Study of alternative approaches to modulate the endocannabinoid system: focus on memory and motor coordination

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A tots els que m'han donat suport durant
aquesta gran ultra.

“ Ningú ens va dir què érem.
Ningú ens va dir que hi anéssim.
Ningú ens va dir que seria fàcil.
Algú va dir que som els nostres somnis.
Que si no somiem, estem morts. “

Kilian Jornet

Abstract

The endocannabinoid system (ECS) is an endogenous neuromodulatory system widely expressed in the central nervous system and peripheral organs, where it fine-tunes numerous physiological functions. The ECS is under study as a therapeutic target, but its pharmacological modulation may trigger unwanted adverse effects due to its widespread distribution. Using novel pharmacological approaches in mice we studied particular outcomes of targeting the ECS and its associated intracellular signaling pathways. Specifically, we identified the peripheral ECS as a relevant modulator of memory persistence in wildtype mice and in a mouse model of fragile X syndrome. Additionally, we revealed the sensitivity of a dual mTOR inhibitor to prevent the amnesic-like effects of Δ9-tetrahydrocannabinol. Finally, we discovered a paradoxical inflammatory effect restricted to the cerebellar area due to the increase in endocannabinoid tone.
The multifaceted responses of systemic ECS modulation reveal the complexity that should be systematically considered in order to better profit its therapeutic potential.

Resum

El sistema endocannabinoid (SEC) és un sistema neuromodulator endògen que es troba àmpliament expressat tant en el sistema nerviós central com en els òrgans de la periferia, on regula varies funcions fisiològiques.
El SEC s'ha proposat com a diana terapèutica, però la seva modulació farmacològica pot desencadenar efectes adversos centrals degut a la seva distribució generalitzada i diversitat de funcions. Aquesta tesi se centra en l'estudi dels efectes de la modulació del SEC i les seves vies de senyalització intracel·lular mitjançant noves aproximacions farmacològiques. Concretament, hem identificat el SEC perifèric com a un modulador important de la persistència de la memòria en ratolins genotípicament controls i en un model ratolí de la síndrome del cromosoma X fràgil. També hem identificat la sensibilitat d'un inhibidor dual de la via de senyalització mTOR per prevenir els efectes deleteris del Δ9-tetrahydrocannabinol sobre la memòria. Finalment, hem descobert un efecte inflamatori paradoxal al cerebel, resultat d'augmentar el to endocannabinoid.
En general, mitjançant un enfocament multidisciplinari hem demostrat respostes complexes polivalents de la modulació del SEC que s'haurien de considerar de forma sistèmica per tal de poder aprofitar millor el seu potencial terapèutic.

Abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
ABHD12	Alpha/beta-hydrolase domain containing 12
ABHD4	Alpha/beta-hydrolase domain type-4
ABHD6	Alpha/beta-hydrolase domain containing 6
AC	Adenylyl cyclase
AEA	N-arachidonoylethanol-amide
AMPAR	3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF	Brain-derived neurotrophic factor
CA	*Cornu ammonis*
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase ii
cAMP	Cyclic adenosine monophosphate
CB1R	Cannabinoid type-1 receptor
CB2R	Cannabinoid type-2 receptor
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CREB	Camp-response element-binding protein
DAG	1,2-diacylglycerol
DAGL	Diacylglycerol lipase
DHPG	3,5-dihydroxyphenylglycerine
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
eCB-LTD	Endocannabinoid-mediated long-term depression
eCB-STD	Endocannabinoid-mediated short-term depression
ECS	Endocannabinoid system
eIF4E	Eukaryotic initiation factor 4e
E-LTP	Early long-term potentiation
EPSP	Excitatory postsynaptic potential
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAAH	Fatty acid amide hydrolase
FMR1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GPCR	G-protein coupled receptor
GPR110	G-protein coupled receptor 110
GPR18	G-protein coupled receptor 18

GPR55	G-protein coupled receptor 55
GSK-3	Glycogen synthase kinase 3
HETE-EA	Hydroxyeicosatetraenoic-ethanolamide
IFN- γ	Interferon-γ
IL	Interleukin
KO	Knockout
LC	*Locus coeruleus*
L-LTP	Late long-term potentiation
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAGL	Monoacylglcerol lipase
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NADA	N-arachidonoyldopamine
NAGly	N-arachidonoylglycine
NAPE	N-acylphosphatidyl-ethanolamine
NAT	Calcium-dependent n-acyltransferase
NFkB	Nuclear factor kb
NMDAR	N-methyl-d-aspartate receptor
NORT	Novel object-recognition test
NTS	Nucleus tractus solitarius
PG	Prostaglandin
PG-EA	Prostanglandin-ethanolamides
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptor
PRAS40	Proline-rich akt substrate of 40kda
P70S6K	P70S6 kinase

Raptor	Regulator associated protein of mTOR
Rictor	Rapamycin insensitive companion of Mtor
THC	Δ9-tetrahidrocannabinol
TNF-α	Tumor necrosis factors-α
TrkB	Tropomyosin-related kinase b
TRPV1	Transient receptor potential vanilloid receptor 1
VGCC	Voltage-gated Ca^{2+} channels
β-AR	β-adrenergic receptors

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INTRODUCTION

1. The endocannabinoid system

For centuries *Cannabis sativa* plant and its derivates, marijuana and hashish, have been used for recreational and medicinal purposes. Major efforts were focused on the isolation of the active compounds of the plant. At least, 120 cannabinoids, known as phytocannabinoids, have been isolated from the plant (Morales *et al.,* 2017). Among them, **Δ^9-tetrahydrocannabinol** (Δ^9-THC) discovered in 1964 (Gaoni and Mechoulam, 1964), is the main psychoactive component. Since then, several biologically active analogs of THC were synthetized to mimic the cannabinoid compound properties. All these molecules are called cannabinoids, due to their cannabimimetic properties.

Few years later, cannabinoid receptors were identified and cloned (Matsuna *et al.,* 1990). This discovery was followed by the characterization of its endogenous ligands, named as endocannabinoids (Devane *et al.,* 1992; Mechoulam *et al.,* 1995), and the main enzymes related on their biosynthesis and degradation. Altogether these components were grouped in an endogenous modulatory system, known as the endocannabinoid system (ECS).

1.1. Components of the endocannabinoid system

The ECS is composed by the cannabinoid receptors, the endocannabinoids and the enzymes involved in their synthesis and inactivation.

1.1.1. Cannabinoid receptors

Initially, it was assumed that cannabinoids would act through a nonspecific membrane-associated mechanism. However, the very high stereospecificity of action of some synthetic cannabinoids pointed to a more selective mechanism (Mechoulam *et al.,* 1988). This hypothesis was supported by the finding that cannabinoids inhibit adenylyl cyclase (AC) activity proportionally to their pharmacological effects (Howlett *et al.,* 1986). Further research led to the discovery of cannabinoid binding sites in the brain (Devane *et al.,* 1988) and their distribution corresponded to the pharmacological properties of psychotropic cannabinoids (Herkenham *et al.,* 1990).

Cannabinoids exert their pharmacological functions acting through the activation of cannabinoid receptors, being the most important the cannabinoid type-1 receptor (CB1R) and the cannabinoid type-2 receptor (CB2R). CB1R was the first cannabinoid receptor cloned in 1990 (Matsuda *et al.,* 1990), while CB2R was cloned three years later (Munro *et al.,* 1993)

Both are seven-transmembrane domain receptors and belong to the family of G-protein coupled receptors (GPCR), mainly coupled to Gi/o proteins (Childers and Deadwyler, 1996). They are differentially distributed through the organism. In general terms, CB1R is highly expressed on the central nervous system (CNS), while CB2R is mainly expressed on the immune system (Svíženská *et al.,* 2008).

In the following decades, diverse studies pointed to the existence of other receptors that bind cannabinoid ligands and mediate their effects. These included some orphan GPCRs, such as the GPR55, GPR18 and GPR110 (Kohno *et al.,* 2006; Pertwee, 2007; Desai *et al.,* 2016), the

transient receptor potential vanilloid receptor 1 (TRPV1) (Marzo and Petrocellis, 2010; Perluigi *et al.,* 2015), sphingosine-1-phosphate lipid receptors GPR3, GPR6 and GPR12 (Morales and Reggio, 2017) and the peroxisome proliferator-activated receptors (PPAR) (O'Sullivan, 2007). In addition, several studies indicate that CB1R, as other GPCRs, could form homodimers (Wager-Miller *et al.,* 2002; Mackie, 2005) and heterodimers with other GPCRs including CB2R (Callén *et al.,* 2012), dopamine D2 (Kearn *et al.,* 2005), opioid (Rios *et al.,* 2009), orexin (Ellis *et al.,* 2006), adenosine (Carriba *et al.,* 2007; Aso *et al.,* 2019) and serotonin receptors (Viñals *et al.,* 2015), contributing to the diversity of signaling pathways and cellular functions of CB1R.

1.1.1.1. Cannabinoid receptor type-1

CB1R is considered the most abundant GPCRs **in the CNS** and responsible for the psychoactive effects produced by THC and other cannabinoid agonists (Kano *et al.,* 2009). Its distribution has been well characterized both in rodents (Herkenham *et al.,* 1991; Tsou *et al.,* 1998) and humans (Westlake *et al.,* 1994). In the CNS, the highest density of CB1R was found in **cerebellum**, **hippocampus**, olfactory regions, basal ganglia, cortex and amygdala. Some other regions present moderate density of CB1R, such as medial hypothalamus, solitary nucleus and spinal cord. Finally, other areas like thalamus and brainstem exhibit low levels of CB1R (Figure 1). Interestingly, the main distribution of this receptor in specific brain regions is related to its role in the control of **learning and memory**, **motor coordination, inflammation**, body temperature, pain perception and appetite regulation.

It is worth mentioning that besides this standard CB1R distribution, its mRNA and protein levels could vary during development or in pathological conditions (Laprairie *et al.,* 2012).

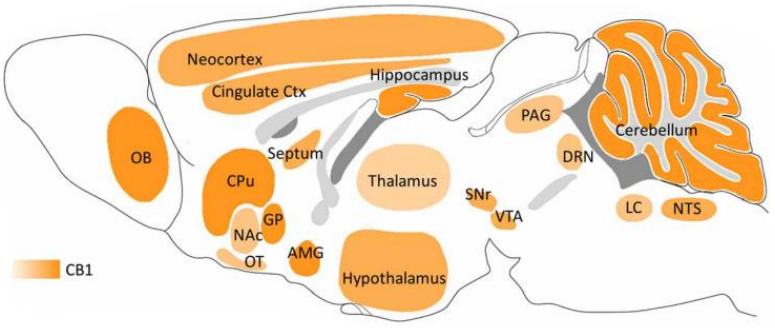

Figure 1 Schematic distribution of CB1R in the brain. Sagittal mouse brain section representing CB1R location (shading densities indicates expression level). AMG, amygdala; ctx, cortex; Cpu, caudate-putamen; DRN, dorsal raphe; GP, globus pallidus; LC, *locuscoeruleus*; NAc, nucleus accumbens; NTS, nucleus tractus solitarius; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; SN, substantia nigra; VTA, ventral tegmental area (Flores *et al.,* 2013)**.**

Moreover, CB1R is also expressed **in multiple peripheral tissues**, where it contributes to modulation of the activity and physiological control of the gastrointestinal tract (Izzo and Sharkey, 2010), cardiovascular system (Liu *et al.,* 2000), liver, pancreas, fat tissue, (Cota *et al.,* 2003), retina (Porcella *et al.,* 2000), immune system (Jean-Gilles *et al.,* 2015), bone (Idris *et al.,* 2005), skeletal muscle (Cavuoto *et al.,* 2007) and adrenal glands (Hofer *et al.,* 2015), among others (Figure 2). Interestingly, recent studies have revealed the cross-talk between peripheral CB1R and CNS functions. Notably, the peripheral sympathetic nervous system, sensitive to peripheral CB1R modulation, is crucial for central functions including hypophagia and anxiety-like effects (Bellocchio *et al.,* 2013).

In agreement, CB1R control of adrenocortical hormones from adrenal glands, was found necessary for the stress-dependent regulation of

non-emotional memory consolidation (Busquets-Garcia *et al.,* 2016). Moreover, peripheral CB1Rs were found relevant for gut-microbiota in obesity and metabolic disorders (Di Marzo, 2018).

At the cellular level, CB1R is mainly located at **presynaptic terminals** controlling neurotransmitter release, particularly GABA and glutamate, but also other neurotransmitters, such as noradrenaline, dopamine, serotonin, acetylcholine and cholecystokinin (Pertwee and Ross, 2002). Additionally, different studies support the presence of somatodendritc CB1R in some brain regions (Bacci *et al.,* 2004; Leterrier *et al.,* 2006; Marinelli *et al.,* 2009; Simon *et al.,* 2013). For instance, post-synaptic CB1R activation in the hippocampus regulates synaptic plasticity and memory through the hyperpolarization-activated cyclic nucleotide-gated channels (Maroso *et al.,* 2016).

Although CB1Rs are widely distributed among different brain areas, their density varies depending on cell populations. In general, GABAergic cells express higher levels of CB1R than glutamatergic cells (Kano *et al.,* 2009). However, the levels of CB1R expression does not correlate with its functionally. Indeed, studies with CB1R knockout (KO) mice in GABAergic or cortical glutamatergic neurons demonstrated that CB1R in glutamatergic neurons is more efficiently coupled to G protein signaling (Steindel *et al.,* 2013).

Apart from their heterogenous distribution in neurons, CB1Rs have been also detected in astrocytes (Navarrete and Araque, 2008) and microglial cells (Cabral and Marciano-Cabral, 2005) with relevant contributions to synaptic plasticity (Navarrete and Araque, 2010; Han *et al.,* 2012) and inflammatory processes (Stella, 2010), respectively. In addition, some studies located CB1R in intracellular organelles such as

the endosomal-lysosomal compartments (Leterrier *et al.,* 2004; McDonald *et al.,* 2007) and the mitochondria (Bénard *et al.,* 2012; Gutiérrez-Rodríguez *et al.,* 2018). Actually, it is demonstrated that mitochondrial CB1R in the hippocampus modulates memory formation through the regulation of neuronal energy metabolism (Hebert-Chatelain *et al.,* 2016).

The diverse distribution, density and expression of CB1R between different areas, cell types and subcellular compartments helps explaining the variety of effects produced by compounds acting on CB1R that will be presented below in section 1.3.

1.1.1.2. Cannabinoid receptor type-2

CB2R is predominantly expressed **in the immune system**, including the spleen, tonsils, thymus, mast cells and blood cells, where it controls inflammatory responses (Svíženská *et al.,* 2008) (Figure 2). Besides the cells of the immune and hematopoietic system, CB2R is also present in other **peripheral organs**, such as muscle, liver, intestine and testis (Liu *et al.,* 2009). CB2R expression has also been detected to lower levels than CB1R in the healthy brain (Van Sickle *et al.,* 2005; Gong *et al.,* 2006). However, there are several reports showing CB2R expression in various regions of the brain, including the retina, striatum, cortex, ventral tegmental area, hippocampus, amygdala, brainstem and cerebellum (Chen *et al.,* 2017). Importantly, CB2R expression levels rapidly increase under pathological conditions (Miller and Devi, 2011), such as neuropathic pain (Svízenská *et al.,* 2013), traumatic brain injury (Lopez-Rodriguez *et al.,* 2015), stroke (Yu *et al.,* 2015) and some neurodegenerative disorders (Aso and Ferrer, 2016; Concannon *et al.,*

2016). Several studies demonstrated that the increase of CB2R in these pathological conditions has a neuroprotective role restraining the inflammatory responses (Palazuelos *et al.,* 2009; Viscomi *et al.,* 2009; Cassano *et al.,* 2017).

At the cellular level, CB2R in the brain is expressed in neurons, specifically on the **postsynaptic region**, where it controls neuronal excitability (Gong *et al.,* 2006; Onaivi *et al.,* 2006). Some studies also located CB2R in glial cells, such as astrocytes and microglial cells after specific insults, such as neuroinflammation (Van Sickle *et al.,* 2005; Cabral and Griffin-Thomas, 2009) (Figure 2). In fact, CB2R **in microglia** has a crucial role in the control of central immune function and neuroinflammation-associated to pathologies. Additionally to their **role in neuroinflammation**, recent studies using pharmacological and genetic approaches demonstrated that CB2R is involved in hippocampal synaptic plasticity and controls the rewarding properties of some addictive drugs (Xi *et al.,* 2011; Aracil-Fernández *et al.,* 2012; Navarrete *et al.,* 2013; Ortega-Álvaro *et al.,* 2015; Stempel *et al.,* 2016). Altogether, it seems that targeting CB2R could be a promising therapeutic strategy for the treatment of some CNS alterations, avoiding the psychotropic effects mediated by adverse CB1R.

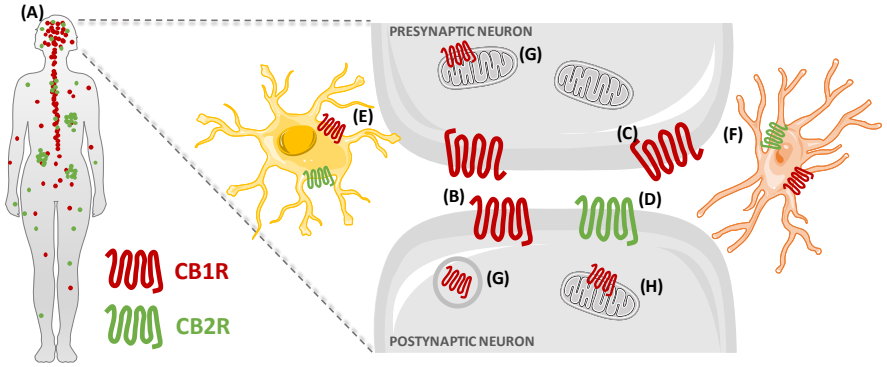

Figure 2. Expression of CB1R and CB2R. (A) CB1R is predominantly expressed on the CNS, but it is also widely expressed in different peripheral tissues, where it can control periphery-brain crosstalk. Contrary, CB2R is preferentially expressed on peripheral immune tissue and into minor extend in the CNS. At the cellular level, CB1R is mainly expressed at the **(B)** presynaptic terminals but it can be also found in **(C)** postsynaptic terminals. **(D)** CB2R in neural cells is located in the postsynaptic terminals. Both CB1R and CB2R could be present in glial cells, both in **(E)** astrocytes and **(F)** microglia. Moreover, some evidences supported the presence of CB1R in **(G)** endosomes and **(H)** mitochondria.

1.1.2. Cannabinoids

Cannabinoids are referred to the molecules that bind to the cannabinoid receptors and alter the neurotransmitter release. They can be classified into two major groups: endogenous cannabinoids (**endocannabinoids**) and exogenous cannabinoids. Exogenous cannabinoids can be divided into **phytocannabinoids** and **synthetic cannabinoids**.

1.1.2.1. Endocannabinoids

The identification of the cannabinoid receptors prompted the existence of endogenous ligands that could bind to these receptors, the endocannabinoids. The first endocannabinoid identified was N-arachidonoylethanol-amide (AEA), also named **anandamide** (Devane *et al.,* 1992). Three years later, another endocannabinoid was discovered, the **2-arachidonoylglycerol** (2-AG) (Mechoulam *et al.,* 1995; Sugiura *et*

al., 1995) (Figure 3). There are other endogenous compounds that could bind to CB1R and CB2R, including 2-arachidonoylglyceryl ether (noladin ether, 2-AGE), O-arachidonoylethanolamine (virodhamine), N-arachidonoyldopamine (NADA), N-arachidonoylglycine (NAGly) and Cis-9,10-octadecanoamide (oleamide or ODA). However, some issues such as their biosynthesis, inactivation and physiological function relevance are still under study (Fonseca *et al.,* 2013).

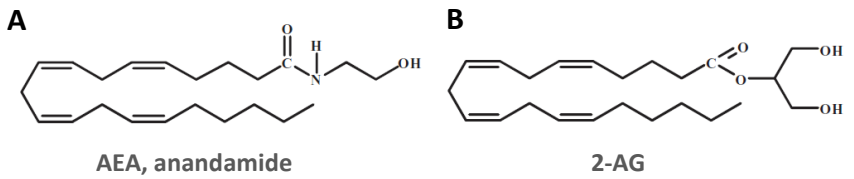

Figure 3. Chemical structure of the main endocannabinoids. (A) AEA, anandamide**. (B)** 2-AG, 2-arachidonoylglycerol

Both, AEA and 2-AG are lipid molecules that are not stored on secretory vesicles, since they are synthesized and released on demand in the postsynaptic terminals in an **activity-dependent manner** (Di Marzo *et al.,* 2005). However, this classical hypothesis is under debate, as some data demonstrated that AEA has been detected associated with cytosolic compartments, called adiposomes, in specific cell lines (Oddi *et al.,* 2008).

AEA acts as a partial agonist of CB1R and CB2R and it has also affinity for TRPV1 (Zygmunt *et al.,* 1999) and PPAR receptors (Bouaboula *et al.,* 2005; O'Sullivan, 2007). 2-AG is considered as a full agonist for both CB1R and CB2R (Stella *et al.,* 1997), but it can also binds to PPAR (Kevin R. Kozak *et al.,* 2002; Du *et al.,* 2011). Moreover, 2-AG levels in the brain are much higher than AEA (Stella *et al.,* 1997) (Table 1). Thus, 2-AG is recognized to be the most important endogenous ligand of CB1R and

CB2R and responsible for the main effects associated with cannabinoid agonist (Sugiura *et al.,* 2006).

In the synapse, upon endocannabinoid release from the postsynaptic neuron, endocannabinoids travel backward and activate cannabinoid receptors on presynaptic terminals to produce a **transient decrease of neurotransmitter release**. Thus, endocannabinoids act as retrograde synaptic messengers (Wilson and Nicoll, 2002; Ohno-Shosaku *et al.,* 2012).

Some lipids with structural resemblances to the endocannabinoids have been identified, but they present low affinity for cannabinoid receptors. These substances are the **endocannabinoid-like compounds**. Interestingly, these compounds share some synthesis and degradation enzymes with the endocannabinoids, interfering on endocannabinoid metabolism and potentiating the cannabinoid signalling. This, concept has been referred as the "entourage" effect. Classical endocannabinoid-like compounds comprise two large distinct families: the **N-acylethanolamines** that include N-stearoylethanolamine (SEA), N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA), and **2-monoacylglycerols** composed of 2-linoleoylglycerol (2-LG), 2-oleoylglycerol (2-OG) and 2-palmitoylglycerol (2-PG) (Fonseca *et al.,* 2013; Kleberg *et al.,* 2014).

1.1.2.2. Phytocannabinoids

Phytocannabinoids are referred to the natural C21 terpenophenolic compounds found in *Cannabis sativa* plant. Until now, over 120 phytocannabinoids have been isolated from the plant, such as Δ^9-THC, Δ^8-THC, cannabinol, cannabidiol, cannabigerol, cannabichromene, Δ^9-

tetrahydrocannabivarin, cannabivarin and cannabidicarin, among others. The two most abundant in the plant are **Δ^9-THC** and **cannabidiol** (Figure 4).

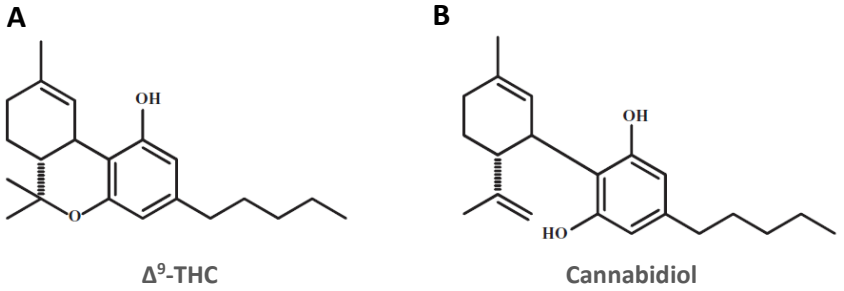

Figure 4. Chemical structure of the two most abundant phytocannabinoids in *Cannabis* plant. (A) Δ9-THC, Δ9-Tetrahydrocannabidiol. **(B)** Cannabidiol.

Δ^9-THC is a partial agonist of CB1R and CB2R and responsible for the psychotropic effects of cannabis, whereas cannabidiol is considered a non-psychotropic substance (Morales *et al.,* 2017b; Turner *et al.,* 2017). It is reported that cannabidiol lacks affinity for CB1R and CB2R, but *in vitro* studies demonstrated that cannabidiol acts as a weak CB1R and CB2R antagonist (Table 1). Other studies revealed that it can also binds to GPR55 (Ryberg *et al.,* 2009) and GPR18 (McHugh *et al.,* 2012) receptors antagonizing their activity. Recently, it has been described that cannabidiol could also bind to adenosine A_{2A} and CB1R heteromers mitigating the cognitive impairment induced by THC (Aso *et al.,* 2019). Currently, cannabidiol is on emerge of interest due to its beneficial anti-inflammatory, analgesic, anti-anxiety and anti-tumour properties, among others. However, the mechanistic bases of cannabidiol effects remain to be elucidated (Morales and Reggio, 2017).

1.1.2.3. Synthetic cannabinoids

Apart from the natural cannabinoids, several synthetic cannabinoids have been designed differing in their intrinsic activity, selectivity and affinity for the cannabinoid receptors. Hence, compounds may act as specific agonists or antagonist for these receptors (Table 1). The most used agonists acting specifically for CB1R/CB2R are HU-210, CP55940 and R-(+)-WIN55212, which have **similar affinity for both receptors**.

Moreover, selective antagonists for CB1R and CB2R have been synthesized. **CB1R-selective antagonists**, being the most relevant rimonabant (SR141716A), AM281, LY320135 and taranabant (MK-0364), act as competitive CB1R antagonists against other endogenous or exogenous ligands (Pertwee *et al.,* 2010). However, in some cases these compounds behave as inverse agonists, producing the opposite effects of those induced by CB1R agonists. Indeed, it was proposed that rimonabant, AM251, AM281, LY320135 and taranabant at micromolar concentrations could act as inverse agonist, whereas at nanomolar concentrations behave as neutral CB1R antagonist (Pertwee, 2005; Howlett *et al.,* 2011). Even thought, it has been recently reported that rimonabant at micromolar concentrations is not acting as an inverse agonist of CB1R, since it may inhibit Gαi/o subunit of heterotrimeric G proteins (Porcu *et al.,* 2018).

Efforts have been dedicated to develop compounds that **avoid the opposite cannabimimetic responses**. Several **neutral CB1R antagonists** have been designed, such as NES0327 (Ruiu, 2003) and O-2050 (Thomas *et al.,* 2004), which only block the agonist-induced effects. Another approach is the use of **allosteric modulators**, compounds that bind to CB1R in a different binding site modifying its

conformation, affinity and/or efficacy of orthosteric ligands (Maroso *et al.,* 2016). In addition, in the last years, the study of peripheral CB1R has emerged and prompted the development of **peripherally-restricted CB1R antagonists** as therapeutic drugs for obesity. Some examples of these compounds are AM6545, TM38837, JD5037, RTI-12 (Chorvat, 2013; Tam *et al.,* 2018).

Besides selective CB1R antagonists, competitive **CB2R-selective antagonists** were also synthetized, such as AM630, SR144528 and JTE-907. Although these compounds display high affinity for CB2R, it is thought that they can also behave as inverse agonists producing inverse cannabimimetic responses (Rinaldi-Carmona *et al.,* 1998).

	Ki (nM)	
Cannabinoid receptor ligand	**CB1R**	**CB2R**
Agonists for CB1R and CB2R		
(-)-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
AEA	61 - 543	279 - 1940
2-AG	58.3, 472	145, 1, 400
Selective agonists for CB1R		
ACEA	1.4 - 5.29	195 > 2000
Arachidonyleyclopropylamide	2.2	715
R-(+)-methanandamide	17.9 - 28.3	815 - 868
Noladin ether	21.2	>3000
Selective agonists for CB2R		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
Competitive antagonist for CB1R		
Rimonabant (SR141716A)	1.8 - 12.3	514 - 13.200
AM251	7.49	2,290
AM281	12	4,200
LY320135	141	14,900
Taranabant	013, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
Competitive antagonist for CB2R		
SR144528	50.3 --> 10.000	0.28 - 5.6
AM630	5152	31.2
JTE-907	2370	35.9
Other compounds		
Cannabinol	120 - 1130	96 - 301
Cannabigerol	81	2600
Cannabidiol	4350 →10,000	2399 →10,000
Virodhamine	912	N.D.

Table 1 Ki values of cannabinoid receptor ligands for the *in vitro* displacement of a tritiated compound (i.e [3H] CP55,940, [3H]SR141716A, [3H]WIN55,212-2) from specific binding sites on rat, mouse or human CB1R and CB2R. Adapted from (Pertwee *et al.,* 2010).

1.1.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids

The availability of the endocannabinoids is continuously controlled by different enzymes implicated in their synthesis and degradation, which are positioned in the **synaptic cleft** (Figure 5).

Both AEA and 2-AG are lipid derivates from arachidonic acid (AA), produced by the hydrolysis of membrane phospholipid precursors. AEA is produced by two main enzymatic reactions. The first step is the transacylation of phosphatidyl-ethanolamine to form N-acylphosphatidyl-ethanolamines (NAPEs) by a calcium-dependent N-acyltransferase (NAT). In the second step, NAPE is hydrolyzed by a specific phospholipase NAPE-PLD, generating AEA and phosphatidic acid (Di Marzo *et al.,* 1994, 2005) (Figure 6). Besides this classical pathway of AEA synthesis, there are alternative pathways responsible for the generation of AEA, such as through the phospholipase C (PLC) (Liu *et al.,* 2006) and the α/β-hydrolase domain type-4 (ABHD4) (Simon and Cravatt, 2006) (Figure 6).

2-AG also results from two enzymatic reactions. First, PLC hydrolyze the membrane phospholipid sn-2-arachidonoyl-PIP2 to produce 1,2-diacylglycerol (DAG). Secondly, DAG is degraded by either two diacylglycerol lipases, DAGL-α and DAGL-β, generating 2-AG (Prescott and Majerus, 1983) (Figure 6).

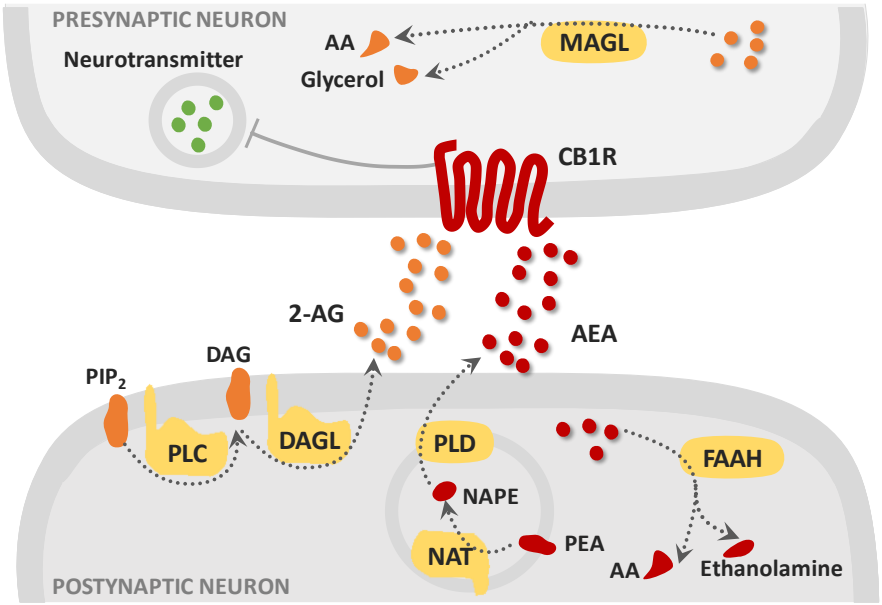

Figure 5. Subcellular location of the main anabolic and catabolic pathways of endocannabinoids in the synaptic cleft. The enzymes for 2-AG biosynthesis are phospholipase C (PLC) and a selective diacylglycerol lipase (DAGL), mainly localized on the membrane of postsynaptic terminals. The enzymes related to the synthesis of AEA are N-acyl transferase (NAT) and a specific phospholipase D (PLD), which are localized on intracellular membranes of postsynaptic neurons. Upon endocannabinoid release to the synaptic cleft, they travel retrogradely and act on the CB1R at the presynaptic terminals producing a transient decrease on neurotransmitter release. After that, AEA is mostly inactivated on neurons postsynaptic to CB1R by the cytosolic fatty acid amide hydrolase (FAAH), whereas 2-AG is metabolized through the monoacylglycerol lipase (MAGL) situated on the cytosol of presynaptic neurons. PIP2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; PEA, phosphatidyl-ethanolamine; NAPE N-arachidonoyl-phosphatidyl-ethanolamine; AA, arachidonic acid. Adapted from (Marzo *et al.,* 2004).

Once endocannabinoids are released from cells and upon activation of their targets, they are rapidly inactivated. In order to be degraded, first they are taken up from the extracellular space to inside the cell. This process is not well defined and several mechanisms have been proposed, including the endocytosis mediated by caveola/lipid rafts (McFarland *et al.,* 2004) or the simple plasma membrane diffusion

(Kaczocha *et al.,* 2006). Currently, the most prevalent hypothesis is that cellular endocannabinoids transport involves the use of "endocannabinoid membrane transporters" , but the identity of these proteins remains to be elucidated (Fowler, 2012, 2013).

After cellular reuptake, endocannabinoids are degraded by their specific enzymes (Figure 6). AEA is mainly hydrolyzed by the fatty acid amide hydrolase (FAAH) into AA and ethanolamine (Deutsch and Chin, 1993; Cravatt *et al.,* 1996). Moreover, AEA can also serve as substrate for the inducible cyclooxygenase-2 (COX-2) and different lipoxygenases (LOXs) to generate prostanglandin-ethanolamides (PG-EA) and hydroxyeicosatetraenoic-ethanolamide (HETE-EA) respectively (Deutsch and Chin, 1993; Cravatt *et al.,* 1996). Regarding 2-AG degradation, approximately 85% of 2-AG is metabolized by the enzyme monoacylglycerol lipase (MAGL), producing AA and glycerol as products (Dinh *et al.,* 2002; Savinainen *et al.,* 2012). The 15% remaining is metabolized by α/β-hydrolase 6 (ABHD6) and α/β-hydrolase 12 (ABHD12) (Blankman *et al.,* 2007). Moreover, 2-AG can be also oxygenated by COX-2 and LOX, resulting in prostaglandin-glycerol esters (PG-Gs) and hydroxyeicosatetraenoic-glycerol ester (HETE-G) (Kozak *et al.,* 2000; K R. Kozak *et al.,* 2002).

It is worth noting that both AEA and 2-AG can also be oxygenated by cytochrome P450 (Chen *et al.,* 2007).

In addition, these AEA and 2-AG metabolic enzymes are also shared with the other members of monoacylglycerol and N-acylethanolamine families, respectively (Fowler *et al.,* 2017).

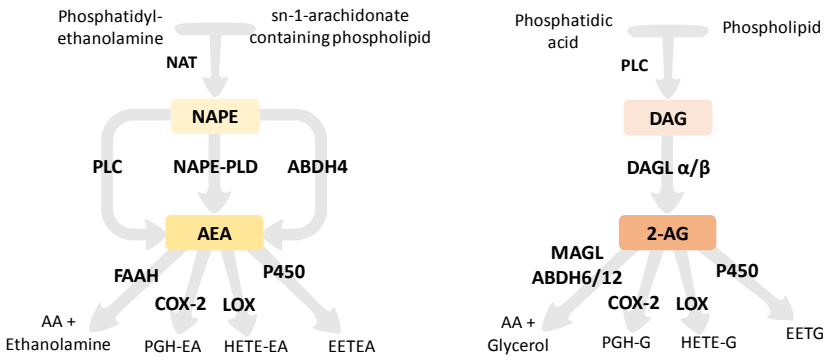

Figure 6. Main and alternative synthesis and degradation pathways of AEA and 2-AG. ABHD: α/β-hydrolase domain; COX2, cyclooxygenase 2; DAGL, diacylglycerol lipase; EETEA, epoxyeicosatrienoic acid-ethanolamide; EETG, epoxyeicosatrienoic acid-glycerol ester; FAAH, fatty acid amide hydrolase; HETE-EA, hydroxyeicosatetraenoic-ethanolamide; HETE-G, hydroxyeicosatetraenoic-glycerol ester; LOX, lipoxygenase; MAGL, monoacylglycerol lipase; NAPE, N-acyl-phosphatidylethanolamine; NAPE-PLD, N-acyl-phosphatidylethanolamine-specific phospholipase D; NAT, N-acyltransferase; PG-EA, prostanglandin-ethanolamides; PG-G, prostaglandin-glycerol ester; PLC, phospholipase C. Adapted from (Iannotti *et al.,* 2016).

The identification of the enzymes implicated in the degradation of endocannabinoids prompted the research of **inhibitory compounds targeting these enzymes** in order to increase the endocannabinoid tone. AEA levels can be enhanced by FAAH enzyme inhibitors, such as URB532, URB597, OL-135, OL-92 and PF-3845, whereas 2-AG levels are increased with selective MAGL inhibitors including JZL184, URB602, SAR127303 or OMDM169 (Tuo *et al.,* 2017).

1.2. Cannabinoid intracellular signaling pathways

The stimulation of the cannabinoid receptors results in the modulation of a wide variety of cellular functions through the activation of different signal transduction pathways (Figure 7). It is thought that there are three distinct signaling waves. The first wave is mediated through the activation of heterotrimeric Gi/o type G proteins (Gα, Gβ and Gγ) and it is observed within seconds and up to min after receptor stimulation.

CB1R coupling to Gαi/o leads the inhibition of the AC activity, which is accompanied by a subsequent reduction in cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA) activity (Howlett and Fleming, 1984; Howlett *et al.,* 1986). However, CB1R coupling to Gβγi/o produces the phosphorylation and activation of different members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinases (Howlett, 2005). Noteworthy, under certain circumstances, CB1R could associate to Gs and Gq proteins (Glass and Felder, 1997; Lauckner *et al.,* 2005).

Some *in vivo* studies demonstrated that CB1R stimulation through THC administration in mice can also activate other kinases, such as the protein kinase C (PKC) (Hillard and Auchampach, 1994; Busquets-Garcia *et al.,* 2018), and the phosphoinositide-3 kinase (PI3K)/Akt (Gómez del Pulgar *et al.,* 2002) with the subsequent activation of glycogen synthase kinase 3 (GSK-3) (Ozaita *et al.,* 2007) and mammalian target of rapamycin (mTOR) (Puighermanal *et al.,* 2009) transduction pathways.

Furthermore, CB1R activation can also modify several types of ion channels inhibiting N- and P/Q-type Ca^{2+} currents (Mackie and Hille, 1992; Twitchell *et al.,* 1997), activating A-type and inwardly rectifying K^{+} channels (Deadwyler *et al.,* 1995; Mackie *et al.,* 1995). These ion conductance changes trigger the repolarization of the plasma membrane and decrease neurotransmitter release (Zou and Kumar, 2018). On the other hand, CB1R coupling to Gi/o or Gq proteins can induce elevations in intracellular Ca^{2+} influx through the activation of phospholipase C (PLC) (Bosier *et al.,* 2010).

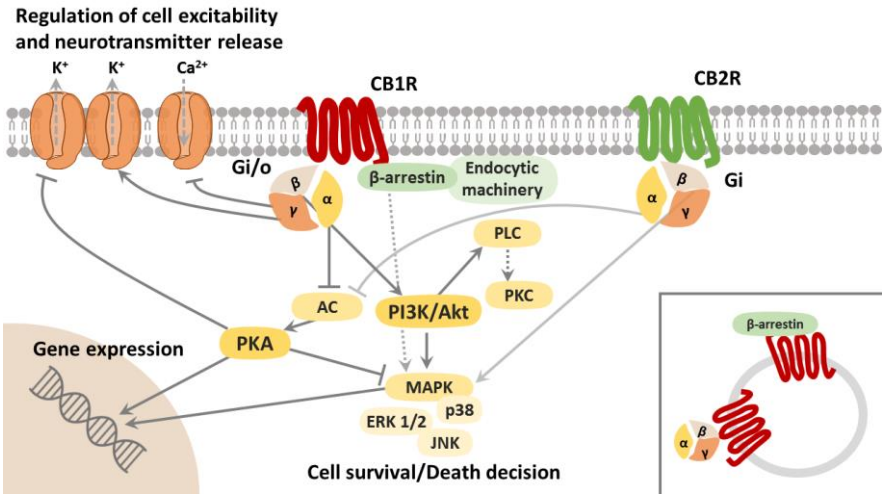

Figure 7. Complexity of cannabinoid receptor signaling. Activation of cannabinoid receptors results in the modulation of multiple cellular responses through three distinct signaling waves. The first wave depends on G proteins. Both CB1R and CB2R are associated with Gαi/o producing the inhibition of the adenylate cyclase (AC) and protein kinase A (PKA) signaling. Moreover, their coupling with Gβγi/o activate different mitogen-activated protein kinase (MAPK) cascades. CB1R negatively regulated voltage-gated Ca^{2+} channels and positively regulate inwardly rectifying K^+ channels, thereby inhibiting neurotransmitter release. Crosstalk among the different pathways activated by the CB1R is illustrated by the variety of responses required on protein kinase A (PKA) inhibition. The second wave stars with β-arrestins coupling with CB1R, with the recruitment of endocytic machinery and internalization of the receptor and the activation of MAPK signaling. After receptor internalization, a third signaling wave can continue the activation of effectors associated with both G proteins and β-arrestins. PI3K, phosphoinositide-3 kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2 (ERK1/2); JNK, c-Jun N-terminal kinases p38, PLC, phospholipase C; PKC, protein kinase C. Adapted from (Bosier *et al.,* 2010; Nogueras-Ortiz and Yudowski, 2016).

After G protein-dependent signaling, CB1R, in association with β-arrestin as scaffold protein begins a second signaling wave. The effect of β-arrestin recruitment is the desensitization and internalization of CB1R. In addition, it can activate different signaling proteins, such as the MAPK cascade (Turu and Hunyady, 2010; Nogueras-Ortiz and Yudowski, 2016). Moreover, CB1R coupling to the adaptor protein FAN leads to *de novo* synthesis or accumulation of ceramide, which

regulates metabolic functions and cell survival/death decision (Galve-Roperh *et al.,* 2000; Sánchez *et al.,* 2001).

Finally, a third wave occurs at intracellular compartments, such as endosomes or lysosomes, where it could be exerted either by G proteins or β-arrestins (Nogueras-Ortiz and Yudowski, 2016).

Other critical factors for the regulation of signaling pathways upon CB1R stimulation include, the lipid composition around CB1R, in particular cholesterol content (Maccarrone, 2010), and the possible formation of CB1R homodimers and heterodimers with other GPCRs (Pertwee *et al.,* 2010).

Thus, the complexity of the entire response initiated by CB1R activation is not only due to the wide range of cannabinoid ligands and effectors, but also to the interconnectivity between signaling pathways.

1.3. Synaptic plasticity mediated by the endocannabinoid system

Specific patterns of synaptic activity produce changes in the strength or the efficacy of synaptic transmission and contribute to behavioral changes, a process called **synaptic plasticity** (Martin *et al.,* 2000). The components of the ECS are predominantly expressed on the synaptic terminal modulating synaptic transmission and plasticity in different cell populations (Kano *et al.,* 2009).

The main mechanism by which endocannabinoids modulate synaptic function is through their retrograde signaling suppressing neurotransmitter release. This suppression can be transient, producing endocannabinoid-mediated short-term depression (eCB-STD), or long-lasting leading to endocannabinoid-mediated long-term depression (eCB-LTD) (Kano, 2014). To date, eCB-STD has been observed in

different brain areas, but it is better studied in the hippocampus and cerebellum, where it influences behavior by modifying neuronal excitability. In the hippocampus, postsynaptic pyramidal cell depolarization induces the release of endocannabinoids and presynaptic CB1R activation, mainly in GABAergic interneurons and to a minor extent in glutamatergic neurons (Kano *et al.,* 2009). CB1R activation results in two main forms of eCB-STD, the depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku *et al.,* 2001; Wilson and Nicoll, 2001) and depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001) in GABAergic and glutamatergic synapses, respectively. Both DSI and DSE are induced by postsynaptic depolarization which produces Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC) and therefore endocannabinoid release (Ohno-Shosaku *et al.,* 2012) (Figure 8A). Other forms of eCB-STD occur in the hippocampus independent to postsynaptic terminal. In these cases, the release of endocannabinoids can be induced by the activation of Gq/11-coupled receptors like metabotropic glutamate receptors (mGluRs) (Varma *et al.,* 2001) and M1/M3 muscarinic acetylcholine receptors (Kim *et al.,* 2002). Although it is known the contribution of these forms of eCB-STD in neuronal modulation, its functional relevance *in vivo* remains to be clarified (Augustin and Lovinger, 2018).

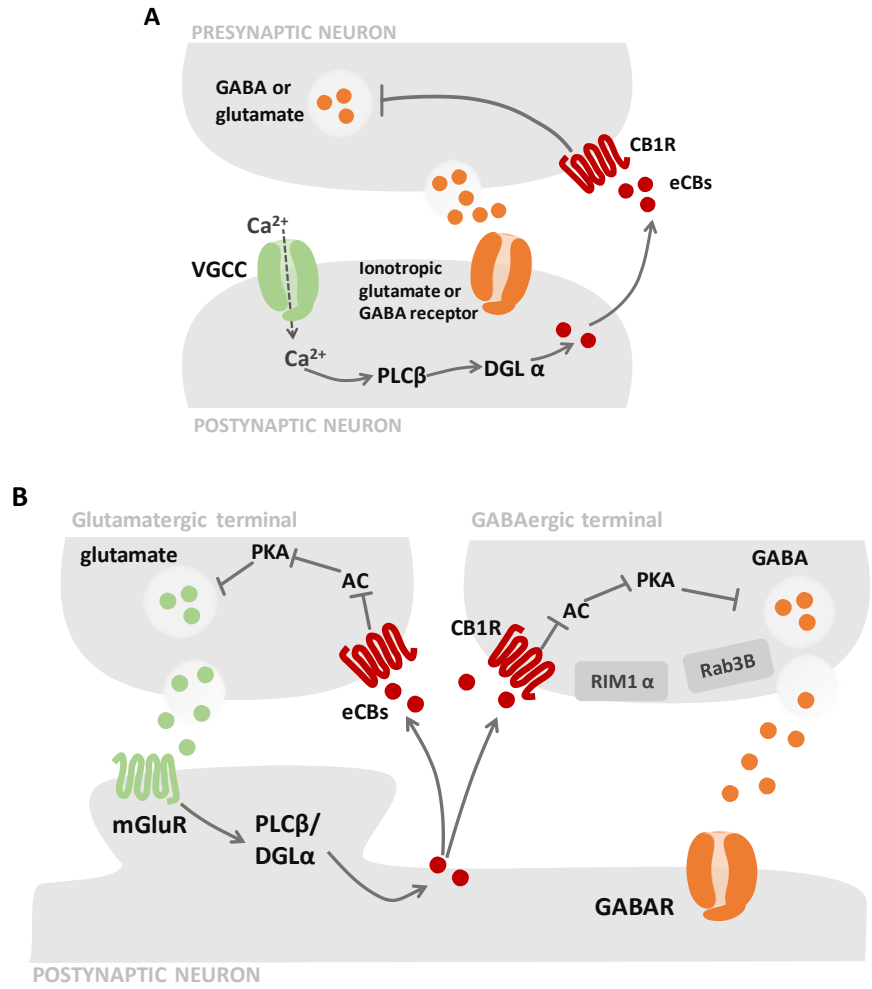

Figure 8. Synaptic plasticity induced by ECS. (A) Short-term depression. Postsynaptic activity triggers Ca^{2+} influx via voltage-gated Ca^{2+} channel (VGCC). Postsynaptic Ca^{2+} promotes diacylglycerol lipase (DGLα)-mediated eCBs production, which retrogradely targets presynaptic CB_1Rs to reduce neurotransmitter release. **(B)** eCB-mediated excitatory long-term depression (LTD) and inhibitory LTD (iLTD). Patterned presynaptic stimulation releases glutamate, which activates postsynaptic mGluRs coupled to PLCβ and DGLα. eCBs homosynaptically target CB_1Rs localized to excitatory terminals and heterosynaptically engages CB_1Rs at inhibitory terminals. A $G_{\alpha i/o}$-dependent reduction in adenylyl cyclase (AC) and protein kinase A (PKA) activity suppresses transmitter release. The active zone protein RIM1α and the vesicle-associated protein Rab3B are also necessary for iLTD. Adapted from (Castillo *et al.,* 2012)

eCB-LTD in the hippocampus is mainly induced at synapses of cholecystokinin-containing GABAergic interneurons, where CB1R is predominantly expressed (Heifets and Castillo, 2009). There, endocannabinoids inhibit GABA release and trigger LTD in inhibitory terminals (iLTD) (Carlson *et al.,* 2002; Chevaleyre and Castillo, 2003) (Figure 8B). iLTD is induced by repetitive activity inputs from surrounding excitatory synapses, which produce the activation of mGluRs coupled to PLCβ and DGLα leading to endocannabinoid mobilization to travel retrogradely and bind to CB1R (Chevaleyre and Castillo, 2003). The molecular mechanisms of iLTD seem to involve cAMP/PKA signaling pathway, the active zone protein RIM1α, vesicle associated protein Rab3B and VGCC, although the exact mechanisms remain to be clarified (Chevaleyre *et al.,* 2007; Tsetsenis *et al.,* 2011). Furthermore, CB1R activation increases protein synthesis through mTOR signaling, suggesting the implication of this signaling pathway in eCB-LTD induction (Younts *et al.,* 2016).

Both DSI and iLTD produce the inhibition of GABA release, facilitating the induction of long-term potentiation (LTP) at excitatory CA1 hippocampal neurons (Carlson *et al.,* 2002; Chevaleyre and Castillo, 2003) and this facilitation is crucial for learning and memory (Xu *et al.,* 2014). Besides the classical endocannabinoid retrograde signaling, some evidences show that endocannabinoids can regulate LTP in the hippocampus by unconventional non-retrograde manner through the activation of postsynaptic CB1R (Maroso *et al.,* 2016), CB1R on astrocytes (Han *et al.,* 2012; Robin *et al.,* 2018) or TRPV1 (Chávez *et al.,* 2010).

1.4. Physiological role of the endocannabinoid system

The ubiquitous cannabinoid receptor distribution, the multiplicity of cannabinoid ligands and the complexity of their signaling pathways contribute to the large number of physiological functions controlled by the ECS. Extensive research using pharmacological and genetical approaches allowed to clarify the physiological functions regulated by the ECS at both central and peripheral tissues.

1.4.1. Central functions regulated by the endocannabinoid system

At the central level, the ECS plays an important role modulating **synapse homeostasis** and the physiological control of multiple brain functions. The components of the ECS, and specifically CB1Rs, are highly expressed in different brain areas and understanding their distinct distribution leads to elucidate ECS function (Hu and Mackie, 2015) (Table 2). The expression of CB1R in the hippocampus and cortex is associated with the control of **learning and memory**, whereas CB1R in the cerebellum and basal ganglia is responsible for fine **motor coordination** control and cerebellar learning performance (Kishimoto and Kano, 2006; Hu and Mackie, 2015). CB1R distribution in prefrontal cortex, hypothalamic nuclei and amygdala is related to the regulation of stress, anxiety and fear responses (Lutz *et al.,* 2015). Moreover, CB1R in the striatum and ventral tegmental area is involved in reward processing and addiction (Maldonado *et al.,* 2006; Parsons and Hurd, 2015). Nociception under different types of acute and chronic pain is also regulated by the ECS mainly expressed in the spinal cord and inhibitory descending pathways (Guindon and Hohmann, 2009; La

Porta *et al.,* 2014). Finally, ECS also controls food intake and energy balance, being the main areas involved the hypothalamus and mesolimbic regions (Di Marzo and Matias, 2005).

CNS structure	Physiological role	Examples of pathological roles
Hippocampus	Learning and memory	Memory impairment
Basal ganglia	Movement control	Slowed reaction time
Cerebellum	Motor coordination balance	Motor coordination impairment
Neocortex	Higher cognitive functions	Altered cognitive functions (judgement, consciousness)
Nucleus Accumbens	Motivation and reward	Drug addiction
Hypothalamus	Body housekeeping functions (body temperature regulation, reproductive function)	Neuroendocrine alterations (increase appetite)
Brain stem	Sleep and arousal, motor control	Alterations on heart rate and blood pressure
Amygdala	Emotional response and fear	Anxiety and paranoia
Spinal cord	Nociception	Altered pain sensitivity

Table 2. Physiological and pathological ECS function in brain regions with higher CB1R expression. CB1R is localized in multiple brain regions where it plays a variety of physiological roles and participates in different pathological states (From (Kano *et al.,* 2009; Hu and Mackie, 2015)).

1.4.2. Peripheral functions regulated by the endocannabinoid system

The components of the ECS are also present in the peripheral nervous system (PNS) as well as in the peripheral tissues controlling several physiological functions (Maccarrone *et al.,* 2015) (Table 3).

In the PNS, CB1R is mainly expressed on the **sympathetic nerve terminals** controlling adrenaline and noradrenaline release into different peripheral regions (Ishac *et al.,* 1996; Tam *et al.,* 2008). In peripheral tissues, the ECS plays an important role in the immune, gastrointestinal, cardiovascular and reproductive systems (Maccarrone *et al.,* 2015). Regarding the immune system, CB2R controls cell migration and the release of several inflammatory factors (Liu *et al.,* 2013; Haskó *et al.,* 2014). Modulation of endocannabinoid signaling in the gastrointestinal tract contributes to the regulation of intestinal motility, barrier permeability, immune functions and the control of food intake and energy balance through both CB1R and CB2R. Although the ECS in the liver is normally quiescent, the overactivation of CB1R has a critical role in pathological conditions, such as liver disease and obesity (Osei-Hyiaman *et al.,* 2005). Similarly, ECS in the cardiovascular system is involved in pathological situations, where it contributes to the progression of its associated-disorders (Steffens and Pacher, 2012). Finally, endocannabinoid signaling in the reproductive system is crucial both in male and female contributing to the correct embryo implantation, oocyte maturation and sperm quality (Wang *et al.,* 2006). Besides the previously mentioned systems, the expression of the CB1R has been reported in skeletal muscle, bone, skin, adipose tissue and adrenal glands, among others (Maccarrone *et al.,* 2015).

At the cellular level, in addition to modulating neurotransmitter release, the ECS regulates different processes including neurogenesis, neural progenitor proliferation and lineage segregation (Galve-Roperh *et al.,* 2013). Moreover, the ECS also mediates **synaptic plasticity**, neuronal survival and neuroprotection against damage (Panikashvili *et*

al., 2001; Kano *et al.,* 2009). Overall, this huge contribution of the ECS as a major homeostatic mechanism allows the modulation of a large amount of central and peripheral responses. Thus, alterations on the components of the ECS could produce different neurological and metabolic disorders (Table 2 and 3).

Peripheral tissue	Physiological role	Examples of pathological roles
PNS	Adrenergic tone control and nociception	Pain, migraine
Immune	Cell migration and cytokines production	Rheumatoid arthritis
Gastrointestinal	GI tract: mobility, gastric acids secretion, neurotransmitter and hormones release, barrier function and energy balance	Celiac disease, Inflammatory bowel disease
	Liver: Quiescent	Hepatic insulin resistance, fibrosis, and lipogenesis
Cardiovascular	Quiescent	Cardiovascular dysfunction, oxidative stress and inflammation
Reproductive	Male: Preservation normal sperm function	Infertility
	Female: Oocyte maturation, embryo implantation, embryonic development	Infertility
Adipose tissue	Thermogenesis and lipogenesis control, mitochondrial biogenesis	Obesity
Locomotor system	Muscle: Energy metabolism (Glucose oxidation) and muscular fibers formation	Muscular dystrophy
	Bone: elongation and remodeling	Osteoporosis
Skin	Proliferation, Differentiation, cell survival, and immune responses	Acne, seborrhea, Allergic dermatitis, itch and psoriasis, pain, psoriasis
Adrenal gland	Catecholamines and aldosterone synthesis and release	HPA axis and blood pressure alterations

Table 3. Physiological and pathological roles of ECS in the periphery. CB1R and CB2R are present in a wide variety of peripheral tissues controlling diverse physiological functions, whose alteration produce different pathological states.

1.5. Potential therapeutic applications of the endocannabinoid system

Cannabis preparations have been employed for medical uses over centuries. Nowadays, cannabinoids are still under investigation in the search for therapeutic applications. Some of the beneficial properties derived from the use of **cannabinoid agonists** include analgesia, stimulation of appetite, antiemesis, immunosuppression, antineoplastic and **anti-inflammatory effects** (Pertwee, 2012). However, CB1R activation, through direct or indirect agonists, also produces different **negative consequences**, such as **cognitive deficits**, **motor impairment** or sedation (Pertwee, 2009). Furthermore, alteration in the components of the ECS, including cannabinoid receptor expression or their coupling efficiency, endocannabinoid metabolizing enzymes or endocannabinoid levels, have been described in different pathological states. These alterations, in some cases, appeared as a protective mechanism to suppress unwanted symptoms and the progression of the disorder (Pertwee, 2009). Notably, preclinical studies have demonstrated that the activation of CB1R might be beneficial in neuropathic and inflammatory pain (Donvito *et al.,* 2018); neuropsychiatric disorders such as depression and anxiety (Hillard *et al.,* 2012); neurodegenerative diseases including multiple sclerosis, Huntington's disease (Sagredo *et al.,* 2012) and Alzheimer's disease (Aso and Ferrer, 2014); brain ischemia (Hasenoehrl *et al.,* 2017) and inflammatory bowel disorders (Hasenoehrl *et al.,* 2017). Contrary, **CB1R blockade** has been proposed for those conditions where CB1R activity contribute to the progression of the disease, for example in obesity, diabetes type-2 (Richey and Woolcott, 2017), reproductive

disorders (Battista *et al.,* 2015), schizophrenia (Saito *et al.,* 2013), Parkinson's disease (Brotchie, 2003) and intellectual disabilities such as Fragile X syndrome (Jung *et al.,* 2012; Busquets-Garcia *et al.,* 2013; Gomis-González *et al.,* 2016) and Down syndrome (Navarro-Romero *et al.,* 2019).

Hence, the goal of cannabinoid-based drugs is to explore their promising therapeutic applications minimizing the adverse consequences.

1.5.1. Cannabinoid agonism

Different CB1R/CB2R agonists have already been developed (Table 4). First Δ^9-THC (dronabinol; Marinol®) and then its synthetic analogue nabilone (Cesamet®) were approved for the suppression of nausea and vomiting produced by chemotherapy. Afterward, dronabinol was applied as an appetite stimulant for patients suffering cachexia induced by chemotherapy and AIDS (Pertwee, 2009, 2012). Nowadays, Sativex®, which contains approximately equal proportion of Δ^9-THC and cannabidiol, is used for the treatment of spasticity in multiple sclerosis patients and for neuropathic pain (Urits *et al.,* 2019). Moreover, Epidolex®, a purified extract containing cannabidiol, is used to treat refractory epilepsies associated with Lennox-Gastaut syndrome and Dravet syndrome (Sekar and Pack, 2019).

However, these compounds exert some unwanted side-effects, principally caused by the **psychoactive properties** of CB1R activation. In order to minimize them, some **emerging strategies** have been developed. One approach is the use of the **peripherally-restricted compounds**. Thus, SAB378 (also named CB13) and NEO1940, both

incapable to cross the blood barrier have been generated and investigated for pain or cancer-induced cachexia, respectively (Di Marzo, 2018).

Taking account the predominantly CB2R expression in immune tissues, targeting CB2R is a promising approach to treat inflammatory and autoimmune diseases. Notably, different **CB2R agonists** have been used in pre-clinical studies of pain, arthritis, cancer, Parkinson's, Huntington's and Alzheimer's disease, among others (Cassano *et al.,* 2017) and some of them are under clinical evaluation with different therapeutic purposes (Table 4).

Besides these "direct strategies" through specific agonists, other "indirect mechanisms" have been assessed. Among them, the **inhibition of the endocannabinoid-degrading enzymes**. FAAH and MAGL inhibitors have been synthetized to increase the levels of AEA and 2-AG, respectively. Moreover, pharmacological or genetic inactivation of FAAH and MAGL reduces AA, product of AEA and 2-AG degradation, and therefore the production of inflammatory mediators (Leishman *et al.,* 2016). Thus, FAAH and MAGL inhibitors are of interest due to their potential anti-inflammatory properties. Nowadays, none of these compounds have been approved for therapeutic use, but there are several under clinical trials for the treatment of multiple disorders. Unfortunately, the phase I clinical trial of the FAAH inhibitor BIA 10-2474 was interrupted due to the death of one volunteer and brain damage in four others. Nevertheless, these severe adverse effects occurred due to the interaction of the drug with another target different from FAAH (van Esbroeck *et al.,* 2017). To date, some FAAH

inhibitors are under clinical trials including PF-04457845 and V158866, which have demonstrated good tolerance in humans.

Preclinical studies with the MAGL inhibitor JZL184 have demonstrated beneficial properties under brain injury and inflammatory conditions (Nomura *et al.,* 2011; Katz *et al.,* 2015; Pihlaja *et al.,* 2015). Moreover, the MAGL inhibitor, ABX-1431, has entered phase II clinical trial with promising results for the treatment of Tourette syndrome (Leishman *et al.,* 2016). However, the MAGL inhibitor PF-06818883, investigated for the treatment of intracerebral hemorrhage, revealed safety problems in phase I clinical trial (NCT03020784).

In addition, the inhibition of the endocannabinoid-degrading enzymes could affect other metabolic pathways, such as the oxygenation of AEA and 2-AG by COX-2 promoting the increase of prostamides and PG-Gs, which exert several inflammatory effects (see section 5.1.2) (Urquhart *et al.,* 2015).

1.5.2. Cannabinoid antagonism

SR141716A (rimonabant, Acomplia®), a CB1R antagonist/inverse agonist, was marketed in Europe in 2006 to treat obesity and cardiometabolic disease. **Rimonabant** demonstrated effectiveness decreasing weight in obese patients, improving their lipid profile and glucose control (Patel and Pathak, 2007). Unfortunately, in 2008 rimonabant **was withdrawn** from the market because of the appearance of psychiatric side effects including depression, anxiety and suicidal ideation (Samat *et al.,* 2008).

It is thought that the undesired effects produced by rimonabant were due to its CB1R inverse agonist properties in the CNS (Meye *et al.,*

2013). Despite this result, the design of CB1R antagonist to treat metabolic disorders could be still on interest. **Alternative approaches** have been developed to devoid the CNS side effects: the use of neutral CB1R antagonists, peripherally-restricted CB1R antagonists or allosteric CB1R modulators. In addition, to avoid CNS side effects, the ability of the ECS to control peripheral functions has received a considerable attention as a target for many peripheral disorders. Thus, the interest of developing peripherally-restricted antagonists has increased. Among them, TM38857, JD5037 and AM6545 have been demonstrated promising preclinical results to reduce body weight and improve metabolic profile in obese mice (Receveur *et al.,* 2010; Tam *et al.,* 2010, 2012).

In this thesis we will mainly focus in studying the **antagonism of peripheral CB1R** and its possible effects on memory in wildtype mice and in an animal model of intellectual disability, the **fragile X syndrome**, as well as, the **inhibition of MAGL** and its effects on motor coordination and cerebellar neuroinflammation. Thus, specific sections about, memory, fragile X syndrome, motor coordination and neuroinflammation will be discussed.

Drug	Action	Indications
Cannabinoid agonists:		
Nabilone	CB1R and CB2R agonism	Cachexia in cancer and AIDS patients; chemotherapy-induced nausea and vomiting
Sativex	CB1R and CB2R agonism and cannabidiol actions	Spasticity in multiple sclerosis patients; neuropathic pain
Epidiolex	Cannabidiol actions	Refractory epilepsies in Lennox-Gastaut and Dravet syndromes
GW842166	CB2R agonism	Pain
S-777469	CB2R agonism	Atopic dermatitis
JBT-101	CB2R agonism	Systemic lupus, erythematosis, scleroderma, dermatomyositis and cystic fibrosis
APD371	CB2R agonism	Abdominal pain in Crohn's disease
SAB378	Peripheral CB1R and CB2R agonism	HIV-associated neuropathy
NEO1940	Peripheral CB1R and CB2R agonism	Cancer and anorexia or cachexia associated with cancer
Inhibitors of degradation endocannabinoid enzymes:		
PF-04457845	FAAH inhibiton	Osteoarthritic pain
URB597	FAAH inhibiton	Symptoms of schizophrenia
V158866	FAAH inhibiton	Spinal cord injury-induced neuropathic pain
JNJ-42165279	FAAH inhibiton	Social anxiety disorders, major depressive disorder with anxious distress
BIA 10-2474	FAAH inhibiton with other targets	Anxiety, Parkinson disease, chronic pain, cancer and hypertension
PF-06818883	MAGL inhibition	Cerebral hemorrhage
ABX-1431	MAGL inhibition	Tourette syndrome, neuralgia, myelitis, neuropathies and multiple sclerosis
Cannabinoid antagonist:		
Rimonabant	CB1R inverse agonism and/or antagonism	Obesity, type-2 diabetes and dyslipidemia

Table 4. Clinical tested ECS-based drugs. Adapted from (Di Marzo, 2018).

2. Memory

Memory is a type of cognitive function that classifies, encodes, stores and retrieves past or present relevant information for the subject (Kandel, 2001). It represents an adaptative mechanism to react in the future based on past and present information (Tetzlaff *et al.,* 2012).

2.1. Memory stages

Memory can be classified depending on its temporal or content dimension. According to the **temporal dimension**, which is referred to as the time that the information is available for the subject, memory can be classified in four general types (Figure 9):

- **Sensory memory** is the ability to retain impressions of sensory information after the stimuli have ended. It corresponds to the initial milliseconds after an item is perceived and involves the visual and auditory senses (Sperling, 1960).
- **Working memory** is referred as a brain system that allows a temporal storage and manipulation of information in mind, which can then be necessary for the execution of complex cognitive tasks including language comprehension, learning and reasoning (Baddeley and Logie, 1999). This memory includes different components: the central executive and the phonologically and visuospatial based store (Baddeley and Hitch, 1974). The time scale of working memory goes from milliseconds to min (Tetzlaff *et al.,* 2012) and the main region involved is the prefrontal cortex (Funahashi, 2017).

- **Short-term memory** is the capacity to retain information from the immediate past. Although short-term memory is more susceptible to perturbations, it can last from min to days in humans and from min to 3-4 hours in rodents. In this kind of memory, the main region implicated is the hippocampus (Walker and Davies, 2003; Kumaran, 2008).
- **Long-term memory** operates on a time scale from days to years in humans, sometimes entire lifetime, and from hours to days in mice. Long-term memory involves protein synthesis, synaptic modifications and structural plasticity changes and implicates several brain areas (McGaugh, 2000; Frankland *et al.,* 2004; Costa-Mattioli *et al.,* 2009; Xu *et al.,* 2009).

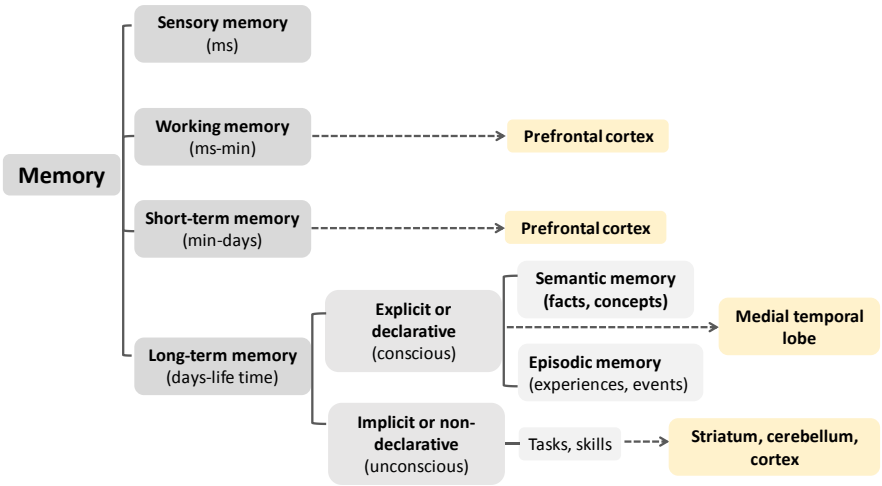

Figure 9. Schematic representation of the different memory types depending on the temporal and content dimension.

Regarding the **content dimension**, long-term memory can be divided in explicit and implicit memories. Firstly, explicit memory, also named **declarative memory**, consists on the conscious integration of facts and events in humans. In animals, this memory is described as the processing of spatial, configural, contextual and relational information (Richter-Levin, 2004). Declarative memory requires the contribution of the medial temporal lobe structures, as their damage triggers amnesia in patients (Squire *et al.,* 2004). This memory is subclassified into **semantic memory**, which comprises the knowledge of facts and concepts about the world, and **episodic memory**, which implicates experiences and events of personal life (Squire, 1992; Squire and Zola, 1998). Secondly, implicit or **non-declarative memory** involves the collection of non-conscious abilities that are expressed through performance. It is referred to the learning of motor and perceptual skills. This kind of memory depends mostly on striatum, cerebellum and cortical association areas (Squire, 2004).

Memory is a dynamic process that involves different stages, being the most important acquisition, consolidation, retrieval and reconsolidation (Figure 10). **Acquisition**, also named encoding, consists on getting and associating sensory information to convert them in a memory (Abel and Lattal, 2001). **Consolidation**, or storage, occurs when a labile memory passes into a more stable, long-lasting form (Squire *et al.,* 2015). Several studies have demonstrated that this process involves gene transcription and protein translation (Suzuki *et al.,* 2004). Regarding **retrieval**, this process implicates the recall of the memories previously stored. Interestingly, when memories are retrieved, they return to a labile state becoming susceptible to

disruption or **reconsolidation**, this is a process that involves gene transcription and protein synthesis (Lee, 2010; Nader, 2015).

Another aspect of memory dynamism is that retrieval can induce the suppression of the memory previously consolidated. This process is named **extinction** and it is considered a new learning, since a new association inhibits the expression of the previous one (Abel and Lattal, 2001). Furthermore, extinction also requires protein synthesis (Suzuki *et al.,* 2004).

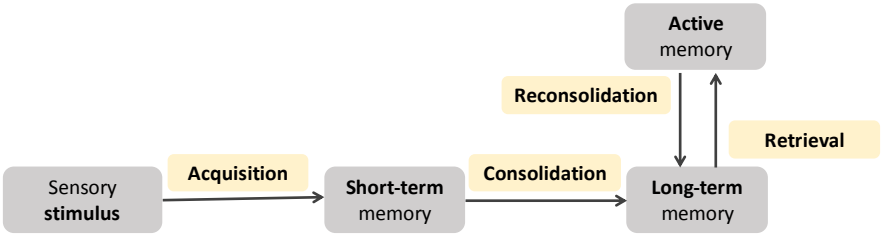

Figure 10. Memory stages. After receiving a sensory input, this information could be converted as a short-term memory. This process is named acquisition. At this moment, memory is considered labile, transient and sensitive to disruption. If this transient information undergoes a process of consolidation, memory becomes resistant to disruption, stabilized as long-term memory. Retrieval convert long-term memory into a labile/active state susceptible to be disrupted or reconsolidated. Adapted from (Drumond *et al.,* 2017)

In addition, memories are continuously modified and **modulatory systems**, including mood, motivation and attention may influence memory consolidation, retrieval, reconsolidation and extinction (Roozendaal and McGaugh, 2011). For instance, novelty produces an increase in arousal, an emotional state that can modulate the intensity and duration of memories during a limited time window (Gold and Korol, 2012).

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2.2. Neuroanatomical substrates of memory

Several studies performed in patients during the 19th century demonstrated that medial temporal lobe resection or damage produced amnesia, pinpointing this area as the main neuroanatomical substrate of memory, specifically for explicit memory (Scoville and Milner, 1957; Squire and Zola, 1998). Subsequent studies with animal models confirmed that the anatomical medial temporal lobe components involved in memory system are hippocampal and parahippocampal regions (Zola-Morgan *et al.,* 1989; Squire and Zola, 1998; Cipolotti *et al.,* 2001; Squire *et al.,* 2004). The **hippocampal formation** is considered the center of memory formation network and it is highly conserved across mammal species (Scoville and Milner, 1957). We will focus in the hippocampal formation of rodents, as it is the model and brain area studied in this thesis.

The rodent hippocampal formation is a C-shaped structure situated in the caudal region of the brain. It includes three different parts: the dentate gyrus, the hippocampus (*cornu ammonis* (CA), CA1 and CA3) proper and the *subiculum* (van Strien *et al.,* 2009). The cortex of the hippocampal formation has three layers called differently among the hippocampal regions. The deeper layer comprises afferent and efferent fibers and interneurons (dentate gyrus: hilus; CA: *stratum oriens*), a more superficial layer is composed of principal neurons and interneurons (dentate gyrus: granule layer; CA and subiculum: *stratum pyramidale* or pyramidal cell layer) and the most superficial layer is considered as the *stratum moleculare* or molecular layer. In CA regions the molecular layer is divided into different sublayers, comprising the *stratum lucidum*, which is exclusively from CA3 and receives the input

from the dentate gyrus, the *stratum radiatum* composed of the apical dendrites of pyramidal neurons and the *stratum lacunosum-moleculare* containing their apical thufts (van Strien *et al.,* 2009) (Figure 11).

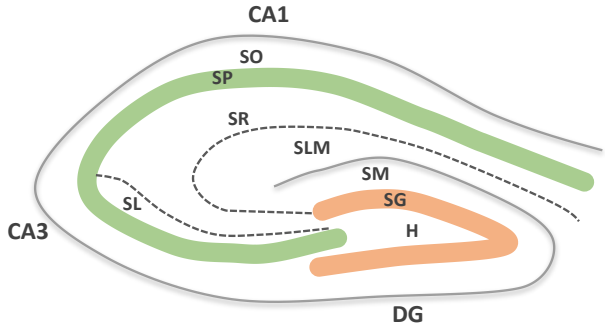


Figure 11. Schema of hippocampal rodent structure and lamination. CA, *cornu ammonis*; DG, dentate gyrus; H, hilus; SO, *stratum oriens*; SP,*stratum pyramidale*; SR, *stratum radiatum*: SLM, *stratum lacunosum-moleculare*; SL, *stratum lucidum*; SM, *stratum moleculare*; SG, *stratum granulosum*.

The **parahippocampal formation** is situated adjacent to the hippocampal formation and is divided into five regions; the pre-subiculum, the prasubiculum, the entorhinal cortex, the perirhinal cortex and the postrhinal cortex (van Strien *et al.,* 2009).

Altogether the **hippocampal-parahippocampal** regions contain a diversity of neural circuits and cell-types that interact to generate a **circuit of information** (Figure 12). The standard circuit is integrated by the cerebral cortex regions joined to the parahippocampal region, which in turn aimed to the hippocampal formation through two parallel projection routes: the perirhinal cortex sends non-spatial and sensory information to the lateral entorhinal cortex, whereas the posthrinal cortex transmits spatial information to the medial entorhinal cortex.

From the entorhinal cortex, these projections reach to the hippocampal formation through two routes, the direct and the indirect pathways. The most well-characterized is the indirect stream of information via the **trisynaptic circuit** (ECII→DG→CA3→CA1) (Figure 12). In this route, the stellate cells from the entorhinal cortex layer II synapse through the perforant pathway to the granule cells of the dentate gyrus, which in turn sends excitatory inputs to CA3 via mossy fibers pathway. Then, the axons from CA3 pyramidal neurons project to CA1 pyramidal neurons via Schaffer collaterals. Beyond the trysinaptic pathway, the entorhinal layer III projects directly to CA1 pyramidal neurons trough the temporoammonic pathway (ECIII →CA1). These direct and indirect corticohippocampal projections target different regions of CA1, since the indirect pathway rinse to the apical pyramidal dendrites from the *stratum radiatum* layer, whereas the direct inputs synapse on apical pyramidal dendrites from the *stratum lacunosum molecular* layer. Lastly, CA1 pyramidal neurons back projections to the deeper layer of the entorhinal cortex to close the cortex-hippocampal loop. In addition, CA3 axons send collaterals synapses to other CA3 neurons (van Strien *et al.,* 2009; Deng *et al.,* 2010).

Although parahippocampal and hippocampal formation are brain regions clearly involved in declarative memories, **other cortical and subcortical regions are also involved**. Noteworthily, it is though that memories are retained in the hippocampus during a period, then the information is transferred to the neocortex, where it can be stored for a longer period. This process produces a network reorganization, changing the center of network from the hippocampus to medial

prefrontal/anterior cingulate cortex (Frankland and Bontempi, 2005; Insel and Takehara-Nishiuchi, 2013).

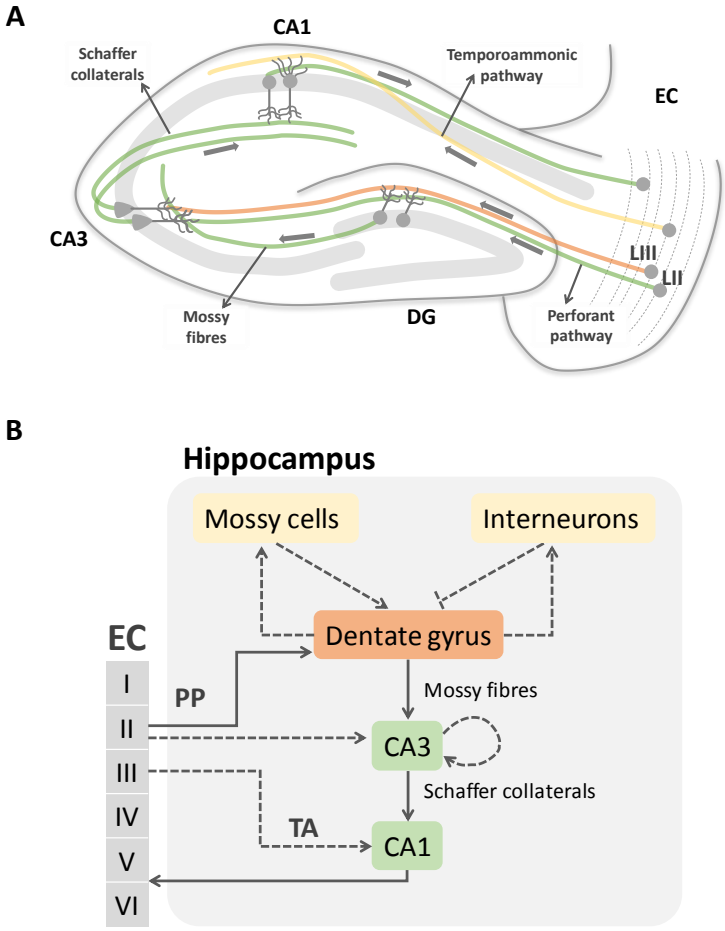


Figure 12. The parahippocampal-hippocampal network. (A) Illustration of the parahippocampal-hippocampal circuit. **(B)** Diagram of the hippocampal network. Solid arrows depicted the traditional excitatory trisynaptic loop: Neuronal axons from the layer II in the enthorinal cortex (EC) project to the dentate gyrus through the perforant pathway (PP) that includes the lateral and medial perforant pathways. The dentate gyrus sends projections to CA3 pyramidal cells through mossy fibers. CA3 pyramidal neurons project the information to CA1 pyramidal neurons by Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. Dashed arrows depicted other alternative projections: CA3 receives direct neuronal axons from the EC layer II through the PP and CA1 receives direct projections from the EC layer III through the temporoammonic pathway (TA). In addition, 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 dentate gyrus. CA1, CA2 and CA3: *cornu ammonis*. Adapted from (Deng *et al.,* 2010).

Another aspect is that **emotional arousal experiences** can produce the activation of hormonal and brain regions that regulate memory consolidation and therefore memory strength. This effect requires **noradrenergic activation** and their interaction with different brain areas including the amygdala and *locuscoeruleus* (LC) (Roozendaal and McGaugh, 2011) (see section 2.6).

2.3. Behavioural paradigms to study memory in mouse models

Behavioral studies using rodents as animal model have widely contributed to understand the neuroregulatory mechanism of human learning and memory in healthy and disease. There are several protocols and mazes to perform these behavioral tasks (Table 5). Usually, the motivation of mice to perform these different tasks is driven by their **instinctive behavior**. For example, the exploratory behavior is challenged in the Y-maze alternation task or the novel object-recognition task, whereas social interactions are presented in the social-recognition test (Paul *et al.,* 2009; Arakawa and Iguchi, 2018). More complex tasks involve the use of **reinforcers**, either positive (food and water) in the radial arm maze, or negative (electric shock) in the case of passive or active avoidance tests.

This thesis is focused on the study of non-emotional memory, specifically memory related to novel stimulus. Thus, we use the **novel object-recognition test** to asses memory in mice.

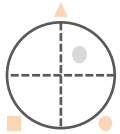
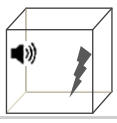
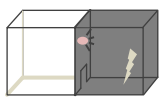
Test	Description	Brain areas	Scheme
Morris Water Maze	It is considered a spatial learning test based on finding the location of a hidden platform submerged in a pool of water. The pool is surrounded by different spatial cues. Rodents are trained during several consecutive days and the time/path length they take to find the platform is measured as a learning index.	Hippocampus	
Radial arm maze	It is a spatial learning task with multiple versions. The apparatus commonly consists of eight arms baited with a reward, and food-deprive rodents are required to remember which arms have already visited. The number of errors when animal re-enter in an arm previously visited are counted.	Hippocampus and prefrontal cortex	
Spontaneous alternation task (Y-maze)	It is used to evaluate working memory through the willingness of rodents to explore. The task is performed in a Y-shaped maze which rodents are allowed to freely explore. The number of arm entries and the number of trials are recorded to calculate the percentage of alternation.	Hippocampus and prefrontal cortex	
Fear conditioning	It is a Pavlovian aversive learning task where animals associate a non-aversive conditioned stimulus (light or sound) with an aversive unconditioned stimulus (electrical foot-shock). Conditioned response (freezing) can be measured as memory indicator.	Amygdala, hippocampus and prefrontal cortex	
Passive avoidance	It is based on the inhibition of the natural tendency of animals to hide in an apparently safer place (dark compartment) which has been previously paired with an electrical foot-shock.	Amygdala, hippocampus and prefrontal cortex	
Active avoidance	It is an avoidance task in which mice are placed in a box with two compartments separated by an open door. Mice have to learn that after a cue light, they will receive an electrical foot-shock unless they change to the other compartment to avoid negative reinforcer.	Amygdala, striatum and prefrontal cortex	

Table 5. Non-operant behavioral tests to study learning and memory in rodents. Description, main brain regions involved and scheme of each test (Adapted from (Lee and Silva, 2009)).

2.3.1. Novel object-recognition test

Object-recognition memory is considered a kind of declarative memory that is commonly impaired in humans during neurodegenerative disorders or brain damage (Winters *et al.,* 2008).

The novel object-recognition test (**NORT**) (Ennaceur and Delacour, 1988) is used to study object-recognition memory in mice and assesses the judgment of a previously encountered item as familiar. Object-recognition memory evaluated in the NORT is based on mice **innate preference to explore a novel object** rather than another object previously presented (Berlyne, 1950). Usually, in the NORT animals are exposed to two identical objects in a familiar context. Then one of these objects is replaced by a novel one. Thus, an animal that remembers the familiar object will spend more time exploring the novel object. Object-recognition memory can also be studied in humans through visual paired comparisons, pointing the use of NORT in rodents as a useful translational instrument to explore the effect of different drugs or evaluate the efficacy of novel therapeutic targets (Bengoetxea *et al.,* 2015; Grayson *et al.,* 2015).

NORT differs from other memory tests because it is one-trial task, without involving learning rules. This allows studying the effects of a drug on different stages of memory depending if it is administered before or after the familiarization session. Another advantage of this test is the absence of reinforcers, avoiding stressful factors and being more translational to human declarative memory tests. Moreover, this test has been replicated in different laboratories using different maze designs, animal strains and objects (Ennaceur and Delacour, 1988; Sik *et al.,* 2003; Bevins and Besheer, 2006; Puighermanal *et al.,* 2009).

In mice, the test commonly consists in three different phases including habituation, familiarization and test (Figure 12). During the habituation phase, the mouse is placed into the arena and is allowed to freely explore it and get used to the new environment. In the familiarization phase, two identical objects are presented, and the mouse explore them. In the V-shaped maze, the objects are placed at the end of each corridor. Finally, the test session could be performed after 3 hours to evaluate **short-term memory**, 24 hours to study **long-term memory,** or 48 hours to assess **memory persistence** (Gold and Korol, 2012). In this last session, one of the familiar objects is replaced for a novel one. Total exploration time of each object is measured and then a discrimination index is calculated as a measure of memory performance. The preference for the exploration of the novel object requires the encoding, consolidation and retrieval of the information for the familiar object (Cohen and Stackman, 2015).

It is worth mentioning the presence of some variables that might influence test performance such as the presence of spatial cues, the duration of trials, the illumination of the arena or the type of objects (Antunes and Biala, 2012). It is important to test the objects and check that mice do not have preference when both objects are considered novel for the mice. Although NORT is classically performed in an open-field arena, some studies have used other environment, such as Y-shaped or **V-shaped mazes**. These alternatives allow reducing contextual and spatial information and consequently increasing total exploration times, the limiting factor of this test (Busquets-Garcia *et al.,* 2013; Vallée, 2014; Gomis-González *et al.,* 2016; Navarro-Romero *et al.,* 2019).

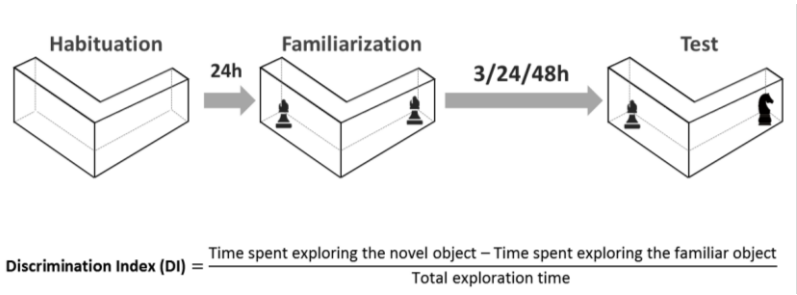


Figure 13. Schematic representation of the protocol used in this thesis, V-shaped maze, and the formula used to perform the novel object-recognition test in mice.

Two main brain areas are necessary for object-recognition memory: **the hippocampus** and **perirhinal cortex.** Although several studies reported object-recognition impairment in rodents (Baker and Kim, 2002; Gaskin *et al.,* 2003; Broadbent *et al.,* 2004; Cohen *et al.,* 2013) and humans (Pascalis *et al.,* 2004; Squire *et al.,* 2007) with hippocampal disfunction, in other cases no substantial or lasting deficits were described (Winters *et al.,* 2004; Forwood *et al.,* 2005). Similar studies only support the role of perirhinal cortex in the object-recognition memory (Barker *et al.,* 2007; Olarte-Sánchez *et al.,* 2015). These contradictory results consisted in brain-regional lesions or in local pharmacological inactivation of relevant brain areas, and the opposed results may be due to the lesion size, dose administration time, or retention time between the training and the test. Indeed, it is though that the perirhinal cortex supports memory for objects explored for a brief period or in the absence of contextual cues, whereas the hippocampus is crucial for longer retention times and with the presence of contextual and spatial information (Cohen and Stackman, 2015).

Noteworthy, both hippocampus and perirhinal cortex are critical for acquisition, consolidation and retrieval in object-recognition memory (Winters and Bussey, 2005; Winters *et al.,* 2008; Cohen *et al.,* 2013).

2.4. Hippocampal synaptic plasticity in memory

At the neurobiological level, learning and memory involves the specific synaptic communication between neurons and cellular factors. It is widely demonstrated that neurons in the brain areas involved in learning and memory processes are **highly plastic**. Upon experience, the pattern of neuronal activity can change and modify their function (synaptic transmission) and structure (synaptic connections and morphology). Many neurobiological mechanisms are involved in these dynamic processes. Notably, there are different post-translational modifications, such as the phosphorylation of ionotropic receptors or the activation of different signaling cascades, including the **mTOR signaling pathway**. Moreover, long-term memory requires controlled changes in gene expression and new protein synthesis. In this section some of these aspects will be discussed.

2.5. Synaptic plasticity and memory

Synaptic plasticity was experimentally demonstrated in 1973, when Bliss and Terje Lomo observed in a rabbit hippocampus that high-frequency patterns of stimulation induced LTP (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). **LTP** is an experimental paradigm of synaptic plasticity, in which high-frequency stimulation (100Hz) triggers a long-lasting increase in the synaptic strength of synaptic transmission.

LTP can be divided into two different phases, the early-phase (E-LTP) independent of protein synthesis and lasting 1-3h and the late-phase (L-LTP) that requires new protein synthesis it lasting longer than 3h, even weeks (Bliss and Collingridge, 1993; Abraham, 2003). Since its first discovery in the hippocampus, LTP has been described in several brain areas of different animal models such as, the cortex (Artola and Singer, 1987), cerebellum (Salin *et al.,* 1996) and amygdala (Clugnet and LeDoux, 1990). Among them, the **hippocampal CA3-CA1 LTP** is the most studied and the best-characterized synapse in terms of synaptic plasticity (Korte and Schmitz, 2016).

CA3-CA1 LTP in the hippocampus **depends on N-methyl-D-aspartate receptor (NMDAR)**. NMDAR is a ligand-coupled ion channel, permeable for Na^+ and Ca^{2+}. Under basal synaptic conditions, 3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) regulates the influx of Na^+ in the postsynaptic terminal and the NMDAR ion channel pore is occluded by Mg^{2+} ions. Postsynaptic depolarization removes Mg^{2+} allowing the influx of Na^+ and Ca^{2+} into the cell. Elevation in Ca^{2+} concentration inside the postsynaptic neuron activates several kinases involved in the induction of LTP, such as the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and PKA (Kumar, 2011) (Figure 14). CaMKII phosphorylates specific AMPAR subunits leading the **facilitation of AMPAR activity and its trafficking** (Huganir and Nicoll, 2013). Interestingly, mRNAs encoding AMPAR subunits, such as GluR1 and GluR2, have been found in the dendrites of hippocampal pyramidal neurons, where they are locally translated after synaptic activity (Grooms *et al.,* 2006). Moreover, the rise of Ca^{2+} can increase cAMP production through AC enzymes, and therefore enhances the

activation of PKA. Then, PKA activates MAPK signaling including ERK, that will phosphorylate different transcription factors, such as cAMP-response element-binding protein (CREB). CREB induces changes in gene transcription and triggers new protein synthesis (Benito and Barco, 2010). Notably, the relevance of newly synthesized proteins in synaptic plasticity has been thoroughly studied and different signaling cascades have been postulated as key regulators, such as the **mTOR signaling pathway**.

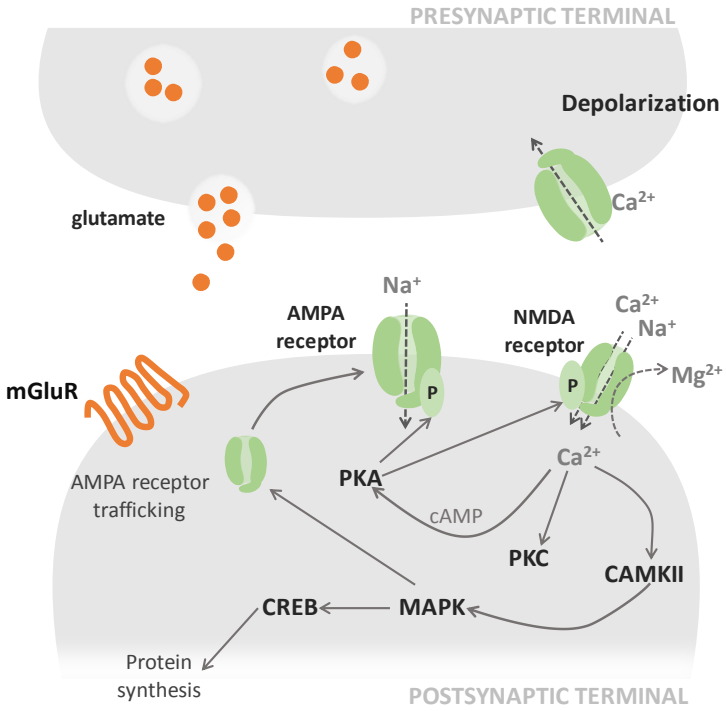


Figure 14. Synaptic plasticity in glutamatergic terminals. The arrival of a series of impulses at the presynaptic terminal triggers the release of glutamate, which binds to glutamate receptors at the postsynaptic membrane. On activation, AMPAR and kainate receptors conduct Na^+ ions, which initiate postsynaptic depolarization. Membrane potential changes initiate the release of Mg^{2+} ions that block NMDAR. Calcium influx through NMDA channels sets off a chain of events that establish long-term potentiation. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CaMKII, calcium/calmodulin-dependent kinase II; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C.

Besides LTP, other models of activity-dependent plasticity have been described, such as the LTD (Lynch *et al.,* 1977), excitatory postsynaptic potential-spike potentiation (Abraham *et al.,* 1985) or spike-timing-dependent plasticity (Dan and Poo, 2004). However, LTP presents different properties that makes it an ideal mechanism for long-term memory storage. These elements include: associativity, by which LTP induction at one synapse could be simultaneously regulated by other pathways; cooperativity, which refers to the observation that presynaptic stimulation has to be paired with postsynaptic depolarization for LTP induction; and specificity that refers only activated synapses could be potentiated and persistence which means that LTP lasts beyond the initial stimulation (Abel and Lattal, 2001). Nowadays strong evidences have demonstrated that altered LTP correlates with behavioral memory impairments. The first experimental proof was that the blockade of NMDARs in the hippocampus impaired learning (Morris *et al.,* 1986). Later, pharmacological and genetic experiments altering directly LTP or its molecular targets confirmed the role of LTP in different learning and memory behavioral tasks (Grant *et al.,* 1992; Tsien *et al.,* 1996; Rogan *et al.,* 1997; Giese *et al.,* 1998; Moser *et al.,* 1998; Shimizu *et al.,* 2000; Whitlock *et al.,* 2006). Indeed, it was observed that NORT in mice is accompanied with an increase of the strength of the CA3-CA1 synapse and its saturation by external high-frequency stimuli disrupted object-recognition memory, suggesting the importance of the CA3-CA1 synapse for a properly NORT performance (Clarke *et al.,* 2010).

2.5.1. Neurotrophic factors

Neurotrophins are key signaling molecules that can modify both synaptic transmission and structure in the hippocampus playing a crucial role in learning and memory (Lu *et al.,* 2014; Gibon and Barker, 2017). The **brain-derived neurotrophic factor (BDNF)** is the most predominant neurotrophin expressed in the hippocampus, where it has a crucial role for synaptic activity and plasticity at the CA3-CA1 synapse. *In vitro* studies with exogenous BDNF administration in hippocampal slices indicate that BDNF **facilitates LTP induction** (Figurov *et al.,* 1996). In parallel, genetic studies with both homo- and heterozygous BDNF KO mice show that BDNF deletion or reduction produces hippocampal-LTP impairment that is prevented with the exogenous application of BDNF (Korte *et al.,* 1995; Patterson *et al.,* 1996). At the behavioral level, post-training infusion of blocking anti-BDNF antibodies into CA1 region impairs object-recognition memory (Furini *et al.,* 2010). In addition, specific BDNF mice KO in hippocampal cells present memory deficits in the NORT (Heldt *et al.,* 2007).

Most of these hippocampal BDNF functions depend on its interaction with the tropomyosin-related kinase B (TrkB) receptor (Figurov *et al.,* 1996; Kang *et al.,* 1997; Chen *et al.,* 1999; Edelmann *et al.,* 2014). BDNF is expressed and released both pre- and pro-synaptically in an activity-dependent manner (Zafra *et al.,* 1990; Zakharenko *et al.,* 2003; Malenka and Bear, 2004; Edelmann *et al.,* 2015). At the presynaptic terminal, BDNF-dependent TrkB activation increases glutamate release into the synaptic cleft through the activation of synapsin I and II (Jovanovic *et al.,* 2000). At the postsynaptic compartment, TrkB activation through BDNF increases **phosphorylation of NMDAR**

subunits 1 and 2B (Suen *et al.,* 1997; Lin *et al.,* 1998), activates **PLCγ/CaMKII/CREB** signaling pathway (Minichiello *et al.,* 2002) and recruits the **AMPAR subunits GluR1 and GluR2** to the plasmatic membrane (Nakata and Nakamura, 2007). Moreover, BDNF also activates mTOR and ERK signaling cascades to enhance global and local mRNA translation in neurons (Takei *et al.,* 2001; Schratt *et al.,* 2004). Altogether, the above molecular mechanisms involving BDNF regulate LTP induction and maintenance.

Beyond its role in synaptic activity, BDNF **modulates dendritic spine morphology** changes increasing spine density, length or its head size (Ji *et al.,* 2010). However, the molecular mechanisms by which BDNF modulates spine morphology remain to be elucidated.

2.5.2. mTOR signaling pathway

mTOR is a serine/threonine kinase that acts as a downstream mediator of the PI3K/Akt pathway. The mTOR pathway controls multiple biological processes including cell growth and protein synthesis in response to different extracellular and intracellular signals (Boutouja *et al.,* 2019). Thus, over the last years much effort has focused on understanding how and where mTOR is regulated in the cell. Although most data have been obtained in non-neuronal cells and *in vitro* models, they could be potentially applicable to the neuronal and synaptic context as well.

In neurons, mTOR could be activated through several extracellular mediators, including BDNF (Takei *et al.,* 2004), insulin, insulin-like growth factor 1 (Quevedo *et al.,* 2002), vascular endothelial growth factors (Kim *et al.,* 2008), ciliary neurotrophic factor (Yokogami *et al.,*

2000) and glutamate (Lenz and Avruch, 2005). Once activated, mTOR interacts with different accessory proteins to form two distinct multi-protein complexes, named mTOR complex 1 (**mTORC1**) and mTOR complex 2 (**mTORC2**). Both complexes share some proteins, including the mammalian lethal with SEC13 protein 8 (mLST8) and DEP domain-containing mTOR-interacting protein (DEPTOR), but differ on others. Additionally, each complex phosphorylates distinct substrates to regulate different cellular processes (Laplante and Sabatini, 2013) (Figure 15).

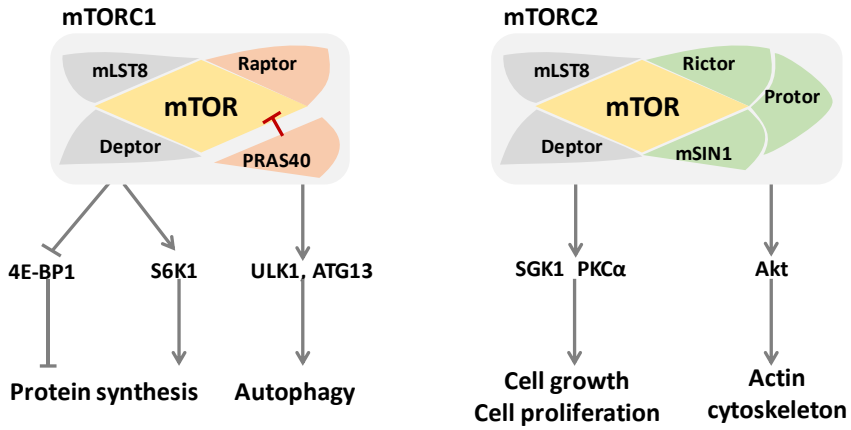


Figure 15. Major distinct characteristics of the two known mTOR signaling complexes, mTORC1 and mTORC2, in composition, substrates, and cellular functions.

2.5.2.1. mTORC1

mTORC1 is activated by binding of the small GTP-binding protein Ras homolog enriched in brain (RHEB) when it has the GTP-binding state activated. Activated mTORC1 contains two specific subunits, the regulator associated protein of mTOR (Raptor) and proline-rich Akt substrate of 40kDa (PRAS40) (Peterson *et al.,* 2009). mTORC1 is **sensitive to the specific inhibitor rapamycin** and regulates cell growth, proliferation and survival, through the modulation of protein

translation, autophagy and lipid and nucleotide synthesis. Interestingly, each protein in the complex plays a specific role in mTORC1 functions (Laplante and Sabatini, 2013) (Figure 15). Raptor recruits the substrates binding to the TOR signaling (TOS) motif present in several mTORC1 substrates (Nojima *et al.,* 2003) and mLST8 associates with the catalytic domain of mTOR to stabilize the kinase activation loop (H. Yang *et al.,* 2013). Contrary, PRAS40 and DEPTOR are two endogenous negative modulators of mTORC1. PRAS40 binds to and inhibits Raptor and consequently avoids substrates recruitment, and DEPTOR has an inhibitory domain that interacts with mTOR protein and negatively modulates its activity (Sancak *et al.,* 2007; Peterson *et al.,* 2009).

As mention above, the main functions of mTORC1 are the regulation of **protein translation and autophagy**. mTORC1 controls protein synthesis through the phosphorylation of P70S6 kinase 1 (P70S6K1) and eukaryotic initiation factor 4E (eIF4E) Binding Protein 1 (4E-BP1). On the one hand, the activation of P70S6K1 by mTORC1 phosphorylates the ribosomal protein S6, involved in mRNA translation (Biever *et al.,* 2015). On the other hand, 4E-BP1 binding to eIF4E avoids the formation of the eIF4F complex, necessary to recognize capped mRNA and initiate translation. In this case, the phosphorylation of mTORC1 dissociates 4E-BP1 from eIF4E allowing mRNA translation (Brunn *et al.,* 1997; Gingras *et al.,* 1999).

mTORC1 also suppresses protein turnover through the regulation of autophagy and the ubiquitin-proteasome system. Autophagy is inhibited by mTORC1 through the phosphorylation of unc-51-like kinase (ULK1) and autophagy-related protein 13 (ATG13), both proteins

involved in the autophagosome formation (Lee *et al.,* 2010). However, how mTORC1 promotes proteolysis via UPS is still unknown (Zhao and Goldberg, 2016). Furthermore, mTORC1 also increases protein turnover through the enhancement of the transcription factor erythroid-derived 2-related factor 1 (NRF1) (Zhang and Manning, 2015).

2.5.2.2. mTORC2

Different to mTORC1, mTORC2 is composed by **rapamycin insensitive** companion of mTOR (Rictor), mammalian stress-activated MAPK-interacting protein 1 (mSin1), and protein observed with Rictor 1 and 2 (Protor1/2). mTORC2 controls **cell survival** and **cytoskeleton reorganization** (Laplante and Sabatini, 2013) (Figure 15).

The main substrates of mTORC2 are the serum/glucocorticoid regulated kinase 1 (SGK1), PKC-α and Akt. mSin1 and Protor1/2 have a crucial role in mTORC2 Akt and SGK1 recognition and phosphorylation. Akt and SGK1 phosphorylation controls cell survival, cell growth and cell proliferation while PKC-α activation controls actin cytoskeleton (Dai and Thomson, 2019).

2.5.2.3. mTOR and synaptic plasticity

mTOR pathway is considered an important signaling cascade in the cell. In the brain, mTOR has an **important role for synaptic plasticity**, and therefore **learning and memory processes** (Costa-Mattioli *et al.,* 2009). Interestingly, an alteration of mTOR signaling pathway is observed in several pathological conditions, such as neurological and psychiatric disorders (Ryskalin *et al.,* 2018). mTOR signaling activation is also

involved in the **memory impairment produced by THC** administration (Puighermanal *et al.,* 2009).

According to the importance of mTORC1 controlling protein synthesis, it is not surprising that its inhibition with rapamycin blocks long-lasting synaptic changes, including NMDA-dependent LTP and protein synthesis, and memory consolidation in different hippocampal paradigms (Tang *et al.,* 2002; Dash *et al.,* 2006; Gafford *et al.,* 2011; Stoica *et al.,* 2011; Deli *et al.,* 2012; Halloran *et al.,* 2012; Jobim *et al.,* 2012). Some studies also reveal the implication of mTORC1 in hippocampal mGluR-dependent LTD (Huber 2001). In fact, mTORC1 is activated after 3,5-dihydroxyphenylglycerine (DHPG), a mGluR1/5 agonist that induces mGluR-dependent LTD (Hou and Klann, 2004; Lebeau *et al.,* 2011). Noteworthy, recent data show that hippocampal mGluR-LTD is normal in the conditional Raptor KO mouse, whereas in the conditional Rictor KO mouse hippocampal mGluR-LTD and its related behaviours are impaired (Zhu *et al.,* 2018).

Although most of the studies have been focused on the role of mTORC1 on synaptic plasticity and memory, **the interest of mTORC2 in this field is increasing.** In fact, the conditional Rictor KO mouse also presents impaired long-term memory and LTP due to deficits in actin polymerization. Moreover, the increase of mTORC2 activity results in the facilitation of L-LTP and long-term memory enhancement, suggesting mTORC2 as a therapeutic target for cognitive dysfunction (Huang *et al.,* 2013). Noteworthy, these studies are hampered by the lack of specific mTORC2 inhibitors.

2.6. Peripheral-to-central modulators of memory: focus on sympathetic adrenal system

Besides central molecular mechanisms involved on memory consolidation, **peripheral hormonal regulators** also can influence memory strength including gonadal hormones, steroids and adrenal catecholamines, such as dopamine, noradrenaline and adrenaline (Gold and Korol, 2012).

One of the first identified neuromodulators with high relevance on memory is adrenaline, also named epinephrine (Gold and Van Buskirk, 1975; Gold and van Buskirk, 1978). In response to **novel experience**, **adrenaline** and **noradrenaline** are released from the adrenal medulla into the blood in a graded manner that depends on the arousal and emotion produced by the experience (McCarty and Gold, 1981). Notably, adrenaline effects on memory behave as an **inverted-U**, which means that low and high levels of arousal result in poor memory performance, but moderate levels improve memory (Yerkes and Dodson, 1908).

A growing body of evidences demonstrate the efficacy of exogenous adrenaline administration enhancing memory in rodents in emotional-related paradigms, such as the active avoidance test (McCarty and Gold, 1981) and non-emotional tests including the Y-maze (Talley *et al.,* 2000) and NORT (Dornelles *et al.,* 2007). Moreover, adrenaline also drives memory improvement in humans (Cahill and Alkire, 2003).

Interestingly, circulating **adrenaline has low brain penetrance** (Axelrod *et al.,* 1959; Hardebo and Owman, 1980) and direct adrenaline infusions into the brain fail to enhance memory (de Almeida *et al.,* 1983). Thus, adrenaline effects on memory appear to be initiated by

peripheral mechanisms that transduce its actions into the brain. There are at least **two peripheral mechanisms**, not mutually excluding, that have been point forward to explain the mnemonic effect of peripheral adrenaline. First, a mechanism involving the activation of β-adrenergic receptors (β-ARs) expressed on the afferent fibers of the vagus nerve. Second, a mechanism involving the activation of hepatic adrenoreceptors and the subsequent increase of blood glucose levels (Gold and Korol, 2012).

According to the involvement of the **vagus nerve**, its fibers are densely embedded with β-ARs that could bind circulating adrenaline producing an increase of the nerve activity (Schreurs *et al.,* 1986; Lawrence *et al.,* 1995) (Figure 16). Then, vagus nerve fibers project to a specific cluster of cells in the brainstem known as the nucleus tractus solitarius (NTS). Direct or indirect efferent fibers from NTS arrives to the LC, the main source of noradrenaline in the brain (Ennis and Aston-Jones, 1988; Van Bockstaele *et al.,* 1998; Reyes and Van Bockstaele, 2006). Indeed, the **NTS-LC-CA1 pathway is necessary for object-recognition memory consolidation** (Mello-Carpes and Izquierdo, 2013).

It is important to mention that in addition to noradrenaline, vagal stimulation also increases the release of dopamine (Szczerbowska-Boruchowska *et al.,* 2012) and serotonin (Manta *et al.,* 2012) across multiple brain areas that could also contribute to memory strength.

Noradrenaline in the hippocampus is involved on the storage of new memories through the regulation of **neural excitability** and **synaptic plasticity** (Hagena *et al.,* 2016). Although noradrenaline can bind to both α- or β- ARs, synaptic information and plasticity in the hippocampus depends mainly on the **activation of β-ARs** (Kemp and

Manahan-Vaughan, 2008). In fact, it is thoroughly demonstrated that LC stimulation modulates hippocampal synaptic strength and improves memory through a β-adrenergic-dependent mechanism (Kemp and Manahan-Vaughan, 2008, 2012; Hansen and Manahan-Vaughan, 2015; Hagena *et al.,* 2016).

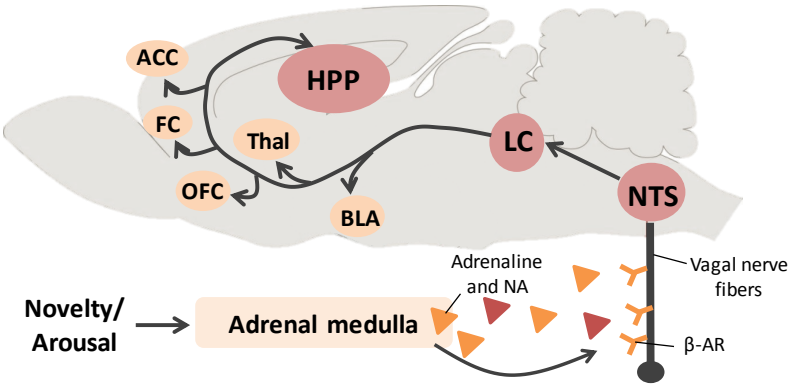


Figure 16. Adrenergic modulation of memory through the vagus nerve mechanism. After arousal experience, such as novelty, adrenal medulla release adrenaline and noradrenaline into the blood. As adrenaline does not cross the blood-brain barrier, it binds to peripheral adrenergic receptors on the vagus nerve. When vagus nerve is activated, it releases glutamate on neurouns in the nucleus tractus solitarius (NTS), which in turn sends glutamatergic projections onto neurons in the *locuscoeruleus* (LC). LC neurons release noradrenaline that binds to different brain areas including the hippocampus (HPP). BLA, basolateral amygdala; Thal, thalamus; OFC, orbitofrontal cortex; FC, frontal cortex; ACC, anterior cingulate cortex. Adapted from (Hagena *et al.,* 2016).

Different subtypes of β-ARs are present in the hippocampus (Lands *et al.,* 1967), being β1-AR and β2-AR mainly involved in synaptic plasticity (Yang *et al.,* 2002; Gelinas *et al.,* 2008; Kemp and Manahan-Vaughan, 2008). *In vitro* studies show that both receptors, once activated, promote AC activity to increase intracellular cAMP and the subsequent stimulation of PKA signaling (Seeds and Gilman, 1971; Maguire *et al.,* 1977; Hausdorff *et al.,* 1989). Additionally, β2-AR can also potentiate ERK/MAPK/CREB signaling pathway regulating protein synthesis

(Daaka *et al.,* 1997; Maudsley *et al.,* 2000). These signaling cascades could mediate synaptic plasticity and long-term memory through β-ARs (Kandel, 2012).

Despite the strong evidences supporting adrenergic memory modulation through the vagus nerve mechanism, several aspects still remain controversial. For example, the presence of β-AR in vagal nerve fibers is unclear, as well as, the possibility that circulating adrenaline could reach to these receptors or, even if it does, adrenaline binding to β-AR could not increase vagal afferent neural activity (Berthoud and Neuhuber, 2000; Mravec, 2006, 2011). Moreover, the efficacy of adrenaline to enhance memory is blunted in food-restricted rats, suggesting the relevance of blood glucose levels to enhance memory (Talley *et al.,* 2000).

For these reasons, a second hypothesis involving the **increase of blood glucose** was proposed. This mechanism is based on the idea that, after novelty experience the increase of circulating adrenaline improves memory performance through the enhancement of blood glucose levels. It is observed that adrenaline could bind to hepatic adrenoreceptors and consequently promote the breakdown of glycogen stores, increasing blood glucose levels (Sutherland and Rall, 1960). The increase in blood glucose could provide **additional energy** substrates to the brain to supply the processes needed for memory consolidation. In fact, glucose administration enhances learning and memory in different memory paradigms in both rodents and humans (Messier, 2004).

2.7. Role of the endocannabinoid system in memory

The ECS has an important role in various aspects of learning and memory. In agreement, CB1R is highly expressed in the hippocampus where it is involved in synaptic plasticity (see section 1.3).

Cannabis consumption in humans produces deficits in short-term and long-term episodic and working memories, without affecting the retrieval of information previously consolidated (Ranganathan and D'Souza, 2006). Interestingly, cannabidiol prevents acute THC-induced memory deficits in humans. Thus, the ratio THC/cannabidiol in the plant may modify cannabis effects over memory in human cannabis consumers (Morgan *et al.,* 2010). However, assuming conclusions from human studies are controversial because of differences on methodologies and vulnerability to cannabis-related memory deficits. Thus, the study of the consequences of the modulation of the ECS on learning and memory rely upon experimental investigation in animal models (Mechoulam and Parker, 2013).

Similar to the findings in humans, most studies in rodents demonstrated that administration of **CB1R agonists produces memory impairment** in several paradigms including the NORT (Schneider and Koch, 2002; O'Shea *et al.,* 2004; Puighermanal *et al.,* 2012), radial arm maze (Rubino *et al.,* 2009), Morris water maze (Varvel *et al.,* 2001; Niyuhire *et al.,* 2007) and delayed matching/non-matching to position task with lever presentation (Heyser *et al.,* 1993). Another aspect is that these effects seem to be mainly due to CB1R, as its blockade with the CB1R antagonist rimonabant prevent such memory impairment (Zanettini *et al.,* 2011). In addition, cannabinoid agonists

administration prevents LTP in a CB1R-dependent manner (Stella *et al.,* 1997; Hoffman *et al.,* 2007; Abush and Akirav, 2009).

Although these results are obtained after systemic administration of CB1R agonists, their intrahippocampal administration also impairs memory performance in the NORT (Clarke *et al.,* 2008), radial arm maze (Lichtman *et al.,* 1995; Wegener *et al.,* 2008) and Morris water maze (Abush and Akirav, 2009).

Besides cannabinoid agonists, the effects of elevating the endocannabinoid tone on memory are not clarified. Pharmacological inhibition or genetic deletion of FAAH, which increases endogenous AEA levels, enhances memory in different paradigms including the Morris water maze (Varvel *et al.,* 2005, 2007) and passive-avoidance tasks (Mazzola *et al.,* 2009; Hasanein and Teimuri Far, 2015). However, FAAH inhibition also results in memory impairment in the NORT, contextual fear conditioned and Y/T-maze tasks (Seillier *et al.,* 2010; Busquets-Garcia *et al.,* 2011; Basavarajappa *et al.,* 2014). These controversies could be explained by a CB1R-independent mechanism, as some effects are not prevented after CB1R blockade with rimonabant. Indeed, FAAH inhibition not only produces the elevation of AEA levels, but also other fatty acids, such as oleoyl-ethanolamine and palmitoyl-ethanolamine, which could bind to PPAR-α and improve memory (Campolongo *et al.,* 2009; Mazzola *et al.,* 2009).

Some studies have reported not significant effect in the NORT after MAGL inhibition (Busquets-Garcia *et al.,* 2011), whereas in other emotional-dependent task, such as extinction in the fear conditioning test (Hartley *et al.,* 2016) and the spatial memory retrieval after stressful event (Morena *et al.,* 2015), MAGL inhibition produces

memory deficits. Moreover, MAGL KO mice show improved learning in the NORT and Morris water maze task (Pan *et al.,* 2011; Kishimoto *et al.,* 2015). Thus, there are several evidences supporting that both AEA and 2-AG enhance or impair memory under different conditions.

Contrary to CB1R agonists, the **blockade of CB1R** or its genetic deletion is associated to **memory enhancement**. Pharmacological studies using rimonabant as CB1R antagonist show memory improvements in olfactory social memory task (Terranova *et al.,* 1996), the elevated T-maze (Takahashi *et al.,* 2005) and the radial arm maze (Lichtman, 2000; Wolff and Leander, 2003). However, in other memory tests, such as the spatial delayed-non-match-to-sample, CB1R antagonist treatment does not show any significant effect (Mallet and Beninger, 1998).

These discrepancies could be due to the temporal requirements of the tasks, as CB1R antagonism enhances memory processes that last min or hours rather than seconds. Thus, it seems that CB1R blockade prolongs the duration of a memory rather than facilitating its learning (Varvel *et al.,* 2009). Consistent with pharmacological studies, CB1R KO mice show memory enhancement in different cognitive tasks, such as improved memory in the NORT for at least 48 hours (Reibaud *et al.,* 1999; Maccarrone *et al.,* 2002), the contextual fear conditioning (Jacob *et al.,* 2012) and the active avoidance task (Litvin *et al.,* 2013). However, these mice present extinction impairments in the fear-conditioning and Morris water maze tasks (Marsicano *et al.,* 2002; Varvel and Lichtman, 2002).

Electrophysiological studies with CB1R antagonists produce both facilitation or impairment of LTP depending on the experimental conditions (Slanina *et al.,* 2005; de Oliveira Alvares *et al.,* 2006),

whereas CB1R KO mice exhibit an enhanced LTP (Bohme *et al.,* 2000; Jacob *et al.,* 2012). Furthermore, CB1R contribution to LTP depends on the cell type population, as CB1R KO in GABAergic cells leads to a decrease hippocampal LTP, while CB1R KO in glutamatergic cells leads to an increased hippocampal LTP (Monory *et al.,* 2015).

CB1R in the periphery could also contribute to memory performance. Notably, CB1R in adrenergic and noradrenergic cells is necessary and sufficient for stress-induced impairment of NORT consolidation (Busquets-Garcia *et al.,* 2016).

2.8. Possible mechanisms underlying memory impairment produced by cannabinoids

The mechanisms that underly the memory impairment produced by cannabinoids have been widely studied through pharmacological, genetic and electrophysiological approaches. The ECS modulates different neurotransmitter systems involved in this memory impairment (Puighermanal *et al.,* 2012). Cannabinoid-induced memory impairment has been related to the inhibition of the cholinergic system in the CNS (Braida and Sala, 2000) or cholecystokinin release (Harro and Oreland, 1993). CB1R in the hippocampus is highly expressed on GABAergic cells (Kawamura *et al.,* 2006) and its activation with THC leads to the suppression of GABA release and consequently an increase of the excitatory firing (Katona and Freund, 2012). This deregulation of excitatory/inhibitory neurotransmission could be a possible mechanism involved in the cannabinoid-induced memory impairment. Moreover, the activation of CB1R in the hippocampus leads to the stimulation of different signaling cascades including the PI3K/Akt

pathway (Ozaita *et al.,* 2007). Some downstream effectors of Akt are the MAPK/ERK and **mTOR signaling pathways**, both important for memory formation. In fact, THC administration produces the over-activation of mTORC1 signaling in the hippocampus, which is associated to the THC amnesic-like effects observed in the NORT (Puighermanal *et al.,* 2009). However, the specific contribution of **mTORC2 signaling in THC amnesic effects remains to be clarified** and represents one of the objectives of this thesis.

3. Fragile X syndrome

3.1. General features of fragile X syndrome

Fragile X syndrome (FXS) is the most common monogenic cause of inherited human **intellectual disability** and autism (de Vries *et al.,* 1998; Penagarikano *et al.,* 2007). It is a X-linked dominant disorder caused by a trinucleotide CGG expansion in the 5'-untranslated region of the fragile X mental retardation gene (FMR1) that encodes for the fragile X mental retardation protein (FMRP) (Verkerk *et al.,* 1991; Penagarikano *et al.,* 2007). In humans, the number of CGG repeats is highly polymorphic. According to these repeats, the *Fmr1* gene has been classified into four allelic forms: normal allele (5-44 repeats), intermediate allele (45-54 repeats), premutation allele (55-200 repeats) and full mutation allele (>200 repeats) (Dean *et al.,* 2016) (Figure 17). Although premutation alleles do not cause FXS, the overexpression of mRNA containing the CGG expansion can produce RNA toxicity (Pretto *et al.,* 2014). Moreover, individuals with the premutation can develop fragile X associated tremor ataxia syndrome (FXTAS), which is characterized by progressive intention tremor (Hagerman and Hagerman, 2015), gait ataxia and dementia. A 20% risk for females carrying the premutation exists to develop the fragile X associated premature ovarian insufficiency, a form of ovarian dysfunction (Hagerman and Hagerman, 2015). Finally, the presence of the full mutation allele leads to the hypermethylation of *Fmr1* gene, and consequently its transcriptionally silencing and the absence or deficiency of FMRP (Sutcliffe *et al.,* 1992; Coffee *et al.,* 1999).

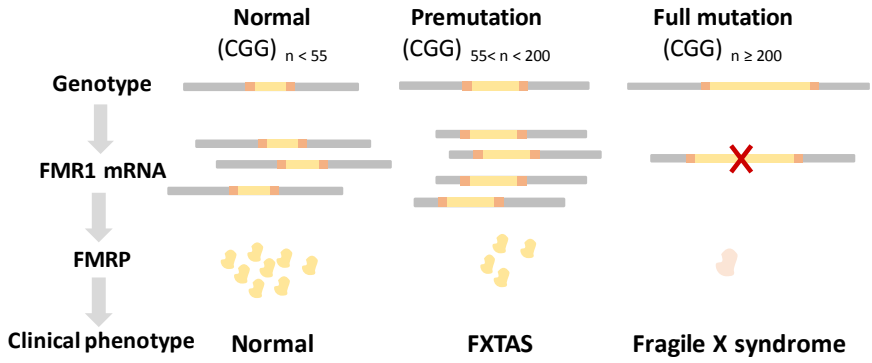


Figure 17. Schematic representation of *Fmr1* expression depending on trinucleotide CGG expansions and its associated clinical phenotype. Adapted from (Hagerman *et al.,* 2002).

FMRP is an **RNA-binding protein** highly expressed in the brain, predominantly at synapses where it acts as a **negative regulator of translation** (Darnell *et al.,* 2011). The loss of this protein impairs normal synaptic plasticity and seems to be the cause of intellectual disability in FXS patients (Penagarikano *et al.,* 2007). Due to the X-linked dominant inheritance, the condition is less severe in females than males because of the X chromosome inactivation. Notably, the prevalence of the full mutation is estimated as 1 in 5000 males and 1 in 8000 females (Hagerman *et al.,* 2017).

3.2. Mouse models of fragile X syndrome

Animal models of FXS have been developed over the years to understand the genetic and cellular mechanism underlying this disorder, and therefore to develop specific therapies. The *Fmr1* gene is highly conserved between mouse and human (95% homology) (Ashley *et al.,* 1993) and the discovery of the genetic cause of FXS led to the generation of the first full mutant mouse model, **the *Fmr1* KO mouse**.

The *Fmr1* KO mouse is the most widely studied mouse model of FXS, generated by homologous recombination where *Fmr1* is selectively knocked out (Bakker *et al.,* 1994).

Nowadays, several mouse models of FXS are available including, conditional and knock-in mouse models that reproduce some of the most important features of the disorder (Table 6).

Genetic approach	Mouse model	Modification	References
Knockout model	Fragile X knockout mice	*Fmr1* knockout	(Bakker *et al.,* 1994)
		Fmr2 knockout	(Mientjes *et al.,* 2006)
Paralogous genes	FXR1	FXR1 knockout	(Siomi *et al.,* 1995)
	FXR2	FXR2 knockout	(Bontekoe *et al.,* 2002)
Repeat expansion	Transgenic	$(CGG)_{60}$	(Bontekoe *et al.,* 1997)
		$(CGG)_{43}$	(Lavedan *et al.,* 1997)
		$(CGG)_{97}$	(Lavedan *et al.,* 1998)
	Knock-in	$(CGG)_{98}$	(Bontekoe, 2001)
	Transgenic rescue	FMR1 cDNA	(Bakker *et al.,* 2000)
		FMR1 YAC	(Peier *et al.,* 2000)

Table 6. Mouse models of fragile X syndrome The *Fxr1* and *Fxr2* genes are autosomal homologs 1 and 2 of *Fmr1* (Adapted from Kooy, 2003 and Wijetunge *et al.,* 2013).

3.3. Pathological and psychological aspects in fragile X syndrome

Patients affected by FXS present different physical and behavioural manifestations that can vary considerably within individuals. Regarding the **physical characteristics**, individuals suffering this syndrome show a long face with large and prominent ears and high broad forehead (Hagerman *et al.,* 2017). Macroorchidism is commonly seen in postpubescent male patients. Other physical features include increased joint laxity, hypotonia and mitral valve prolapse (Katerina Bambang *et al.,* 2011).

According to the **behavioural abnormalities**, the most prominent phenotype in FXS is **intellectual disability** showing IQ values usually between 20 and 70, with alterations in working and short-term memory, executive functioning, mathematical and visuo-spatial abilities and speech delay (Penagarikano *et al.,* 2007; de Esch *et al.,* 2014). Moreover, some patients present hyperactivity, hypersensitivity to sensorial stimuli, anxiety, attention deficit, epileptic seizures and autistic features (Penagarikano *et al.,* 2007).

The *Fmr1* KO mouse model reproduces some of the previously described characteristics including the hyperactivity, macroorchidism and increased sensitivity to auditory stimuli, leading to epileptic seizures, in addition to a diminished acoustic startle reflex. Noteworthy, the *Fmr1* KO mouse shows mild cognitive deficits in the NORT (Busquets-Garcia *et al.,* 2013), the Morris water maze task (D'Hooge *et al.,* 1997), the radial arm maze task (Mineur *et al.,* 2002), avoidance task (Brennan *et al.,* 2006) and in the trace fear-conditioning test (Zhao *et al.,* 2005).

3.3.1. Cellular and molecular alterations in fragile X syndrome

Post mortem studies in FXS patients have revealed no pathological brain anomalies (Reyniers *et al.,* 1999; He and Portera-Cailliau, 2013). Both FXS patients and *Fmr1* KO mice show microanatomy abnormalities that include alterations in dendritic spine density and maturation related to deficits in synaptic plasticity (Bakker *et al.,* 1994; He and Portera-Cailliau, 2013). These **dendritic spine alterations** consist on an increase of immature dendritic spines, also known as filopodia, usually accompanied by a less number of mushroom

morphology, typical of mature synapses (Levenga *et al.,* 2010). Interestingly, this immature spine morphology profile is observed in several brain regions, such as the CA1 region of the hippocampus (Levenga *et al.,* 2010; Busquets-Garcia *et al.,* 2013), cerebellum (Koekkoek *et al.,* 2005) and neocortex (Nimchinsky *et al.,* 2001). However, studies evaluating this phenotype show some discrepancies depending on the experimental design, genetic background, age of the animals and the brain region evaluated (He and Portera-Cailliau, 2013). These observations in dendritic spines may be a consequence of the absence of FMRP, which causes a defect in spine maturation and pruning altering correct synaptic transmission (Nimchinsky *et al.,* 2001). Moreover, the loss of FMRP leads to an increase in protein synthesis in total brain homogenates and isolated synaptoneurosomes from *Fmr1* KO mice (Dölen *et al.,* 2007). In addition, the disturbances in synaptic function and spine morphology have been associated to aberrant signalling of the excitatory group I mGluR (mGluR1 and mGluR5) (Levenga *et al.,* 2010). Notably, an **uncontrolled activity of mGluR5** has been described in FXS (Bear *et al.,* 2004; Michalon *et al.,* 2012) and genetic reduction of mGluR5 expression is sufficient to normalize some features of the *Fmr1* KO mouse model (Dölen *et al.,* 2007). In normal conditions, activation of group I mGluR triggers local mRNA translation and the internalization of AMPARs from the postsynaptic density of dendrites that are proposed to potentiate synaptic plasticity, specifically mGluR-LTD. As a negative feedback mechanism, FMRP represses the translation of a subset of mRNAs important for AMPAR internalization. An exaggerated LTD in response to mGluR activation occurs in FXS due to the absence of FMRP, which

weakens the synapse (Bear *et al.,* 2004; Pop *et al.,* 2014) (Figure 18). This theory is known as the **mGluR theory of FXS**.

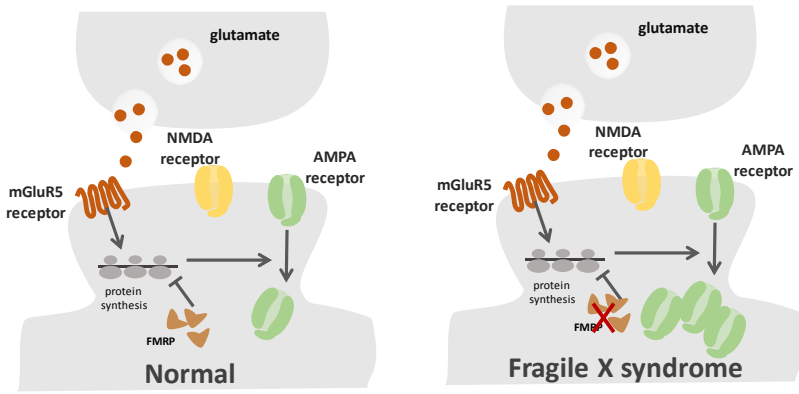


Figure 18. The mGluR theory of fragile X syndrome. **(A)** In normal conditions, FMRP acts modulating mGluR activity and thus reducing AMPAR internalization. **(B)** In fragile X syndrome, the absence of FMRP leads to an excessive AMPAR internalization producing an exaggerated mGluR-LTD (*Levenga et al., 2010*).

In addition to the mGluR theory, several alterations in the **GABAergic system** have been detected in the hippocampus of *Fmr1* KO mouse, including a reduction in the expression of GABAR subunits and decreased mRNA expression of glutamate decarboxylase-67, both leading to a reduced GABAergic signalling (D'Hulst and Kooy, 2009; Paluszkiewicz *et al.,* 2011).

Both, alterations in the GABAergic system and the mGluR theory, lead to the hypothesis that symptoms of FXS may result from a disturbance of the **excitatory-inhibitory imbalance** (Paluszkiewicz *et al.,* 2011).

Many other mechanisms have been described to be involved in FXS pathophysiology. Downstream mGluR signaling effectors have been reported to be altered in *post mortem* samples of FXS patients, such as increased phosphorylation of EIF4E (Gkogkas *et al.,* 2014) and P70S6K (Sawicka *et al.,* 2016). Both proteins could be responsible for the excess of protein synthesis observed in *Fmr1* KO mice. In this regard,

inhibition of translation rescue some features in the *Fmr1* KO mouse phenotype (Richter *et al.,* 2015). Other proteins involved in the pathology of FXS include the increased expression of phosphatidylinositol 3-kinase enhancer (PIKE) (Gross *et al.,* 2015), GSK3 (Guo *et al.,* 2012) and the amyloid precursor protein (APP) (Westmark *et al.,* 2011). Furthermore, FMRP protein is associated with the MMP9 mRNA, encoding an endopeptidase important for dendritic spine maturation and synapse formation (Stawarski *et al.,* 2014). These MMP9 levels are enhanced in Fmr1 KO mice (Dziembowska *et al.,* 2013).

Finally, the mTOR signalling pathway has been found overactivated in the hippocampus of *Fmr1* KO mice, leading to **aberrant mTOR-dependent protein synthesis** (Levenga *et al.,* 2010; Busquets-Garcia *et al.,* 2013).

3.4. Therapeutic targets in preclinical fragile X syndrome models

The identification of cellular and molecular alterations in FXS, prompted the use of treatments acting on these targets to prevent the main features in FXS. The first strategies considered the mGluR hypothesis. Both genetic reduction or pharmacological blockade of these receptors ameliorated several phenotypes of the *Fmr1* KO mouse, such as neuronal structural alterations, susceptibility to audiogenic seizures and hyperactivity (Santoro *et al.,* 2012). Moreover, studies in animal models of FXS demonstrated that the **mGluR5 inhibitors** MPEP, CTEP or fenobam corrected some phenotype alterations (Krueger and Bear, 2011; Michalon *et al.,* 2012). In clinical studies, an initial phase I/II trial of the mGluR5 negative modulator

AFQ056 showed improvement in hyperactivity, stereotypic behavior and inappropriate speech in patients with FXS, but in the subsequent phase IIb trials of AFQ056 and a similar mGluR5 modulator RO4917523 did not show such an amelioration (Hagerman *et al.,* 2018).

Treatment with some **$GABA_BR$ agonists** also reduced susceptibility of *Fmr1* KO mice to audiogenic seizures (Pacey *et al.,* 2009) and corrected the enhanced protein synthesis in the hippocampus and social behavior in *Fmr1* KO mice (Henderson *et al.,* 2012; Qin *et al.,* 2015). In a phase II trial, treatment with $GABA_BR$ arbaclofen improved social withdrawal in FXS patients (Berry-Kravis *et al.,* 2012), but in a subsequent phase III trials, FXS patients treated with arbaclofen did not show such an improvement (Berry-Kravis *et al.,* 2017). The increase of GABAergic tone can be also achieved through $GABA_AR$ agonists. The administration of $GABA_AR$ agonists resulted in the amelioration of anxiety, hyperactivity, rotarod performance and incidence of audiogenic seizures in *Fmr1* KO mice (Heulens *et al.,* 2012). Gaboxadol, a **$GABA_AR$ agonist** in a phase II trial showed improvement in anxiety in a sub-group of FXS patients (Ligsay *et al.,* 2017). Importantly, dual agonist of both $GABA_AR$ and $GABA_BR$ have also demonstrated improvement in hyperactivity and social behavior in FXS patients (Ligsay *et al.,* 2017). Other therapeutic strategies in FXS may include the inhibition of MMP9. MMP9 inhibition through **minocycline** is used in the clinic to ameliorate anxiety and attention problems in children FXS patients (Leigh *et al.,* 2013). Furthermore, the inhibition of mTOR with **temsirolimus**, a specific mTOR inhibitor, reversed object-recognition memory deficits in *Fmr1* KO mice (Busquets-Garcia *et al.,* 2013).

In conclusion, FXS is a complex disorder with different cellular and molecular processes involved. The combination of different strategies targeting different pathways altered in the disorder could ameliorate the pathophysiological and psychological aspects of FXS (Hagerman *et al.,* 2017).

3.5. Targeting the endocannabinoid system in fragile X syndrome

The absence of FMRP also dysregulates the ECS. It has been described that mGluR5 and CB1R present a functional interaction to regulate several physiological and pathological conditions (Olmo *et al.,* 2016). Some studies demonstrate that *Fmr1* KO mice have enhanced endocannabinoid signaling leading to 2-AG-dependent and mGluR-dependent synaptic plasticity abnormalities, such as enhanced LTD at inhibitory synapses and decreased LTD at excitatory synapses (Maccarrone *et al.,* 2010; Zhang and Alger, 2010). On the one hand, the increase of 2-AG signaling through MAGL inhibition normalized synaptic plasticity and behavioral alterations in the FXS mouse model (Jung *et al.,* 2012). On the other hand, the blockade of CB1R has been proposed as a therapeutic target of FXS. In accordance, CB1R antagonist rimonabant or CB1R genetic attenuation in *Fmr1* KO mice has normalized object-recognition memory deficits, susceptibility to audiogenic seizures, altered spine morphology in CA1 hippocampal region and the aberrant mGluR5-LTD (Busquets-Garcia *et al.,* 2013). Moreover, a recent study has demonstrated that low doses of rimonabant or a CB1R neutral antagonist equally normalize the object-recognition memory deficits in *Fmr1* KO mice (Gomis-González *et al.,* 2016).

4. Motor coordination

Motor coordination is the combination of different body movements that results in a planned action. Motor coordination is achieved when subsequent parts of the same movement, or different body parts movements are combined in a well-timed, smooth and efficient manner in accordance to the intended goal. This function integrates proprioceptive information from the musculoskeletal system to the CNS, to manage, plan and deliver motor commands back to the musculoskeletal system (Diedrichsen *et al.,* 2010). Several regions in the CNS are involved in motor function including the **cerebellum**, striatum, motor cortex brainstem and the spinal cord (Laforce and Doyon, 2001). In this thesis, we will focus on the cerebellum as a major substrate to motor coordination.

4.1. Behavioural paradigms to study motor coordination in mouse models

Behavioral studies in animal models have widely contributed to understand the neurobiological basis of motor coordination under healthy and pathological states. In rodents, motor coordination can be assessed to characterize the motor phenotype of genetically modified animals and to evaluate the effects of pharmacological compounds or other experimental manipulations (Carter *et al.,* 2001). Several well-established and widely used protocols are available for measuring cerebellum-dependent motor coordination in rodents, such as the rotarod, beam walking, footprint analysis and the coat-hanger test, among others (Table 6). It is important to mention that each test tightly change between different laboratories.

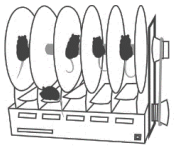
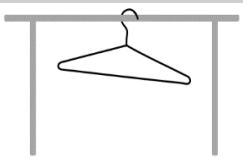
Test	Description	Scheme
Rotarod	It is used to assess the ability of an animal to balance on a rotating rod. The maximum speed reached by the animal or the time that it takes to fall at a range of different speeds is measured.	
Beam walking test	This test evaluates the ability of an animal to traverse a series of elevated narrow beams to reach an enclosed escape platform. During this test, the number of footslips performed and the latency to traverse the beam are measured.	
Footprint test	It is used to analyze animal's gait. It consists on painting the animal's paws with non-toxic color inks and the animal is allowed to walk along a paper-covered corridor. Once, footprints are dried, the following measures can be taken: front base, hind base, left overlap, right overlap, left forelimb stride, right forelimb stride, left hindlimb stride, right hindlimb stride.	
Coat-hanger test	It measures the ability of an animal to walk along and remain on a coat hanger. The measures taken during the test are the fall latency time, the distance walked by the animal and the time that the animal spends to reach the end of the hanger.	

Table 6. Main tests used to evaluate motor coordination (Carter *et al.,* 2001)**.**

4.2. The cerebellum as a neuroanatomical substrate of motor coordination

The cerebellum has a crucial role in the neural control of movement using sensory inputs from the periphery to regulate fine-tune movement and posture (Doyon *et al.,* 2003). The cerebellum is located at the caudal part of the brain and its structure and circuits are highly conserved across different species of mammals (Sultan and Glickstein, 2007). In general terms, the cerebellum receives sensory inputs and generates motor-related outputs determined by cerebellar neuronal networks. This area is anatomically divided into two hemispheres through a narrow midline zone, called *vermis* (Figure 19). The cerebellum consists of a cortical lamina, the cerebellar cortex, which covers a white matter core. The **cerebellar cortex** is enriched in neurons (gray matter), whereas the **white matter core** is mainly composed of myelinated nerve fibers coming to and from the cerebellar cortex. The **cerebellar nuclei** are buried within the white matter and receive information from the cerebellar cortex sending it to the thalamus and brainstem (Ashida *et al.,* 2018).

Although voluntary and involuntary movements can be initiated without cerebellar participation, cerebellum is crucial for the proper performance of smooth and accurate goal-directed movements, elaborating postural corrections to control balance and learning new motor skills (De Zeeuw *et al.,* 2011). It is worth to mention recent studies that have supported the role of cerebellum in emotion and several non-motor developmental disorders, such as autism, attention deficit hyperactivity disorders, and developmental dyslexia (Stoodley, 2016; Flace *et al.,* 2018).

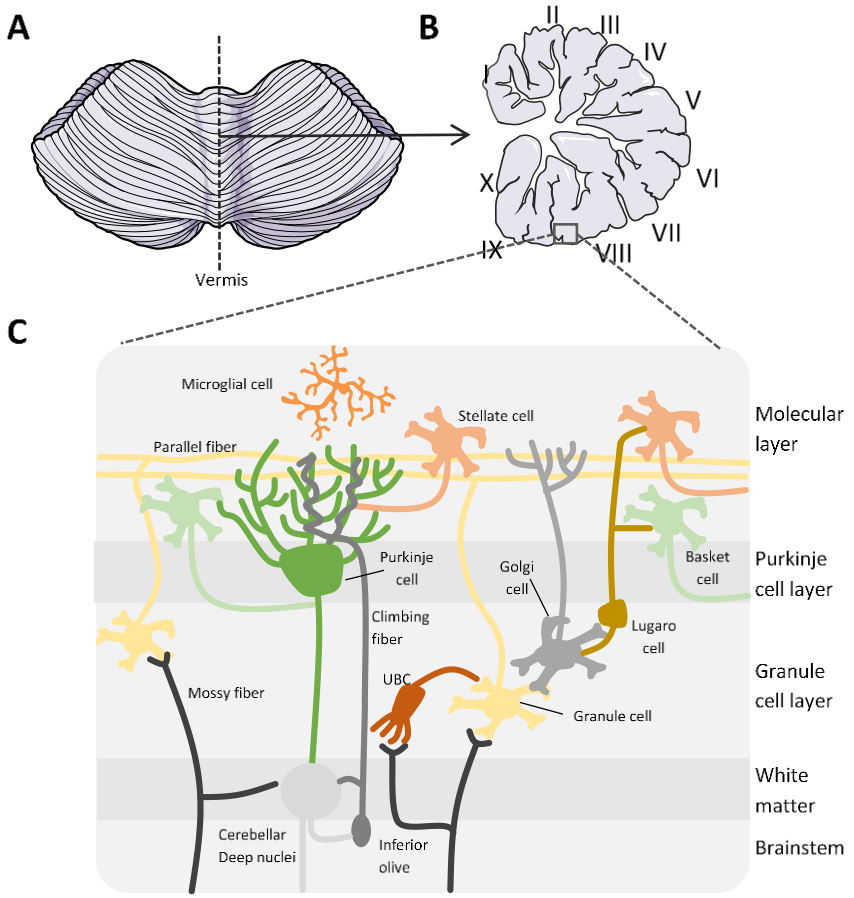


Figure 19. Anatomical architecture of the cerebellum. (A) Posterior view of the cerebellum **(B)** Drawing of midsagittal cross-section through the cerebellum, showing lobular organization, which each lobe demarcated with Roman numerals (I-X). **(C)** Representation of the cytoarchitecture of the cerebellar cortex. UBC, unipolar brush cells. Adapted from (Ashida *et al.,* 2018).

4.3. Cerebellar cortex organization

Several anatomical aspects in the cerebellum make this structure extremely powerful for processing information. First, the conserved regular laminar and simple cellular organization of the cerebellar cortex. Second, its connectivity with other brain areas (Ito, 2006).

The cerebellar cortex throughout its extent is divided in **three different layers**: the molecular layer, the Purkinje cell layer and the granular layer (from outer to inner) (Figure 19C). These layers contain five main cell types (Purkinje, stellate, basket, Golgi and granule cells) that have specific roles within the cerebellar circuit including a characteristic synaptic organization (Ito, 2006).

Purkinje cells are GABAergic neurons and they are considered the key players of the cerebellar cortex, providing its unique information output. They are distributed in a monolayer that projects the dendrites into the molecular layer through an extensive fan-like dendritic tree. In the molecular layer, Purkinje cell dendrites receive two main excitatory fiber inputs, from **mossy/parallel fibers** and **climbing fibers** (Figure 19). On the one hand, mossy fibers convey sensory and motor information from different parts of the body through afferents from the spinal cord (Matsushita, 1999a, 1999b), pontine nuclei (Serapide *et al.,* 2001), and several other brainstem structures (Päällysaho *et al.,* 1991). Mossy fibers synapse indirectly to Purkinje cells via granule cells. The axons of **granule cells** ascend to the molecular layer, where they bifurcate in two perpendicular processes known as parallel fibers that synapse on dendritic trees of Purkinje cells. On the other hand, climbing fibers are originated from the inferior olive and convey somatosensory, visual and other cortical information, and synapse directly to Purkinje cells (Palay and Chan-Palay, 1976).

Thus, Purkinje cells combine sensory information and motor commands through mossy/parallel fiber and climbing fiber inputs. Each Purkinje cell can receive excitatory inputs from more than 100.000

parallel fibers, whereas only from one single climbing fiber (Fox and Barnad, 1957).

The remaining cell types in the cerebellar cortex are interneurons, including Golgi cells, stellate cells and basket cells (Figure 19). These interneurons form glutamatergic synapses with parallel fibers. In addition, **stellate cells** and **basket cells** also originate inhibitory synapses with Purkinje cells. Otherwise, **Golgi cells** form inhibitory synapses on the synaptic terminals of the mossy fibers (Pellionisz and Szentágothai, 1973). Furthermore, two other types of interneurons are present in the granular layer, the **Lugaro cells** that make inhibitory contact with Golgi cells (Dieudonné and Dumoulin, 2000), and the **unipolar brush cells** that receive excitatory inputs from a single mossy fiber terminal and synapse with granular cells (Diño *et al.,* 1999).

Besides neuronal cells, glial cells including **Bergmann glial cells** and **microglia** are also present in the cerebellar cortex critically influencing synaptic excitability and cerebellar functions. Bergmann glial cells are specialized astrocytes that interact closely with Purkinje cells (Grosche *et al.,* 2002; Bellamy, 2006). These glial cells are important during cerebellum development, controlling migration, cell's maturation and synaptogenesis. Moreover, they express glutamate receptors and transporters involved in the clearance of excessive extracellular glutamate concentrations, avoiding Purkinje cells' excitotoxicity (Bellamy, 2006; Takayasu *et al.,* 2009). Microglial cells have an important role in the cerebellar cortex, where they are more expressed in the granular layer than in molecular layer (Vela *et al.,* 1995). We will dedicate the section 5.1.1 to describe more in detail the main features of microglial cells.

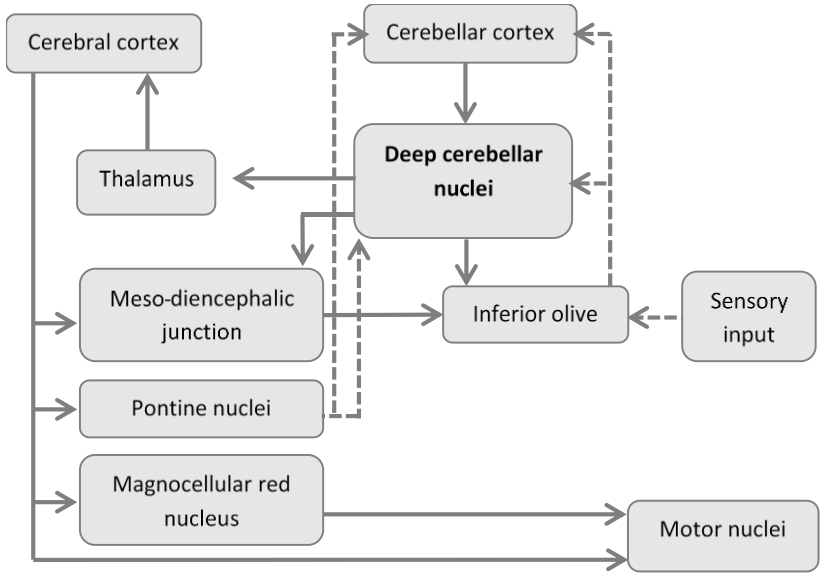


Figure 20. Representative diagram of the main cerebellar connectivity. Doted arrows indicate inputs to the cerebellum, whereas continue arrows the outputs. Adapted from (Gao *et al.,* 2012).

Once Purkinje cells have combined all the information from mossy/parallel fibers (pontine nuclei) and climbing fibers (inferior olive), their axons project to the deep cerebellar nuclei via GABAergic synapse (Figure 20).

Beyond the cerebellar cortex, deep cerebellar nuclei also receive inputs from collateral axons of mossy fibers and climbing fibers directly. Then, deep cerebellar nuclei neurons send the integrated signals to the inferior olive, via inhibitory feedback, and the thalamus, which in turn feeds information to other extracellular areas for the control of motor and/or cognitive functions (Figure 20). In addition, the cerebellum seems to count with independent anatomical modules that send and receive projections from a specific area of the cerebral cortex originating a closed anatomical loop. Thus, the cortico-ponto-cerebellar projections originate a closed loop system in which,

cerebellum returns projections to the cerebral cortex through the thalamus (Gao *et al.,* 2012).

Altogether, this well-defined organization of the laminar cerebellar cortex and their specific connections make the cerebellum an interesting area to study neuronal circuitries and synaptic plasticity mechanisms involved in the control of motor coordination. It is also noteworthy that alterations in the cerebellar organization and circuits are associated with motor coordination deficits and several brain disorders.

4.4. Role of the endocannabinoid system in motor coordination

As previously mentioned, the components of the ECS are strongly expressed in the cerebellum modulating its function. Indeed, **cannabis consumption is associated with motor impairment** and deficits in cerebellar circuitry both in human and rodents (Skosnik *et al.,* 2008; Cutando *et al.,* 2013; Steinmetz and Freeman, 2016).

The cerebellum is one of the brain regions with highest CB1R expression (Tsou *et al.,* 1998; Egertová and Elphick, 2000; Freund *et al.,* 2003). CB1R expression is very low in Purkinje cell bodies and climbing fiber terminals but, it is highly expressed at excitatory parallel fibers terminals into Purkinje cells (Kawamura *et al.,* 2006). Moreover, CB1R is predominantly expressed at higher levels on presynaptic terminals of inhibitory interneurons, including basket and stellate cells (Tsou *et al.,* 1998; Kawamura *et al.,* 2006; Rodríguez-Cueto *et al.,* 2014).

According to CB1R distribution in the cerebellar cortex, its activation inhibits action potential-evoked and spontaneous inhibitory postsynaptic currents at interneuron-Purkinje cell synapses or

excitatory postsynaptic currents at parallel fiber-Purkinje cell and climbing fiber-Purkinje cell synapses (Takahashi and Linden, 2000; Szabo *et al.,* 2004; Kano *et al.,* 2009). In the cerebellar cortex, CB1R activation has been associated with multiple forms of synaptic plasticity modulated by CB1R, eCB-STD and eCB-LTD (Kano *et al.,* 2009; Ohno-Shosaku and Kano, 2014). Notably, endocannabinoid release regulates DSI at interneuron terminals or DSE at parallel and climbing fibers (Szabo *et al.,* 2006; Tanimura *et al.,* 2009). Thus, the ECS has an important role in the function and control of overall output of the cerebellar cortex and, consequently, a proper motor coordination function.

Activation of CB1R by synthetic cannabinoids promote **cerebellar dysfunction**, causing severe motor incoordination (Lichtman *et al.,* 1998; DeSanty and Dar, 2001; Patel and Hillard, 2001). In these studies, pre-treatment with CB1R antagonist prevented this phenotype suggesting the blockade of CB1R as an interesting target to cerebellar ataxias. The activation of the endocannabinergic tone also modifies cerebellar activity. Thus, the endogenous increase of 2-AG levels with MAGL inhibitor treatment (Pan *et al.,* 2009) or in MAGL KO mice (Zhong *et al.,* 2011; Tanimura *et al.,* 2012) produces prolonged DSI and DSE in rodent cerebellar slices. Interestingly, MAGL KO mice exhibit normal locomotor activity and rotarod performance (Chanda *et al.,* 2010).

However, it remains to be elucidated whether inactivation or deletion of MAGL produce cerebellum-related alterations in other more demanding behavioral paradigms.

In spite of the motor coordination alterations widely demonstrated with CB1R ligands, the consequences of CB1R deletion is somewhat

controversial. It is reported that young CB1R KO mice present normal motor coordination (Steiner *et al.,* 1999; Kishimoto and Kano, 2006), whereas mature and older CB1R KO mice exhibit deficits in motor function in rotarod tests (Bilkei-Gorzo *et al.,* 2005). In this regard, previous studies performed in our laboratory revealed that CB1R KO mice show motor coordination impairments and cerebellar alterations that are related to local cerebellar inflammation (Cutando *et al.,* 2013).

5. Neuroinflammation

Neuroinflammation is defined as the activation of the innate immune system in response to an inflammatory event in the CNS. It is characterized by different cellular and molecular changes that play an important role in both physiological and pathological conditions. In this regard, neuroinflammation is a hallmark of several neurologic disorders, including chronic pain, traumatic brain injury, neurodegenerative diseases, and stroke (Di Vito *et al.,* 2017). Previous studies performed in our laboratory revealed that motor impairments observed during THC withdrawal condition and in CB1R KO mice were associated with an increase of both microglial activity and mRNA levels of some pro-inflammatory genes, such as COX-2, in the cerebellum (Cutando *et al.,* 2013).

In this section some of the most relevant cellular and molecular mechanism for this thesis will be accounted.

5.1. Cellular and molecular mechanisms of neuroinflammatory processes

After brain damage or insult to neural tissue, rapid cascade of molecular events emerges to initially potentiate and later mainly reduce the inflammatory response.

Inflammatory responses in the CNS are typically initiated by the identification of the new stranger stimulus by the receptors expressed in the surface of microglial cells, but also by the **migration of microglia** to the affected site.

This recognition of novel stimuli leads to the activation of signal transduction pathways that control the transcription of several **cytokine** and **chemokine genes** that serve to recruit additional immune cells (Colonna and Butovsky, 2017). Alternatively, these inflammatory mediators may stimulate the **synthesis of inflammatory genes**, such as *Cox2* (Chen *et al.,* 2018).

5.1.1. Microglial cells

Microglial cells are the immune effector cells in the CNS, where they represent between 5-20% of total glial cells in rodents, depending on the specific region (Lawson *et al.,* 1990) and being more abundant in the gray matter compared to the white matter (Kofler and Wiley, 2011). They have an hematopoietic origin from the invasion of peripheral mesodermal primitive macrophages (Alliot *et al.,* 1999). During embryogenesis, these cells migrate, differentiate and proliferate into the CNS, where they originate a dense network along the parenchyma contributing to brain homeostasis (Saijo and Glass, 2011).

Microglial cells are considered the pivotal players in inflammation **restoring homeostasis** upon injury or infection (Kempermann and Neumann, 2003). In addition, in a healthy CNS microglia can control proliferation and differentiation of neurons, as well as the formation of new synapses (Graeber and Streit, 2010). Thus, alterations in microglia functionality have been implicated in brain development and aging, as well as in the progression of several neurodegenerative diseases and neuropathologies (Colonna and Butovsky, 2017).

5.1.1.1. Microglial activation process

Two-photon imaging studies have demonstrated that **microglial morphology is dynamic**. They are currently classified into two groups: "**resting state**" and "**activated state**" depending on their morphology and the expression of activation markers (Colonna and Butovsky, 2017) (Figure 21).

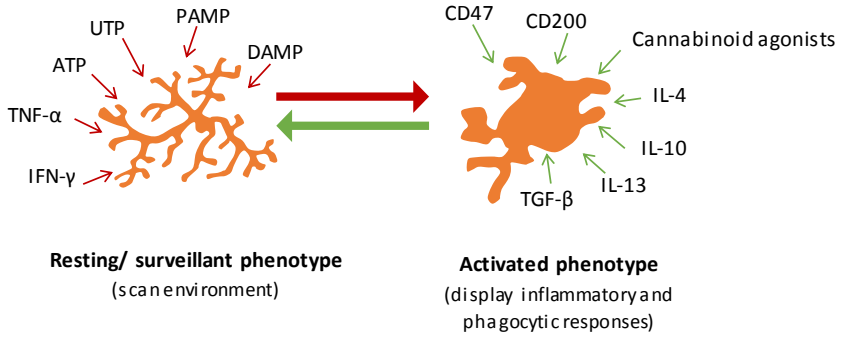


Figure 21. Classical stages of microglial cells activation from a morphological point of view. Several "on" factors activate surveillant microglia cells (red arrows), which shapes from a tiny soma and highly ramified conformation to a more amoeboid and less ramified state. Activated microglia returns to a resting state through different "off" factors (green arrows).

Under normal conditions, without brain injury or alteration, microglial cells are ramified with multiple branches and processes that extend from the soma (Kettenmann *et al.,* 2011; Benarroch, 2013). In this resting state, microglial ramifications contact with neurons, astrocytes and blood vessels monitoring the functional state of synapses. Notably, microglial cells are in continuous movement scavenging their surrounding area and their branches are extending and retracting more than 1 to 3 µm/min (Nimmerjahn *et al.,* 2005). Thus, the term "resting" microglia has been exchanged in the healthy CNS by "**surveillant**", as they act as sensors monitoring their local environment.

After brain injury or inflammatory stimuli, microglial cells become activated and change the surveillant morphology for an amoeboid-like shape. This morphology is characterized by an enlargement of the soma and a retraction of microglial branches. During this process, microglial cells move to the site of lesion or to the invader pathogen following chemotactic gradients. Activated microglia could exert different adaptative responses, depending on the type of stimulus or the environmental factors that activate them (Benarroch, 2013). Activated microglia could adopt different morphologies, such as ameboid, rod or multinucleated and may exert pro-inflammatory, cytotoxic, immunoregulatory and repair functions (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Ransohoff and Cardona, 2010).

Microglial cells control their surveillance and effector function according to the environmental signals recognized through different adhesion molecules, receptors, ion channels, transporters and intracellular enzymes expressed in their surface. These receptors convey different signals and control the transition from surveillance to the different activated states (Hanisch, 2013; Lee, 2013). These signals have been classified into “On” and “Off” (Biber *et al.,* 2007).

“On” signals induce microglial activity and are obtained from pathogens including the bacterial wall lipopolysaccharide (LPS), bacterial and viral RNA or DNA, and viral structures envelopes. Intracellular elements derived from necrotic cells and heat shock proteins can also activate microglial cells. In addition, interferon-γ (IFN-γ) and tumor necrosis factors-α (TNF-α) cytokines derived from lymphocytes activate the nuclear factor kB (NFkB) with the subsequent

transcriptional activation of proinflammatory genes (Hanisch, 2013). Furthermore, injured or activated neurons or astrocytes release ATP and UTP that stimulate the purinergic receptors expressed on microglial cells, predominantly the P2X ionotropic receptors P2X4 and P2X7, as well as the P2Y metabotropic receptors (P2Y2, P2Y6 and P2Y12). These receptors are important for the role of microglia in surveillance, synaptic plasticity and response to injury (Kettenmann *et al.,* 2011; Hanisch, 2013) (Table 7).

Moreover, microglial cells present adenosine and cytokine receptors, as well as neurotransmitter receptors including adrenergic, cholinergic and dopamine receptors that also modulate microglial activity (Hanisch, 2013).

"On signal"	Source	Microglial receptor
Pathogen-activated and Danger-associated molecular patterns	Bacterial wall lipopolysaccharides, viral, fungal or bacterial DNA or RNA, intracellular constituents from necrotic cells, heat shock proteins	Pattern recognition receptors (toll-like receptors)
IFN-γ	Released from Th1 lymphocytes	IFN-γ receptor
TNF-α	Released from Th2 lymphocytes	TNF receptor
ATP, UTP	Released from damaged neurons and astrocytes	Purinergic receptors (P2X4, P2X7, P2Y12)

Table 7. Main "On" signals in microglial cells with their respective source and microglial receptor (Benarroch, 2013)

In contraposition, microglial cells also receive **"Off" signals** to maintain them in a surveillant state (Table 8). Some of these signals are the secreted chemokine CX3CL1 by neurons and the neuronal CD200 and CD47 proteins that interact with their receptors CX3CR1, CD200R or SIRP-α receptors, respectively. In addition, the interaction between the anti-inflammatory interleukins IL-4, IL-10, IL-13 and the transforming

growth factor-β (TGF-β) with the respective receptors expressed on microglial cells reduces the microglial activated state. Moreover, microglial cells express CB1R and CB2R, as well as other cannabinoid-like receptors including GPR55 or TRPV1 (Carlisle *et al.,* 2002; Stella, 2009). Although the constitutive expression of these receptors is nearly undetectable in resting microglial cells, upon microglial activation an increase of CB2R expression is observed, and their stimulation reduces microglial activation and neurotoxicity (Stella, 2010).

"Off signal"	Source	Microglial receptor
CX3CL1 or Fraktalkine	Secreted by neurons	CX3CR1
CD200	Neuronal surface	CD200R
CD47	Neurons	SIRP-α
IL-4, IL-10, IL-13 and TGF-β	Th2 lymphocytes	Interleukin receptors
Cannabinoid agonists	Neurons	CB1R and CB2R

Table 8. Main "Off" signals in microglial cells with their respective source and microglial receptor (Benarroch, 2013).

Once activated, microglia can adopt different phenotypes, mainly classified into M1 and M2 states. The **M1 phenotype** is referred to the classical activation associated with the transcriptional stimulation of NFkB, leading to the production and release of **proinflammatory** cytokines and chemokines, as well as prostaglandins (PGs) (Martinez and Gordon, 2014). The **M2 phenotype** is considered an alternative activation state that exerts **anti-inflammatory** responses and promotes tissue repair through the release of anti-inflammatory cytokines and extracellular matrix proteins, respectively. These dichotomic patterns of microglial activation represent a wide range of responses. Microglial activation can start adopting a M1 phenotype to mediate an innate

response and then restrain the extent of microglial activation changing to the M2 phenotype (Benarroch, 2013).

In summary, microglial cells respond to several types of transforming factors that mediate the exchange from a surveillance state to activated state involving cell morphology, gene expression and functional changes. Microglial activation depends on the combination of the appearance of new "On" signals or the loss of restraining "Off" signals.

5.1.2. Cyclooxygenase-2

Under neuroinflammatory conditions, different signaling cascades become activated, such as the MAPK and NFkB pathways, inducing the transcription of several inflammatory genes including *Cox2* (Chen *et al.,* 2018).

COX-2 is an inducible enzyme that plays a key role in neuroinflammatory processes (Vane and Botting, 1998). In the CNS, COX-2 is constitutively expressed in postsynaptic terminals of neurons (Kaufmann *et al.,* 1996), where it has been implicated in the regulation of synaptic plasticity (Chen *et al.,* 2002). Other cellular factors, different from the transcription factor NFkB (Schmedtje *et al.,* 1997), also induce *Cox2* upregulation, including multiple growth factors, IL1, TNF-α, LPS, and elevated Ca^{2+} concentrations (Rojas *et al.,* 2019).

COX-2 catalyzes the conversion of AA and endocannabinoids (AEA and 2-AG) into PGs through different sequential steps. First, COX-2 oxygenates AA to generate PG-G_2. Next, the peroxidase action of the COX-2 enzyme rapidly converts PG-G_2 to PG-H_2. Finally, PG-H_2 is metabolized by other tissue-specific synthases to different PGs and

thromboxane, that constitute the biologically active products (Smith *et al.,* 2011) (Figure 22).

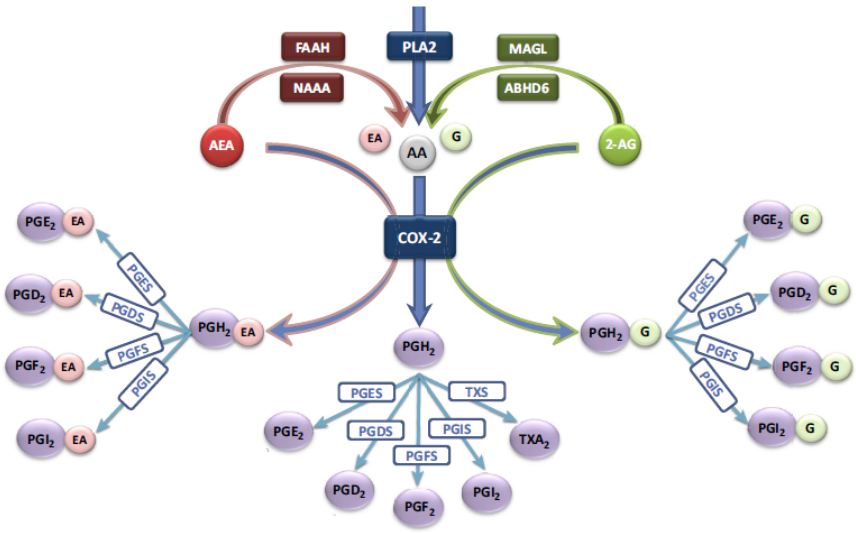


Figure 22. Main pathways of generation of eicosanoids through COX-2. Two enzymes are responsible for the hydrolysis of 2-arachidonoylglycerol (2-AG) into arachidonic acid (AA) and glycerol (G): the monoacylglycerol lipase (MAGL) and α/β hydrolase domain 6 (ABHD6). Fatty acid amide hydrolase (FAAH) and N-acylethanolamine hydrolyzing acid amidase (NAAA) degrade N-arachidonoylethanolamine (AEA) into AA and ethanolamine (EA). During inflammation, AA is produced from membrane phospholipids by phospholipase A2 (PLA2). AA is metabolized by cyclooxygenase-2 (COX-2) to give PG-H_2, which in turn is taken up by specific PG synthases (PGS) to give the PGs (PG-D_2, PG-E_2, PG-F_2, PG-I_2). PGs act through specific G protein-coupled receptors. Classically, during inflammatory conditions, COX-2 is upregulated leading to increased formation of PGs. 2-AG and AEA can be metabolized by COX-2, similarly to AA, to give PG-glycerol esters (PG-Gs) and PG-ethanolamides (PG-EAs), respectively. Adapted from (Alhouayek and Muccioli, 2014).

PGs are lipid structures released by neuronal and glial cells in **response to inflammatory processes** (Quan *et al.,* 1998) and exert their actions by binding to different types of prostanoid receptors (Boie *et al.,* 1997; Breyer *et al.,* 2001). PGs binding to their receptors expressed on microglial cells affect the **neuroinflammatory progression** (Lima *et al.,* 2012). Moreover, it is reported that PGs could alter synaptic plasticity and produce functional alteration in particular brain structures, such as

the hippocampus, where they produce memory deficits (Hein and O'Banion, 2009).

AEA and 2-AG metabolism by COX-2 leads to the production of PG-EA and PG-G respectively (Kozak *et al.,* 2002). Some studies failed to detect these endocannabinoid-derived PGs *in vivo* due to their rapid hydrolysis into PGs (Hu *et al.,* 2008; Ritter *et al.,* 2012). It is reported that PG-EA and PG-G are not ligands for the classical prostanoid receptors, and that alternative receptors mediate their effects (Hu *et al.,* 2008). Although the biological activities of these endocannabinoid-derived prostaglandins remain to be elucidated, some evidences indicate that they are important modulators of neurotransmission and synaptic plasticity and they induce neuroinflammation and neurotoxicity (Nirodi *et al.,* 2004; Sang *et al.,* 2006, 2007; Yang *et al.,* 2008).

5.2. Role of endocannabinoid system in neuroinflammation

The ECS plays an important role in immunomodulation and inflammation (Mecha *et al.,* 2016). Indeed, several components of the ECS are upregulated during inflammation in order to protect cells from damage and to counteract the massive release of toxic cytokines and inflammatory mediators from microglia (Sánchez and García-Merino, 2012).

As mentioned above, CB2R is low expressed on surveillant/resting microglial cells, but its expression is enhanced in activated cells (Cabral *et al.,* 2008). **CB2R activation inhibits cytokine production** (Ehrhart *et al.,* 2005), reduces antigen presentation (Buckley, 2008) and **modulates immune cell migration** (Miller and Stella, 2008). Moreover,

it has been demonstrated that endocannabinoids induce the anti-inflammatory M2 microglial phenotype (Sánchez and García-Merino, 2012; Mecha *et al.,* 2015). Thus, stimulation of CB2R has been proposed for the treatment of neuroinflammation in neurodegenerative diseases.

CB1R is also involved in inflammatory processes. **CB1R stimulation inhibits** the release of nitric oxide and some **pro-inflammatory cytokines** (Waksman *et al.,* 1999). In this regard, 2-AG administration inhibits the expression of the pro-inflammatory cytokines IL-6, IL-1β, IL-1α and TNF-α after brain injury in mice (Puffenbarger *et al.,* 2000; Panikashvili *et al.,* 2006). In addition, other cannabinoid-like receptors including GPR55 (Kallendrusch *et al.,* 2013), PPAR-γ (Lee and Won, 2014) and TRPV1 (Raboune *et al.,* 2014) are expressed in microglial cells and respond to endogenous or synthetic cannabinoids to modulate microglial activation, migration, and proliferation processes.

Finally, MAGL has a main role in inflammation, acting as a rate-limiting enzyme in the production of free AA that serves as a precursor for proinflammatory mediators. It has been demonstrated that **MAGL inhibitors act as anti-inflammatory agents** increasing 2-AG levels and decreasing AA levels and consequently the main source of PGs production (Nomura *et al.,* 2011).

In summary, cannabinoid agonists modulate several processes in microglial cells including activation, proliferation, cytokine expression, migration and phagocytosis, postulating the ECS system may represent a key target in the treatment of neuroinflammation.

OBJECTIVES

General objective

Growing data support the modulation of the ECS and its associated intracellular signaling pathways as a therapeutic target for many disorders. However, targeting this system has important central adverse effects. Some alternatives have been proposed to minimize these side effects, such as the use of inhibitors of endocannabinoid-degradation enzymes, and peripherally-restricted cannabinoid agonists or antagonists. Therefore, the main goals of this thesis are to evaluate the effects of targeting two alternative strategies: the antagonism of CB1R and the inhibition of MAGL.

Specific objectives

Objective 1

To study the involvement of the peripheral CB1R in the modulation of object-recognition memory in wildtype and FXS mice.

Article #1

The peripheral endocannabinoid system modulates memory persistence through an adrenergic-dependent mechanism in mice

Martínez-Torres S, Bergadà-Martínez A, Martínez-Gallego I, Ortega J, Galera-López L, Ortega Álvaro A, Remmers F, Beat Lutz B, Ruiz-Ortega J.A, Meana J.J, Maldonado R, Rodríguez-Moreno A, Ozaita A

Supplementary results #1

Peripheral CB1R antagonism rescues cognitive Fragile X syndrome phenotype

Martínez-Torres S, Losada-Puiz P, Martínez-Gallego I, Rodríguez-Moreno A, Maldonado R, Ozaita A

Objective 2

To study the mTORC1/mTORC2 involvement in the amnesic-like effects produced by THC.

Supplementary results #2

The dual mTORC1/mTORC2 inhibitor P529 blocks the amnesic-like effects produced by THC

Martínez-Torres S, Maldonado R, Ozaita A

Objective 3

To study the effects of the monoacylglycerol lipase enzyme inactivation in the regulation of the motor coordination tasks and its downstream mechanisms.

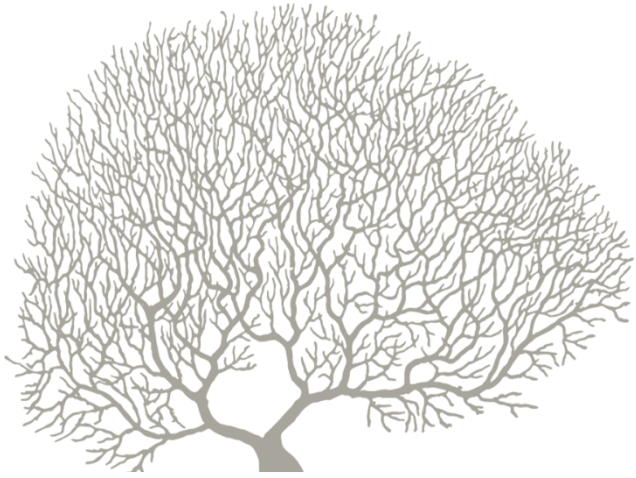
Article #2

Monoacylglycerol lipase blockade impairs fine motor coordination and triggers cerebellar neuroinflammation through cyclooxygenase-2

Martínez-Torres S, Cutando L, Pastor A, Kato A, Sakimura K, de la Torre R, Valjent E, Maldonado R, Kano M, Ozaita A

Brain Behav Immun. 2019. 81: 309-409

RESULTS



Objective 1

To study the involvement of the peripheral CB1R in the modulation of object-recognition memory in wildtype and FXS mice.

Article #1

The peripheral endocannabinoid system modulates memory persistence through an adrenergic-dependent mechanism in mice

Sara Martínez-Torres, Araceli Bergadà-Martínez, Irene Martínez-Gallego, Jorge Ortega, Lorena Galera-López, Antonio Ortega-Álvaro, Floor Remmers, Beat Lutz, José Ángel Ruiz-Ortega, Jorge Javier Meana, Rafael Maldonado, Antonio Rodríguez-Moreno, Andrés Ozaita

Supplementary results #1

Peripheral CB1R antagonism rescues cognitive fragile X syndrome phenotype

Sara Martínez-Torres, Pilar Losada-Ruiz, Irene Martínez-Gallego, Antonio Rodríguez-Moreno, Rafael Maldonado, Andrés Ozaita

Martínez-Torres S, Bergadà-Martínez A, Martínez-Gallego I, Ortega J, Galera-López L, Ortega-Álvaro A, Remmers F, Beat Lutz B, Ruiz-Ortega J.A, Meana J.J, Maldonado R, Rodríguez-Moreno A, Ozaita A. Peripheral inhibition of cannabinoid CB1 receptor enhances memory persistence through an adrenergic dependent-mechanism in mice. Submitted

The peripheral endocannabinoid system modulates memory persistence through an adrenergic-dependent mechanism in mice

Abbreviated title: Peripheral CB1 receptors modulate memory persistence

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Keywords: memory persistence; CB1 cannabinoid receptor; peripheral-to-central, synaptic plasticity,

Abstract

Peripheral inputs continuously shape brain function and modulate memory performance. In this regard, our group revealed that the peripheral cannabinoid type-1 receptor (CB1R) was involved in the memory consolidation impairments induced by stress. We now used the novel object-recognition test (NORT) to study the possible peripheral CB1R modulation on non-emotional memory persistence. We found that the peripherally-restricted CB1R antagonist AM6545 showed a mnemonic effect in the NORT that was absent in adrenalectomized mice or when mice were pre-treated with the peripherally restricted β-adrenergic antagonist sotalol. Genetic CB1R deletion in dopamine β-hydroxylase-expressing cells also facilitated memory persistence further supporting a role of the adrenergic tone modulated by the endocannabinoid system. *Locus coeruleus* activity as well as extracellular noradrenaline levels in the hippocampus were increased after AM6545 treatment. Such enhancement in the noradrenergic axis was relevant to the mnemonic effect of AM6545 since intra-hippocampal injection of the β-adrenergic antagonist propranolol prevented the memory improvement induced by peripheral AM6545. Moreover, sub-chronic AM6545 treatment produced neuronal plastic and functional changes in the hippocampus as well as increased expression of neurotrophic factors and AMPA receptors. These data reveal that the peripheral CB1R contributes to the modulation of memory persistence and hippocampal synaptic plasticity involving peripheral and central adrenergic/noradrenergic mechanisms.

Introduction

Most everyday facts create a short lived recall that fades away, whereas others, with the intervention of the hippocampus, are retained through unpredictable periods of time that may last a life-time, even if they are created from similar sensorial stimuli (Morris, 2006). Therefore, persistence of stimuli-driven memories involves a discrimination/selection of worth-memorizing stimuli. This is mainly relevant for non-emotional memories that are retained during shorter time periods than those associated to a stressful situation (Roozendaal *et al.,* 2009). The endocannabinoid system (ECS), highly expressed in the central nervous system (CNS) and peripheral tissues (Kano *et al.,* 2009; Maccarrone *et al.,* 2015), plays a key role in learning and memory (Kano *et al.,* 2009). The cannabinoid type-1 receptor (CB1R) is heavily expressed in the brain (Pacher *et al.,* 2006), predominantly localized at presynaptic sites of different neuronal cell types, where it suppresses neurotransmitter release depending on local synaptic activity (Ohno-Shosaku *et al.,* 2001). Exogenous compounds with affinity for CB1R modify memory function (Ohno-Shosaku *et al.,* 2001; Hoffman *et al.,* 2007; Niyuhire *et al.,* 2007; Puighermanal *et al.,* 2012). In accordance, pharmacological blockade of CB1R and genetic CB1R inactivation in mice increase memory persistence in the novel object-recognition test (NORT) (Reibaud *et al.,* 1999; Maccarrone *et al.,* 2002), although the mechanisms involved are largely unknown. Such regulation of memory by CB1R blockade has been previously assumed to occur solely through centrally located receptors (Zanettini *et al.,* 2011). However, previous results have challenged this central view. Indeed, peripheral CB1Rs, through their physiological activation after a stressful situation, were

responsible for the stress-induced amnesia over object-recognition memory (Busquets-Garcia *et al.,* 2016). Genetic and pharmacological approaches revealed that such deficits in object-recognition memory consolidation were mediated by peripheral CB1R in dopamine beta-hydroxylase positive cells (DBH+ cells) (Busquets-Garcia *et al.,* 2016), where CB1R controls the release of adrenaline and noradrenaline (Niederhoffer *et al.,* 2001). Notably, systemic administration of adrenaline, a monoamine with limited brain penetration, produces mnemonic effects in rats (Dornelles *et al.,* 2007). We therefore hypothesized that peripherally-acting CB1R inhibitors could be responsible for the adrenergic tone necessary for proper memory consolidation.

In the present study, we have investigated the impact of peripheral CB1R blockade on an hippocampal-dependent non-emotional memory task to reveal the critical interplay of peripheral inputs modulated by the ECS in memory persistence.

Materials and methods

Animals

Young adult (10-12 weeks old) male Swiss albino (CD-1) mice (Charles River, France) were used for pharmacological approaches on behavioural, microdialysis, electrophysiological and biochemical approaches. For genetic approaches to reduce CB1R expression, heterozygous mice for the *Cnr1* gene and their wild-type littermates in C57BL/6J genetic background were used (Zimmer *et al.,* 1999). Conditional KO mice for the *Cnr1* gene lacking CB1R exclusively in D□H-expressing cells were generated as previously detailed (Busquets-Garcia *et al.,* 2016) in C57BL/6J genetic background. The transgenic line Tg(Thy1-EGFP) MJrs/J (*Thy1-EGFP* mice) in C57BL/6J background (Stock # 007788, The Jackson Laboratories, USA) was used to study the density and morphology of dendritic spines.

Mice were housed in plexiglas cages (2-4 mice per cage) and maintained in a temperature (21 ± 1 °C) and humidity (55 ± 10%) controlled environment. Food and water were available *ad libitum*. All the experiments were performed during the light phase of a 12 h cycle (light on at 8 am; light off at 8 pm). Mice were habituated to the experimental room and handled for 1 week before starting the experiments. All behavioural experiments were conducted by an observer blind to experimental conditions.

All animal procedures were conducted following "Animals in Research: Reporting Experiments" (ARRIVE) guidelines and standard ethical guidelines (Kilkenny *et al.,* 2010) (European Communities Directive 2010/63/EU). Animal procedures were approved by the local ethical

committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB).

Drugs and treatments

Rimonabant (Axon Medchem) and mifepristone (Sigma-Aldrich) were dissolved in 5% ethanol, 5% cremophor-EL and 90% saline (0.9% NaCl). AM6545 (Tocris-Bio-Techne) was dissolved in 0.26% DMSO, 4.74% ethanol, 5% cremophor-EL and 90% saline (0.9% NaCl). Sotalol (Sigma-Aldrich) was dissolved in 90% saline (0.9% NaCl). Propranolol (Sigma-Aldrich) was dissolved in 90% saline (0.9% NaCl). All intraperitoneally (i.p.)-injected drugs were administered in a volume of 10 ml/kg of body weight at the doses and time points indicated.

Bilateral adrenalectomy

Mice were anesthetized by isoflurane inhalation, 5% v/v induction and 3% v/v for maintenance, with oxygen (0.8 L/min). A small incision of 1 cm was made in the left and right flanks, and the adrenal glands were identified and removed from the surrounding tissue. Wounds were closed in two layers using 4/0 silk sutures (Alcon). All animals were given access to saline after surgery to ensure adequate salt balance. The experiments were resumed following a recovery period of 10 d.

Bilateral intra-hippocampal cannula implantation

Mice were anesthetized by i.p. injection of a mixture of ketamine hydrochloride (75 mg/kg) and medetomidine hydrochloride (1 mg/kg) in saline. During surgery, mice were placed in a stereotaxic frame and a bilateral 26-gauge guide cannula (Plastics One) was implanted into the dorsal hippocampus to be used to guide a bilateral injection

cannula (33-gauge internal cannula, Plastics One). The bilateral guide cannula was held in place using dental cement (Dentalon plus, Heraeus Kulzer GmbH) and 2 stainless-steel screws. The placement was set at 1 mm above the target injection site and the guide cannula was sealed with a dummy of stainless-steel wire with 0.5 mm of projection to prevent obstruction. The target injection site coordinates were as follows: anteroposterior, −1.80 mm; mediolateral, ±1.00 mm; dorsoventral, 2.00 mm (Paxinos and Franklin, 2001). Animals were kept on a 37 °C heating pad during the surgery, and until recovery from anaesthesia. After surgery, anaesthesia was reversed by a subcutaneous (s.c.) injection of atipamezole hydrochloride (2.5 mg/kg). Mice also received an i.p. injection of gentamicine (1 mg/kg) and a s.c. injection of meloxicam (2 mg/kg). The behavioural experiments started between 7 d and 21 d after surgery. To verify cannula implantation after behavioural experiment, mice were euthanized, and the brains removed, frozen and stored at -80 °C until sectioning. Brains were cut in coronal sections (30 µm) on a cryostat and mounted on slides. Then, slides were stained with cresyl violet and the injection sites were verified under a light microscope by an experimenter blind to experimental conditions (Supplementary Figure S3A). Mice with cannula location outside of the hippocampus were excluded from the study.

Intra-hippocampal drug administration

After familiarization phase of NORT, mice received a bilateral intra-hippocampal injection of 0.50 µl of propranolol (0.5, 1 or 2 µg) or saline at a constant rate of 0.25 µl/min by using a microinfusion pump during 2 min. The injection cannula projected 1.00 mm below the ventral tip of the implanted guide cannula. The displacement of an air bubble inside the length of the polyethylene tubing that connected the Hamilton syringe to the injection needle was used to monitor the microinjections. After infusion, the injection cannula was left for an additional period of 2 min to allow the fluid to diffuse and to prevent reflux before withdrawal.

Behavioural studies

*NORT: O*bject-recognition memory was assayed in the V-maze in a sound-attenuated room with dim illumination (4-7 lux). A digital camera on top of the maze was used to record the sessions. This task consists in 3 different phases (habituation, familiarization and test) performed on different days for 9 min. On day 1, mice were habituated to the empty V-maze. In the familiarization phase, mice were introduced the next day in the V-maze were 2 identical objects were presented. Finally, the test was performed 3 h, 24 h or 48 h later, where 1 of the familiar objects was replaced for a novel object and mice were allowed to explore them. The total time spent exploring each of the 2 objects (novel and familiar) was recorded. Object exploration was defined as orientation of the nose toward the object at a distance < 2 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf)

object divided by the total time spent exploring both objects: (Tn + Tf): DI = (Tn − Tf)/(Tn + Tf). DI was interpreted as a quantification of novel object-recognition memory persistence.

Locomotor activity: Locomotor activity was assessed for 120 min after acute administration of AM6545. Individual locomotor activity boxes (9 × 20 × 11 cm) (Imetronic) were used in a low luminosity environment (5 lux). The total activity and the total number of rearings were detected by infrared sensors to detect locomotor activity and infrared plane to detect rearings.

Tissue preparation for immunofluorescence

Mice were deeply anesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) prior to intracardiac perfusion of cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH7.4 (PB). Brains were removed and post-fixed overnight at 4 °C in the same fixative solution. The next day, brains were moved to PB at 4 °C. Coronal brain sections (30 µm for immunofluorescence staining or 60 µm for dendritic spine study in *Thy1-EGFP mice*) were made on a freezing microtome and stored in a 5% sucrose solution at 4 ºC until use.

Immunofluorescence and cell quantification

Antibodies against the proliferation marker Ki-67 (anti-Ki67, rabbit, ab15580, 1:300, Abcam) were used as primary antibody and anti-rabbit (donkey, Alexa Fluor-488, A21206, 1:600, Life Technologies) as secondary antibody. Coronal sections (1 every 6) per animal were selected, covering the rostral to caudal extension of the hippocampus

(from 1.3 and 2.5 mm posterior to Bregma). Four brain sections per animal were used for performing the immunofluorescence. Slices were blocked in a solution containing 3% normal donkey serum and 0.3% Triton X-100 in PB (NDS-T-PB) at room temperature for 2 h and incubated in the same solution with primary antibodies at 4 °C. Slices were rinsed 24 h later with PB and incubated with secondary antibodies in NDS-T-PB for 2 h at room temperature. Then, sections were rinsed and mounted onto gelatin-coated slides with Mowiol mounting medium. Images from Ki67 labelling were obtained using a confocal microscope (Leica TCS Sp5 STED) with 10×/0.40 dry lens and 2x zoom with a sequential line scan at 1024 × 1024 pixel resolution. The images were obtained choosing a representative 10 µm z-stack from the slice with 1.01 µm depth intervals. Quantification of Ki67+ cells in the subgranular zone (SGZ) of the hippocampus was performed using the Fiji software (ImageJ). The number of positive cells was calculated as the mean of total number of cells counted referred to the volume of the SGZ (μm^3). Positive cells density was referred to that calculated for the control group.

Dendritic spine analysis

Secondary and tertiary apical dendrites of pyramidal neurons from the *stratum radiatum* of CA1 region of the dorsal hippocampus were analysed in *Thy1-EGFP mice*. Images were acquired with a confocal microscope (TCS SP5 STED Leica) using a glycerol immersion lens plus 3 times magnification (63X/3) with a sequential line scan at 1024 × 1024 pixel resolution. Serial optical sections were acquired with a 0.13 µm step size. A minimum of 8 dendrites per animal were selected from 4

different slices. A maximum of 2 dendrites were selected from the same neuron. Confocal images were deconvoluted using Huygens Essential software and dendritic spines were analysed using the semi-automated software NeuronStudio. Spine density was calculated by expressing the average number of spines in a 10 µm portion of the dendrite. NeuronStudio calculated for each spine the following parameters based on its morphology: head/neck ratio (threshold = 1.100 pixel), length/head ratio (threshold = 2.5 pixel) and head size (threshold = 0.350 µm). Then, software classified spines into 3 major morphologic types: mushroom (if the head/neck ratio and the mushroom head size were above the threshold), stubby (if the head/neck and the length/head ratios were below the threshold) and thin (in the remaining cases). Each spine was checked manually by an observer blind to experimental conditions for unbiased classification.

***In vivo* microdialysis**

Animals were anesthetized with isofluorane (1.5-2.5 % v/v for induction and maintenance) and placed in a Kopf stereotaxic frame. Intracerebral probe (cuprophan membrane of 2 mm) was implanted in the hippocampus and fixed to the skull. The coordinates were AP -3.4 mm, L +2.6 mm, DV –4.2 mm (Franklin and Paxinos, 1997). The next day, mice were placed in a plastic bowl and connected to a fraction collection system for freely-moving animals (Raturn, BASi, USA). The input tube of the dialysis probe was connected to a syringe pump (BeeHive and BabyBee, BASi), which delivered a modified cerebrospinal fluid (CSF) containing NaCl 148 mM, KCl 2.7 mM, CaCl2 1.2mM and MgCl2 0.85 mM (pH 7.4) to the probe at a rate of 1 µl/min. The output

tubes from the animals were attached to a refrigerated fraction collector (HoneyComb, BASi). Samples were collected every 35 min for the analysis of the different neurotransmitters on vials containing 5 µl of acetic acid 0.1 M. Eight baseline samples were collected from each animal, but only the last 6 ones were used for subsequent analysis.

NA, DA and 5-HT chromatographic analysis

Neurotransmitter concentrations were measured immediately after samples collection by Ultra Performance Liquid Chromatography (UPLC) coupled to an electrochemical detector (Alexys analyser, Antec Leyden, Holland). The mobile phase consisted of 100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA, 950-1500 mg/l 1-octanesulfonic acid (OSA), 5% v/v acetonitrile; the pH was adjusted to 6.0 with 50% NaOH/ 45% KOH solution. The flow rate of the mobile phase was 0.075 ml/min and the temperature for the analytical column (Acquity UPLC BEH C18, 1.7 µm, 1x100 mm; Waters, Milford, USA) was 37 ºC.

Tissue for biochemical and mRNA analysis

Mice were treated with AM6545 (1 mg/kg, i.p.) or vehicle for 7 d. Twenty four h after the last administration, hippocampal tissues were dissected on ice, frozen on dry ice and stored at -80 ºC until used.

Immunoblot analysis

Hippocampal tissues were homogenized in 30 volumes of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 µg/mL aprotinin, 1 µg/mL leupeptine, 1 µg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 5 mM sodium pyrophosphate, and 40 mM beta-

glycerol phosphate) and 1% Triton X-100 using a Dounce homogenizer. After 10 min incubation at 4 ºC, samples were centrifuged at 16,000 g for 30 min to remove insoluble fragments. Protein content in the supernatants was determined by DC-micro plate assay (Bio-Rad), following manufacturer's instructions. Equal amounts of brain lysates were separated in 10% SDS-polyacrylamide gels before electrophoretic transfer onto nitrocellulose membrane overnight at 4 ºC. Nitrocellulose membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.4) with 0.1% Tween-20 (T-TBS) and 3% bovine serum albumin. Afterwards, nitrocellulose membranes were incubated for 2 h with the primary antibodies. Anti-BDNF (mouse, 1:150, Qiagen) and anti-actin (mouse, 1:20,000, Millipore) were used as primary antibodies. Then, nitrocellulose membranes were washed 3 times (5 min each) and subsequently incubated with the corresponding secondary antibody for 1 h at room temperature. Anti-rabbit (1:15,000, Cell Signaling) or anti-mouse (1:15,000, Cell Signaling) were used as secondary antibodies. After 3 washes (5 min each), immunoreactivity was visualized by enhanced chemiluminescence detection (Luminata Forte, Amersham). Optical densities of relevant immunoreactive bands were quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v 4.6.9 (Bio-Rad). For quantitative purposes, the optical density values were normalized to β-actin values in the same sample and were expressed as a percentage of control treatment.

RNA extraction and reverse transcription

Isolation of total RNA from hippocampal tissues was performed using a RNeasy Mini kit (tissue; QIAGEN) according to the manufacturer's instructions. Total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 100 ng of total RNA from each animal to produce cDNA in a 20 µl reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Real-time PCR was carried out in a 10 µl reaction using SYBR Green PCR Master Mix (Roche) according to the manufacturer's protocol with a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems). Specific primers for mouse were used (Table 1).

Gene	Forward	Reverse
*Bdnf (*BDNF)	5'-CAGGTGAGAAGAGTGATGACC-3'	5'-ATTCACGCTCTCCAGAGTCCC-3'
Gria1 (GluA1)	5'-TCCGCAAGATTGGTTACTGG-3'	5'-CAGATCTCGTAGGCCAAAGG-3'
Gria2 (GluA2)	5'-AATAGAAAGGGCCCTCAAGC-3'	5'-ATTCCAAGGCTCATGAATGG-3'
Ngf (NGF)	5'-CAAGGACGCAGTTTCTATACTG-3'	5'-CTTCAGGGACAGAGTCTCCTTCT-3'
Ntf3 (NT-3)	5'-CCAGGCGGATATCTTGAAAAA-3'	5'-AGCGTCTCTGTTGCCGTAGT-3'
*Actb (*β-actin)	5'-CGTGAAAAGATGACCCAGATCA-3'	5'-CACAGCCTGGATGGCTACGT-3'

Table 1. Primer sequences used in this study.

Quantification was performed by using the comparative CT Method (ΔΔCT Method). All the samples were tested in triplicate and the relative expression values were normalized to the expression value of β-actin. The fold change was calculated using the eq. $2(^{-\Delta\Delta Ct})$ formula, as previously reported (Livak and Schmittgen, 2001).

In vivo electrophysiological recording in the locus coeruleus

Mice were anaesthetized with chloral hydrate (400 mg/ kg, i.p.) and placed in the stereotaxic frame with the skull positioned horizontally. A burr hole was drilled and the recording electrode was placed 1.5 mm posterior to lambda and 0.2–1.2 mm from the midline and lowered into the LC usually encountered at a depth of between 2.7 and 4.0 mm from the brain surface (Gobbi *et al.,* 2007). A catheter (Terumo Surflo1; Teruma Medical Products) was then inserted in the peritoneo for additional administrations of anaesthetic by a pump and systemic drug. The body temperature was maintained at 37 ºC for the entire experiment using a heating pad.

Single-unit extracellular recordings of mouse LC neurons were performed as previously described (Gobbi *et al.,* 2007). The recording electrode was filled with 2% solution of Pontamine Sky Blue in 0.5% sodium acetate and broken back to a tip diameter of 1–2 mm. The electrode was lowered into the brain by means of a hydraulic microdrive (model 640; David Kopf Instruments). LC neurons were identified by standard criteria, which included spontaneous activity displaying a regular rhythm and firing rate between 0.5 and 5 Hz, characteristic spikes with a long-lasting (>2 ms), positive–negative waveform action potentials and the biphasic excitation–inhibition response to pressure applied on contralateral hind paw (paw pinch), as previously described in mice (Gobbi *et al.,* 2007) and rats (Cedarbaum & Aghajanian, 1976). The extracellular signal from the electrode was pre-amplified and amplified later with a high-input impedance amplifier and then monitored on an oscilloscope and on an audio monitor. This activity was processed using computer software (Spike2

software; Cambridge Electronic Design) and firing rate was calculated. Basal firing rate and other electrophysiological parameters were measured for 3 min. Changes in firing rate were expressed as percentages of the basal firing rate (mean firing rate for 3 min prior to drug injection) and were measured after 35 min until the end of the experiment. Only 1 cell was studied in each animal when any drug was administered.

***Ex vivo* electrophysiological recordings in the hippocampus**

Hippocampal slices were prepared as described in detail elsewhere (Andrade-Talavera *et al.,* 2016). Briefly, mice were anesthetized with isoflurane (2% v/v) and brains were rapidly removed into ice-cold solution (I) consisting of (in mM): 126 NaCl, 3 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (pH 7.2, 300 $mOsmL^{-1}$), and positioned on the stage of a vibratome slicer and cut to obtain transverse hippocampal slices (350 mm thick), which were maintained continuously oxygenated for at least 1 h before use. All experiments were carried out at room temperature (22–25 ºC). For experiments, slices were continuously perfused with the solution I described above. For LTP studies, whole-cell patch-clamp recordings were made from pyramidal cells located in the CA1 field of the hippocampus. CA1 pyramidal cells were patched under visual guidance by infrared differential interference contrast microscopy and verified to be pyramidal neurons by their characteristic voltage response to a current step protocol. Neurons were recorded using the whole-cell configuration of the patch-clamp technique in voltage-clamp mode with a patch-clamp amplifier (Multiclamp 700B), and data were

acquired using pCLAMP 10.2 software (Molecular Devices). Patch electrodes were pulled from borosilicate glass tubing, and had resistances of 4–7 MΩ when filled with (in mM): CsCl, 120; HEPES, 10; NaCl, 8; $MgCl_2$, 1; $CaCl_2$, 0.2; EGTA, 2 and QX-314, 20 (pH 7.2–7.3, 290 mOsm L^{-1}). Cells were excluded from analysis if the series resistance changed by more than 15% during the recording. Recordings were low-pass filtered at 3 kHz and acquired at 10 kHz.

In paired-pulse experiments, 2 consecutive stimuli separated by 40 ms were applied at the beginning of the baseline recording and again 30 min after applying the LTP protocol. Data were filtered at 3 kHz and acquired at 10 kHz. A stimulus-response curve (1–350 µA, mean of 5 excitatory postsynaptic current, EPSC, determination at each stimulation strength) was compiled for each experimental condition. Paired-pulse ratio was expressed as the slope of the second field excitatory postsynaptic potential (fEPSP) determination divided by the slope of the first fEPSP. Data were analyzed using the Clampfit 10.2 software (Molecular Devices). The last 5 min of recording were used to estimate changes in synaptic efficacy compared to baseline.

Statistical analysis

Results are reported as mean ± standard error of the mean (s.e.m). Data analysis were performed using with GraphPad Prism software (GraphPad Software). Statistical comparisons were evaluated using unpaired Student's t-test for 2 groups comparisons or two-way ANOVA for multiple comparisons. Subsequent Bonferroni *post hoc* was used when required (significant interaction between factors). Comparisons were considered statistically significant when $p < 0.05$.

Results

CB1R inhibition enhances memory persistence in the novel object-recognition test

Object-recognition memory is a labile non-emotional type of memory that can be revealed 3 h or 24 h after the familiarization session, when naïve mice readily discriminate novel and familiar objects (Figure 1A). This memory type is susceptible of modulation by post-familiarization treatments using CB1R agonists (Puighermanal *et al.,* 2009; Busquets-Garcia *et al.,* 2011; Busquets-Garcia *et al.,* 2018) or stress (Busquets-Garcia *et al.,* 2016). Notably, discrimination values significantly decrease when object-recognition memory is assessed 48 h after the familiarization phase (one-way ANOVA, interaction: $F(2,19) = 8.55$, $p = 0.002$; *post hoc* Bonferroni, 3h vs 48h $p = 0.004$; 24h vs 48h $p = 0.007$) (Figure 1A). We used this memory paradigm assayed 48 h after the familiarization phase to evaluate the role of CB1R in memory persistence. We found that acute post-familiarization treatment with a low dose of the systemic CB1R specific antagonist rimonabant (1 mg/kg, i.p.) showed better memory persistence than vehicle-treated mice (Student's t-test: $p = 0.02$) (Figure 1B). In addition, heterozygous mice for the *Cnr1* gene (Student's t-test: $p = 0.004$) also showed improved memory persistence compared to their respective wild-type littermates (Figure 1C), indicating that such a modulation in memory persistence is CB1R dependent. Post-familiarization administration of the peripherally-restricted CB1R antagonist AM6545 also enhances object-recognition memory at 48 h (Student's t-test: $p = 0.002$) (Figure 1D). No differences in total exploration time were detected between genotypes or pharmacological treatments in any of the experimental

groups (Figure S1). Furthermore, AM6545 treatment did not affect locomotor activity analyzed for 120 min post administration (Figure S2).

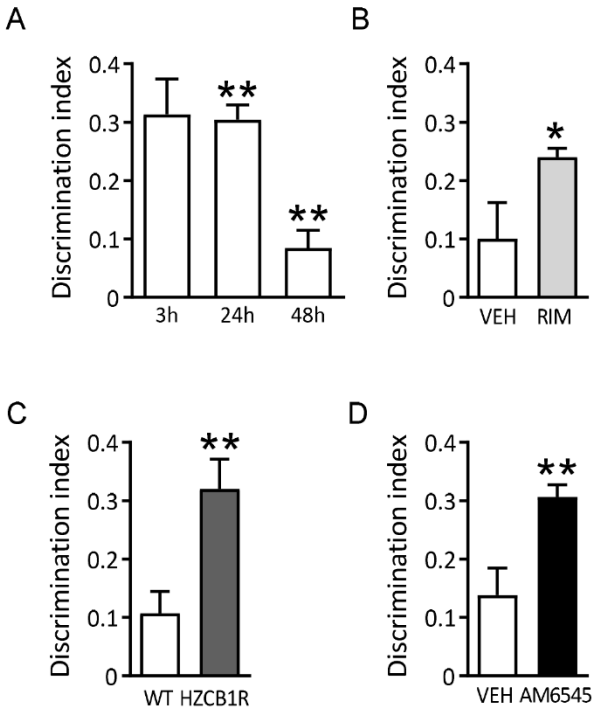

Figure 1. Systemically or peripheral CB1R blockade improves memory persistence in the novel object-recognition test. (A) Discrimination index values obtained at 3 h, 24 h and 48 h after the familiarization phase (n = 5-8). **(B-D)** Discrimination index values in NORT at 48h **(B)** after acute post-familiarization treatment with rimonabant vehicle (VEH) or rimonabant (RIM) (1 mg/kg) (n = 7-11) **(C)** in HZCB1R and WT mice (n = 6-8) **(D)** after acute post-familiarization treatment with vehicle (VEH) or AM6545 (1 mg/kg) (n = 7-8). Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA followed by Bonferroni *post hoc* or Student's t-test.

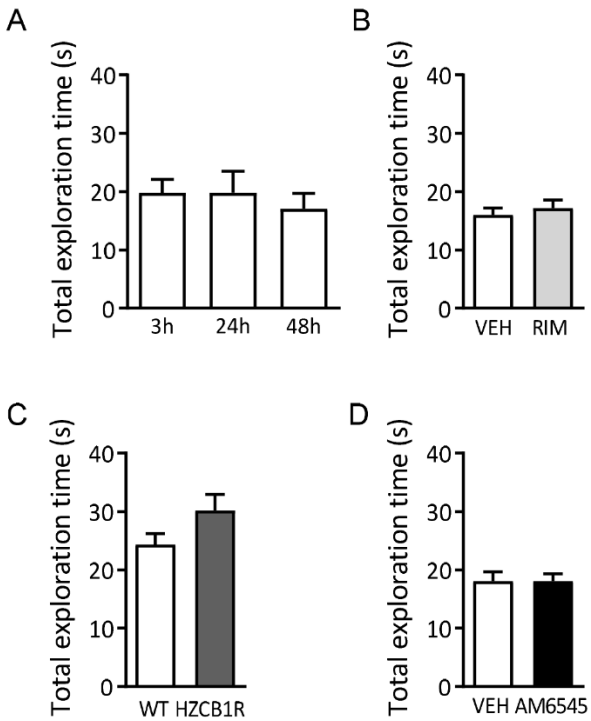

Figure S1. Total exploration times of systemically or peripheral CB1R blockade are not affected. (A) Total exploration times obtained at 3 h, 24 h and 48 h after the familiarization phase (n = 5-8). **(B-D)** Total exploration times in NORT at 48h **(B)** after acute post-familiarization treatment with rimonabant vehicle (VEH) or rimonabant (RIM) (1 mg/kg) (n = 7-11) **(C)** in HZCB1R and WT mice (n = 6-8) **(D)** after acute post-familiarization treatment with vehicle (VEH) or AM6545 (1 mg/kg) (n = 7-8). Data are expressed as mean ± s.e.m.

Peripheral CB1R inhibition enhances novel-object recognition memory in a peripheral β-adrenergic dependent mechanism

We hypothesized that a peripherally located tissue such as the adrenal glands, that express CB1R (Hillard, 2015) could be a relevant player for memory consolidation (McIntyre *et al.,* 2012) for object-recognition memory. Therefore, we evaluated the effect of post-familiarization AM6545 in bilaterally adrenalectomized mice. Memory improvement mediated by acute AM6545 was significantly blocked in mice without adrenal glands (two-way ANOVA, interaction: $F(1,22) = 4.42$, $p = 0.047$; *post hoc* Bonferroni, naive-VEH vs naive-AM6545 $p = 0.040$; naive-

AM6545 vs ADX-AM6545 p = 0.047) (Figure 2A), supporting the role of CB1R in this peripheral tissue. Adrenal glands release glucocorticoids and catecholamines into the blood, both relevant for memory (McIntyre *et al.,* 2012). To figure out which hormones are responsible of the mnemonic effects produced by peripheral CB1R blockade, mice were pre-treated after the familiarization phase with the glucocorticoid receptor antagonist mifepristone (50 mg/kg) or the peripheral β-adrenergic antagonist sotalol 20 min prior to AM6545 injection (Figure 2B). Mifepristone pre-treatment did not prevent the AM6545 memory enhancement (two-way ANOVA, interaction: $F(1,21) = 0.038$, $p = 0.846$; mifepristone/vehicle effect: $F(1,21) = 0.707$, $p = 0.409$; AM6545/vehicle effect: $F(1,21) = 25.11$, $p<0.001$) (Figure 2C). In contrast, mice pre-treated with sotalol did not show the memory improvement observed in AM6545-treated mice (two-way ANOVA, interaction: $F(1,31) = 7.58$, $p = 0.009$; *post hoc* Bonferroni, Saline-VEH vs Saline-AM6545 $p = 0.01$; Saline-AM6545 vs Sotalol-AM6545 $p = 0.002$) (Figure 2D).

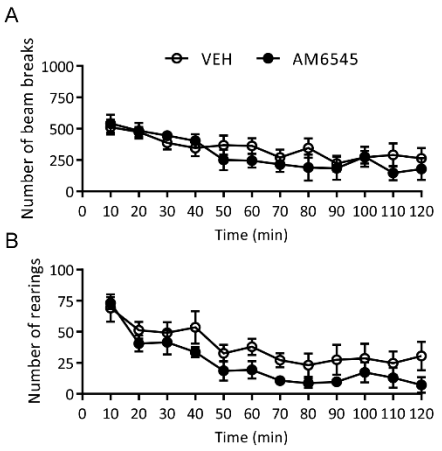

Figure S2. Locomotor activity after acute AM6545 administration. (A) Total activity and **(B)** number of rearings performed in locomotor activity boxes for 120 min by mice treated with vehicle (VEH) or AM6545 (1 mg/kg) (n = 6). Data are expressed as mean ± s.e.m.

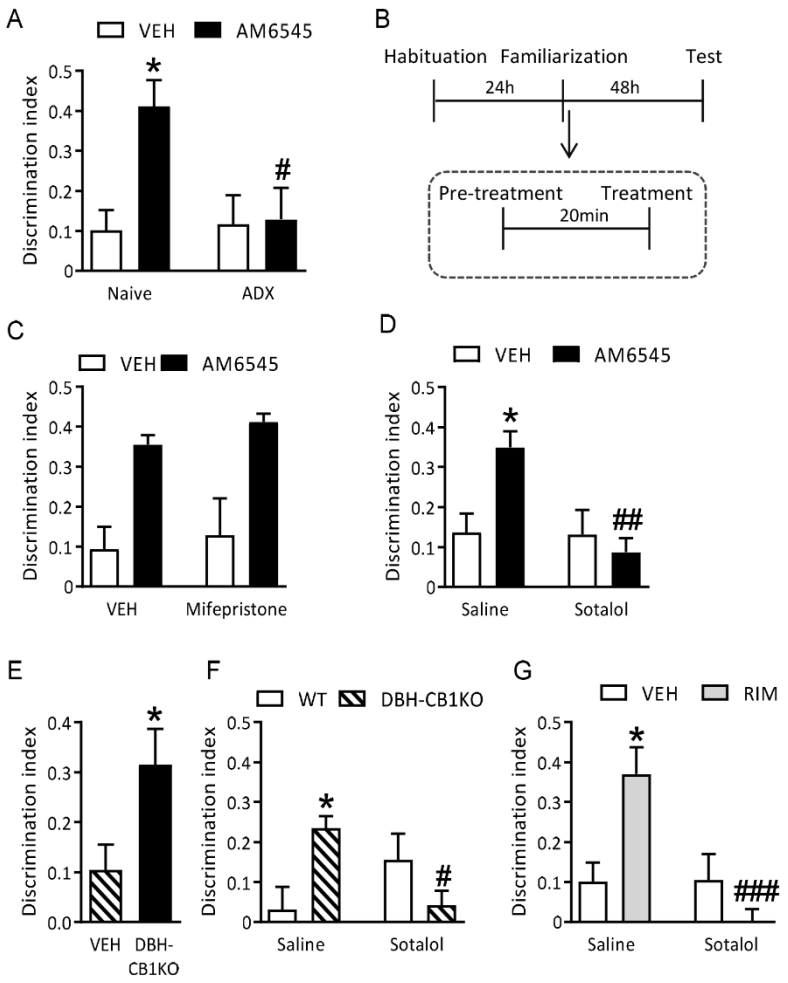

Figure 2. AM6545 enhances novel object-recognition memory through a peripheral β-adrenergic mechanism. (A) Discrimination index values obtained in the NORT performed at 48 h of adrenalectomized (ADX) or naive mice treated with vehicle (VEH) or AM6545 (1 mg/kg) (n = 6-8). **(B)** Schematic representation of acute drug pre-treatment and treatment after the familiarization phase. **(C-D)** Discrimination index values obtained in the NORT performed at 48 h of mice treated with vehicle (VEH) or AM6545 (1 mg/kg) after pre-treatment with **(C)** vehicle (VEH) or mifepristone (50 mg/kg) (n = 6-7) **(D)** saline or sotalol (10 mg/kg) (n = 8-10). **(E)** Discrimination index values in WT or DBH-CB1KO mice in NORT at 48 h **(F)** and after saline or sotalol (10 mg/kg) treatment (n = 6-8). **(G)** Discrimination index values for mice pre-treated with saline or sotalol (10 mg/kg) prior to rimonabant (RIM) (1 mg/kg) or vehicle (VEH) in the NORT at 48 h (n = 9-11). Data are expressed as mean ± s.e.m. * $p < 0.05$ (treatment effect) $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (pre-treatment effect) by two-way ANOVA test followed by Bonferroni *post hoc*.

Given the relevant role of circulating adrenaline/noradrenaline released by dopamine beta-hydroxylase cells (DBH+) in the adrenal medulla, we assessed whether inhibition of CB1R exclusively in DBH+ cells could mimic the mnemonic effect of systemic and peripheral CB1R antagonists. We used a combination of genetic and pharmacological approaches to show that conditional knock-out mice lacking the CB1R in DBH+ cells (DBH CB1-KO mice) displayed enhanced object-recognition memory persistence compared to wild-type controls (Student's t-test: $p = 0.04$) (Figure 2E). Interestingly, enhanced memory persistence in DBH CB1-KO mice was abolished by sotalol administration (two-way ANOVA, interaction: $F(1,22) = 10.41$, $p = 0.004$; *post hoc* Bonferroni, Saline-WT vs Saline-DBH CB1-KO $p = 0.02$; Saline- DBH CB1-KO vs Sotalol- DBH CB1-KO $p = 0.04$) (Figure 2F), pointing to a relevant role of CB1R-modulated peripheral adrenergic/noradrenergic tone in memory persistence. Sotalol pre-treatment similarly prevented the cognitive improvement elicited by systemically-acting rimonabant supporting the relative prominence of peripheral CB1R (two-way ANOVA, interaction: $F(1,38) = 10.78$, $p = 0.002$; *post hoc* Bonferroni, Saline-VEH vs Saline-rimonabant $p = 0.03$; Saline-rimonabant vs Sotalol-rimonabant $p = 0.0004$) (Figure 2G). No differences in total exploration time were detected between genotypes or pharmacological treatments in any of the experimental groups (Figure S3).

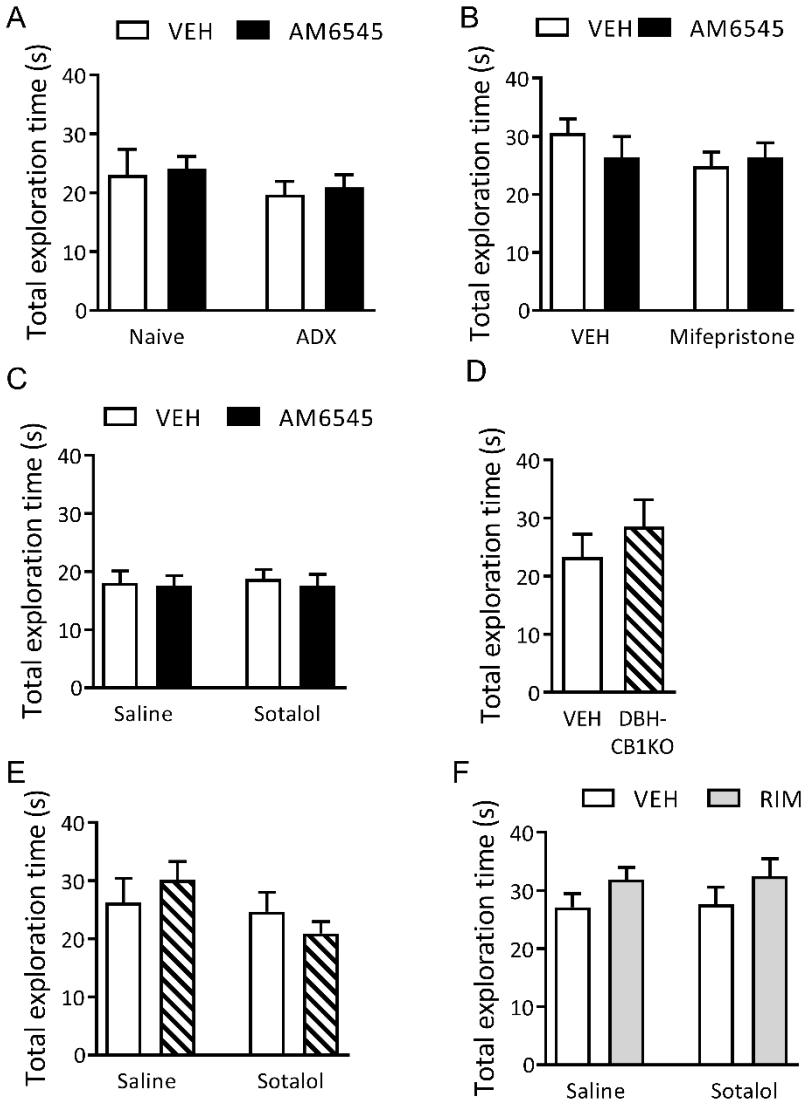

Figure S3. Total exploration times of systemically or peripheral CB1R blockade are not affected. (A) Total exploration times obtained at 3 h, 24 h and 48 h after the familiarization phase (n = 5-8). **(B-D)** Total exploration times in NORT at 48h **(B)** after acute post-familiarization treatment with rimonabant vehicle (VEH) or rimonabant (RIM) (1 mg/kg) (n = 7-11) **(C)** in HZCB1R and WT mice (n = 6-8) **(D)** after acute post-familiarization treatment with vehicle (VEH) or AM6545 (1 mg/kg) (n = 7-8). Data are expressed as mean ± s.e.m.

Increased hippocampal noradrenaline mobilization by peripheral CB1R blockade induces memory improvement

We measured LC neural activity to assess the CNS effects of CB1R peripheral modulation with AM6545. Systemic AM6545 enhanced LC firing rates compared to vehicle treated mice (Figure 3A). Next, we performed extracellular microdialysis analysis in the hippocampus after systemic AM6545 treatment. Analysis of NA, DA and 5-HT extracellular levels after AM6545 administration revealed a specific transient increase in NA in comparison to the vehicle-treated mice (two-way repeated measure ANOVA, interaction: $F(1,14) = 2.19$ $p = 0.009$) (Figure 3B and Figure S4),

We then tested whether β-adrenergic receptors in CA1 hippocampal region were necessary for the increased memory persistence mediated by AM6545. Thus, local intra-hippocampal microinjection of a dose of propranolol that did not affect memory performance (1 µg per 0.5 µl per side, see Figure S5) blocked the mnemonic effects produced by systemic AM6545 administration (two-way ANOVA, interaction: $F(1,119) = 5.03$ $p = 0.04$; *post hoc* Bonferroni, Saline-VEH vs Saline-AM6545 $p = 0.005$; Saline-AM6545 vs Propranolol-AM6545 $p = 0.01$) (Figure 3C), without affecting the total exploration time (Figure 3D). These data indicate the functional relevance of noradrenergic hippocampal activation in the effect of peripheral CB1R blockade over memory persistence.

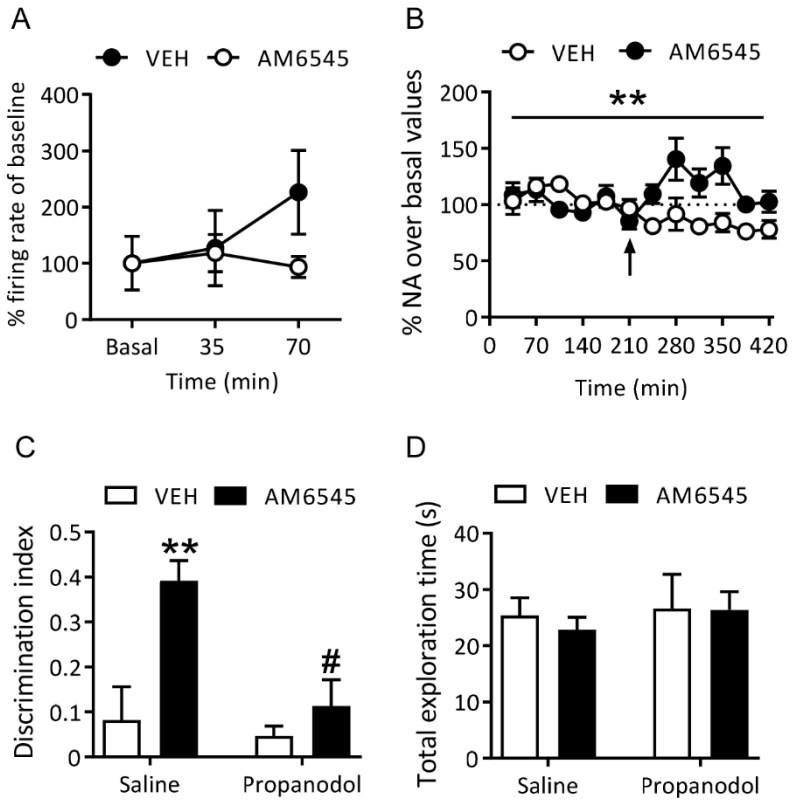

Figure 3. Acute AM6545 treatment increases central noradrenergic activity. (A) Percentage of mean firing rate in the LC after acute vehicle (VEH) or AM6545 (1 mg/kg) administration respect to baseline values (n = 2-3). **(B)** Percentage of extracellular noradrenaline (NA) levels in the hippocampus after acute vehicle (VEH) or AM6545 (1 mg/kg) administration respect to baseline values (n = 6-7). The arrow indicates the time of administration**. (C)** Discrimination index values and **(D)** total exploration time obtained in the NORT performed at 48 h mice treated with AM6545 (1 mg/kg) or vehicle (VEH) after bilateral intrahippocampal injection of saline or propranolol (1 µg/µl 0.5 µl per side) (n = 5-6). Data are expressed as mean ± s.e.m. For microdialysis * $p < 0.05$ two-way repeated measures ANOVA followed by Bonferroni post hoc was performed. For NORT ** $p < 0.01$ (treatment effect) $^{\#}p < 0.05$ (pre-treatment effect) by two-way ANOVA test followed by Bonferroni *post hoc*.

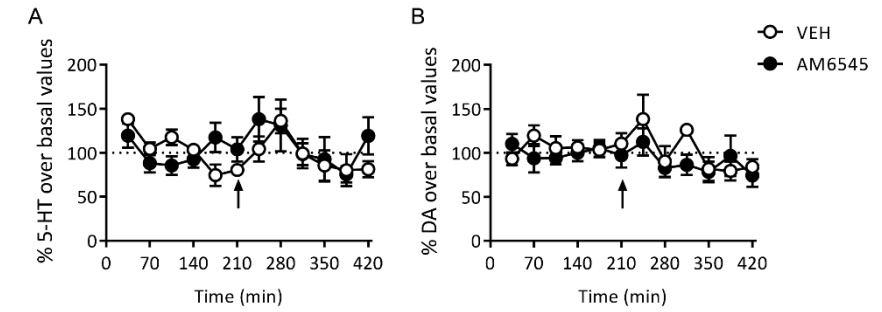

Figure S4. Acute AM6545 administration does not modify other monoamine extracellular levels in the hippocampus. (A-B) Extracellular **(A)** serotonin (5-HT) and **(B)** dopamine (DA) levels in the hippocampus after acute AM6545 (1 mg/kg) or vehicle (VEH) administration respect to baseline values (n = 6-7). Arrow indicates the time of administration. Points represent mean ± s.e.m and are expressed as percentages of baseline.

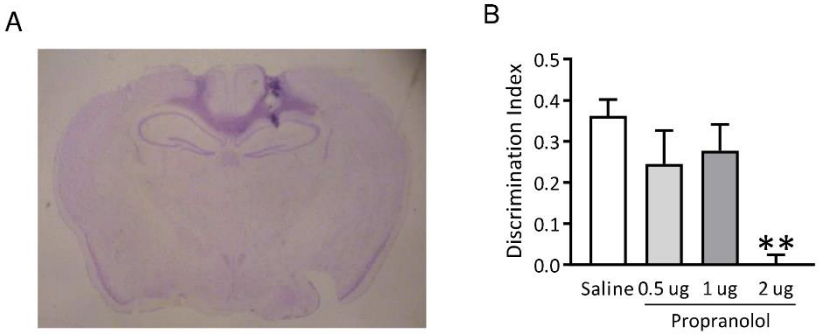

Figure S5. Intrahippocampal propranolol injection. (A) Cannula placement in the dorsal hippocampus. A brain coronal section from a representative mouse showing cannula placement in the dorsal hippocampus. Brain slices were stained with cresyl violet. **(B)** Discrimination index values of different doses of intrahippocampal propranolol infusion or saline in the NORT at 24 h (n = 4). Data are expressed as mean ± s.e.m. ** $p < 0.01$ by one-way ANOVA followed by Bonferroni *post hoc*.

Sub-chronic AM6545 treatment enhances object-recognition memory and induces hippocampal synaptic plastic changes

We performed a sub-chronic treatment with AM6545 for 7 d in CD-1 mice to further study the sustained effects of peripheral CB1R blockade. The last administration of AM6545 was injected 6 h after the familiarization phase to avoid the acute behavioral effect of AM6545

on the NORT. We observed that sub-chronic AM6545 treatment enhanced memory persistence in the NORT (Student's t-test p = 0.002), indicating the lack of tolerance to this mnemonic effect (Figure 4A).

Then we assessed whether sub-chronic AM6545 treatment produces neuronal plastic changes in the hippocampus. Adult neurogenesis was studied through the quantification of the number of cells expressing the endogenous marker of cell proliferation Ki67 in the subgranular zone (SGZ) of the dentate gyrus. Sub-chronic treatment with AM6545 did not modify the number of Ki67+ cells (Student's t-test p = 0.39) (Figure 4B). Taking advantage of a transgenic mice expressing the EGFP fluorescent protein under Thy1 promoter (*Thy1-EGFP* mice), we analysed spine density of apical dendrites from CA1 pyramidal neurons at the *stratum radiatum* after sub-chronic AM6545 treatment. Although no differences were observed on the total number of spines (Student's t-test p = 0.42) (Figure 4C), spine morphology analysis revealed an increase in the number of mushroom (mature) spines in *Thy1-EGFP* mice treated with AM6545 (Student's t-test p = 0.02) (Figure 4D).

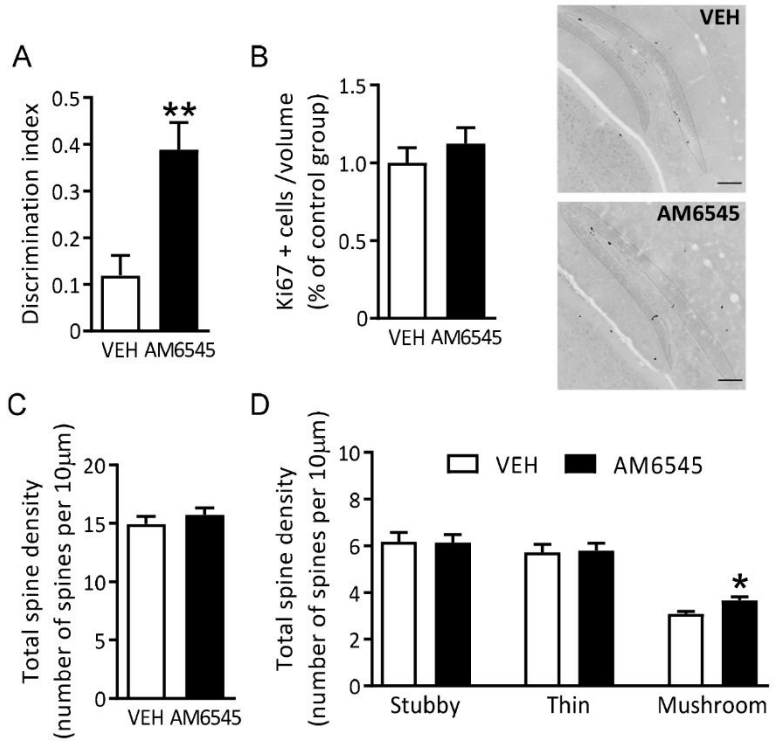

Figure 4. Sub-chronic AM6545 treatment improves object recognition-memory and induces hippocampal synaptic plastic changes. (A) Discrimination index values in NORT at 48h after sub-chronic 7 d treatment with vehicle (VEH) or AM6545 (1 mg/kg) (n = 7-11). **(B)** Average density of Ki67+ cells and representative grey scale confocal images in the subgranular zone of dentate gyrus of mice treated for 7 d with AM6545 (1 mg/kg) or vehicle (VEH) (n = 7-8). **(C)** Quantification of the total number of spines in 10 µm of CA1 pyramidal dendrites of mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). **(D)** Analysis of spine morphology classification. Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$ by Student's t-test.

Sub-chronic treatment with peripheral CB1R antagonist occludes long term potentiation in the CA1 region of the hippocampus

To investigate the functional consequences of peripheral CB1R blockade in the hippocampus, we studied LTP at Schaffer collateral-CA1 synapses in slices from mice treated for 7 d with AM6545 (1 mg/kg) or vehicle. The last administration was performed 24 h prior to slice collection, the time when object-recognition memory was assessed.

LTP was induced by stimulating *Schaffer collaterals* at 100-Hz during 1s. Slices from vehicle-treated mice showed robust LTP (168 ± 10 %), whereas LTP was completely occluded in slices from mice treated with AM6545 (96 ± 8 %) (Student's t-test p = 0.002) (Figure 5A-B). *To* determine the site of expression of LTP, we analyzed PPR during baseline and 60 min after the protocol. The analysis of PPR before and after LTP showed no differences in control slices (1.41 ± 0.11 after LTP vs 1.67 ± 0.16 in baseline) (Figure 5C) confirming the established postsynaptic expression of this form of LTP. Treatment with AM6545 did not induce changes in PPR (1.51 ± 0.18 after LTP vs 1.89 ± 0.17 in baseline).

We also compiled a stimulus-response curve (50-350 μA) to examine whether basal synaptic transmission was affected in mice treated with AM6545 for 7d. we found that slices from AM6545-treated mice presented increased amplitude of EPSCs (Figure 5D). Next, we evaluated the expression of neurotrophic factors in the hippocampus to assess the mechanism of AM6545 treatment in synaptic plasticity. RT-qPCR analysis showed a significant enhancement in the mRNA levels of *Ngf* (Student's t-test p = 0.018) (Figure 5E) and *Bdnf* (Student's t-test p = 0.009) (Figure 5F) after sub-chronic 7 d AM6545 treatment, whereas no significant changes were observed in the mRNA levels of the *Ntf3* (Figure S6). Interestingly, immunoblot analysis supported the increase of BDNF levels in hippocampal homogenates of mice treated for 7 d with AM6545 (Student's t-test p = 0.04) (Figure 5G). We also examined the expression of AMPA receptor subunits in the hippocampus. RT-qPCR analysis showed a trend to enhance the mRNA levels of *Gria1* subunit after sub-chronic 7 d AM6545 treatment

(Student's t-test p = 0.08) (Figure 5H) that was significantly increased when evaluating the *Gria2* subunit (Student's t-test p = 0.002) (Figure 5I).

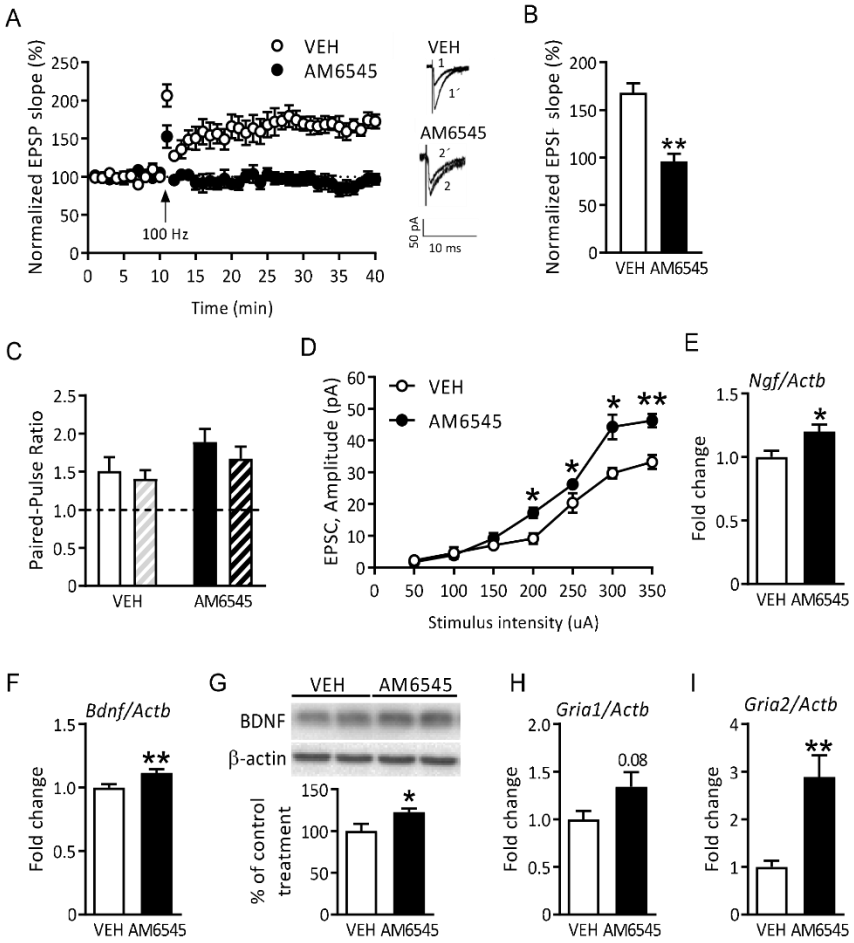

Figure 5. Sub-chronic peripheral CB1R blockade prevents LTP in CA1 hippocampal region. **(A)** Average time courses of the change in the slope of the fEPSP in hippocampal slices from mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). Traces represent samples of fEPSPs recorded for each experimental group before (1,2) and 30 min after (1',2') LTP induction (n = 6). **(B)** Average LTP of the last 10 min of recordings (n = 6). **(C)** Paired-pulse ratio before and after (fill pattern bars) LTP induction in hippocampal slices from mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). **(D)** Stimulation input/output curves in hippocampal slices from mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). **(E-F)** Hippocampal mRNA levels of the neurotrophic factors **(E)** *Ngf* and **(F)** *Bdnf* of mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). **(G)** Representative immunoblot and quantification of BDNF total expression in the hippocampus of mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg). **(H-I)** Hippocampal mRNA levels of

the AMPAR subunits (n = 7) **(H)** *Gria1* **(I)** and *Gria2* from mice treated with vehicle (VEH) or AM6545 (1 mg/kg) for 7 d (n = 7-8). Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$ by Student's t-test.

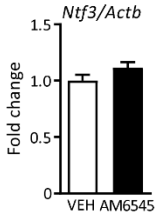

Figure S6. Sub-chronic peripheral CB1R blockade does not change *Ntf3* mRNA levels in hippocampal homogenates. Hippocampal mRNA levels of the neurotrophic factor *Ntf3* of mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg) (n = 7-8). Data are expressed as mean ± s.e.m.

Discussion

Our study identifies a relevant role of the peripheral ECS in modulating non-emotional memory persistence through the mobilization of central and peripheral adrenergic/noradrenergic mechanisms.

We choose to study object-recognition memory, an hippocampal-dependent test (Cohen and Stackman, 2015), since this is a model of non-emotional memory, which persistence might be modulated by post-training manipulation. In this regard, our group has observed deficits in object-recognition memory by CB1R agonists (Puighermanal *et al.,* 2009), endocannabinoid build-up (Busquets-Garcia *et al.,* 2011) or stress (Busquets-Garcia *et al.,* 2016), all administered after the familiarization phase. In the present study, we analyzed object-recognition memory recall 48 h after the familiarization phase since such interval shows impaired object discrimination compared to shorter intervals. Using this 48 h interval, we found that overall CB1R blockade, through pharmacological or genetic approaches, significantly increased discrimination indexes. In agreement, several previous studies have demonstrated that both pharmacological or genetic CB1R inactivation improve memory in different hippocampal-dependent tasks (Reibaud *et al.,* 1999; Lichtman, 2000; Maccarrone *et al.,* 2002; Wolff and Leander, 2003; Takahashi *et al.,* 2005; Jacob *et al.,* 2012).

We used the peripherally-restricted CB1R neutral antagonist AM6545 to evaluate the role of peripheral CB1R in object-recognition memory persistence. Post-familiarization treatment with AM6545 significantly enhanced memory persistence in mice. This drug shows no significant blood-brain barrier permeability compared to the systemic antagonist rimonabant (Tam *et al.,* 2010), suggesting that AM6545 effects derived

from a peripheral mechanism. As previous results identified adrenal glands important for the stress-induced amnesia over NORT (Busquets-Garcia *et al.,* 2016), we used adrenalectomized mice to evaluate whether adrenal glands were relevant for the memory persistence enhancement produced by AM6545 treatment. Adrenalectomized mice treated with AM6545 did not present a memory enhancement, pointing to a relevant role of adrenal glands in this response. Adrenaline/noradrenaline and corticosteroids secreted by the adrenal glands have a significant impact in memory consolidation (Roozendaal and McGaugh, 2011; McIntyre *et al.,* 2012; C. Yang *et al.,* 2013). We then gathered several evidences that pointed to the mobilization of the adrenergic transmission, compared to the mobilization of corticosteroids, by peripheral CB1R blockade: i) AM6545-induced memory persistence enhancement was prevented by the peripherally restricted β-adrenergic receptor antagonist sotalol, but not by the corticosteroid receptor antagonist mifepristone; and ii) mice lacking CB1R in DBH+ cells, the cells releasing adrenaline/noradrenaline from adrenal glands, showed enhanced object-recognition memory persistence compared to wild-type littermates, an improvement that was sensitive to peripheral β-adrenergic receptor inhibition.

A growing body of evidences have demonstrated the efficacy of exogenous adrenaline administration to enhance hippocampal-dependent memory in rodents (Talley *et al.,* 2000; Dornelles *et al.,* 2007). Interestingly, adrenaline effects on memory appear to be initiated by the activation of β-adrenergic receptors expressed on the afferent fibers of the vagus nerve (Schreurs *et al.,* 1986; Lawrence *et al.,* 1995). Vagus nerve fibers project to the NTS, which projects to

different brain areas, such as the LC (Ennis and Aston-Jones, 1988; Van Bockstaele *et al.,* 1998; Reyes and Van Bockstaele, 2006). Our data demonstrate that AM6545 administration increased LC firing and NA extracellular levels in the hippocampal region, suggesting an increased activity of LC projections to the hippocampus. LC activity is physiologically increased after novelty experience and it is considered to prime the persistence of hippocampal-based long-term memories (Sara, 2009; Hansen, 2017). Moreover, NA in the hippocampus is involved on the storage of new memories through the regulation of neural excitability and synaptic plasticity (Hagena *et al.,* 2016). Although NA can bind to both α- or β- adrenergic receptors, synaptic information and plasticity in the hippocampus depend largely on the activation of β-adrenergic receptors (Kemp and Manahan-Vaughan, 2008). Indeed, LC stimulation modulates hippocampal synaptic strength and improves memory through a β-adrenergic-dependent mechanism (Kemp and Manahan-Vaughan, 2008, 2012; Hansen and Manahan-Vaughan, 2015; Hagena *et al.,* 2016). In agreement, we observed that the intra-hippocampal blockade of β-adrenergic receptors with propranolol prevented the increase of object-recognition memory persistence produced by AM6545. In addition to NA, vagal stimulation also increases the release of dopamine (Szczerbowska-Boruchowska *et al.,* 2012) and serotonin (Manta *et al.,* 2012) across multiple brain areas, which could also contribute to memory strength. However, AM6545 administration did not affect neither dopamine nor serotonin extracellular levels in the hippocampus.

Furthermore, we demonstrated that AM6545 did not produce tolerance effects on memory since the increase in memory persistence was maintained after 7 d of treatment. To assess the possible mechanisms involved, we evaluated neuronal progenitor proliferation. Indeed, increasing evidences linked adult neurogenesis of the dentate gyrus to the establishment of hippocampal-dependent memory (Saxe *et al.,* 2006; Dupret *et al.,* 2008; Jessberger *et al.,* 2009), and NA is recognized as a mediator of cell proliferation in the hippocampus (Kulkarni *et al.,* 2002; Masuda *et al.,* 2012). However, sub-chronic AM6545 treatment did not modify the number of cells expressing the cell proliferation marker Ki67 in the subgranular zone of the dentate gyrus.

At the structural level, analysis of dendritic apical spines from CA1 pyramidal neurons revealed that AM6545 increased the number of mushroom spines, the most mature/functional form of dendritic spines (Hayashi and Majewska, 2005). Changes on the number of mushroom spines may impact on synaptic plasticity enhancing synaptic strength (Attardo *et al.,* 2015), and mushroom spine density has also been associated to the improvement of other memory types (Mahmmoud *et al.,* 2015).

Electrophysiological studies demonstrated that sub-chronic AM6545 treatment increased the amplitude of EPSCs in the hippocampal CA1 region. As the experiments were performed at -70 mV, EPSCs were basically mediated by the activation of AMPA receptors. No changes in paired-pulse ratio were observed after AM6545 treatment, discarding a presynaptic component in the facilitation of neurotransmitter release and suggesting a postsynaptic increase in AMPA receptor mediated

currents. This could be explained by the significant increase of mRNA levels of the AMPA receptor Gria2 in the hippocampus of AM6545-treated mice. In this region, the activation of β-adrenergic receptors can trigger the phosphorylation of AMPAR facilitating their traffic to extrasynaptic sites (Vanhoose and Winder, 2003; Rouach *et al.,* 2005; Joiner *et al.,* 2010) and reinforcing LTP (Oh *et al.,* 2006).

These neuronal plasticity changes could be mediated by the increase of mRNA level of the neurotrophins Bdnf and Ngf in the hippocampus of AM6545-treated mice. Both neurotrophins have an important role in the regulation of long-term synaptic plasticity and memory (Gibon and Barker, 2017). We also revealed a concomitant increase of BDNF protein levels in the hippocampus of AM6545-treated mice. In agreement, the inhibition of the BDNF signaling pathway in the hippocampus has been reported to hinder memory consolidation and reconsolidation (Bekinschtein *et al.,* 2007).

Altogether, our study identifies peripheral CB1R as a relevant target to enhance memory persistence and regulate long-term synaptic plasticity through a central and peripheral adrenergic/noradrenergic mechanism. This peripheral target could represent an interesting approach to avoid some of the unwanted side effects reported after systemic CB1R blockade (Samat *et al.,* 2008) and to avoid blood-brain barrier penetration problems in designing new therapeutic compounds (Pardridge, 2012).

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Supplementary results #1

Peripheral CB1R antagonism rescues cognitive fragile X syndrome phenotype

FXS is the most common cause of intellectual disability (Penagarikano *et al.,* 2007). Several therapeutic approaches have been suggested for the treatment of the cognitive symptoms of this disorder. In accordance, previous results in our group revealed that the systemically-acting CB1R antagonist rimonabant improved memory performance and restored the altered CA1 spine density and morphology as well as the aberrant mGluR5-LTD in the hippocampus of *Fmr1* KO mice (Busquets-Garcia *et al.,* 2013; Gomis-González *et al.,* 2016). However, rimonabant was found to produce psychiatric adverse effects when used as a strategy to treat obesity (Christensen *et al.,* 2007).

In the framework of this project, we have explored the potential use of the peripherally acting CB1R antagonist AM6545 for cognitive enhancement, an strategy that will prevent the possibility of developing central psychiatric adverse effects (Silvestri and Di Marzo, 2012).

Materials and methods

Animals

Male *Fmr1* KO mice in Friend Virus B (FVB) background (*Fmr1* KO, FVB.129P2-Pde6b+ Tyrc-ch Fmr1tm1Cgr/J) and wild-type mice (WT, FVB.129P2-Pde6b+ Tyrc-ch/AntJ) were purchased from The Jackson Laboratory and crossed to obtain *Fmr1* KO and WT littermates.

To observe hippocampal pyramidal neurons, double transgenic mice (Thy1-EGFP/*Fmr1* KO) were obtained by backcrossing transgenic Thy1-EGFP males (Jackson Laboratory) to *Fmr1* hetetozygous females.

Mice were housed 4 per cage and maintained in standard environment conditions of temperature (21ºC ± 1ºC) and humidity (55% ± 10%) with food and water *ad libitum*.

All the experiments were performed during the light phase of 12 h light/dark cycle (lights on at 8 a.m. and off at 8 p.m.). Mice were habituated in the experimental room and handled for 1 week before starting the experiment. All animal procedures were conducted following ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny *et al.,* 2010) and standard ethical guidelines (European Communities Directive 2010/63/EU). Procedures were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB).

Drugs

AM6545 (1 mg/kg) from Tocris was dissolved in 0.26% DMSO, 4.74% ethanol, 5% cremophor-EL and 90% saline (0.9% NaCl). Sotalol (10 mg/kg) from Sigma-Aldrich was dissolved in 90% saline (0.9% NaCl). All

drugs were injected intraperitoneally (i.p.) in a volume of 10 ml/kg of body weight for acute or sub-chronic (7 d) treatment.

Novel object-recognition test (NORT)

The NORT was performed as previously described in article #1. Animals were treated immediately after or 6 h after the familiarization phase and the memory test phase was evaluated at 24 h later.

Slice preparation for electrophysiological recordings

Wild-type (WT) and *Fmr1* KO mice were anesthetized with isofluorane (2%) and decapitated for slice preparation. Hippocampal slices were prepared as described in detail elsewhere (Andrade-Talavera *et al.,* 2016). After decapitation, the whole brain with the two hippocampi was removed into ice-cold solution containing (in mM): 126 NaCl, 3 KCl, 1.25 KH_2PO4, 2 MgSO4, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (pH 7.2, 300 mOs/mL) positioned on the stage of a vibratome slicer and cut to obtain transverse hippocampal slices (350 μm thick). The slices were maintained continuously oxygenated (95% O_2/5% CO_2) in this solution for at least 1 h before use. All experiments were carried out at room temperature (22–25ºC). For experiments, slices were continuously superfused with the solution described above.

Electrophysiological recordings

Whole-cell patch-clamp recordings were made from pyramidal cells located in the CA1 field of the hippocampus. CA1 pyramidal cells were patched under visual guidance by infrared differential interference contrast microscopy and verified to be pyramidal neurons by their characteristic voltage response to a current step protocol. Neurons

were recorded in either voltage-clamp configuration with a patch-clamp amplifier (Multiclamp 700B), and data were acquired using pCLAMP 10.2 software (Molecular Devices). Patch electrodes were pulled from borosilicate glass tubing, and had a resistance of 4–7 MΩ when filled with (in mM): caesium chloride, 120; HEPES, 10; NaCl, 8; MgCl2, 1; CaCl2, 0.2; EGTA, 2 and QX-314, 20 (pH 7.2–7.3, 290 mOsm L−1). Cells were excluded from analysis if the series resistance changed by more than 15% during the recording. Recordings were low-pass filtered at 3 kHz and acquired at 10 kHz. mGluR5-LTD was induced by treating slices with DHPG (100 µM) for 10 min. LTD was quantified by comparing the mean EPSC amplitude over the 30 min post-drug period with the mean EPSC amplitude during the baseline period and calculating the percentage change from 5 last min.

Brain perfusion and slices preparation for dendritic spine analysis

Twenty-four hours after the last administration of AM6545 (1 mg/kg), mice were deeply anesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine (100 mg/kg)/xylazine (20 mg/kg) prior to intracardiac perfusion of cold 4 % paraformaldehyde. Brains were removed and stored in a solution of 30 % sucrose at 4 °C. Coronal frozen sections were made at 60 µm on a freezing microtome (Leica) and stored in a 5 % sucrose solution.

Dendritic spines analysis

Secondary and tertiary apical dendrites of CA1 pyramidal neurons from the *stratum radiatum* of the dorsal hippocampus were analyzed in Thy1-EGFP/*Fmr1* KO mice as previously described in article#1.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 using unpaired Student's t-test for two groups comparisons or two-way ANOVA for multiple group comparisons. Subsequent Bonferroni *post hoc* was used when significant interaction between factors. Comparisons were considered statistically significant when $p < 0.05$.

Results

Acute AM6545 ameliorates object-recognition memory in *Fmr1* KO mice

We first evaluated the effect of the peripherally-restrictive CB1R neutral antagonist, AM6545 in the NORT. Acute administration of AM6545 (1 mg/kg) immediately after the familiarization phase of NORT restored the object-recognition memory deficits in *Fmr1* KO mice at 24 (two-way ANOVA, interaction: $F(1,42) = 10.83$, $p = 0.002$; *post hoc* Bonferroni, WT-VEH vs *Fmr1* KO-VEH $p<0.001$; *Fmr1* KO-VEH vs *Fmr1* KO-AM6545 $p<0.001$) (Figure 23A). However, when animals were treated with acute AM6545 (1 mg/kg) 6 h after the familiarization phase of NORT, once memory has been already consolidated, memory deficits in *Fmr1* KO mice were not prevented (two-way ANOVA, interaction: $F(1,43) = 1.623$, $p = 0.209$; treatment effect: $F(1,43) = 57.77$, $p<0.001$) (Figure 23B). No differences in total exploration time were detected between genotypes or pharmacological treatments in any of the experimental groups (Figure 23C-D).

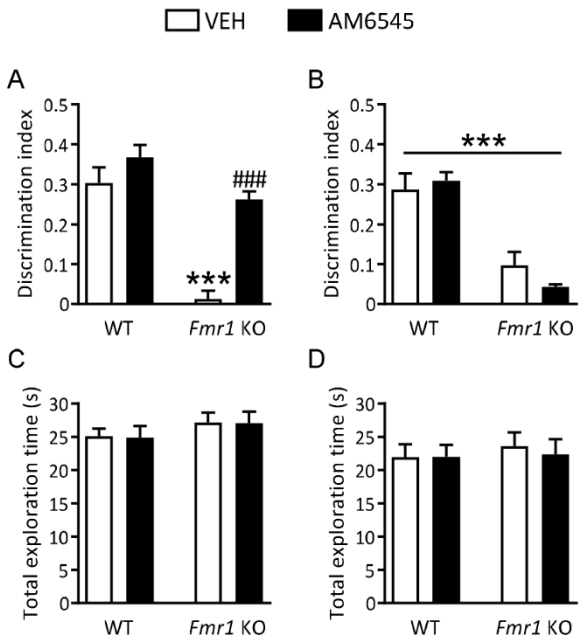

Figure 23. Acute AM6545 treatment ameliorate NORT performance in *Fmr1* KO mice. (A-B) Discrimination index of WT and *Fmr1* KO mice after an acute administration of vehicle (VEH) or AM6545 (1 mg/kg) **(A)** immediately after (n = 11-12) and **(B)** 6 h after the familiarization phase of NORT (n = 10-13). **(C-D)** Total object exploration time during test phase of NORT for WT and *Fmr1* KO mice treated with acute vehicle (VEH) or AM6545 (1 mg/kg) administration **(C)** immediately after familiarization phase (n = 11-12) and **(D)** 6 h after familiarization phase (n = 10-13). (Data are expressed as mean ± s.e.m. ***p < 0.001 (genotype effect) ###p < 0.001 (treatment effect) two-way ANOVA followed by Bonferroni *post hoc.*

Peripherally-restricted β-adrenergic receptor antagonist blocked the memory improvement produced by acute AM6545 administration in *Fmr1* KO mice

Given the role of peripheral and central adrenergic system in memory persistence mediated by peripheral CB1R (Martínez-Torres *et al.,* in preparation), we assessed whether this mechanism was also involved in the normalization of object-recognition memory deficit by CB1R peripheral blockade in *Fmr1* KO mice. We found that the mnemonic effect of AM6545 in *Fmr1* KO mice was prevented by the pre-treatment

with the β-adrenergic peripherally-restricted antagonist sotalol (10 mg/kg) (two-way ANOVA, interaction: $F(1,28) = 34.05$, $p<0.001$; *post hoc* Bonferroni, *Fmr1* KO-Saline-AM6545 vs *Fmr1* KO-Sotalol-AM6545 $p<0.001$) (Figure 24A). These results indicate that blocking peripheral CB1R in *Fmr1* KO mice enhances object-recognition memory through the activation of peripheral β-adrenergic receptors. No differences in total exploration time were detected between genotypes or pharmacological treatments (Figure 24B).

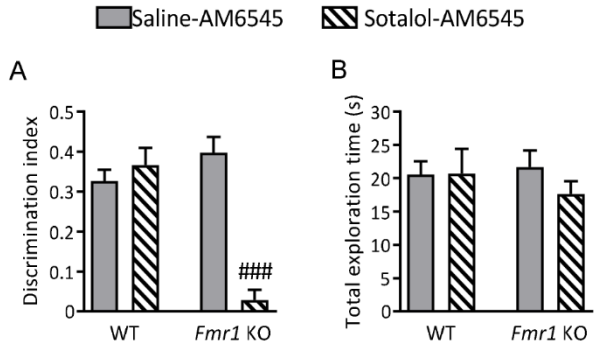

Figure 24. Memory improvement of Fmr1 KO mice after acute AM6545 administration is blocked with sotalol pre-treatment. (A) Discrimination index of WT and *Fmr1* KO mice pre-treated with saline or sotalol (10 mg/kg) prior to vehicle (VEH) or AM6545 (1 mg/kg) administration after the familiarization phase of NORT (n = 7-9). **(B)** Total object exploration time of *Fmr1* KO mice during test phase of NORT mice treated with saline or sotalol (10 mg/kg) before AM6545 (1 mg/kg) administration (n = 7-9). Data are expressed as mean ± s.e.m. ###$p < 0.001$ (treatment effect) two-way ANOVA followed by Bonferroni *post hoc*.

Sub-chronic AM6545 treatment prevents object-recognition memory deficits and cellular alterations in *Fmr1* KO mice

We then tested whether a sub-chronic pharmacological intervention was suitable to improve memory deficits and restore hippocampal cellular alterations in the *Fmr1* KO mice using the peripherally-restricted CB1R antagonist AM6545 (1 mg/kg). WT and *Fmr1* KO mice were treated during 7 d and the test phase of the NORT was performed 24 h after the last administration (Figure 25A). The last AM6545 administration was performed 6 h after the familiarization phase to avoid acute behavioral effects of AM6545. AM6545 treatment for 7 d restored object-recognition memory impairment in *Fmr1* KO mice (two-way ANOVA, interaction: $F(1,44) = 5.702$, $p = 0.021$; *post hoc* Bonferroni, WT-VEH vs *Fmr1* KO-VEH $p = 0.0009$; *Fmr1* KO-VEH vs *Fmr1* KO-AM6545 $p = 0.0007$) (Figure 25B) without altering total exploratory times in the NORT (Figure 25C).

Elevated mGuR5-dependent long-term depression (mGuR5-LTD) is considered a characteristic phenotype of *Fmr1* KO mice. Genetic and pharmacological manipulations that normalize cognitive alterations often also correct this form of synaptic plasticity (Bhattacharya *et al.,* 2012; Michalon *et al.,* 2012; Busquets-Garcia *et al.,* 2013; Sidhu *et al.,* 2014; Gomis-González *et al.,* 2016). We tested whether sub-chronic AM6545 treatment could reduce the elevated mGluR5-LTD in hippocampal slices of *Fmr1* KO mice. WT and Fmr1 KO animals received a daily dose of rimonabant (1 mg/kg) or vehicle for 7 d until 24 h before brain extraction and hippocampal slice preparation. After 10 min baseline recording, bath application of DHPG (100 µM, 10 min) induced a transient acute depression, which is considered an

electrophysiological readout of group I mGluR activation, followed by a small LTD in WT mice (Figure 25D). Here we observed that the LTD induced by DHPG was significantly enhanced in the vehicle (VEH)-treated *Fmr1* KO mice, consistent with previous reports. Sub-chronic AM6545 treatment normalized the enhanced mGluR5-LTD in hippocampal slices of *Fmr1* KO mice (two-way ANOVA, interaction: $F(1,23) = 4.769$ $p = 0.043$; *post hoc* Bonferroni, WT-VEH vs *Fmr1* KO-VEH $p = 0.002$; *Fmr1* KO-VEH vs *Fmr1* KO-AM6545 $p = 0.003$) (Figure 25D-E).

In addition, FXS patients and *Fmr1* KO mice show alterations in dendritic spine density and morphology (de Vries *et al.,* 1998; Busquets-Garcia *et al.,* 2013). In this regard, Fmr*1* KO mice treated with AM6545 (1 mg/kg) for 7 d showed a tend to reduce total dendritic spine density of CA1 pyramidal neurons (Student's t-test $p = 0.07$) (Figure 25F). When spines were classified depending on their morphology, sub-chronic AM6545 treatment significantly decreased the number of thin (immature) spines compared to vehicle (VEH)-treated *Fmr1* KO mice (Student's t-test $p = 0.02$) (Figure 25G).

Altogether, these results reveal the involvement of the peripheral CB1R in object-recognition memory and hippocampal cellular manifestations of FXS.

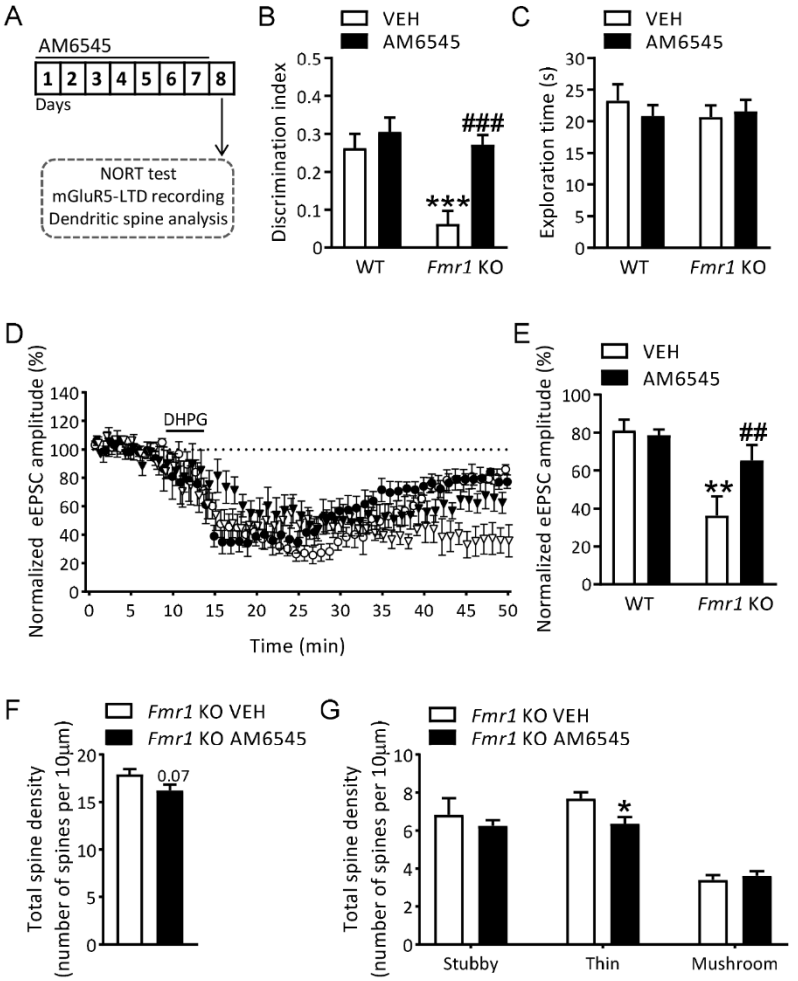

Figure 25. Sub-chronic AM6545 treatment ameliorates object-recognition memory, mGluR5-dependent LTD and dendritic spine alterations in *Fmr1* KO mice. (A) Schematic representation of the experimental protocol. **(B)** Discrimination index after a sub-chronic administration of vehicle (VEH) or AM6545 (1 mg/kg) in WT and *Fmr1* KO mice (n = 11-13). **(C)** Total object exploration time during test phase of NORT of WT and *Fmr1* KO mice treated for 7d with vehicle (VEH) or AM6545 (1mg/kg) administration (n = 11-13). **(D)** Average time courses of the change in the amplitude of evoked excitatory postsynaptic currents (eEPSC) in hippocampal slices from mice treated for 7 d with vehicle (VEH) or AM6545 (1 mg/kg) after bath application of DHPG (n = 6-7). **(E)** Mean EPSC amplitude values of the last 5 min of recordings in (D) figure (n = 6-7). **(F)** Quantification of the total number of spines in 10 µm of CA1 pyramidal dendrites of *Fmr1* KO mice treated for 7 d with vehicle (VEH) or AM6545 (1mg/kg) (n = 4-5). **(G)** Morphological analysis of dendritic spines in the CA1 pyramidal dendrites in *Fmr1* KO mice after pharmacological treatments (n = 4-5). Data are expressed as mean ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001 (genotype effect) ##p < 0.01, ###p < 0.001 (treatment effect) by Student's t-test (F and G) or two-way ANOVA followed by Bonferroni *post hoc* (B,C and E).

Objective 2

To study the involvement of mTORC1/mTORC2 in the amnesic-like effects produced by THC using the dual inhibitor P529

Supplementary results #2

The dual mTORC1/mTORC2 inhibitor P529 blocks the amnesic-like effects produced by THC

Sara Martínez-Torres, Rafael Maldonado, Andrés Ozaita

Supplementary results #2

The dual mTORC1/mTORC2 inhibitor P529 blocks the amnesic-like effects produced by THC

THC administration produces long-term memory deficits in human and in animal models (Ranganathan and D'Souza, 2006; Zanettini *et al.,* 2011), and the hippocampus plays a crucial role in these memory deficits (Barna *et al.,* 2007). In this regard, THC administration increases specific signaling pathways in the hippocampus including the MAPK (Derkinderen *et al.,* 2003), Akt/GSK3 (Ozaita *et al.,* 2007), PKC (Busquets-Garcia *et al.,* 2018) and mTORC1 pathways (Puighermanal *et al.,* 2009, 2013), which are involved in its amnesic effects. Previous results from our group revealed that rapamycin and temsirolimus, both specific mTORC1 inhibitors, prevented the memory impairment produced by THC (Puighermanal *et al.,* 2009, 2013). mTORC2 is another signaling complex sharing several proteins with mTORC1 that has been found to be relevant for learning and memory processes (Huang *et al.,* 2013). However, its contribution to the amnesic-like effects of THC has not been yet investigated. Interestingly, the phosphorylation of the mTORC2 downstream effector Akt (Sarbassov *et al.,* 2006) is enhanced after THC treatment (Ozaita *et al.,* 2007; Puighermanal *et al.,* 2009) suggesting that mTORC2 could have a role in the amnesic-like effects of THC.

Nowadays, specific mTORC2 inhibitors are not currently available. Therefore the study of mTORC2 function has been limited due to the lack of specific pharmacological tools. However, the dual mTORC1/mTORC2 inhibitor Palomid 529 (P529) has been described to block both mTOR complexes activity *in vivo* and it has demonstrated good brain penetrance (Lin *et al.,* 2013), being a proper pharmacological compound to study THC effects on memory.

The main aim of this project was to characterize the participation of both mTORC1 and mTORC2 in the deficits in hippocampal function after THC administration in mice. For this purpose, we first evaluated the capacity of the dual mTORC1/mTORC2 inhibitor, P529, to alter the performance of mice in the novel object-recognition memory test and to modify the amnesic responses mediated by THC. Additionally, we studied whether the phosphorylation of downstream effectors for both mTORC1 and mTORC2 complexes in the hippocampus would be affected by such pharmacological interventions.

Materials and methods

Animals

Swiss albino CD1 male mice were purchased from Charles River and tested at 8-12 weeks of age. Mice were housed in cages of 4 and maintained at a controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %) environment. Food and water were available *ad libitum*. Lighting was maintained at 12 h cycles (on at 8 am and off at 8 pm). All experiments were performed during the light phase of the dark/light cycle. The animals were habituated to the experimental room and handled for 1 week before starting the experiments. All behavioral

experiments were performed under experimental conditions blind to the observer.

All animal procedures were conducted following ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny *et al.,* 2010) and standard ethical guidelines (European Communities Directive 2010/63/EU), and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB).

Drugs and treatments

Delta9-tetrahydrocannabinol (THC) was purchased from THC Pharm GmbH and Palomid 529 (P529) from Sigma-Aldrich. THC was diluted in 5 % ethanol, 5 % Cremophor-EL and 90 % saline (0.9% NaCl) and administered i.p. in a volume of 10 ml/kg. P529 was diluted in olive oil and orally administered by gavage in a volume of 5 ml/kg.

Novel object-recognition test (NORT)

The NORT at 24 h was performed as previously described in article #1.

Immunoblot

Hippocampal tissue was rapidly dissected, immediately frozen on dry ice and stored at −80 °C until used. The preparation of the samples and the immunoblot procedure was performed as previously described in article #1 to obtain cytosolic and solubilized membrane proteins. The primary antibodies used are detailed in Table 9. Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and visualized by chemiluminescence detection

(Luminata Forte Western HRP substrate, MerckMillipore). Digital images were acquired on a ChemiDoc XRS System (Bio-rad) and quantified by The Quantity One software v.4.6.3. (Bio-rad). For quantitative purposes, optical density values of active phosphospecific antibodies were normalized to their non-phosphorylated specific antibodies or gapdh as loading controls in the same sample and expressed as a percentage of control treatment (VEH).

Antigen	Host	Source/Identifier
p-mTOR (S2448)	Rabbit	Cell Signaling Technology (#2971)
mTOR	Rabbit	Cell Signaling Technology (#2972)
p-AKT (S473)	Rabbit	Cell Signaling Technology (#9271)
AKT	Mouse	Cell Signaling Technology (#4691)
p-P70S6K (T389)	Rabbit	Cell Signaling Technology (#9205)
P70S6K	Rabbit	Cell Signaling Technology (#9202)
p-Rictor (T1135)	Rabbit	Cell Signaling Technology (#3806)
Rictor	Rabbit	Cell Signaling Technology (#2140)
p-(Ser) PKC Substrate	Rabbit	Cell Signaling Technology (#2261)
GAPDH (loading control)	Mouse	Santa Cruz (sc-32233)

Table 9. Primary antibodies used for immunoblot.

Statistical analysis

Data were analyzed with GraphPad Prism 7.0 using unpaired Student's t-test for two groups comparisons or two-way ANOVA for multiple groups comparisons. Subsequent Bonferroni *post hoc* was used when significant interaction between factors. Comparisons were considered statistically significant when $p < 0.05$.

Results

The dual mTORC1/mTORC2 inhibitor P529 produces dose-related memory deficits in the novel object-recognition test

To assess the effects of dual mTORC1/mTORC2 inhibition in the NORT, post-training administration of different doses of P529 were evaluated in the NORT at 24 h (Figure 26A). P529 administration at doses of 0.3, 1 and 10 mg/kg produced significant long-term memory deficits in comparison to the vehicle (VEH)-treated group (one-way ANOVA, interaction: $F(4,27) = 5.487$ $p = 0.002$; post hoc Bonferroni, VEH vs P529 (0.3 mg/kg) $p = 0.018$; VEH vs P529 (1 mg/kg) $p = 0.004$; VEH vs P529 (10 mg/kg) $p = 0.0006$). P529 administered at 0.1 mg/kg did not produce significant effect in the NORT in comparison to the control group. The doses assessed did not alter total exploration time, discarding a general effect on locomotion as a cause of the poor memory performance (Figure 26B).

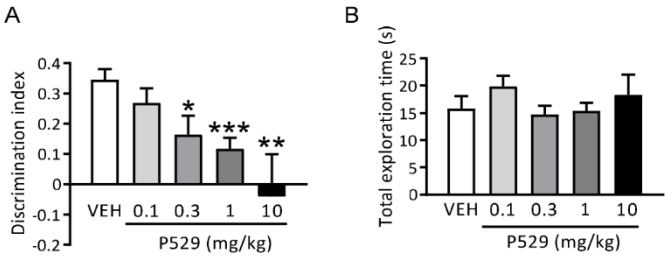

Figure 26. Post-training administration of different doses of the dual inhibitor mTORC1/mTORC2 in the NORT. (A) Discrimination index and **(B)** total exploration time of vehicle (VEH) or P529 treated mice in the test phase of NORT (n = 5-6 per group). Data are expressed as mean ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA followed by Bonferroni *post hoc*

P529 pre-treatment prevents the amnesic-like effects produced by acute THC administration

After identifying the dose of 0.1 mg/kg of P529 without significant effects on long-term memory in the NORT, we next studied whether the amnesic-like effects produced by THC (3 mg/kg) were sensitive to P529 pre-treatment. Acute THC (3 mg/kg) administration after the familiarization phase of the NORT produced long-term memory deficits (Figure 27A-B). When mice were pre-treated with P529 (0.1 mg/kg) 20 min before THC administration, THC-memory deficits were slightly prevented (two-way ANOVA, interaction: $F(1,15) = 1.56$ $p = 0.245$; pre-treatment (P529) effect: $F(1,15) = 4.605$ $p = 0.048$; treatment (THC) effect: $F(1,15) = 27.94$ $p<0.001$) (Figure 27A). As the peak of P529 plasma levels increase between 30 min and 1 h post-administration (Lin *et al.,* 2013), we decided to pre-treat mice with P529 1 h before THC administration . Using this time schedule, P529 significantly prevented the NORT deficits mediated by THC (two-way ANOVA, interaction: $F(1,19) = 16.22$ $p = 0.0007$; post hoc Bonferroni: VEH-VEH vs VEH-THC $p = 0.009$; VEH-THC vs P529-THC $p = 0.0002$) (Figure 27B). No differences in total exploration time were detected in any treatment schedule (Figure 27C-D).

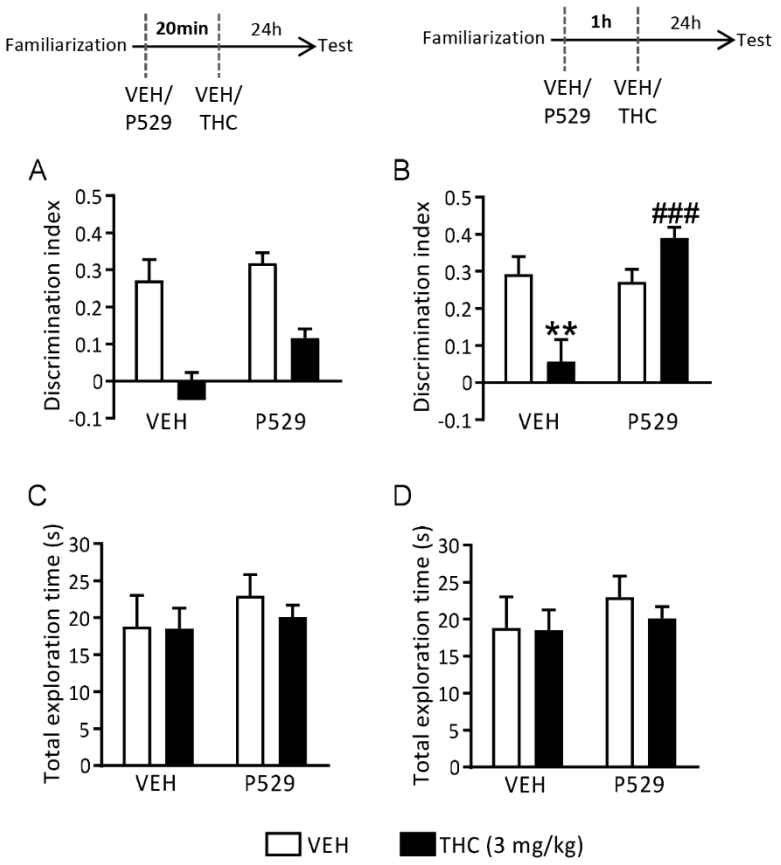

Figure 27. Pre-treatment with the mTORC1/mTORC2 inhibitor (P529) blocked the amnesic-like effects produced by THC administration in the NORT. (A) Discrimination index values in the NORT of mice treated with vehicle (VEH) or THC (3 mg/kg) after 20 min pre-treatment with VEH or P529 (0.1 mg/kg) (n = 4-5). **(B)** Discrimination index values in the NORT of mice treated with vehicle (VEH) or THC (3 mg/kg) after 1 h pre-treatment with P529 (0.1 mg/kg) (n = 5-6). **(C)** Total exploration time in the test phase of NORT of mice treated with vehicle (VEH) or THC (3 mg/kg) after 20 min pre-treatment with P529 (0.1 mg/kg) (n = 4-5). **(B)** Total exploration time in the test phase of NORT of mice treated with vehicle (VEH) or THC (3 mg/kg) after 1 h pre-treatment with vehicle (VEH) or P529 (0.1 mg/kg) (n = 4-5). Data are expressed as mean ± s.e.m. **p < 0.01 (THC treatment effect) ###p < 0.001 (P529 treatment effect) by two-way ANOVA followed by Bonferroni *post hoc.*

P529 pre-treatment does not modify the overactivation of mTOR induced by THC treatment

To evaluate the activation of the kinase mTOR, the phosphorylation levels of mTOR(S2448) were analyzed in the hippocampus of mice treated with P529 1 h before THC administration. Immunoblot analysis revealed that THC increased p-mTOR (S2448) levels in the hippocampus in comparison to the vehicle group (Student's t-test p = 0.04). However, P529 pre-treatment did not prevent such an enhancement (Figure 28).

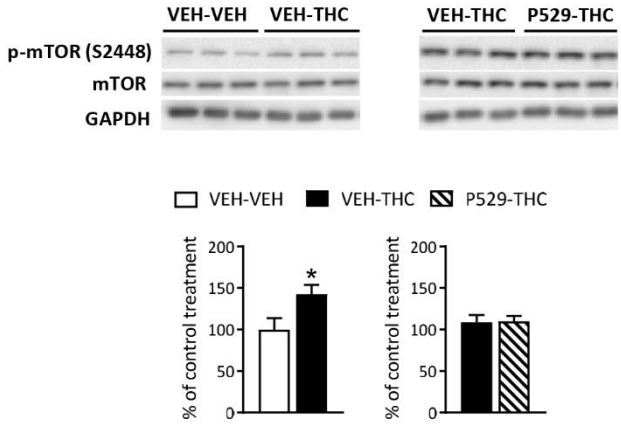

Figure 28. Phosphorylation of mTOR in the hippocampus of mice treated with P529 1 h beforeTHC treatment. Immunoblot analysis of p-mTOR (S2448) levels 1 h after treatment with vehicle (VEH) or THC (3 mg/kg), in mice that had been pre-treated with VEH or P529 (0.1 mg/kg) (n = 6-7). Data are expressed as mean ± s.e.m. *p < 0.05 by Student's t-test.

P529 pre-treatment does not modify the overactivation of mTORC1 and mTORC2 substrates produced by THC treatment

mTORC1 and mTORC2-specific substrates were evaluated in the hippocampus 1 h after THC (3 mg/kg) administration in the presence of P529 (0.1 mg/kg) pre-treatment. Immunoblot analysis of hippocampal samples revealed increased levels of p-P70S6K (T389), which is considered as a readout of mTORC1 activity (Acosta-Jaquez *et al.,*

2009), in THC-treated mice (Student's t-test p = 0.034). However, the pre-treatment with P529 did not affect such an increase (Figure 29A).

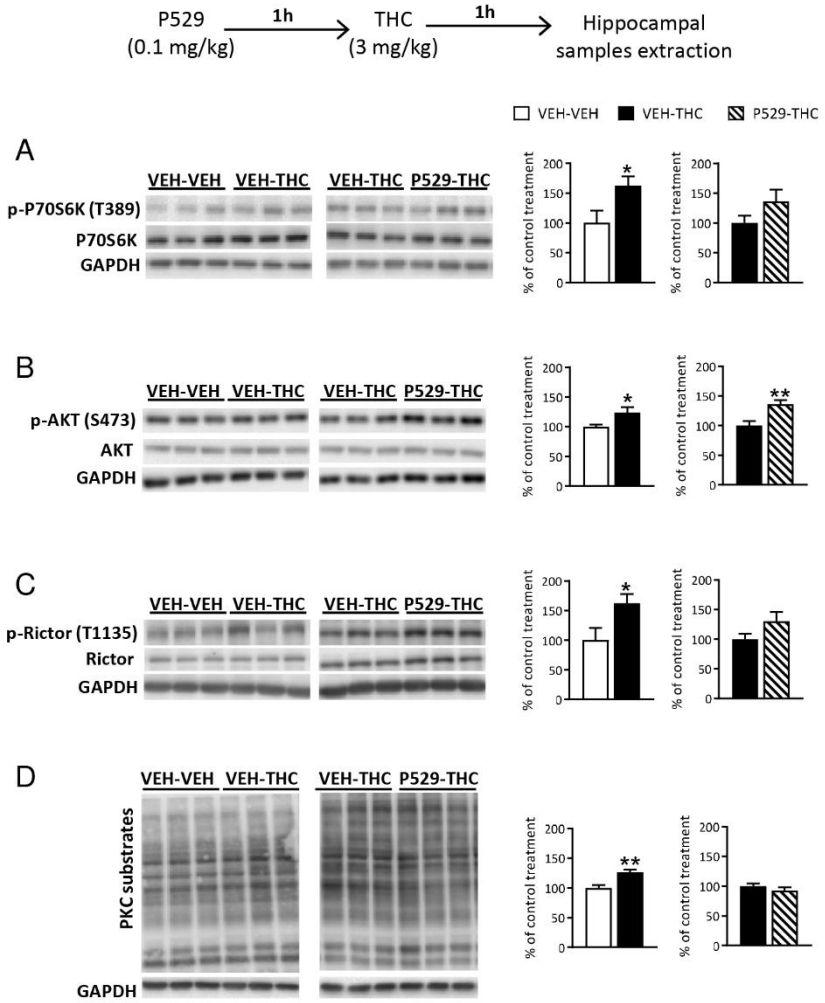

Figure 29. Phosphorylation of direct and indirect substrates of mTOR in the hippocampus of mice 1 h after P529-THC treatment. (A) p-P70S6K(T389), **(B)** p-AKT(S473), **(C)** p-Rictor (T1135), and **(D)** PKC phosphorylated substrates were analyzed in hippocampal samples 1 h after treatment with vehicle (VEH) or THC (3 mg/kg), in mice that had been pre-treated with vehicle (VEH) or P529 (0.1 mg/kg) (n = 6-7). Data are expressed as mean ± s.e.m. *p < 0.05, **p < 0.01 by Student's t-test.

Interestingly, the levels of p-AKT (S473), considered as a readout of mTORC2 activity (Sarbassov *et al.,* 2006), were enhanced after THC treatment (Student's t-test p = 0.036) and P529 pre-treatment

increased even more those phosphorylation levels (Student's t-test p = 0.007) (Figure 29B).

Phosphorylation of Rictor (T1135), a direct target of P70S6K, does not lead to major changes in mTORC2-kinase activity (Dibble *et al.,* 2009). THC administration increased p-Rictor (T11335) levels in the hippocampus and P529 pre-treatment did not modify Rictor phosphorylation after THC treatment (Figure 29C).

Taking into account that PKC-α (Ikenoue *et al.,* 2008) is another target of mTORC2 and PKC signaling is enhanced after THC administration (Busquets-Garcia *et al.,* 2018), we measured PKC kinase activity. We used an antibody that recognizes the consensus domain for the conventional PKCs isoforms phosphorylation (including the PKC-α): Ser residues surrounded by Arg or Lys at the −2 and +2 positions and a hydrophobic residue at the +1 position. The immunoblot analysis of hippocampal samples revealed a significant enhancement in phosphorylated PKC substrates after THC administration (Student's t-test p = 0.003). However, P529 pre-treatment did not modify the increase in phosphorylated PKC substrates mediated by THC (Figure 29D).

Our results revealed that acute THC (3 mg/kg) administration impaired NORT and enhanced mTORC1 and mTORC2 downstream substrates in the hippocampus. P529 pre-treatment prevented the memory impairment induced by THC, but no signs of mTORC1/mTORC2 inhibition were observed in the canonical pathways analyzed in hippocampal homogenates.

Objective 3

To study the effects of the monoacylglycerol lipase enzyme inactivation in the regulation of the motor coordination tasks and its downstream mechanisms.

Article #2

Monoacylglycerol lipase blockade impairs fine motor coordination and triggers cerebellar neuroinflammation through cyclooxygenase-2

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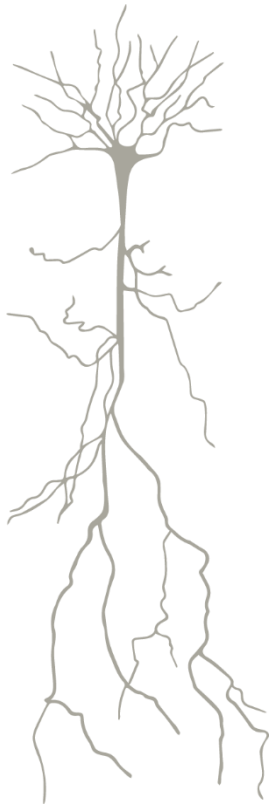
Brain Behav Immun. 2019; 81:399-409

Martínez-Torres S, Cutando L, Pastor A, Kato A, Sakimura K, de la Torre R, et al. Monoacylglycerol lipase blockade impairs fine motor coordination and triggers cerebellar neuroinflammation through cyclooxygenase-2. Brain, behavior, and immunity. 2019;81:399–409. DOI: 10.1016/j.bbi.2019.06.036



DISCUSSION

The ECS and its associated intracellular signaling pathways are widely expressed in the CNS and in peripheral organs, where it is involved in the regulation of multiple functions including memory, motor coordination and inflammatory responses of particular relevance for the aim of this Doctoral Thesis. Although the ECS has been proposed as a therapeutic target for numerous disorders, the modulation of this system implies several caveats derived from the wide distribution and diversified role of the ECS at different organs and cell types.

Thus, the main aim of this thesis was to evaluate the effects of targeting the ECS and its associated intracellular signaling pathways using alternative strategies beyond classical systemic agonists and antagonists of the cannabinoid receptors. In particular, we studied the effects on mouse behavioral, cellular, biochemical and molecular responses of **(1)** a well-characterized peripherally-restricted CB1R antagonist, **(2)** the inhibition of MAGL, and **(3)** an inhibitor of mTORC1/mTORC2 signaling. These three different strategies to modulate ECS activity collectively reveal the complexity of targeting the ECS. Indeed, apart from those expected outcomes, other unexpected results may also appear and must be taken into consideration.

The results described in this thesis are the first to report a persistent memory improvement mediated by a peripherally-restricted CB1R antagonist, which modulated central and peripheral adrenergic/noradrenergic pathways. In addition, we identified the peripheral CB1R as a target to prevent the memory deficits and hippocampal functional and structural abnormalities in a mouse model of FXS. Furthermore, we revealed that THC increased both mTORC1 and mTORC2 activity and the pre-treatment with a dual

mTORC1/mTORC2 inhibitor prevented the amnesic-like effects mediated by THC. Finally, we revealed the specific cerebellar neuroinflammation and motor coordination impairment produced by the pharmacological inhibition and genetic deletion of the MAGL enzyme. In this section we will further discuss the main results obtained in each objective of the present thesis.

1. The peripheral endocannabinoid system contributes to memory persistence by affecting the peripheral and central adrenergic/noradrenergic tone

The peripheral ECS has been hypothesized to represent an interesting alternative target for obesity (Tam *et al.,* 2018) that could prevent the negative adverse effects of previous approaches using systemically acting antagonists for the CB1R (Silvestri and Di Marzo, 2012). In this regard, peripherally-restricted CB1R reduce body weight in experimental models of overweight and obesity (Cluny *et al.,* 2010; Boon *et al.,* 2014; Hsiao *et al.,* 2015; Han *et al.,* 2018, 2019). Whether such peripherally-restricted CB1R antagonists could similarly modulate cognitive responses has not been yet explored. Although memory performance is much assumed to depend on centrally occurring processes (Kruk-Slomka *et al.,* 2017), some reports describe a role of peripherally-relevant mediators in cognitive performance (Roozendaal and McGaugh, 2011; Jenkins *et al.,* 2016). The understanding of those peripheral factors that facilitate or prevent memory formation is crucial in order to take advantage of these mechanisms to improve memory in pathological conditions.

In the present study, we have used the NORT to evaluate memory persistence. NORT is used to assess short-term and long-term memory depending on the interval between the familiarization session and the test session (retention time). We observed that retention intervals of 10 min to 24 h showed clear memory persistence based on the high discrimination indexes obtained, whereas an interval of 48 h did not show signs of object-recognition memory persistence. Thus, we reasoned that NORT assessment at 48 h could be used to evaluate a labile memory susceptible to be enhanced.

Administration of the systemically-acting CB1R antagonist rimonabant or the genetic downregulation of CB1R increased object-recognition memory persistence assessed 48 h after the familiarization phase. Our data is consistent with several evidences demonstrating hippocampal-dependent memory facilitation in CB1R KO mice (Reibaud *et al.,* 1999; Maccarrone *et al.,* 2002; Jacob *et al.,* 2012) or after rimonabant treatment (Lichtman, 2000; Wolff and Leander, 2003; Takahashi *et al.,* 2005). Interestingly, one study demonstrated that rimonabant improved memory in the elevated T-maze test only when it was administered just after the training session, but not 20 min before the test, indicating that this antagonist specifically enhanced the consolidation of memory but not its acquisition (Takahashi *et al.,* 2005).

1.1. Peripheral CB1R blockade mobilizes peripheral and central adrenergic/noradrenergic signaling to modulate memory persistence

Previous results in our group demonstrated that CB1R at peripheral locations were responsible for the amnesia induced by stress on the

NORT (Busquets-Garcia *et al.,* 2016). These results pointed to an interplay between central and peripheral mechanisms in the control of non-emotional memory performance. Indeed, the peripherally-restricted CB1R antagonist AM6545 prevented novel object-recognition memory deficits under stressful situations (Busquets-Garcia *et al.,* 2016). In our study, we evaluated the role of peripheral CB1R in modulating memory persistence and we hypothesized that adrenal glands play a relevant role in such a function. This was confirmed by the fact that adrenalectomized mice did not show the memory enhancement produced by AM6545. Since acute AM6545 administration transiently increases blood levels of corticosterone, adrenaline and NA (Busquets-Garcia *et al.,* 2016), we performed additional experiments to block these peripherally-produced factors. We found that pre-treatment with the glucocorticoid receptor antagonist mifepristone did not affect AM6545-mediated memory enhancement. Instead, the peripherally-restricted β-AR antagonist sotalol blocked the increased object-recognition memory persistence mediated by AM6545. Sotalol also prevented the enhancement in memory persistence produced by the systemic CB1R antagonist/inverse agonist rimonabant. This result indicates that the memory-enhancing effects of rimonabant may have an up-to-now disregarded important peripheral component. Both the peripherally-restricted antagonist AM6545 and the systemic antagonist rimonabant showed similar profiles in this regard.

CB1R in DBH+ cells modulate the release of circulating adrenaline and NA (Niederhoffer *et al.,* 2001). Thus, we hypothesized that CB1R in this cell type may have an important role in the mnemonic effects induced

by AM6545. We used a conditional CB1R knockout on DBH+ cells (DBH-CB1KO) to directly assess the relevance of CB1R in DBH+ cells. This mouse model has been previously used to evaluate the role of the ECS on the amnesia induced by stress (Busquets-Garcia *et al.,* 2016) and in bone formation (Deis *et al.,* 2018). We found a significant increase in object-recognition memory persistence in DBH-CB1KO mice, which was sensitive to sotalol, further pinpointing the role of CB1R in the control of the peripheral adrenergic tone to modulate memory persistence. Adrenaline mnemonic effects seem to be initiated by the activation of β-ARs present on the afferent fibers of the vagus nerve and the stimulation of NTS-LC pathway (Schreurs *et al.,* 1986; Lawrence *et al.,* 1995). Indeed, the activation of the NTS-LC pathway is necessary for the consolidation of hippocampal dependent-tasks, including the novel object-recognition (Mello-Carpes and Izquierdo, 2013). We demonstrated that acute AM6545 administration transiently increased LC firing rate accompanied with an enhancement of NA extracellular levels in the CA1 hippocampal region. These data indicate that targeting peripheral CB1R overactivates LC projections to the hippocampus, which could also participate in the mnemonic effects produced by AM6545. Although the mnemonic effect produced by LC stimulation depends on hippocampal β-ARs (Hagena *et al.,* 2016), it could also involves the activation of dopamine receptors (Takeuchi *et al.,* 2016), consistent with the idea that tyrosine hydroxylase positive cells can also release dopamine (Devoto and Flore, 2006). However, our data showed that acute AM6545 administration did not alter dopamine extracellular levels in the hippocampus. Indeed, we observed that intra-hippocampal injection of the β-AR antagonist propranolol

prevented the mnemonic effects induced by AM6545, supporting the importance of NA and hippocampal β-ARs activation to increase memory persistence.

Its worthy to mention that recent evidences highlight the key role of the peripheral ECS for metabolic regulation leading to the preclinical development of several compounds that selectively block peripheral CB1R, including AM6545 (Hirsch and Tam, 2019). Further studies will be necessary to discard other CB1R peripheral actions that could participate in the mnemonic effects produced by AM6545.

In summary, our results support that peripheral CB1R antagonism enhances the release of adrenaline and NA into the blood. Both hormones can bind to peripheral β-ARs including those expressed in the vagus nerve. The vagus nerve sends projections to the NTS and subsequently to LC, which increases its activity and releases NA to the hippocampus. Finally, NA release in the hippocampus would strengthen memory consolidation/persistence (Figure 30).

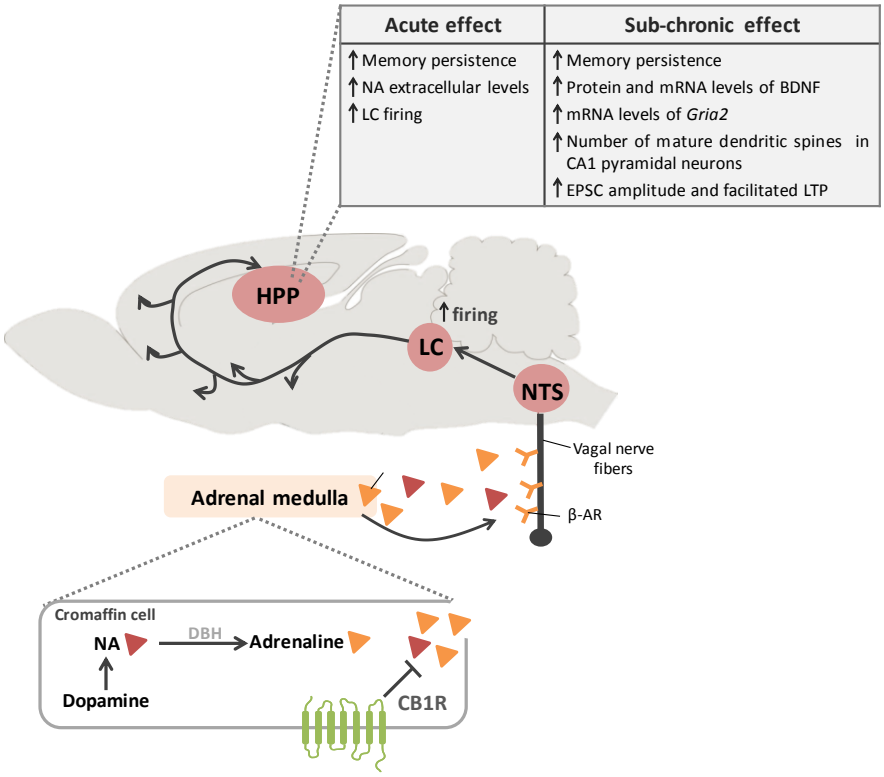


Figure 30. Summary of the results obtained in the first objective of this thesis and hypothesis proposed. Acute and sub-chronic administration of the peripherally-restricted CB1R neutral antagonist AM6545 increases novel object-recognition memory persistence by the increase of peripheral and central adrenergic/noradrenergic tone. This is supported both by pharmacological and genetic approaches blocking or deleting peripheral CB1R and β-adrenergic receptors. The blockade of peripheral CB1R, could increase the discharge of peripheral adrenaline and noradrenaline released from the dopamine β-hydroxylase positive cells (DβH+) of the adrenal medulla (Niederhoffer *et al.,* 2001). Adrenaline does not cross the blood-brain barrier and binds to peripheral β-adrenergic receptors, such as the ones expressed on the vagus nerve (Chen and Williams, 2012). Vagus nerve projects to the nucleus of the solitary tract (NTS), which in turn sends glutamatergic projections onto neurons in the *locus coeruleus* (LC) increasing its firing (Reyes and Van Bockstaele, 2006). Then, LC neurons release more NA to the (HPP). Sub-chronic AM6545 administration produces several neuronal plasticity changes that could explain the memory persistence improvement. BLA, basolateral amygdala; Thal, thalamus; OFC, orbitofrontal cortex; FC, frontal cortex; ACC, anterior cingulate cortex. Adapted from (Hagena *et al.,* 2016).

1.2. Hippocampal effects of sub-chronic peripheral CB1R blockade

We evaluated long-term object-recognition memory in mice treated for 7 d with AM6545 to assess whether tolerance to AM6545 effects may develop. In a set of experiments, AM6545 was administered 6 h after the familiarization phase, considered as an interval of time when the memory trace for objects has been already consolidated (Busquets *et al.,* 2016). Sub-chronic AM6545 treatment increased memory persistence in NORT in that schedule, suggesting that those effects could be mediated by persistent neuronal plasticity changes derived from the repeated treatment. Neuronal progenitor proliferation was evaluated to assess the possible mechanisms involved. Although, AM6545 treatment did not change the expression of the cell proliferation marker Ki67 in the subgranular zone of the dentate gyrus, other processes relevant for neurogenesis such as cell progenitor turnover (Coradazzi *et al.,* 2016), stem cell differentiation or maturation, have not been investigated after sub-chronic AM6545. As a second mechanistic approach, we focused on the structural plasticity by analyzing the density and morphology of spines on apical dendrites from CA1 pyramidal neurons. Dendritic spines are highly transformed by plasticity mechanisms and are involved in memory performance (Attardo *et al.,* 2015). An increase on the number of mushroom spines, the most mature form of dendritic spine (Hayashi and Majewska, 2005) was observed after 7 d of AM6545 treatment. This change on dendritic spine morphology in CA1 pyramidal neurons may increase synaptic strength in this area and reinforce synaptic connections (Hayashi and Majewska, 2005). Consistent with this neuronal plasticity modification, sub-chronic AM6545 treatment enhanced neurotrophic mRNA levels of

Bdnf and *Ngf* and protein BDNF levels in the hippocampus. Neurotrophins are involved in long-term synaptic plasticity and memory (Gibon and Barker, 2017). Indeed, BDNF signaling inhibition in the hippocampus impairs memory consolidation (Bekinschtein *et al.,* 2007).

Electrophysiological studies in hippocampal slices of mice revealed that sub-chronic AM6545 treatment increased EPSCs amplitude in the hippocampal CA1 region. No changes in paired-pulse ratio were observed after AM6545 treatment discarding a presynaptic component promoting neurotransmitter release. Thus, the increase in EPSCs amplitude seems to be mediated through a postsynaptic increase in AMPAR-mediated currents. In this regard, hippocampal samples of AM6545-treated mice showed a significant increase in mRNA levels of the AMPAR subunit *Gria2*. A potential link between these findings and the enhanced release of NA mediated by AM6545 could involve β-AR. Indeed, hippocampal β-AR activation facilitates AMPAR trafficking to extrasynaptic sites (Vanhoose and Winder, 2003; Rouach *et al.,* 2005; Joiner *et al.,* 2010) reinforcing LTP (Oh *et al.,* 2006). However, the application of a LTP protocol in brain slices after sub-chronic AM6545 treatment did not induce a long-term increase in EPSC amplitude, suggesting that AM6545 treatment already increased the amplitude of these currents saturating or occluding LTP. Consequently, an intrinsic effect of AM6545 altering LTP may be consistent with the observed increased in object-recognition memory persistence.

In summary, our study identifies the peripheral CB1R as a relevant target to increase object-recognition memory persistence and to enhance long-term synaptic plasticity in widtype mice through

combined central and peripheral adrenergic/noradrenergic mechanism (Figure 30).

1.3. Targeting peripheral CB1R as a treatment for the memory deficits that characterize FXS

FXS is an inherited condition coursing with intellectual disability (Penagarikano *et al.,* 2007). Although there is currently no effective treatment for FXS, several drugs have been tested in preclinical models of the syndrome and in clinical trials, including modulators of the mGluR system and GABAergic agents, among others (Munshi *et al.,* 2017).

Previous research in our group identified the ECS as a potential therapeutic target in FXS. In accordance, the most studied mouse model of FXS, the *Fmr1* KO mouse, showed clear improvements in several phenotypes when treated with low doses of systemic CB1R antagonists, such as rimonabant or NESS0327 (Busquets-Garcia *et al.,* 2013; Gomis-González *et al.,* 2016). Indeed, the pharmacological blockade of CB1R by rimonabant or the genetic downregulation of CB1R in *Fmr1* KO mice normalized the memory impairment, the altered dendritic spine morphology, the enhanced mGluR5-LTD and the high susceptibility to suffer epileptic seizures, among other features (Busquets-Garcia *et al.,* 2013; Gomis-González *et al.,* 2016).

Given the peripheral involvement of CB1R in hippocampal memory persistence and the synaptic changes in mice revealed in the previous objective, we studied the possible impact of targeting peripheral CB1R in the *Fmr1* KO mouse model. We have focused our attention on the

memory deficits and hippocampal alterations presented in *Fmr1* KO mice.

We assessed memory performance in the NORT 24 h after familiarization phase, as it is the interval when *Fmr1* KO mice present clear memory deficits. We observed an amelioration of novel object-recognition memory deficits in the *Fmr1* KO mice that received acute AM6545 administration just after the familiarization session. AM6545 administration 6 h after the familiarization period did not prevent memory deficits in the *Fmr1* KO mice, suggesting that acute AM6545 directly affects memory consolidation only during the consolidation period, which approximately occur from 2 to 3 h after the familiarization session. Moreover, sotalol blocked AM6545 mnemonic effects in *Fmr1* KO mice indicating that peripheral β-ARs are involved in memory normalization, similar to the results obtained in wildtype mice receiving AM6545. In this regard, AM6545 treatment could peripherally modify the activity of vagus nerve afferents, a manipulation that when performed through electrostimulation has shown to reduce seizure frequency, improve memory, social abilities and verbal communication and enhance quality of life of autism individuals (Park, 2003; Wilfong and Schultz, 2006; Warwick *et al.,* 2007; Danielsson *et al.,* 2008; Levy *et al.,* 2010). These evidences point to the possibility that acute inhibition of peripheral CB1R using AM6545 would produce a transient enhancement of vagus nerve fiber afferents, a promising approach to improve several phenotypes of FXS individuals.

Besides acute AM6545 treatment, sub-chronic AM6545 treatment in *Fmr1* KO mice normalized NORT deficits even when the last AM6545

administration was delivered 6 h after the familiarization phase. These data support an accumulative effect of repeated AM6545 exposure over relevant brain areas for cognition. Sub-chronic administration of AM6545 also normalized the aberrant mGluR5-LTD in hippocampal slices of *Fmr1* KO mice, a well-described manifestation of the *Fmr1* KO mice (Bear *et al.,* 2004; Michalon *et al.,* 2012). Another relevant characteristic of FXS subjects and *Fmr1* KO mice is the enhanced level of immature dendritic spines (He and Portera-Cailliau, 2013; Jawaid *et al.,* 2018). Sub-chronic AM6545 treatment significantly decreased the number of immature spines of CA1 pyramidal neurons in *Fmr1* KO mice. This effect is somewhat reminiscent of the effect of sub-chronic treatment with rimonabant in reducing the number of immature spines, although rimonabant additionally increased the number of the mature ones in the hippocampus (Busquets-Garcia *et al.,* 2013). These results support the hypothesis that a relevant number of the systemic effects of rimonabant can be obtained through its peripheral targets.

It is worthy to mention that targeting specifically peripheral CB1R could be an interesting approach to avoid the central unwanted side effects reported when systemically CB1R targeting and to avoid blood-brain barrier permeability problems in the designing of new therapeutic compounds (Pardridge, 2012). Moreover, some of the features of the FXS are also observed in several autism spectrum disorders and other neurological disorders suggesting that strategies that normalize these alterations in FXS might also be effective in other brain disorders (Varghese *et al.,* 2017).

2. Involvement of mTORC1 and mTORC2 in the long-term memory deficits produced by THC

Understanding the mechanisms by which exogenous cannabinoid agonists induce memory impairment can be useful to elucidate the role of the ECS in memory. Previous studies from our group revealed that acute high dose of THC (10 mg/kg) triggers the activation of the mTOR pathway in the hippocampus, which correlates with memory impairment in mice. Indeed, pre-treatment with the mTOR inhibitor rapamycin blocked the amnesic-like effects produced by THC (Puighermanal *et al.,* 2009). Additionally, we found that temsirolimus, a rapamycin derivative (Yu *et al.,* 2001), also prevented different effects of THC, including the memory impairment (Puighermanal *et al.,* 2013). More recently, lower doses of THC (3 mg/kg) were shown to modulate PKC activity in the hippocampus, contributing to THC memory deficits (Busquets-Garcia *et al.,* 2018). These data indicate that both mTORC1 and mTORC2 could play a role in the amnesic effects of THC. To assess this possibility, we used a dual mTORC1/mTORC2 inhibitor to block the activity of both complexes under THC effects.

Rapamycin is an immunosuppressant and antifungal compound (Houchens *et al.,* 1983) that binds to a small protein of 12kDa, FK506-binding protein (FKBP12) to form the rapamycin–FKBP12 complex. This complex binds to mTOR protein and prevents mTOR from interacting with its substrates, blocking its activity (Sabatini *et al.,* 1994). Instead, mTORC2 is mainly considered a rapamycin-resistant complex, as it does not interact with the rapamycin-FKBP12 complex (Jacinto *et al.,* 2004). Nevertheless, some evidences have demonstrated that prolonged rapamycin treatment, by capturing mTOR-available protein could also

inhibit mTORC2 signaling (Sarbassov *et al.,* 2006). Although specific mTORC2 inhibitors that could be used *in vivo* are not available, several dual mTORC1/mTORC2 inhibitors have been developed (Waldner *et al.,* 2016). These compounds are ATP-competitive mTOR kinase inhibitors acting on all of the kinase-dependent functions of mTORC1 and mTORC2 and therefore blocking the activation feedback of PI3K/AKT signaling (Figure 31) (Schenone *et al.,* 2011). P529, also named Palomid-529 or RES-529, is a PI3K/AKT/mTOR pathway inhibitor that targets both mTORC1 and mTORC2 through mTOR complex dissociation (Xue *et al.,* 2008) and has shown good brain penetration (Lin *et al.,* 2013). P529 is under clinical development since it has demonstrated safety and effectiveness in a phase I clinical trial (NCT01271270 and NCT01033721) as a treatment for wet age-related macular degeneration.

We found that P529 produced dose-dependent long-term memory deficits in the NORT. These amnesic-like effects were reminiscent of those found with high doses of rapamycin (Puighermanal *et al.,* 2009) or temsirolimus (Puighermanal *et al.,* 2013), and reveal the importance of this signaling pathway for proper cognitive performance. Importantly, these effects over memory performance produced by P529 are observed with a single administration of this compound at the doses of 0.3, 1 and 10 mg/kg. These doses are much lower than the doses proposed for neovascular age-related macular degeneration (Dalal *et al.,* 2013), prostate cancer (Diaz *et al.,* 2009) and osteosarcoma (Hu *et al.,* 2018). In this regard, our data cautions about the central effects of low doses of P529. Indeed, although no drug-related adverse events have been reported during these clinical

studies, probably no CNS effects have been specifically evaluated due to the route of drug administration. Noteworthy, P529 was administered in these clinical studies as an ocular injection. Currently, the oral formulation of P529 that has demonstrated highly blood brain barrier penetration through oral administration is being developed for the treatment of glioblastoma, where it has received orphan designation by the US Food and Drug Administration, and for prostate cancer (Weinberg, 2016). Further studies might be necessary to evaluate the possible memory alterations produced by the dual mTORC1/mTORC2 inhibition.

In order to determine whether the P529 would efficiently prevent the long-term memory deficits produced by THC, we pre-treated mice with P529 (0.1 mg/kg), a dose that devoid intrinsic effects on the NORT. This dose of P529 blocked THC (3 mg/kg) amnesic-like effects. Interestingly, P529 pre-treatment prevented THC impairment in the NORT only when administered 1 h before THC treatment, but not when the administration of P529 was performed 20 min in advance. Consistent with this lack of effect at a short time before THC administration, pharmacokinetics experiments have shown that the highest peak of P529 plasma concentration was obtained between 30 min and 1 h after oral administration (Lin *et al.,* 2013).

Considering that mTOR is overactivated in the hippocampus after THC administration (Puighermanal *et al.,* 2009; 2013; Busquets-Garcia *et al.,* 2018), we wondered whether the alteration of this kinase activity induced by THC could be blocked after P529 pre-treatment. To this end, we evaluated the levels of p-mTOR (S2448), which phosphorylation is related to its kinase activity and regulated by the PI3K/Akt pathway

(Navé *et al.,* 1999) (Figure 31). We observed that THC (3 mg/kg) administration increased p-mTOR (S2448) levels in hippocampal samples, but P529 pre-treatment did not prevent such enhancement. However, whether increase of p-mTOR (S2448) should be taken as a measure of mTOR activity is under debate in some cell types (Figueiredo *et al.,* 2017). Interestingly p-mTOR (S2448) has been shown to be regulated by P70S6K to increase mTOR activity *in vitro*, acting as a positive feedback control (Figure 31) (Holz and Blenis, 2005). mTOR can also be autophosphorylated in residue S2481, which is considered a biomarker of intrinsic mTOR catalytic activity (Soliman *et al.,* 2010). This is an alternative that warrants further exploration.

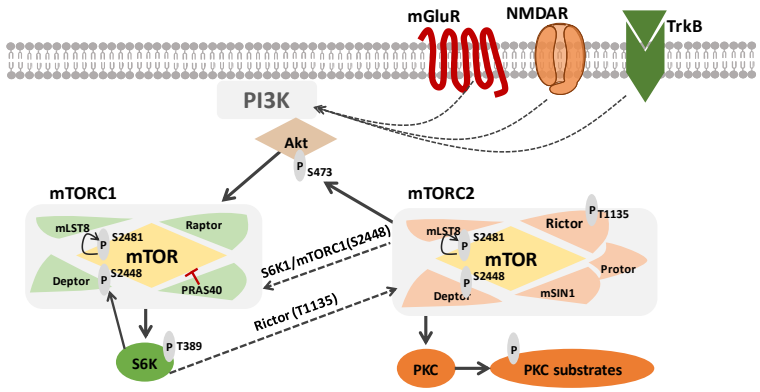


Figure 31. **Schematic representation of the complex signaling of mTORC1 and mTORC2**. Neuronal receptors and channels (mGluR, NMDAR, Trk-B) activate downstream signaling pathways including PI3K/Akt, which leads the activation of mTORC1. The upstream signaling regulating mTORC2 activity in neurons is still unknown. mTORC1 activity regulates several downstream effectors including P70S6K. P70S6K can phosphorylate Rictor triggering a crosstalk between both complexes. mTORC2 may modulate the activity of mTORC1 either directly through mTOR (S2448) or indirectly through Akt (S473). Akt, protein kinase B; PKC, protein kinase C; mLST8, mammalian *lethal with sec 13*; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; NMDAR, N-methyl-D-aspartate receptor ; PI3K, phosphoinositide-3 kinase; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PRAS40, proline-rich Akt/PKB substrate 40 kD; Protor, protein observed with rictor; Raptor, regulatory-associated protein with TOR; Rictor, rapamycin- insensitive companion of mTOR; Sin1, stress-activated protein kinase

interacting protein 1; P70S6K/S6K, ribosomal protein S6 kinase ; TrkB, receptor kinase B. T = threonine; S = serine.

To further investigate mTOR activity, we analyzed the substrates of mTORC1 and mTORC2 in response to P529 intervention (Figure 24). The phosphorylated levels of the mTORC1 target P70S6K (T389) were increased in the hippocampus of mice treated with THC (3 mg/kg). Similarly, previous results have demonstrated that acute high dose of THC (10 mg/kg), but not the low non-amnesic dose of 0.3 mg/kg, increased p-P70S6K (T389) in the hippocampus (Puighermanal *et al.,* 2009,2013). Interestingly, P70S6K regulates mTORC2 activity by direct phosphorylation of the mTORC2 component Rictor at T1135 in HEK-293E cells (Dibble *et al.,* 2009). Rictor is highly expressed in neurons where it plays a key role in brain development and the control of neuronal actin cytoskeleton (Angliker and Rüegg, 2013), as its deletion results in abnormal brain development (Guertin *et al.,* 2006; Shiota *et al.,* 2006). In agreement, Rictor forebrain-specific KO, which selectively affects mTORC2 activity, presents altered actin dynamics and signaling and long-term memory impairment (Huang *et al.,* 2013). Our results revealed enhanced p-Rictor (T1135) levels in the hippocampus of mice treated with acute THC (3 mg/kg). This phosphorylation does not regulate mTORC2 kinase activity *in vitro*, but negatively regulates p-Akt (S473), pointing a cellular mechanism to prevent simultaneous activation of mTORC1 and mTORC2 (Dibble *et al.,* 2009). We found that THC (3 mg/kg) enhanced p-Akt (S473) levels in hippocampal samples, as previously described with THC (10 mg/kg) treatment (Ozaita *et al.,* 2007; Puighermanal *et al.,* 2009). We also evaluated PKC kinase activity since PKC-α was described as another downstream substrate of

mTORC2 (Ikenoue *et al.,* 2008). PKC kinase activity was evaluated by detecting the change in phosphorylation of PKC substrates, which were previously reported to be increased in mice treated with THC (Busquets-Garcia *et al.,* 2018). As expected, THC (3mg/kg)-treated mice presented an overall increase in the phosphorylation levels of PKC substrates in the hippocampus.

Rapamycin or temsirolimus pretreatment, both mTORC1 specific inhibitors, prevented the overactivation of the mTORC1 target p70S6K produced by THC (10 mg/kg), but did not block the increased levels of mTORC2 target p-Akt (S473) (Puighermanal *et al.,* 2009). Strikingly, while the dual mTORC1/mTORC2 inhibitor P529 at a dose of 0.1 mg/kg prevented THC-mediated long-term memory deficit, it did not block neither mTORC1 nor mTORC2 overactivation in the hippocampus after THC administration. Indeed, a paradoxical enhancement over THC-modulated p-Akt (S473) levels were detected. One possible explanation of these paradoxical results could be that the immunoblot analysis was performed in total hippocampal homogenates instead of studying a specific subcellular compartment, such as synaptosomes. In this regard, recent studies have gained enhanced specificity by using a subcellular fractionation previous to immunoblot analysis (Li *et al.,* 2010; Sun *et al.,* 2016).

The above observations support the growing evidence about the relationships between alteration in brain mTOR signaling and aberrant synaptic plasticity, excitatory/inhibitory imbalance and cognitive disorders observed after THC exposure (Puighermanal *et al.,* 2009, 2013). Similar relationships may also be considered in genetic conditions where these pathways are deregulated as it has been shown

for several models of intellectual disability including FXS (Sharma *et al.,* 2010; Busquets-Garcia *et al.,* 2013), tuberous sclerosis (Prather and de Vries, 2004; Meikle *et al.,* 2008; Bateup *et al.,* 2013) and Down syndrome (Troca-Marín *et al.,* 2014; Bordi *et al.,* 2019) as well as, epilepsy (Pun *et al.,* 2012) and depression (Abelaira *et al.,* 2014).

In summary, our results revealed that acute THC (3 mg/kg) produced NORT deficits and hyperactivation of mTORC1 and mTORC2 in the hippocampus. Although P529 pre-treatment clearly prevented the amnesic-like effects produced by THC, no signs of mTORC1/mTORC2 inhibition were observed under those conditions in hippocampal homogenates.

3. Involvement of MAGL inhibition in a paradoxical cerebellar inflammation accompanied with motor incoordination

The ECS plays an important role in neuroinflammatory responses (Mecha *et al.,* 2016). Indeed, endocannabinoid levels are increased after brain injury, which manages to trigger protective functions that reduce microglial reactivity, decrease pro-inflammatory mediators and promote brain homeostasis (Xu and Chen, 2015). Several approaches have been proposed to target the ECS in order to obtain central anti-neuroinflammatory responses (Mestre *et al.,* 2009, 2011; Lourbopoulos *et al.,* 2011; Mecha *et al.,* 2015). In contrast with these observations, previous results from our group have demonstrated an increase of activated microglia and pro-inflammatory mediators selectively in the cerebellum after repeated exposure to a high dose of THC. These effects were accompanied by alterations in motor coordination and deficits in conditioned cerebellar-learning (Cutando

et al., 2013). We hypothesized that the effects of THC could be mimicked by experimental conditions leading to a buildup in endocannabinoids. We therefore focused our attention on studying the effects of increasing the endocannabinoid tone using two approaches directed to MAGL: a pharmacological approach using the MAGL inhibitor JZL184 and a genetic approach using the MAGL KO mouse model. Interestingly, MAGL inhibition and its subsequent 2-AG enhancement has been proposed to be useful against inflammation (Petrosino and Di Marzo, 2010; Alhouayek *et al.,* 2014), neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Nomura *et al.,* 2011; Pihlaja *et al.,* 2015) or oligodendrocyte degeneration (Bernal-Chico *et al.,* 2015). Contrary, we observed increased activated microglial cells in the cerebellum, but not in the hippocampus, after pharmacological inhibition and genetic inactivation of MAGL. Indeed, the previous studies did not consider assessing cerebellar inflammation, which we found is specifically modulated by these interventions on MAGL.

In agreement with other observations (Leishman *et al.,* 2016), we found that MAGL deletion produced an enhancement of 2-AG levels and other 2-acylglycerols, such as 2-OG and 2-LG, both in the cerebellum and the hippocampus, whereas no alteration was observed on the levels of AEA and the other N-acylethanolamines analyzed. These results show the constrained specificity and selectivity of MAGL activity inhibition. While 2-AG is mostly hydrolyzed by MAGL (Bisogno *et al.,* 1999), other enzymes may also participate in 2-AG degradation. This is the case of the α-hydrolases ABHD6 and ABHD12 and, to a minor

extent, the inducible enzyme COX-2 (Blankman *et al.,* 2007; Marrs *et al.,* 2010; Duggan *et al.,* 2011).

In our experimental conditions of MAGL inhibition, microglial reactivity was revealed by the morphology of microglial cells in the cerebellum, but not in other brain areas. This was correlated with significant increases in the expression of *Itgam* mRNA and subtle changes in *Il1b* and *Tnfa* expression. Previous observations in whole brain homogenates after pharmacological and genetic inhibition of MAGL (Nomura *et al.,* 2011) showed somewhat similar results when the expression of mRNA levels for *Il1b*, *Il1a*, *Il6* and *Tnfa* was analyzed.

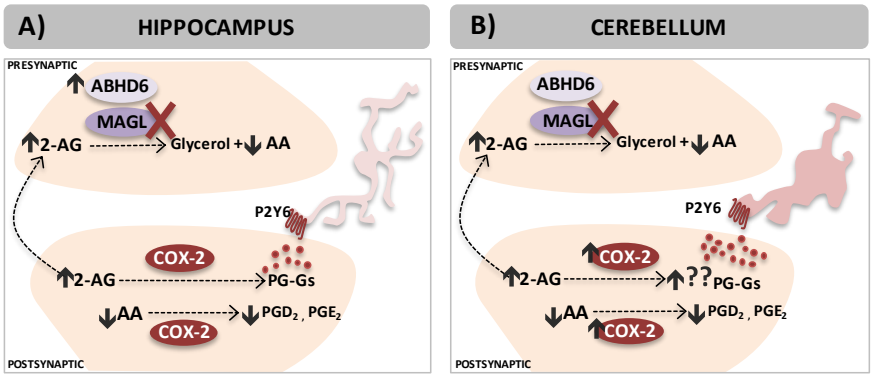
Paradoxically, JZL184 has anti-inflammatory effects, which are not only mediated by CB1R and CB2R (Alhouayek *et al.,* 2011). Instead, these effects were associated to the reduction of pro-inflammatory mediators, specifically the eicosanoids derived from AA (Nomura *et al.,* 2011). In this case, as MAGL turns 2-AG into AA and glycerol, it was observed that MAGL inhibition contributed to reduce the pool of AA, which in turn prevented inflammatory processes by limiting prostanoid production (Nomura *et al.,* 2011). In agreement with these results, we observed reduced AA, PGE_2 and PGD_2 levels in the cerebellum and hippocampus as a consequence of MAGL deletion. This finding did not fit with the reactive microglia selectively detected in the cerebellum. Prostanoid production depends on AA availability but also on the expression of the inducible COX-2 enzyme, whose expression is enhanced upon inflammation (Smith *et al.,* 2011). In the cerebellum, COX-2 activity was found relevant for motor learning and long-term synaptic plasticity of Purkinje cells (Le *et al.,* 2010). In the forebrain, COX-2 expression physiologically contributes to synaptic dendrites

activity (Kaufmann *et al.,* 1996; Chen *et al.,* 2002). While 2-AG levels are increased in the cerebellum and hippocampus of MAGL KO mice and in mice treated with JZL184 (40 mg/kg), we found different response in both brain areas regarding *Cox2* mRNA expression. Both MAGL KO and JZL184 (40 mg/kg)-treated mice showed enhanced *Cox2* mRNA levels in the cerebellum, whereas hippocampal *Cox2* mRNA expression was not modified from basal conditions. This different response between cerebellum and hippocampus may derive from the alternative metabolism of accumulated 2-AG in both brain regions. While *Cox2* mRNA levels were enriched in the cerebellum, *Abhd6* mRNA expression was higher in the hippocampus, suggesting a differential segregation in the cerebellum for the enzymatic pathways involved in prostaglandin synthesis from AA metabolism. In addition, the common expression of COX-2 and ABHD6 proteins at the post-synaptic terminals in the cerebellum and hippocampus (Dinh *et al.,* 2002; Pardue *et al.,* 2003) may suggest a similar role for both enzymes on the rapid degradation of 2-AG after its synthesis to precisely regulate the retrograde synaptic transmission. Further studies are necessary to clarify the participation of COX-2 and ABHD6 enzymes in the control of synaptic transmission and its specific localization under conditions of enhanced 2-AG.

Cerebellar functionality in MAGL KO mice was previously evaluated using the rotarod test and no significant alterations were detected (Chanda *et al.,* 2010). However, we found that JZL184-treated mice and MAGL KO mice showed motor coordination deficits in the beam walking and footprint test, which are highly sensitive tests to measure fine motor coordination (Carter *et al.,* 2001).

As *Cox2* mRNA expression is the principal hallmark that could explain the different neuroinflammatory phenotype between cerebellum and hippocampus in the same animal, we analyzed the consequences of the COX-2 inhibitor NS398 in MAGL KO mice. NS398 sub-chronic administration prevented alterations in cerebellar microglial morphology and motor coordination observed in MAGL KO mice. In addition, COX-2 inhibition did not affect the already decreased levels of PGE_2 and PGD_2 in MAGL KO mice, exempting these mediators from a role in microglial reactivity normalization under NS398 conditions. An alternative possibility comes from the fact that 2-AG can be directly processed by COX-2 to generate PG-Gs. This enzymatic reaction has been described *in vitro* (Kevin R. Kozak *et al.,* 2002; Alhouayek and Muccioli, 2014) and *in vivo* (Kingsley *et al.,* 2005; Hu *et al.,* 2008; Morgan *et al.,* 2018) under conditions of increased 2-AG accumulation. However, PG-Gs are rapid hydrolyzed into PGs *in vivo*, which make difficult its detection under normal conditions (Hu *et al.,* 2008; Ritter *et al.,* 2012) or in our experimental conditions (data not shown). PG-Gs derived from 2-AG metabolism could bind to classical prostanoid receptors (Kozak *et al.,* 2001), but they can also act through binding to alternative receptors (Hu *et al.,* 2008). One of them is the purinergic receptor P2Y6, for which PG-Gs have demonstrated affinities in the picomolar range (Brüser *et al.,* 2017). Interestingly, this receptor is expressed in microglial cells in normal and pathophysiological conditions controlling phagocytosis, inflammation and migration (Koizumi *et al.,* 2007; Barragán-Iglesias *et al.,* 2014). Therefore, this receptor could be a reasonable link between the putative raise of PG-Gs, derived from 2-AG accumulation by increased COX-2 activity, and

the microglial reactivity detected in our study specifically in the cerebellum (Figure 32). Further studies are required to clearly ascertain the differential mechanisms involved in the cerebellar responses produced by the 2-AG buildup.



- Increased 2-AG levels and decreased AA and PG (PGD_2 and PGE_2) levels.
- Enhanced mRNA expression of *Abhd6* but no differences on pro-inflammatory mediators.
- No effect on soma perimeter of microglial cells.
- Correct motor coordination.

- Increased 2-AG levels and decreased AA and PG (PGD_2 and PGE_2) levels.
- Enhanced mRNA expression of *Cox-2* and other pro-inflammatory mediators.
- Increased soma perimeter of microglial cells.
- Motor coordination deficits.

Figure 32. Summary of the results obtained in this aim and proposed model to explain the specific neuroinflammatory phenotype in the cerebellum of JZL184-treated mice and MAGL KO mice. Under control conditions monoacylglycerol lipase (MAGL) and α/β hydrolase domain 6 (ABHD6) are two major enzymes responsible for the hydrolysis of 2-arachidonoylglycerol (2-AG) into glycerol and arachidonic acid (AA). The cyclooxygenase-2 (COX-2) can metabolize AA to originate prostaglandins (PGs), such as PGD_2 and PGE_2 and can also hydrolyze 2-AG to give PG-glycerol esters (PG-Gs), which may bind to the P2Y6 receptor expressed in microglial cells and activate them. **(A-B)** In JZL184-treated mice or MAGL knockout (MAGL KO) mice: **(A)** there is an increase of *Abhd6* mRNA levels in the hippocampus, which is not enough to compensate the increased 2-AG levels, the AA depletion and the decreased levels of PGD_2 and PGE_2; **(B)** in the cerebellum, there is an increase of mRNA levels of neuroinflammatory markers including *Cox*-2 that may increase PG-Gs levels and consequently produce the activation of microglial cells through the P2Y6 receptor. This microglial activation is prevented after sub-chronic administration of the COX-2 inhibitor NS398, suggesting a critical role of COX-2 in the cerebellum.

Furthermore, 2-AG accumulation may also prevent microglial activation through CB2R expressed in reactive microglia. In inflammatory situations, 2-AG has been reported to bind to CB2R expressed in reactive microglial cells modulating their activity (Maresz *et al.,* 2005; Wen *et al.,* 2015). Our results revealed no significant differences in the *Cnr2* expression in the cerebellum or the hippocampus of MAGL KO mice, while a significant enhancement in the expression of *Cnr1* in the cerebellum of MAGL KO mice was found, indicating that the cerebellum responds in a different manner to MAGL inhibition and the subsequent 2-AG increase.

Electrophysiological studies performed in the MAGL KO mice and JZL184-treated mice revealed that increased 2-AG levels modulates DSE and DSI performance and prolong DSE in cerebellar slices (Schlosburg *et al.,* 2010; Tanimura *et al.,* 2010; Zhong *et al.,* 2011). Indeed, juvenile MAGL KO mice present reduced EPSCs amplitude in parallel fiber-Purkinje cell (Zhong *et al.,* 2011). However, enhanced 2-AG levels in the hippocampus has been shown to increase memory persistence leading to slower spatial memory retrieval (Morena *et al.,* 2015) and extinction of fear memory (Hartley *et al.,* 2016) and enhancing memory retention of inhibitory avoidance training (Ratano *et al.,* 2018). Moreover, MAGL KO mice exhibited enhanced memory acquisition in the novel object-recognition test and in the Morris water maze (Kishimoto *et al.,* 2015). These different effects in synaptic transmission and functionality between cerebellum and hippocampus, together with the specific cerebellar increased microglial reactivity, may explain the specific motor coordination impairments revealed in

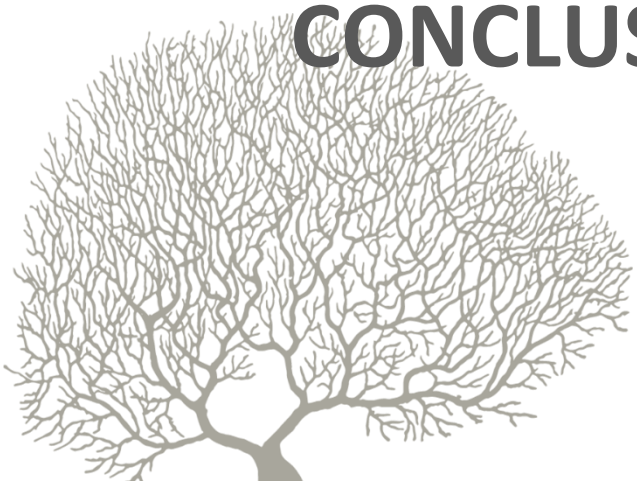
JZL184-treated mice and MAGL KO mice in our experimental conditions.

JZL184 is a potent and selective MAGL inhibitor without off-target *in vitro* effects at nanomolar levels. At micromolar concentrations *in vitro* or at 16 mg/kg *in vivo*, JZL184 suppresses FAAH activity and devoids the increase of AEA levels (Long *et al.,* 2009). Notably, it is necessary more than 80% inhibition of FAAH to observe the accumulation of AEA levels (Fegley *et al.,* 2004). However, JZL184 administration at high doses (40 mg/kg) rather than low doses (4 mg/kg) increase both AEA and 2-AG levels producing cannabimimetic side effects (Long *et al.,* 2009; Kinsey *et al.,* 2013). Here we used an acute high dose of 40 mg/kg, which has been demonstrated anti-inflammatory effects both in brain homogenates and in the hippocampus (Nomura *et al.,* 2011). Additionally, we studied a low dose of 8 mg/kg, which has previously used to study the effects of 2-AG accumulation on memory and anxiety responses (Busquets-Garcia *et al.,* 2011). Although we cannot discard off-target effects with the high dose of JZL184, MAGL KO mice and JZL184 (40 mg/kg)-treated mice showed similar modifications indicating that part of the effects produced by JZL184 administration in the cerebellum are mediated through MAGL inhibition.

Moreover, pharmacological MAGL inhibition using JZL184 (Nomura *et al.,* 2011; Chen *et al.,* 2012; Lysenko *et al.,* 2014) or other highly-selective MAGL-inhibitors, such as KML29 (Pasquarelli *et al.,* 2017a, 2017b) or MJN110 (Niphakis *et al.,* 2013) has revealed neuroprotective effects. However, the possible microglial reactivity that KML29 and MJN110 could induce in the cerebellum remains to be clarified.

Altogether, these results strongly support the sensitivity of the cerebellum to alterations in ECS signaling and highlight a potential disadvantage of strategies that target directly MAGL and produce the resulting accumulation of 2-AG for the treatment of inflammatory responses.

CONCLUSIONS



Novel approaches to target the ECS and its associated intracellular signaling pathways demonstrate complex responses in mouse models, probably derived from its widespread distribution and the intricated complexity of this neuromodulatory system that warrants multifaceted studies to clarify its potential interest as a therapeutic target. The main conclusions of the work presented in this Doctoral Thesis can be summarized as follows:

1. Acute administration of the systemic CB1R antagonist/inverse agonist rimonabant and the peripherally-restricted CB1R neutral antagonist AM6545 increase memory persistence in the novel object-recognition test through a peripheral adrenergic mechanism.
2. Acute AM6545 administration enhances CNS noradrenergic tone by increasing the locus coeruleus firing and hippocampal noradrenaline release.
3. Intra-hippocampal β-adrenergic blockade prevents the memory improvement produced by AM6545.
4. Sub-chronic AM6545 treatment for 7 d increases neurotrophic factors and AMPAR, which could explain both the increase of mature forms of dendritic spines in CA1 pyramidal cells and EPSC amplitude in CA3-CA1 synapses.
5. Acute AM6545 administration normalizes object-recognition memory performance of *Fmr1* KO mice through a peripheral adrenergic mechanism.

6. Sub-chronic administration of AM6545 for 7 d normalizes object-recognition memory impairment and restores the altered mGlur5-LTD and dendritic spine morphology of CA1 pyramidal neurons in the *Fmr1* KO mice.
7. The dual mTORC1/mTORC2 inhibitor P529 produces object-recognition memory impairment in a dose-related manner.
8. A sub-effective dose of P529 (0.1 mg/kg) blocks the amnesic-like effects produced by acute THC (3 mg/kg) administration in the novel object-recognition test.
9. P529 pre-treatment does not apparently reduce the mTORC1/mTORC2 overactivation mediated by THC treatment in the canonical pathways analyzed in the hippocampus.
10. Pharmacological inhibition and genetical deletion of MAGL produce significant motor incoordination, cerebellar microglia activation and cerebellar COX-2 overexpression without affecting the hippocampus.
11. MAGL KO mice present increased levels of the 2-acylglycerols 2-AG, 2-OG and 2-LG in the cerebellum and hippocampus, which are not modulated by COX-2 inhibition.
12. COX-2 inhibition prevents the cerebellar neuroinflammation caused by MAGL deletion without modifying PG levels, suggesting PG-Gs as responsible for the microglial activation differentially occurring in the cerebellum, but not in the hippocampus.
13. Regional brain differences in MAGL inhibition outcome should be considered when evaluating this enzyme as an anti-inflammatory target.

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ANNEX

Article #1

Serotonergic mechanisms involved in antidepressant-like responses evoked by GLT-1 blockade in rat infralimbic cortex

Júlia Gasull-Camós, Sara Martínez-Torres, Mireia Tarrés-Gatius, Andrés Ozaita, Francesc Artigas, Anna Castañé

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Article #2

Use of the Vsoc-maze to study sociability and preference for social novelty in rodents

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