days after THC cessation, p70S6K was slightly, although non-significantly, increased in those mice that still showed memory impairment (Figure 22B). Finally, there were no differences in the phosphorylation of p70S6K after the total recovery of the THC-induced amnesic-like effects compared to vehicle (test 12) (Figure 22B).

To further characterize the involvement of the mTORC1 signaling pathway in the memory impairment produced by chronic exposure to THC, we pretreated mice with temsirolimus (1 mg/kg) 20 min before each THC administration (6 days, once daily) and the object recognition test was performed as previously described. We found that temsirolimus totally prevented the amnesic-like effects produced by THC along all the tests performed (Figure 23). In addition, the chronic treatment of temsirolimus had no intrinsic effects in memory formation (Figure 23).

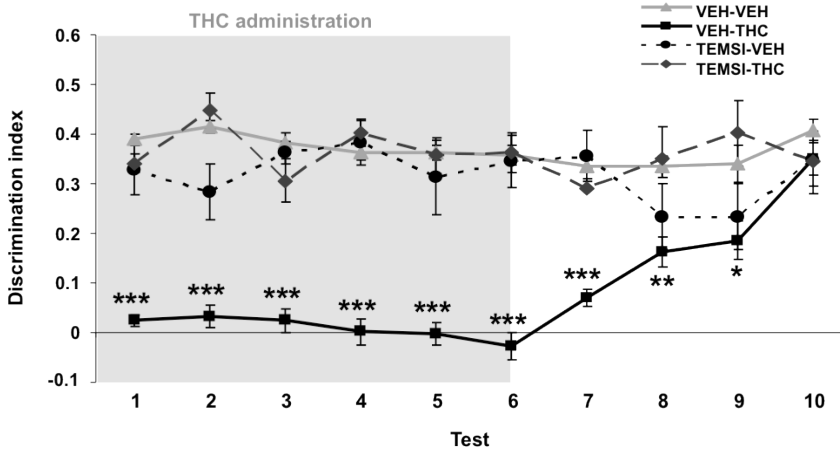
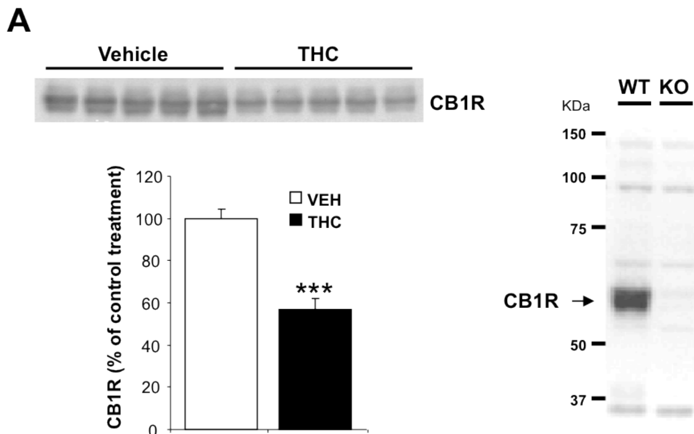


Figure 23. **mTOR inhibition by temsirolimus prevents the THC-induced long-lasting memory impairment.**

Discrimination index values of each test session of mice chronically treated with THC (10 mg/kg, 6 days, once daily) or vehicle (gray lines) in the object recognition memory test. Temsirolimus administration (1 mg/kg) 20 min prior to THC prevents the memory impairment produced by the cannabinoid (n=10-20 mice/group).

In order to further characterize the molecular signaling events triggered after a chronic THC exposure, we analyzed the CB1R expression in the hippocampus of mice that performed the object recognition task and were sacrificed 30 min after the last THC chronic administration. Using immunoblot and immunofluorescence techniques, we found a downregulation of around 40% of the CB1R expression (Figures 24A and 24B). Moreover, mice chronically treated with THC (10 mg/kg, 6 days, once daily) were exposed to several behavioral measurements to evaluate locomotion, anxiety-like responses (elevated plus maze and elevated zero maze), and antinociception (acetic acid test) 4 h after the last THC administration. As expected, a profound tolerance to the hypolocomotor (Figure 25A) and antinociceptive effects (Figure 25B) of THC was found.

Interestingly, no tolerance to the anxiogenic-like responses in the elevated zero maze (Figure 26A) and in the elevated plus maze (Figures 26B and 26C) was observed after a THC (10 mg/kg) chronic treatment. Moreover, the anxiolytic-like effect of the low dose of THC (0.3 mg/kg) was also preserved after a chronic treatment of THC at 0.3 mg/kg (Figure 27A). However, an acute dose of THC (0.3 mg/kg) did not induce anxiolytic-like effects in mice chronically treated with THC (10 mg/kg) (Figure 27B).



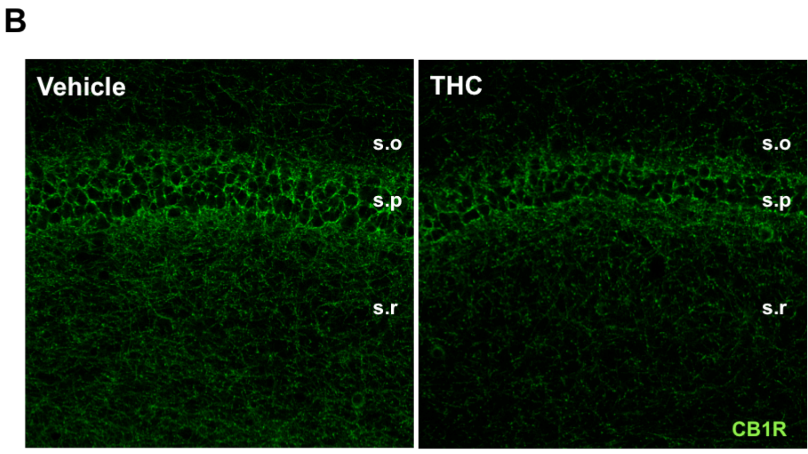


Figure 24. **THC chronic treatment downregulates CB1R in the hippocampus.**

A, Immunoblot and quantitative analysis of CB1R expression of mice chronically treated with vehicle or THC (10 mg/kg, 6 days, once daily). The specificity of the antibody anti-CB1R was determined by immunoblot in WT and CB1R$^{-/-}$ hippocampal mice. B, Immunofluorescence analysis of CB1R expression in the CA1 region of the hippocampus of mice chronically treated with vehicle or THC (10 mg/kg, 6 days, once daily) (n=5 mice/group). s.o, stratum oriens; s.p, stratum pyramidale; s.r, stratum radiatum.

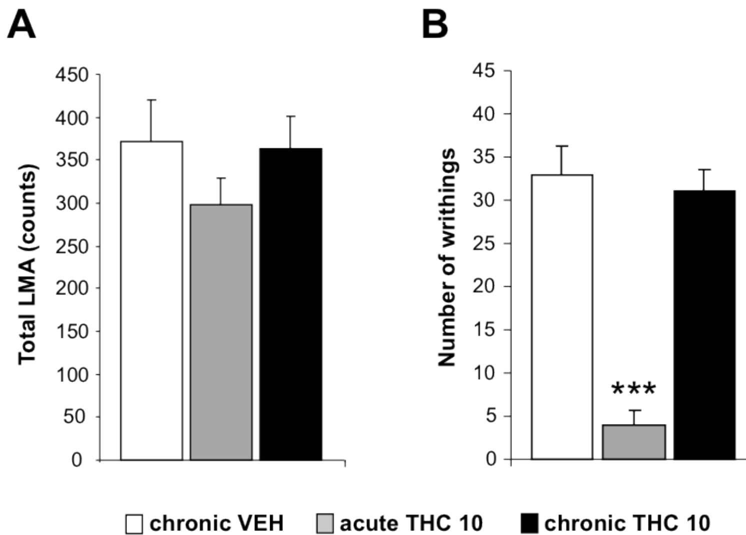


Figure 25. **Tolerance to hypolocomotion and antinociception after chronic THC exposure.**

Tolerance to the hypolomotor effect (A) or antinociceptive effect in the acetic acid test (B) 4 h after the last chronic THC administration (10 mg/kg, 6 days, once daily) (n=10 mice/group). LMA, Locomotor activity.

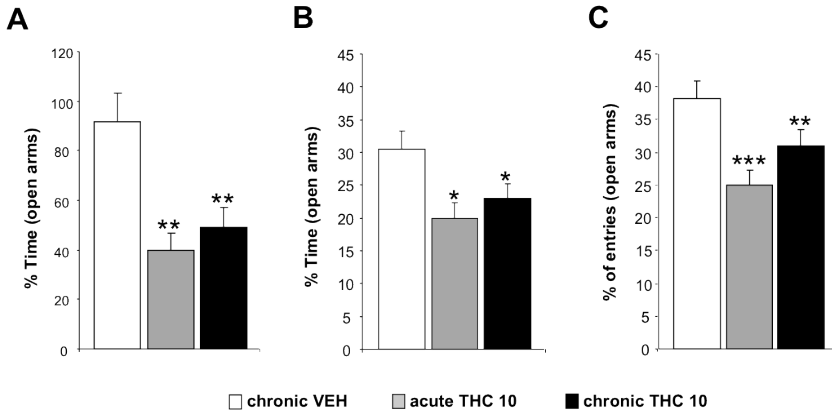


Figure 26. **The anxiogenic-like effect produced by a high dose of THC still persists after a chronic treatment.**

No tolerance to the anxiogenic-like responses appears 4 h after the last THC administration of a chronic exposure (10 mg/kg, 6 days, once daily), as it is shown by a reduction in the time spent in the open arms of the elevated zero maze (A) and the elevated plus maze (B), as well as a decrease in the number of entries in the open arms in the elevated plus maze (n=10 mice/group).

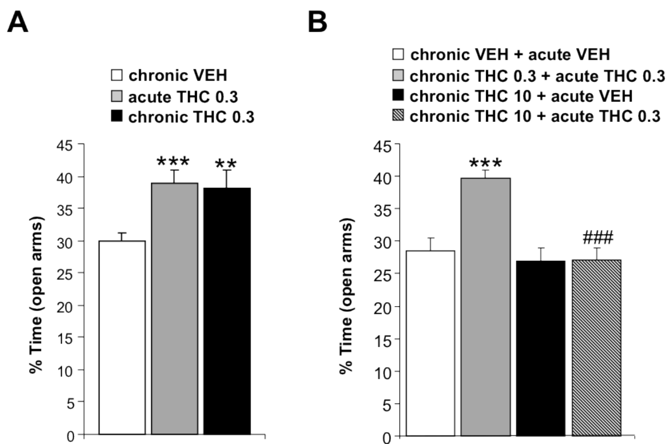


Figure 27. **The anxiolytic-like effect produced by a low dose of THC still persists after a chronic treatment of THC 0.3 mg/kg, but not after a chronic exposure of THC 10 mg/kg.**

A, No tolerance to the anxiolytic-like responses appears 4 h after the last THC administration of a chronic exposure (0.3 mg/kg, 6 days, once daily), as it is shown

by a reduction in the time spent in the open arms of the elevated plus maze. B, Tolerance to the anxiolytic-like effects appears 4 h after an acute THC administration (0.3 mg/kg) following a chronic THC exposure (10 mg/kg, 5 days, once daily) in the elevated plus maze (n=10 mice/group).

Discussion

A. NEUROBIOLOGICAL SUBSTRATES UNDERLYING THE MEMORY IMPAIRMENT PRODUCED BY CANNABINOIDS

Molecular basis of amnesic-like effects produced by CB1R agonists

Understanding the complexity of the signaling pathways related to cannabinoid receptor activation will be helpful to elucidate the neurobiological mechanisms underlying the role of cannabinoids in the regulation of memory processes. We found that CB1R stimulation, either by an exogenous cannabinoid (THC) or by an endocannabinoid (AEA), triggers the activation of the Akt/mTORC1 signaling pathway in the hippocampus. This effect correlated with memory impairment in two different cognition paradigms, the object recognition and the context recognition test, because the memory impairment produced by cannabinoids was prevented by the mTORC1 inhibitor rapamycin. On the contrary, the endocannabinoid 2-AG did not induce mTOR activation and did not produce memory disruption.

We described that a dose-dependent administration of THC produces mTORC1 activation through CB1R. However, mTORC1 can be activated by different manners. We found that THC activates Akt, the upstream regulator of mTORC1, via PI3K as it was blocked by the inhibitor of this kinase, wortmannin. Moreover, THC administration inhibits GSK-3, probably through the Akt-mediated phosphorylation. Once Akt is activated, it may activate mTORC1 by different mechanisms: (i) by direct mTOR phosphorylation (Ser2448), (ii) by inhibiting GSK-3, and thus preventing the activation of the mTORC1 negative regulator TSC2, (iii) by directly inhibiting TSC2, and (iv) by phosphorylating PRAS40, a negative regulator of mTORC1. In

parallel, ERK1/2 signaling pathway activation positively modulates mTORC1 activity[411]. Therefore, we have described some of the kinases that participate in the regulation of mTORC1 in the hippocampus of mice treated which THC.

One of the most important functions attributed to mTORC1 is the control of protein synthesis. mTORC1 contributes to overall cap-dependent translation by phosphorylating the effector 4E-BP, and in combination with the activation of the other target p70S6K, might further enhance the translation efficiency, by up-regulating ribosomal proteins and translational factors[185]. We found that THC promotes the phosphorylation of both effectors in a time-dependent manner. Strikingly, the phosphorylation of p70S6K is the most sensitive effect of THC among the effects on other proteins analyzed, being active between 30 min and 4 h after THC treatment. Apart from the activation of mTORC1 and its two downstream targets, which suggests that protein translation could be promoted, we found that THC induces the phosphorylation of some components of the translational apparatus and factors that participate in the initiation step of translation. These proteins are the ribosomal protein S6, the eukaryotic initiation factors eIF4E, eIF4G, and eIF4B. On the other hand, ERK1/2 also cooperates together with mTOR to promote protein synthesis, as the pretreatment of the ERK1/2 signaling inhibitor SL327 prevented the phosphorylation of eIF4E induced by THC. Altogether, these results indicate that translational processes might occur in neurons upon THC administration. Interestingly, non-amnesic doses of anisomycin prevented the disruptive memory effects that THC produces in the object recognition task.

Immunofluorescence analysis revealed that mTORC1 activation after THC treatment occurs mainly in the CA1 region of the hippocampus, a region tightly involved in memory processes. In particular, the increase in p70S6K phosphorylation takes place in the soma and dendrites of pyramidal neurons, and did not colocalize with the CB1R staining at the presynaptic compartments. As the THC-induced ERK1/2 phosphorylation shows a similar immunostaining pattern in the hippocampus and has been reported to be mediated by NMDARs[403], we have investigated whether glutamatergic mechanisms could play a role in the mTORC1 signaling activation promoted by THC. We found that the pretreatment with MK801, a NMDAR antagonist, partially attenuated the THC-induced phosphorylation of p70S6K at Thr389 site and totally blocked the phosphorylation at Thr421/Ser424 residues, as well as the phosphorylation of eIF4E, probably by preventing ERK1/2 activation. Moreover, pretreatment with MK801 abolished the memory impairment produced by THC in the object recognition test. In agreement, enhanced NMDAR-mediated synaptic transmission in the IRSp53 knockout mice, which exhibits a marked increase in LTP at Schaffer collateral-CA1 pyramidal synapses, is associated with impaired memory in Morris water maze and object recognition tasks[412]. In the same line, mice lacking dystrophin protein, which is enriched in the postsynaptic densities of pyramidal neurons, exhibit enhancement of CA1 hippocampal LTP and impaired long-term memory in the object recognition task, probably due to a decrease in the threshold for NMDAR activation[413]. Several studies support the idea that enhanced LTP is not always correlated with enhanced memory, and thus, numerous mutant mice showing increased LTP display memory

impairments[304]. Altogether, these studies suggest that enhanced NMDAR-mediated LTP could lead to altered memory formation.

As we observed that glutamatergic transmission plays a role in both mTORC1 and ERK1/2 signaling cascades, we evaluated whether CB1Rs present in glutamatergic or GABAergic neurons are responsible of the molecular mechanisms triggered by THC administration. Taking advantage of the CB1R conditional knockout mice that lack CB1R either in glutamatergic or GABAergic cells, we found that only CB1Rs expressed in GABAergic neurons are responsible of the THC-driven p70S6K phosphorylation. In agreement, mice lacking CB1R in GABAergic terminals do not exhibit memory impairment in the object recognition test upon THC administration. This is the first time that a functional role has been attributed to CB1Rs located in GABAergic neurons, as the other pharmacological effects of THC have been linked at the present moment with CB1Rs expressed in principal glutamatergic neurons[414]. CB1Rs located in GABAergic axon terminals are activated by lower concentrations of cannabinoid receptor agonists than CB1Rs expressed in glutamatergic terminals[415]. In addition, CB1R expression is lower in glutamatergic neurons than in GABAergic neurons in the hippocampus[376,377]. However, unexpectedly, GABA does not appear to play a major role in the classical tetrad pharmacological effects (catalepsy, hypothermia, hypolocomotion, and antinociception) produced by acute cannabinoid administration. Strikingly, THC produces full tetrad effects in the conditional knockout mice lacking CB1R in GABAergic terminals[414]. Likewise, the GABA-A receptor antagonist bicuculline, does not block the THC-induced tetrad

effects[416]. Since THC would preferentially decrease GABA release by acting at CB1Rs located on interneurons and has less effect on glutamate release, memory impairment could be a consequence of a disruption of hippocampal network activity, which is mediated by synchronized GABAergic discharges and has been shown to be disrupted by cannabinoids[362,417].

The requirement of both CB1Rs expressed on GABAergic neurons and NMDARs in THC-induced memory impairment points to a possible deregulation of the inhibitory/excitatory transmission promoted by this cannabinoid agonist. Taking into account the differential CB1R density on the terminals of the different neuronal populations in the hippocampus, one possible explanation could be that THC would mainly reduce GABA release, and thereby indirectly increase glutamatergic transmission, which would activate mTOR in the somatodendritic compartment of pyramidal cells through NMDARs. In this regard, both a reduction in GABA and a concomitant increase in glutamate release have been observed in the rat prefrontal cortex after THC administration, which is also involved in memory processes[400]. In agreement, mutant mice overexpressing the GABA transporter type 1, which removes GABA from the synaptic cleft, displayed impaired object recognition[418]. Moreover, conditional mutant mice in which GABA transporter type 1 has been expressed selectively in neurons show memory impairment in the same behavioral test[419]. These results indicate that decreased GABAergic tone, as a consequence of increased clearance of GABA from the synaptic cleft, alters memory in the object recognition task. Electrophysiological studies show that repetitive low-frequency

synaptic stimulation promotes persistent up-regulation of endocannabinoid signaling at CA1 GABAergic synapses. Thus, LTD is induced at inhibitory synapses while LTP is facilitated at glutamatergic synapses[420]. In agreement, THC has been shown to act as a full agonist at CB1R located on GABAergic terminals in the hippocampus, while acting as a partial agonist at CB1R present on glutamatergic terminals[399]. In summary, these results suggest that a possible unbalance between excitatory and inhibitory transmission produced by cannabinoids could promote the activation of mTORC1 signaling and finally lead to memory impairment.

Distinct CB1R ligands differentially control memory processes

We also characterized the different role of the two main endocannabinoids, AEA and 2-AG, in the control of memory consolidation. While AEA behaves similar to THC in terms of altering two memory tasks and activating mTOR in the hippocampus, 2-AG does not produce any of these effects. This dichotomy could be explained by the following mechanisms:

(i) Anatomical segregation of the enzymes involved in the synthesis and degradation of endocannabinoids

The specificity of cannabinoid receptor-mediated intracellular signal transduction might rely on the extracellular environment of the targeted cells. Thus, on-demand release, retrograde trafficking, and rapid degradation of endocannabinoids within a highly localized space provide an effective control of cannabinoid-mediated responses. One

hypothesis that might explain this dichotomy is the anatomical segregation of the enzymes involved in the synthesis and degradation of these endocannabinoids. As THC-induced amnesic-like effects are mediated by CB1R located in GABAergic neurons, one possibility could be that the synthesis and degradation of AEA take place in the vicinity of this neuronal population. In contrast, the metabolism of 2-AG might occur in the vicinity of glutamatergic terminals. Two isoforms of DAGL, the major synthetic enzyme for 2-AG, have been cloned: DAGLα and DAGLβ, although the DAGLα seems to be sufficient for most endocannabinoid signaling[421]. In rodents, DAGLα is mainly expressed in postsynaptic spines directly across from excitatory afferent terminals and it is rarely located near CB1R-expressing perisomatic inhibitory terminals[376,422] at which DSI occurs[423,424]. In agreement, immunostaining of DAGLα in postmortem human hippocampus showed a laminar pattern corresponding to the termination zones of glutamatergic pathways. Electron microscopy analysis revealed that this pattern of staining corresponds to DAGLα present in dendritic spine heads[425]. MAGL, the predominant enzyme inactivating 2-AG, was enriched in hippocampal excitatory axon terminals in agreement with the previous ultrastructural analyses performed in rodents[425]. Together, these studies indicate that 2-AG is synthesized postsynaptically and inactivated presynaptically mainly at glutamatergic synapses, near CB1R, and suggest that the entire molecular architecture of retrograde 2-AG signaling at excitatory synapses is evolutionarily conserved across species.

In contrast, NAPE-PLD, the main enzyme synthesizing AEA, is found in some glutamatergic terminals, but not in postsynaptic pyramidal cell bodies[426,427]. Thus, while DGLα is predominantly found in dendritic spines, NAPE-PLD is mainly located in axons, although an intense labeling has been reported in neuronal perikarya and proximal dendrites of hippocampal pyramidal cells[428]. However, additional metabolic pathways responsible for AEA production have to be taken into account, since the NAPE-PLD knockout mice have similar brain AEA levels as the wildtype mice[48,429]. On the other hand, FAAH, the AEA-metabolizing enzyme, is located selectively in the somatodendritic compartment of principal neurons, mainly on intracellular calcium stores[53,428,430]. The complementary distribution of FAAH and MAGL, together with the distinct distribution of NAPE-PLD and DAGLα, suggest that AEA and 2-AG signaling might regulate different functions that are spatially segregated.

(ii) Functional selectivity of CB1R agonists

Another possible explanation for the dichotomy of both endocannabinoids in the modulation of memory consolidation could be due to a differential functional selectivity. Indeed, it has been reported that selective agonists for a particular receptor might differentially alter coupling to distinct signaling pathways[431,432]. In this sense, distinct intrinsic efficacy or duration of the signal may account for this functional segregation. AEA has been reported to act as a partial agonist at CB1R, while 2-AG acts as a full agonist[433,434]. The efficacy of CB1R stimulation as well as the duration may trigger the activation of distinct intracellular signaling events in the neuron. Thus,

in spite of the possible anatomical segregation of the enzymes, if both endocannabinoids would bind to CB1R located in the same cell type, it is tempting to speculate that AEA, but not 2-AG, would promote mTORC1 signaling activation, probably due to different efficacy to modulate intracellular responses. In this regard, THC would activate CB1R in a similar manner as AEA does.

It is noteworthy that the specificity of cannabinoid receptor-mediated responses largely relies on a wide variety of factors, such as the type of ligand, the precise location of the receptor and its proximity to certain signaling complexes, and the expression of neighboring receptors, G proteins, and other signaling partners. Thus, functional selectivity might account for CB1R agonists-triggering different signaling events in the cell[69]. Electrophysiological data support the fact that in the same target cell, the difference between eCB-LTD and eCB-DSI relies on the duration of CB1R activity, which engages distinct signaling events in the neuron leading to a short or long suppression of neurotransmitter release[45].

On the other hand, distinct ligands acting on CB1R can yield different intracellular mechanisms. Thus, the comparison of the transcriptional regulation mediated by HU-210 and CP-55,940 reveals unpredicted different profiles of these two ligands, which are usually viewed as reference CB1R agonists[435]. In addition, it has been demonstrated that, among the different MAPK family members, HU-210 is more efficacious in increasing ERK1/2 phosphorylation, while CP-55,940 displays a higher efficacy to activate c-Jun N-terminal kinase[436]. Another example of functional selectivity is the difference relying on the potency of the cannabinoid receptor agonist. Whilst 2-AG is more

potent in activating ERK than inhibiting adenylyl cyclase, other two agonists, noladin ether and CP-55,940, preferentially inhibit adenylyl cyclase[437].

Moreover, the same ligand through the same receptor can lead to distinct intracellular outcomes in different cells. Strikingly, the antitumoral action of THC has been attributed to the inhibition of the Akt/mTORC1 signaling axis in different types of tumor cells, including glioma and astrocytoma, without affecting nontransformed cells[68]. However, we have reported an activation of the Akt/mTORC1 signaling cascade in neurons. Thus, depending on the cell type, an even opposite signal transduction response can be triggered by the same CB1R agonist.

(iii) THC and AEA occlude 2-AG-mediated responses

Another possible mechanism could be that THC and AEA may occlude the 2-AG tone due to their lower intrinsic efficacy, as suggested by others[438], and thus preventing 2-AG-mediated effects. This hypothesis is unlikely because the endocannabinoid tone occlusion by pretreatment of rimonabant did not increase mTORC1 activity and did not produce memory impairment.

Although we have segregated the functional role of the two main endocannabinoids in the control of memory consolidation, cannabinoids might play a different role in other stages of memory such as acquisition, retrieval, extinction, or reconsolidation. Moreover, depending on the memory test performed, different brain areas might

be involved and thus, cannabinoids could differentially participate in other memory processes.

Dysregulation of mTOR and protein synthesis lead to memory impairment

We have demonstrated that non-amnesic doses of anisomycin, a protein synthesis inhibitor, prevent the impaired memory produced by THC in the object recognition paradigm. A highly regulated control of protein translation is crucial for an appropriate memory function because either an enhancement or reduction can alter synaptic plasticity and memory. In most cases, excessive protein synthesis results in aberrant, rather than "improved", plasticity and cognition. This indicates that mTORC1 activity has to be tightly regulated acting as a sensor carefully modulating translational rates during precise temporal windows, rather than simply acting as a translational "on/off" switch. One particular issue that has not been yet elucidated is whether translational control regulated by mTORC1 is more important for promoting translation of a specific subset of mRNAs than for general translation. Indeed, the identity of the transcripts translated in response to mTORC1 activation remains poorly characterized. Some of the proteins that are found to be locally upregulated after the induction of LTP or LTD are CaMKIIα, PKMζ, Arc, and MAP1B[238,439-441]. Thus, it is plausible that requirement of mTORC1 in both synaptic plasticity and memory entails translational control of specific mRNAs. Therefore, it would be of interest to match the translational regulatory mechanisms with a subset of translated mRNAs and with mnemonic processes upon CB1R

stimulation. Thus, determining the identity of the proteins synthesized in response to THC administration will shed light on the mechanisms responsible for memory impairment.

mTORC1 signaling seems to be necessary for long-term memory formation. Rapamycin treatment impaired memory performance 24 h, but not 3 h, after training, indicating that the animal could form short-term memory[234]. Therefore, rapamycin particularly disrupts the consolidation of memory in a long-term manner. Moreover, other studies have shown that rapamycin alters memory consolidation in different behavioral tasks that depend on distinct brain areas[346,348,442-444]. In agreement, our results indicate that high doses of rapamycin or temsirolimus produce impaired memory in the object recognition task. Surprisingly, a recent study in humans reports that pharmacological blocking of mTORC1 by everolimus enhances cognition[445]. This is the only current study showing an increase in memory performance produced by pharmacological mTORC1 inhibition.

Taken together all these studies, it is generally assumed that an appropriate mTORC1 activation is necessary for the proper long-term memory storage. However, our results indicate that mTORC1 activation is responsible for the long-term memory deficits produced by THC. These apparently contradictory results could be explained by the following hypotheses:

(i) Overactivation of mTORC1 signaling

The intact function of mTORC1 and the precise control of translation are required for the proper memory storage. Thus, either enhancing or

reducing the activity of this signaling cascade, causes memory disruption. Upon CB1R stimulation by THC or AEA, the mTORC1 signaling pathway could be overactivated, and thus alter memory consolidation. In this regard, $TSC1^{+/-}$ and $TSC2^{+/-}$ mutant mice that exhibit a persistent activation of mTOR display memory impairment[242,243]. In agreement, FKBP12 mutant mice, which show both basal mTOR and p70S6K increased phosphorylation in the hippocampus, display enhanced associative contextual fear memory, although an altered performance in the object recognition task probably due to perseveration[234]. Moreover, FMRP knockout mice, which model Fragil X syndrome and display cognitive deficits, show an overactivation of mTORC1 signaling[268]. However, in all these mutant mice it is unknown whether mTOR-driven translational control leads to an increase of translation of a specific subset of mRNAs or promotes a general translation.

(ii) mTORC1 activation leads to different protein translation outcomes

An interesting issue that remains to be resolved is how mTORC1 activation can promote protein translation required for memory storage and protein translation involved in amnesic-like effects. Another open question to be answered is how NMDAR and mGluR activation can both induce mTOR signaling while producing LTP and LTD, respectively, which are two entirely different outcomes. The fact that mTOR orchestrates the complex cellular events serving as a signal integrator, indicates that mRNA transcripts could be differently translated in response to different stimuli. This fact brings to the next plausible hypothesis where the requirement of mTORC1 in memory

storage might entail translational control of specific mRNAs, whereas the activation of mTORC1 induced by THC engages the translation of different mRNAs that might underlie the amnesic-like effects. In this sense, a specific subset of mRNAs could be notably important to produce memory impairment if we consider amnesia as an active process rather than a product of memory storage alteration.

The next open question related to this hypothesis is how mTORC1 activation can differently regulate mRNA translation. One possible explanation could be the distinct localization of mTOR pools near the tightly organized receptor-signaling regions. In agreement, different trafficking of mRNA transcripts to specific regions where certain membrane receptors are expressed might also be possible. Thus, depending on the neuronal compartment where a surface receptor is activated and the proximity of the mRNA transcripts together with the mTORC1 signaling components, different physiological outcomes might occur. Upon CB1R activation, mTORC1 may promote local translation of a specific subset of mRNAs responsible of amnesia, while after the training phase of a task, mTORC1 may promote the translation of certain transcripts involved in consolidation.

B. MODULATION OF ANXIETY BY CANNABINOIDS

We have demonstrated that AEA, 2-AG and a low dose of THC (0.3 mg/kg) produce anxiolytic-like responses in the elevated plus maze. However, different mechanisms seem to underlie these effects. Our results and previous studies indicate that while AEA and THC drive anxiolytic-like responses through CB1R[90,95], 2-AG acts through CB2R. Thus, two distinct endocannabinoids evoke the same physiological response by acting at different cannabinoid receptors. Moreover, we found that JWH133, a selective CB2R agonist, produces clear anxiolytic-like effects in the same task, pointing to CB2R as a novel target to treat anxiety-related disorders.

CB2R has been considered a peripheral receptor and its expression in neurons has remained controversial[31]. However, compelling anatomical and functional evidences suggest that CB2Rs might be expressed in the CNS. Indeed, CB2R was found in the brainstem of rat, mouse and ferret[30]. In recent studies, CB2R has been identified in different brain areas, including the hippocampus, olfactory nucleus, cerebral cortex, striatum, thalamus, amygdala, and cerebellum[31,446,447]. In addition, functional roles have been attributed to the CB2R expressed in the CNS. For example, CB2R seems to play a role in neurogenesis[448-450], and has also been related to psychiatric disorders[451,452]. The antiemetic effect of 2-AG was blocked by the CB2R antagonist AM630, suggesting an involvement of CB2R in this response[30]. Moreover, transgenic mice overexpressing CB2R display reduced depressive-related behaviors in the tail suspension test and in the novelty-suppressed feeding test[453].

The role of CB2R in anxiety behavior has been previously studied by using mice overexpressing CB2R[454]. These mice show anxiolytic-like responses compared to wildtype in the light and dark box and in the elevated plus maze. This behavioral response has been related to the modulation of the GABergic system and neuroendocrine responses. Thus, overexpression of CB2R prevents the effects of stress on the increase in corticotropin releasing factor gene expression, which may indicate that CB2R plays a role in the control of hypothalamic-pituitary-adrenal axis in addition to CB1R[454]. Moreover, GABA-Aα2 and GABA-Aγ2 receptor subunit gene expression are increased in the hippocampus and amygdala of mice overexpressing CB2R, suggesting that CB2R might contribute to the regulation of the GABAergic system[454]. In agreement, a recent study shows an effect of the CB2R agonist JWH133 on the suppression of GABAergic inhibitory signaling in the hippocampus, which was blocked by prior administration of the CB2R antagonist AM630[455]. Although the expression of CB2R in neurons seems plausible to explain the anxiolytic-like effect produced by 2-AG and JWH133, microglial or astrocytic CB2R expression cannot be discarded.

Our study is the first report showing that an activation of CB2R, by the synthetic agonist JWH133 or by indirectly increasing 2-AG levels, produces anxiolytic-like responses. Although the role of 2-AG in anxiety has been less explored than AEA, a recent study suggested that JZL184 produces a CB1R-dependent anxiolytic-like effect in the marble burying[96], a compulsive-like behavior that mimics the symptoms of obsessive-compulsive anxiety[456]. This is in contrast with our results, which clearly demonstrate that the anxiolytic-like

responses mediated by JZL184 depend exclusively on CB2Rs. One possible explanation could be the different paradigms used to evaluate anxiety. Although the marble burying assay has been used as a model to evaluate anxiolytic-like responses, the data obtained from this assay do not necessarily correlate directly with those obtained on exploratory models of anxiety-like behavior, such as the elevated plus maze, the open field, or the light and dark box. The marble burying assay reflects more a repetitive and perseverative behavior rather than novelty-induced anxiety[457]. Thus, 2-AG might play a role through CB1R in this particular behavioral situation, whereas 2-AG produces anxiolytic-like effects in the elevated plus maze and elevated zero maze through the stimulation of CB2R.

Another possible explanation would take into account the dose of JZL184 used. In the marble burying assay, doses of 16 and 40 mg/kg were used, which have been described to inhibit FAAH activity as well[118], whereas the dose of 8 mg/kg was used in our study. Thus, the inhibition of FAAH activity by high doses of JZL184 would enhance AEA levels in specific brain areas that might promote the anxiolytic-like effect in the marble burying test through CB1R activation.

We have also demonstrated that the anxiogenic-like effects mediated by a high dose of THC (10 mg/kg) in the elevated plus maze require mTORC1 activation. Thus, the administration of this high dose of THC increases the phosphorylation of p70S6K in the hippocampus and amygdala, two brain structures highly related with anxiety responses. In agreement, we found that the pretreatment of the mTOR inhibitor temsirolimus prevents THC-induced anxiogenic-like effects. A low dose of THC (0.3 mg/kg) does not increase mTOR

signaling in those structures and produces the opposite effect on anxiety. Thus, the activation of the mTOR signaling pathway might serve as a molecular mechanism underlying the biphasic effect attributed to cannabinoids on anxiety. Moreover, our studies point to temsirolimus as a pharmacological tool of interest to prevent the negative consequences associated to THC, such as memory impairment and anxiogenic-like effects, while the therapeutic effects, such as antinociception, remain intact.

C. CHRONIC EFFECTS OF CANNABINOIDS

Memory

We have reported in our studies the absence of tolerance to the memory impairment produced in the object recognition test by THC or sustained AEA levels after a 6-days chronic treatment (10 mg/kg of THC or 1 mg/kg of URB597, once daily). On the contrary, chronic enhanced 2-AG levels (8 mg/kg of JZL184, once daily) did not produce impaired memory in this task, in agreement with the differential role that we found between AEA and 2-AG after acute treatment in short- and long-term memory. Thus, acute or chronic administration of URB597 or THC impairs memory consolidation, whereas 2-AG does not participate in this process, at least in the memory processes analyzed in our studies: object recognition and context recognition memory tasks.

The sustained elevated AEA levels after URB597 chronic administration did not produce CB1R downregulation in the hippocampus, which may underlie the lack of tolerance to the amnesic-like effects. However, other mechanisms would also be involved in this lack of tolerance. Indeed, although chronic THC exposure impaired memory along the 6 days of treatment, a notably downregulation of CB1R occurred in the hippocampus. Moreover, THC-treated animals required around 4-5 days to totally recover memory after the cessation of the drug. The fact that one of the major roles of mTORC1 is promoting protein translation[185] and that pretreatment with the protein translation inhibitor anisomycin abolished the acute THC-induced amnesic-like effects, lead to hypothesize that the translation of certain proteins might contribute to

the long-term memory impairment produced by cannabinoids. Therefore, upon the first THC administration, mTORC1 signaling would promote the synthesis of certain proteins, which would alter memory consolidation in a perdurable manner. Along the THC chronic treatment, these proteins would be synthesized in response to mTORC1 activation and, although CB1Rs undergo downregulated and mTORC1 would be therefore less activated by THC than in acute conditions, the levels of these newly translated proteins would be sufficient to maintain the disruption of memory consolidation. After THC cessation, the presence of these proteins during the 4-5 days of recovery period of memory, would underlie the long-lasting memory impairment produced by the cannabinoid.

Another possible explanation for the long-lasting effect of the prolonged THC exposure in memory impairment might be through the accumulation of the drug in the brain due to its lipophilic properties. Thus, even after the cessation of the treatment, the remaining THC would explain the amnesic-like effects that mice show during the following 4-5 days until they completely recover memory. However, this is unlikely because THC accumulation was not found in the rat brain tissue either 30 min or 24 h following a single drug administration or 24 h following a chronic treatment (10 mg/kg, 7 days, once daily)[360].

In the acute experiments, pretreatment with MK801, a NMDAR antagonist, blocks THC-induced amnesic-like effects and attenuates the phosphorylation of p70S6K (Thr389), revealing the crucial role of NMDARs in the effects of THC. It has been shown that chronic THC treatment (10 mg/kg, 7 days, once daily) produces CB1R-

dependent decreases in the expression of NR2A, NR2B, and GluR1 glutamate receptor subunits in mouse hippocampus, which are accompanied by impaired LTP[458]. Therefore, both endocannabinoid and glutamatergic systems interact each other in long-lasting synaptic plasticity. Since the density of CB1Rs is higher in hippocampal GABAergic than glutamatergic neurons[376,377], the hypothesis that THC mainly decreases GABA release and indirectly enhances glutamatergic transmission, which activates mTORC1 through NMDAR, seems plausible to explain the acute effects. In this regard, it is tempting to speculate that after chronic treatment the CB1R-dependent downregulation of NR2A and NR2B, two subunits of NMDARs[458], might be due to the glutamate released in response to THC administration. In addition, the impaired LTP after prolonged *in vivo* exposures to THC might also underlie altered cognitive function.

In another study, repeated THC exposure also alters hippocampal LTP in a long-lasting manner[360]. Thus, LTP induced by theta burst stimulation or HFS, is abolished in rats treated during 3 or 7 days with THC (10 mg/kg, once daily), but not during 1 day. The complete blockade of LTP persists 3 days after the cessation of the chronic THC treatment and is not totally reversed up to 14 days following THC withdrawal[360]. Together, these results demonstrate that chronic THC might cause enduring changes in brain function that interfere with the mechanisms necessary for LTP, a process tightly associated with memory[459]. A persistent memory impairment produced by THC has also been demonstrated in another study showing amnesic-like effects in the Morris water maze in rats after a chronic THC exposure during 24 days (1mg/kg, once daily), although they are tolerant to the

hypolocomotor effect[393]. In agreement, our studies showed that those mice that developed a rapid tolerance to the hypothermic effect did not show tolerance to the cognitive functions. Moreover, tolerance to hypolocomotion and antinociception occurred in mice treated with the same regimen of chronic THC. Thus, memory impairment seems to be resistant to the tolerant effects of repeated cannabis exposure.

Anxiety

We have shown that chronic treatment with URB597 or JZL184 did not produce tolerance to the anxiolytic-like effects produced in the elevated plus maze by AEA and 2-AG, respectively. The lack of tolerance produced by sustained AEA levels is in agreement with the anxiolytic phenotype of the FAAH knockout mice[95].

Our results also indicate the absence of CB1R downregulation after the prolonged blockade of endocannabinoid deactivation, which is in agreement with the lack of tolerance to the anxiolytic-like effects produced by URB597 or JZL184. However, one study has shown that repeated administration of JZL184 during 6 days using a dose 5 times higher than the dose used in our studies (40 mg/kg versus 8 mg/kg) produces tolerance and CB1R desensitization, an effect mimicked by the deletion of the gene encoding MAGL[139]. Thus, the lack of cannabinoid receptor neuroadaptations following chronic treatment with low doses of inhibitors of endocannabinoid-degrading enzymes highlights the promising therapeutic benefits of the endocannabinoid modulation. In addition, the persistent lack of tolerance to the anxiolytic-like effect produced by prolonged JZL184 exposure points

to this MAGL inhibitor as a promising therapeutic tool to reduce anxiety without apparent side-effects, such as memory impairment.

On the other hand, our results also show the absence of tolerance to the anxiety-related responses after chronic treatment with THC, either at low or high doses. The lack of tolerance to the anxiolytic-like effect of a 6-days repeated exposure of THC 0.3 mg/kg is expected considering that this low dose would not produce CB1R downregulation or desensitization. However, acute THC administration (0.3 mg/kg) after a 5-days treatment of THC at high dose (10 mg/kg) does not induce anxiolytic-like responses. On the contrary, acute THC administration of a high dose (10 mg/kg) after a 5-days treatment of THC (10 mg/kg) produces anxiogenic-like effects. The different functional role of CB1R in anxiety modulation depending on the neuronal type where it is expressed might underlie the results obtained in our studies. In agreement, the group of Dr. Beat Lutz presented unpublished results in several scientific congresses, showing the different functional role of CB1R in anxiety-like responses by using CB1R conditional knockout mice. Their studies suggest that CB1R present in GABAergic neurons is responsible for the anxiogenic-like effects produced by high doses of cannabinoids, whereas CB1R located in glutamatergic cells participates in the anxiolytic-like effects induced by low doses of cannabinoids. Taken into account these findings, one possible explanation for our results might be that CB1R suffers distinct neuroadaptations after a chronic THC treatment depending on the neuronal population where the receptor is expressed. Thus, it is plausible to speculate that prolonged THC treatment at high doses (10 mg/kg) mainly reduces

CB1R functional activity in glutamatergic cells, which could explain the tolerance to the anxiolytic-like effects of THC (0.3 mg/kg). On the contrary, the same chronic THC exposure (10 mg/kg) did not develop tolerance to the anxiogenic-like responses because these effects are mediated by CB1R located in GABAergic neurons that would maintain their functional activity in contrast to CB1R present in glutamatergic terminals.

These distinct CB1R neuroadaptations could explain the differential development of tolerance to other pharmacological effects of THC. In this sense, THC effects on the tetrad assay have been described to be mediated mainly by CB1R located in glutamatergic neurons[414]. We found tolerance development to the hypothermic, hypolocomotor and antinociceptive effects following chronic THC exposure. On the contrary, no tolerance to the memory impairment produced by THC treatment appeared after this chronic THC exposure because CB1R present in GABAergic terminals is responsible for this effect.

In summary, our studies have contributed to the characterization of some of the mechanisms underlying several physiological responses of endocannabinoids as well as several pharmacological effects of THC. Thus, we have shown that the administration of inhibitors of endocannabinoid-degrading enzymes may be therapeutically useful to treat anxiety and pain, mainly due to the lack of notably side-effects and tolerance, compared to classical cannabinoid agonists. Moreover, low doses of THC, which do not produce memory impairment, might also be beneficial to obtain analgesic and anxiolytic effects.

One of the main caveats of using cannabis for therapeutical purposes has been the impairment of cognitive functions and the anxiogenic

effects that have been widely reported in recreational consumers of *Cannabis sativa* derivatives. Therefore, the prevention of both memory impairment and anxiogenic-like effects produced by THC using mTORC1 inhibitors (rapamycin or temsirolimus) might help to accept this phytocannabinoid as a potential therapeutic compound to treat several diseases, including cancer, pain, multiple sclerosis, and Huntington's disease, among others.

Conclusions

The main conclusions of the work presented in this thesis can be summarized as follows:

1. Acute THC treatment activates the PI3K/Akt/GSK-3 signaling pathway *in vivo* through CB1R in a dose-dependent manner in different brain areas.

2. Both mTORC1 and NMDAR in the hippocampus are involved in the memory impairment produced by THC or enhanced AEA levels.

3. THC administration activates multiple effectors involved in protein translation, such as the ribosomal protein S6, and the translation initiation factors eIF4E, eIF4G and eIF4B. In addition, the pretreatment of the protein synthesis inhibitor anisomycin prevents the memory impairment produced by THC.

4. CB1Rs located in GABAergic neurons, but not in glutamatergic neurons, are critically involved in THC-induced mTORC1 signaling activation and amnesic-like effects.

5. mTORC1 is also involved in the anxiogenic-like effects produced by a high dose of THC, but not in the anxiolytic-like responses induced by a low dose of THC. The mTORC1 inhibitor temsirolimus blocks the amnesic- and anxiogenic-like effects of THC, while does not affect other pharmacological responses.

6. Chronic THC treatment produces tolerance to the hypothermic, hypolocomotor, and antinociceptive effects, while amnesic- and anxiogenic-like responses persist after prolonged THC exposure.

7. Enhanced AEA levels produce anxiolytic-like responses through CB1Rs, while enhanced 2-AG levels promote a similar effect by acting on CB2Rs. In agreement, direct stimulation of CB2Rs by JWH133 produces anxiolytic-like responses.

8. Chronic pharmacological enhancement of the endocannabinoid tone does not produce tolerance to the anxiolytic and antinociceptive effects, pointing to the endocannabinoid-degradating enzymes inhibitors as promising therapeutic tools.

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