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Role of cellular and sub-cellular CB_1 receptor in Hippocampal D_1-positive cells on Memory Processes

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Rôle du récepteur CB_1 cellulaire et subcellulaire dans les cellules D_1-positives de l'hippocampe sur les processus de mémoire

Sous la direction de Giovanni MARSICANO

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TABLE OF CONTENTS

ACKNOWLEDGMENTS 6

LIST OF PUBLICATIONS 9

Articles published or in process of publication in peer reviewed scientific journals 9

LIST OF COMMUNICATIONS...13

Poster communications 13

Oral communications...14

RÉSUMÉ...15

ABSTRACT...17

LIST OF ABREVIATIONS..19

LIST OF FIGURES 21

Section I – GENERAL INTRODUCTION 22

Part 1 – The endocannabinoid system in the brain 23

I – Introduction 23

II – Cannabinoid receptors 26

III – Localization of CB_1 receptors in the brain...28

IV – Endocannabinoids Synthesis, Transport and Degradation...32

V – Pharmacological and Genetic Tools to study the ECS...35

VI – Pathological roles of the CB_1 receptor..38

Part 2 – Synaptic plasticity and Memory..47

I – How to study Synaptic Plasticity?..48

II – Hippocampal Synaptic Plasticity 51

III – Mechanisms of NMDA receptor-dependent LTP..53

IV – CB_1-mediated Modulation of Synaptic Activity..56

Part 3 – Memory 61

I – Long-term potentiation (LTP): A Synaptic Mechanism for Memory 62

II – CB_1 Receptors and Memory Process..64

III – Novel Object Recognition as Tool to investigate Memory..66

Part 4 – Dopaminergic System and the ECS 68
I – D_1 receptors in Hippocampal-dependent Synaptic Plasticity and Memory................................69
II – D_1 and CB_1 receptors – Linking in memory functions...72
Part 5 – Subcellular Signaling of CB_1 receptor: Mitochondrial CB_1 and Memory.............................74
I – Mitochondrial Functions...76
II – Role of Mitochondrial CB_1 in the Brain...78
III – Mitochondrial CB_1 and Memory...80
Section II – RESEARCH OBJECTIVES 83
Section III – RESULTS 88
Part 1 – Specific hippocampal interneurons shape consolidation of recognition memory.............89
Part 2 – Linking Mitochondrial G-protein Signaling to cannabinoids-induced amnesia: A new Mitochondria-specific chemogenetic Strategy..133
Section IV – GENERAL DISCUSSION...156
Part 1 – Specific hippocampal interneurons shape consolidation of recognition memory...........157
Part 2 – Linking Mitochondrial G-protein Signaling to cannabinoids-induced amnesia: A new Mitochondria-specific chemogenetic Strategy..160
Section V – REFERENCES 163

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The endocannabinoid-alcohol crosstalk: recent advances on a bi-faceted target. *Clinical and Experimental Pharmacology and Physiology.*

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11) BRANCATO A., CASTELLI V., LAVANCO G., TRINGALI G., MICALE V., KUCHAR M., D'AMICO C., PIZZOLANTI G., FEO S. AND CANNIZZARO C. 2021
Binge-like alcohol exposure in adolescence: behavioural, neuroendocrine and molecular evidence of abnormal neuroplasticity... and return. *Biomedicines.*

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Linking mitochondrial G-protein signaling to cannabinoids-induced amnesia: a new mitochondria-specific chemogenetic strategy

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2020 – FENS Virtual Forum of Neuroscience

Specific hippocampal interneurons shape consolidation of recognition memory

Lavanco G.; Oliveira da Cruz J. F.; Busquets-Garcia A.; Bellocchio L.; Zhao Z.; Robin L.; Cannich A.; Varilh M.; Lesté-Lasserre T.; Maitre M.; Marsicano G.; Soria-Gomez E.

Oral communications

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Binge drinking during adolescence as a vulnerability factor for migraine? Focus on Calcitonin gene-related peptide.

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Titre : Rôle du récepteur CB_1 cellulaire et subcellulaire dans les cellules D_1-positives de l'hippocampe sur les processus de mémoire

Résumé :

Via la modulation de l'activité neuronale par les récepteurs cannabinoïdes de type 1 (CB_1), le système endocannabinoïde représente un système modulateur cérébral majeur contrôlant les fonctions de la mémoire. D'autre part, plusieurs rapports soulignent le rôle crucial de la signalisation de la dopamine hippocampique dans la régulation des processus liés à la mémoire. De plus, des preuves récentes suggèrent que les cellules hippocampiques exprimant des récepteurs de dopamine possèdent également des récepteurs CB_1.

Le travail présenté dans cette thèse vise à établir un lien fonctionnel entre les récepteurs CB_1 et la signalisation dopaminergique dans la régulation des processus de mémoire liés à l'hippocampe, en mettant l'accent sur les mécanismes cellulaires et sous-cellulaires impliqués.

Dans la première partie de la thèse, nous avons observé qu'une lignée de souris dépourvue de CB_1 dans les cellules des récepteurs dopaminergiques de type 1 (D_1-CB_1-KO) présentait une déficience de la mémoire de reconnaissance des objets nouveaux à long terme (NOR) et, la réexpression virale de CB_1 dans les cellules D_1-positives de l'hippocampe des souris D_1-CB_1-KO a inversé la déficience NOR présente chez ces souris. En outre, nous avons mis en évidence une activation excessive des récepteurs GABAA de l'hippocampe et une altération de la potentialisation à long terme (LTP) in vivo dans la voie CA3-CA1 comme étant les principaux mécanismes cellulaires à l'origine des troubles de la mémoire chez les souris D_1-CB_1-KO. Nous avons ainsi fourni des preuves fonctionnelles de l'implication d'une petite sous-classe d'interneurones hippocampiques exprimant le récepteur cannabinoïde de type 1 (CB_1) dans la modulation de circuits hippocampiques spécifiques dans les processus de mémoire.

La deuxième partie de la thèse s'est concentrée sur la localisation subcellulaire de l'activation des CB_1 dans les cellules D_1 positives. En effet, outre la régulation canonique de l'activité neuronale par

le récepteur CB_1 de la membrane plasmique, des preuves récentes suggèrent l'implication du récepteur CB_1 mitochondrial ($mtCB_1$) dans la régulation des processus bioénergétiques qui ont un impact sur la transmission synaptique et les effets amnésiques des cannabinoïdes. Nous avons découvert que les récepteurs $mtCB_1$ dans les neurones hippocampiques D_1-positifs ne sont pas nécessaires pour la régulation physiologique de la formation de la mémoire en soi, mais que leur activation est nécessaire pour les troubles de la mémoire induits par le THC. En recherchant la signalisation intracellulaire et intra-mitochondriale de la protéine G impliquée dans ce processus, nous avons développé une nouvelle stratégie chimiogénétique qui module spécifiquement la signalisation mitochondriale de la protéine G et nous avons observé sa contribution dans l'activité mitochondriale du cerveau et les fonctions cognitives. Nous avons observé sa contribution à l'activité mitochondriale du cerveau et aux fonctions cognitives. L'activation chimiogénétique spécifique de la signalisation mitochondriale de la protéine G entraîne une augmentation de la respiration mitochondriale qui, à son tour, résout l'effet amnésique induit par le THC.

Dans l'ensemble, les résultats de cette thèse indiquent les mécanismes reliant la diversité des récepteurs CB_1 cellulaires et subcellulaires dans les fonctions cérébrales supérieures, y compris l'apprentissage et la mémoire, et fournissent la base pour le développement de stratégies thérapeutiques plus sélectives et précises pour les troubles cognitifs.

Mots clés : [Récepteur CB_1, récepteur $mtCB_1$, cellules D_1-positives, mémoire]

INSERM U1215 – NEUROCENTRE MAGENDIE

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Title: Role of cellular and sub-cellular CB1 receptor in Hippocampal D1-positive cells on Memory Processes

Abstract:

Via modulation of neuronal activity by cannabinoid receptor type-1 (CB_1), the endocannabinoid system represents a major brain modulatory system controlling memory functions. On the other hand, several reports point out a crucial role of hippocampal dopamine signaling in the regulation of memory related processes. Furthermore, recent evidence suggests that hippocampal cells expressing dopamine receptors do also posses CB_1 receptors.

The work presented in this Thesis aims at establishing a functional connection between CB_1 receptor and dopaminoceptive signaling in the regulation of hippocampal related memory processes with particular enfasis on the cellular and sub-cellular mechanisms involved.

In the first part of the thesis we observed that a mouse line lacking CB_1 in dopamine receptor type-1 cells (D_1-CB_1-KO) displayed impaired long-term novel object recognition memory (NOR) and, interestingly, viral-mediated re-expression of CB_1 in D_1-positive cells in the hippocampus of D_1-CB_1-KO mice reversed the NOR impairment present in these mice. Furthermore, we pointed out execessive hippocampal GABAA receptor activation and impaired in vivo long-term potentiation (LTP) in the CA3-CA1 pathway as the main cellular mechanisms for memory impairment in D_1-CB_1-KO. Thus, we provided functional evidence for the involvement of a small subclass of type-1 cannabinoid receptor (CB_1)-expressing hippocampal interneurons in the modulation of specific hippocampal circuits in memory processes.

The second part of the Thesis focused on subcellular location of CB_1 activation in D_1 positive cells. Indeed, besides the canonical regulation of neuronal activity by plasma membrane CB_1 receptor, recent evidence suggests the involvement of mitochondrial CB_1 receptor ($mtCB_1$) in the regulation of bioenergetic processes which impacts on synaptic transmission and amnesic effects of cannabinoids. We found that $mtCB_1$ receptors in hippocampal D_1-positive neurons is not required for physiological regulation of memory formation *per se* but its activation is required for THC-induced memory impairment. Looking for the intracellular and intra-mitochondrial G-protein

signaling involved in these processes, we developed a new chemogenetic strategy which specifically modulates the mitochondrial G-protein signaling and we observed its contribution in brain mitochondrial activity and cognitive functions. Specific chemogenetic activation of mitochondrial G-protein signaling results in increased mitochondrial respiration which in turns rescues THC-induced amnesic effect.

Overall, the results of this Thesis indicate the mechanisms linking the diversity of cellular and subcellular CB_1 receptors in higher brain functions, including learning and memory and provide the basis for the development of more selective and precise therapeutic strategies for cognitive disorders.

Keywords: [CB1 receptor, mtCB1 receptor, D1-positive cells, memory]

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LIST OF ABREVIATIONS

2-AG	arachidonoylglycerol
AC	adenylyl cyclase
ACEA	arachidonyl-2′-chloroethylamine
AEA	arachidonoyl ethanolamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
CaMKII	ca2+/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid type-1
CB_2	cannabinoid type-2
CB-LTD	cannabinoid-induced LTD
CCK	cholecystokinin
CNS	central nervous system
CRE	CRE recombinase
CREB	cAMP responsive element binding protein
D_1	dopamine type-1
D_2	dopamine type-2
DAG	diacylglycerol
DI	discrimination Index
DREADDs	designer receptor exclusively activated by designer drugs
DS	dopaminergic system
DSE	depolarization induced suppression of excitation
DSI	depolarization induced suppression of inhibition
eCB	endocannabinoid
eCB-LTD	endocannabinoid mediated LTD
ECS	endogenous cannabinoid system
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
ERT	estrogen receptor
FAAH	fatty acid amid hydrolase
fEPSP	field excitatory post synaptic potentials
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GPCR	G protein coupled receptor

HPA	hypothalamic–pituitary–adrenal
HFS	high frequency stimulation
I-LTD	inhibitory-LTD
IP3	inositol trisphosphate
ISH	in situ hybridization
JNK	c-Jun N-terminal kinase
KO	knockout
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol Lipase
MAPK	mitogen-activated protein kinases
mGLu	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MSN	medium spiny neurons
$mtCB_1$	mitochondrial CB1 receptor
NAPE	N-arachidonoyl phosphatidyl ethanol
NMDA	N-methyl-D-aspartate receptor
NORT	novel object recognition task
OXPHOS	oxidative phosphorylation
PKA	protein Kinase A
PLC	phospholipase C
PLD	phospholipase D
PV	parvalbumin
STED	stimulated emission depletion
THC	Δ9-tetrahydrocanabinol
TRPV-1	transient receptor potential vanilloid 1
VGCC	voltage-gated calcium channel
VTA	ventral tegmental area
WT	wild-type

LIST OF FIGURES

Figure 1 – Distribution of CB_1 Receptors in the adult mouse brain 28
Figure 2 – In the Hippocampus: Presynaptic Terminal Distribution of CB_1 Receptors.....................29
Figure 3 – In the Hippocampus: Intracellular and Subcellular Distribution of CB_1 Receptors...........30
Figure 4 – Different Pathways involved in the Biosynthesis and Degradation of AEA and 2-AG......34
Figure 5 – Generation of a cell-type specific KO mouse 37
Figure 6 – Involvement of the Endocannabinoid System in Different Neuropathologies.................39
Figure 7 – LTP at Hippocampal CA1 Synapses...51
Figure 8 – Molecular Mechanism of Long-Term Potentiation...55
Figure 9 – Intracellular CB_1 Receptor Signaling Pathways...57
Figure 10 – Endocannabinoid-Mediated Short- And Long-Term Synaptic Plasticity Mechanisms....60
Figure 11 – Scheme of the Novel Object Recognition Task...67
Figure 12 – View of the CB_1 receptor functional expression: Classic Vs Current.............................75
Figure 13 – Schematic view of the mtCB_1-dependent Signalin pathway 81

SECTION I – GENERAL INTRODUCTION

PART 1 – THE ENDOCANNABINOID SYSTEM

I – Introduction

The Endogenous Cannabinoid System (ECS) is a complex set of circuits coordinating many other systems of our organism, thus representing a regulator of many physiological processes (Pacher et al. 2006). Because of multitude of effects in both humans and animals, the interest in understanding the action mechanisms of *cannabis sativa* (commonly named marijuana or simply cannabis) started. Originary from Asia, cannabis has been used for medical, spiritual, religious or recreational purposes for at least 5,000 years (a Chinese pharmacological treatise attributed to Emperor Shen Nung, dated 2737 BC, contains the first reference to the use of cannabis as medicine). The Aryans smoked cannabis and they may have been to teach the properties of cannabis to both the Indian people and the ancient Assyrians (Curran et al., 2016, Mechoulam et al., 2014).
The date on which cannabis was introduced into Central, Northern and Western Europe is unknown, but it probably dates back at least 500 years before Christ, since an urn containing cannabis leaves and seeds was found in Berlin about 2,500 years ago. Also a few centuries before Christ, before the advent of the Roman Empire, various European peoples such as the Celts and the Picts cultivated and used cannabis. From then on, cannabis cultivation in Europe has been common, if not massive, for centuries. Europeans also knew, of course, the recreational potential of the plant (Mechoulam and Parker, 2013).
The medical use of cannabis was introduced into Europe only around 1840 by a young Irish doctor named William O'Shaughnessy. He worked in India, for the East India Trading Company, where the medical use of cannabis was widespread. In the following decades, cannabis had a short period of popularity in both Europe and the United States. At the peak of its popularity, different medicinal preparations were available, with cannabis as an active ingredient, which were prescribed for various indications, such as menstrual cramps, asthma, coughs, insomnia, childbirth pains, migraines, throat infections and opium withdrawal symptoms.

The use of cannabis as a medicine disappeared at the beginning of the 20th century due to some of its chemical and physical properties, which made impossible the creation of medicinal preparations standardized and therefore reliable. Therefore, its place was replaced by opium-derived medicines such as morphine and codeine.

Scientific research aimed at understanding the mechanisms underlying the effects of cannabis on the brain started in the 20th century. Indeed, in the two decades that followed the identification and synthesis of Δ9-tetrahydrocanabinol (THC), the psychoactive molecule of Cannabis, by scientists Mechoulam and his colleague Yoel Gaoni in Israel in 1964 (Gaoni and Mechoulam, 1964), scientists collected a huge *plethora* on notions on the pharmacology, biochemistry and clinical effects of Cannabis.

In 1990, a potent THC-like molecule synthesised by Pfizer (CP55,940) enabled researchers to begin mapping the precise positions of cannabinoid receptors in the brain, following the signals emitted by a radioactive tag bound to this molecule (Herkenham et al. 1990).

In the same year, at a conference of the National Academy of Science's Institute of Medicine, Dr. Lisa Matsuda announced that she and her colleagues at the National Institute of Mental Health (NIMH) had made a fundamental discovery by locating the precise sequence of DNA coding for THC-sensitive receptors in the mouse brain. Dr Matsuda also announced that she had successfully cloned the sensitive receptor to Cannabis, and called it Cannabinoid-type 1 receptor (CB_1) (Matsuda, et al. 1990).

The greatest advances in Neurophysiology of cannabinoids started in the 1990s and was followed by 25 years of scientific evidence, which gave a fairly clear picture of the ECS functionality.

In 1992, a collaboration between researchers brought to light a new neurotransmitter, called the "endocannabinoid" (eCB), a molecule that binds with the same receptors in the brain that are sensitive to THC. They called this substance "Anandamide", (AEA) (Devane et al. 1992).

In 1995 the Mechoulam group discovered, in parallel with another group of Japanese researchers, a second important eCB, 2-arachidonylglycerol, (2-AG) (Sugiura et al. 1995).

This eCB binds not only to CB_1 receptors mainly present in the brain, but also to a second type, called CB_2 receptors, which was identified in 1993 (Munro et al. 1993).

In 1998, Professor Vincenzo di Marzo defined the ECS as "A central regulator capable of modulating and balancing the main activities of organisms such as eating, sleeping, relaxing, protecting and forgetting" (Di Marzo et al. 1998).

In the following sections, I will introduce the ECS also making an excursus of the main and important findings that have highlighted the functional relevance of this system in the pathophysiology of the central nervous system.

The aim of the thesis is investigating how the ECS can modulate some of the most important physiological and adaptive functions of our organism: memory, learning and synaptic plasticity. I will focus my attention on hippocampal circuits and dopaminocetive cells in this brain area.

II – Cannabinoid receptors

The isolation and purification of the components of marijuana, cannabidiol and THC, which took place in the '60 years (Mechoulam et al. 1965; Mechoulam and Gaoni, 1965), led to an explosion of cannabinoid research but, paradoxically, their molecular mechanisms of action were still not fully elucidated. One of the first studies that proved the intracellular effects of cannabinoids and their action through a receptor was that carried out by the pioneer Howlett (Howlett, 1984), using neuroblastoma membrane preparations. He demonstrated the decrease in the accumulation of cyclic AMP (cAMP) stimulated by prostanoids, and therefore an inhibition of adenylate cyclase by THC. Later, Howlett and colleagues demonstrated that this effect required a functional Gi protein (Howlett et al. 1986). In 1988 this group was the first to isolate and characterize a cannabinoid receptor in the rat brain (Devane et al. 1988). The gene encoding this receptor was then identified and cloned by Matsuda et al. (1990) and called cannabinoid receptor type 1 (CB_1). A second receptor was identified in the HL-60 cell line in 1993 and named cannabinoid receptor type 2 (CB_2) (Munro et al. 1993).

CB_1 and CB_2 receptors belong to the 7-domain transmembrane receptor family. Most of the biological effects described for cannabinoids are mediated by the coupling of Gi/Go type G proteins (GPCRs). GPCRs are a diverse family of eukaryote-specific membrane receptors which convert external signals, such as light, peptides, lipids and proteins, into specific cellular responses. Their key role in cellular signaling has made them the central focus of modern drug discovery (Hauser et al. 2017). GPCR domains comprise the extracellular N terminus, seven transmembrane alpha helices (TM), loops connecting the TMs, and an intracellular C terminus. The ligand generally binds through a binding site gap formed by the TM bundle, directly by a pocket formed by the extracellular loops, or a combination of extracellular loop and binding site gap residues. Binding induces a conformational change in the receptor, causing activation of a G-protein which then initiates a specific cellular process (Latorraca et al. 2017; Weis and Kobilka, 2014).

The human CB_1 and CB_2 receptors are closely related GPCRs, exhibiting approximately 44% amino acid similarity overall and 68% homology in the TMs (Munro et al. 1993; Hryhorowicz et al. 2019).

It appears that CB_1 can pair to both Gi and Go, while CB_2 is thought to preferentially pair to Go (Glass

and Northup, 1999). This coupling difference could explain the variation in THC's effectiveness in activating these CB_1 and CB_2 receptors: although THC binds with the same affinity to both CB_1 and CB_2 receptors, it activates CB_1 but does not, or only partially, the CB_2 (Bayewitch et al. 1996).
Although CB_1 and CB_2 are well known and characterized, numerous pharmacological studies suggest the existence of additional metabotropic and ionotropic cannabinoid receptors able to respond to the endogenous agonists. Among these, the transient receptor potential vanilloid type 1 (TRPV1) ion channel, which was found to bind some cannabinoid ligands (Caterina et al. 1997), G-protein-coupled receptor 55 (GPR55) and G-protein-coupled receptor 119 (GPR119) as novel potential cannabinoid receptors (Brown, 2007).
GPR55 was cloned in mouse, rat and human (Ryberg et al. 2007). The human GPR55 (hGPR55) gene is located on chromosome 2 and encodes a protein of 319 amino acids, shares only 14% sequence identity with the CB_1 and CB_2 receptors and is mainly expressed in the brain (caudate and putamen) (Ross, 2009). The human GPR119 is encoded by a protein of 335 amino acids and isoforms of this receptor are present in various mammalian species (Fredriksson et al. 2003). Expression profiles of GPR119 mRNA receptor seem to be restricted to the pancreas, foetal liver and gastrointestinal tract in human (Overton et al. 2008).
Because CB_1 receptors are known to mediate the majority of the cannabinoid-induced psychotropic effects, studying the role of CB_1 receptors in brain physiology and pathology is a major topic in cannabinoid research. In next sections, I will thereby concentrate on CB_1 receptor and how its physiology and biology is involved in brain plasticity and behavior and what is its role in the modulation of brain functions.

III – Localization of CB_1 receptors in the brain

The cannabinoid receptor type-1 (CB_1) is considered one of the GPCRs with the highest expression rate in the brain (Busquets-Garcia et al. 2018a). CB_1 receptors are most highly expressed by the axons and presynaptic terminals of neurons in the neocortex, olfactory system, amygdala, striatum, cerebellum, thalamus, substantia nigra, ventral tegmental area (VTA), periaqueductal gray and the spinal cord and hippocampus (Figure 1) (Busquets-Garcia et al. 2016; Hu and Mackie, 2015; Marsicano and Kuner, 2008; Soria-Gomez et al. 2017), the latter, which will be the main region of interest of this thesis.

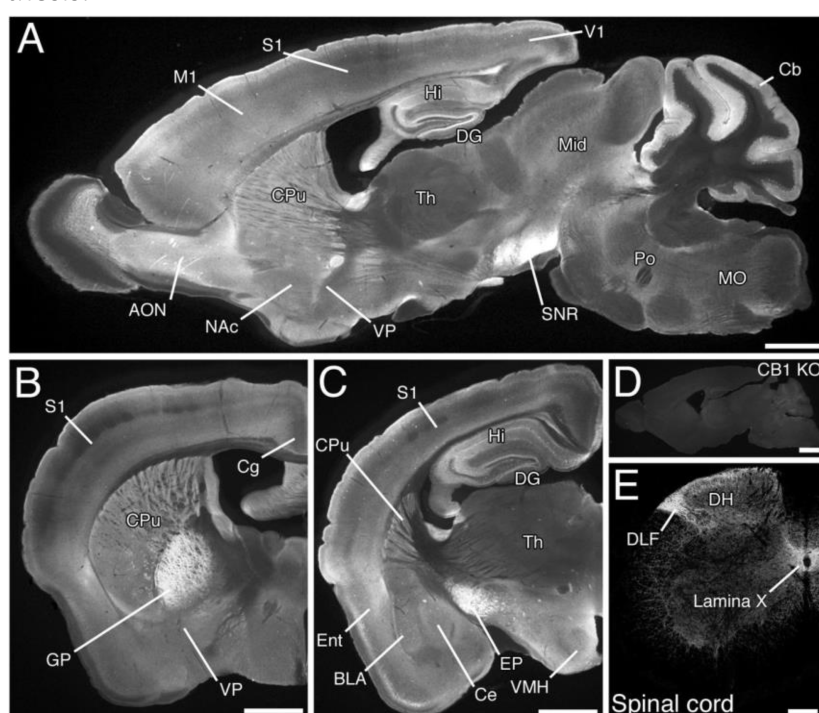

FIGURE 1 – DISTRIBUTION OF CB1 RECEPTORS IN THE ADULT MOUSE BRAIN
Distribution of CB1 receptors in the central nervous system of adult mice. *A–D*: overall distribution in parasagittal (*A* and *D*) and coronal (*B* and *C*) brain sections of wild-type (*A–C*) and CB1-knockout (*D*) mice immunolabeled with a high-titer polyclonal antibody against the COOH terminus of mouse CB1 receptor [443–473 amino acid residues, GenBank accession no. NM007726; Fukudome et al., 2004]. CB1 immunoreactivity is highest along striatal output pathways, including the substantia nigra pars reticulata (SNR), globus pallidus (GP), and entopeduncular nucleus (EP). High levels are also observed in the hippocampus (Hi), dentate gyrus (DG), and cerebral cortex, such as the primary somatosensory cortex (S1), primary motor cortex (M1), primary visual cortex (V1), cingulate cortex (Cg), and entorhinal cortex (Ent). High levels are also noted in the basolateral amygdaloid nucleus (BLA), anterior olfactory nucleus (AON), caudate putamen (CPu), ventromedial hypothalamus (VMH), and cerebellar cortex (Cb). Virtual lack of immunostaining in CB1-knockout (KO) mice indicates the specificity of the CB1 immunolabeling. *E*: CB1 immunolabeling in the spinal cord. Note that striking CB1 immunoreactivity is seen in the superficial dorsal horn (DH), dorsolateral funiculus (DLF), and lamina X.
NAc, nucleus accumbens; VP, ventral pallidum; Ce, central amygdaloid nucleus; Th, thalamus; Mid, midbrain; Po, pons; MO, medulla oblongata; Or, stratum oriens; Py, pyramidal cell layer; Ra, stratum radiatum; Lm, lacunosum moleculare layer; Mo, dentate molecular layer; Gr, dentate granular layer; ML, cerebellar molecular layer; PC, Purkinje cell layer; GL cerebellar granular layer; LI, lamina I; LIIo, outer lamina II; LIIi, inner lamina II. Scale bars: 1 mm (*A–C*, *E*); 200 ∝m (*D*). [(A-E) from Kano et al. 2009].

III.A – CB_1 LOCALIZATION IN DIFFERENT NEURONAL POPULATIONS IN THE HIPPOCAMPUS

The hippocampus is a forebrain structure that participates in cognitive functions such as learning, memory, and sensory integration (Scarante et al. 2017; Galve-Roperh et al. 2013; Mackie, 2005) and is one of the brain regions with the highest expression of CB_1 receptors (Marsicano and Kuner, 2008).

In the hippocampus, the CB_1 receptor is primarily localized in GABAergic neurons, but it can also be found in glutamatergic neurons (Figure 2) (Busquets-Garcia et al. 2018a; Gutierrez-Rodriguez et al. 2018; Hebert-Chatelain et al. 2016; Jimenez-Blasco et al. 2020).

GABAergic inhibitory interneurons are widely distributed throughout the different hippocampal subregions. Although they represent only 10 to 15% of the neuronal population, they provide the inhibitory input necessary to regulate excitation and facilitate neural oscillation. GABAergic cells are classified by their neuroanatomical characteristics, molecular expression profiles (parvalbumin (PV) or cholecystokinin (CCK) expressing cells), developmental origins, or their electrical activity (Pelkey et al. 2017). Notably, most CCK positive interneurons express CB_1 receptors, whereas only a little percentage of PV interneurons do (Katona et al. 1999).

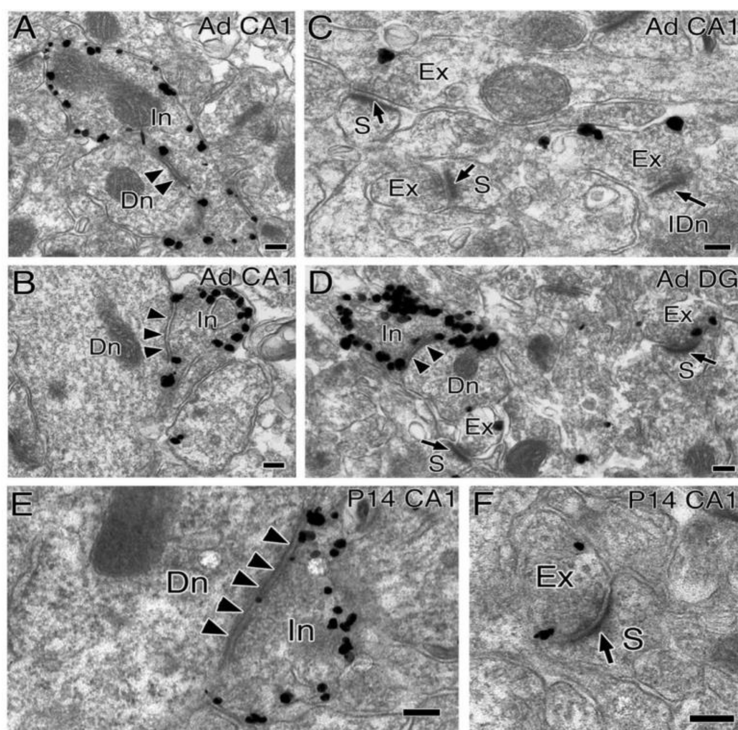

FIGURE 2 – IN THE HIPPOCAMPUS: PRESYNAPTIC TERMINAL DISTRIBUTION OF CB1 RECEPTORS
Immunoelectron microscopy showing presynaptic localization of CB1 receptors in the hippocampus. Ultrathin sections were prepared from adult (*A–D*) or P14 (*E*, *F*) mice. *A–F*: preembedding silver-enhanced immunogold for CB1 in the stratum radiatum of the CA1 region (*A–C*, *E*, *F*) and in the innermost molecular layer of the dentate gyrus (*D*). Arrowheads and arrows indicate symmetrical and asymmetrical synapses, respectively. Dn, dendrite; Ex, excitatory terminal; IDn, interneuronal dendrite; In, inhibitory terminal; S, dendritic spine. Scale bar: 100 nm. [(A-F) from Kano et al. 2009].

CB_1 receptors have been described anatomically and functionally in the glutamatergic pyramidal neurons of the CA1 and CA3 regions, although in considerably less amount as compared to GABAergic neurons (Katona et al. 2006; Marsicano and Lutz, 1999; Marsicano et al. 2003).

Besides the classic excitatory/inhibitory transmission regulation by CB_1 receptors, cannabinoid signaling also controls cholinergic and dopaminergic neurotransmitter release (Marsicano et al. 2003; Degroot et al. 2006).

III.B – CB1 LOCALIZATION IN OTHER CELL TYPES AND INTRACELLULAR ORGANELLES

During the past years, many studies confirmed by both in vitro and in vivo experiments the functional expression of CB_1 receptors in astroglial cells (Figure 3, from Han et al., 2012) (Navarrete and Araque, 2008; Han et al. 2012; Bosier et al. 2013), which can modulate glutamatergic transmission indirectly through astrocyte-dependent mechanisms such as the release of gliotransmitters like glutamate, adenosine and D-serine (Robin et al. 2018; Perea et al. 2009; Navarrete and Araque, 2010).

Although CB_1 receptors are localized primarily at the plasma membrane, more and more evidence suggest the presence of functional intracellular CB_1 receptors such as endosomes (Dudok et al. 2015) and brain mitochondria ($mtCB_1$) (Figure 3) (Bénard et al. 2012; Hebert-Chatelain et al. 2014; Koch et al. 2015; Hebert-Chatelain et al. 2016).

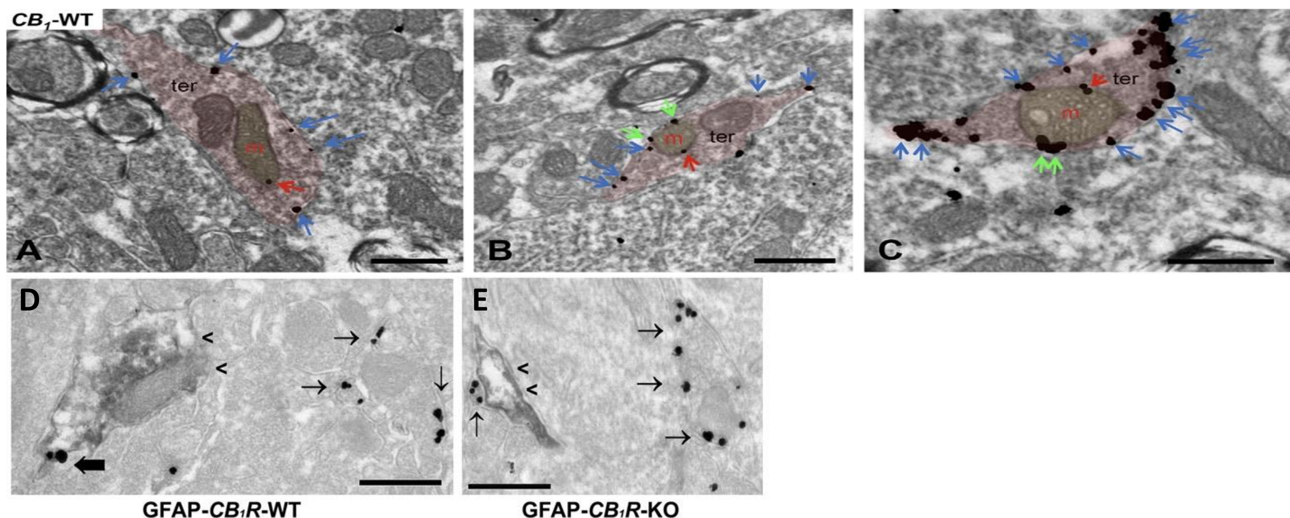

FIGURE 3 – IN THE HIPPOCAMPUS: INTRACELLULAR AND SUBCELLULAR DISTRIBUTION OF CB1 RECEPTORS
A-C. Immunoelectron detection of CB1 receptors in neuronal CA1 hippocampal mitochondria in CB1-WT by a goat anti-CB1 C-ter31 antibody combined with a pre-embedding silver-intensified immunogold method [(A-C) from Benard et al. 2012]. *D-E.* Electron microscopic images show a high density of CB1R immunopositive silver grains (small arrows) in axons/terminals of both tamoxifen-treated GFAP- *CB1R*-WT and GFAP-*CB1R*-KO mice, and a low density of silver grains (large arrow) in DAB-stained astrocytes (arrowheads) of GFAP-*CB1R*-WT mice but not of GFAP-*CB1R*-KO littermates. The scale bar represents 500 nm. [(D,E) from Han et al. 2012]

Mitochondria are a subset of subcellular organelles which play a crucial role in maintaining cellular metabolic and energetic homeostasis. Defects in mitochondrial functions and structure have been associated to different pathologies, including obesity, type-2 diabetes, and neurodegenerative diseases (Bournat and Brown, 2010; Hojlund et al. 2008; Schneeberger et al. 2013; Dietrich et al. 2013). In the central nervous system (CNS), for example in specific neurons of hypothalamus, mitochondria are also important for coordinating energy intake and expenditure regulating diet-induced obesity (Schneeberger et al. 2013; Dietrich et al. 2013).

Sevearl reports showed that different exogenous agonists of CB_1 receptors, such as THC, WIN55,212-2, HU210, or JZL195 (an inhibitor of the two enzymes responsible for the degradation of endocannabinoids) dose-dependently decreased respiration rates of purified brain mitochondria from wild-type (WT) mice, but not from CB_1-KO mice (Bénard et al. 2012) suggesting a direct link between endocannabinoids, CB_1 receptors, and brain cellular bioenergetics, which could constitute a new target for the treatment of biochemical and metabolic disorders.

IV – Endocannabinoids Synthesis, Transport and Degradation

The main endocannabinoids isolated from central and peripheral nervous system tissues are arachidonoylethanolamide (also known as anandamide, AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Figure 4) (Mechoulam et al. 1995). Structurally, endocannabinoids are lipids derived from esters, ethers and amides of long chain polyunsaturated fatty acids (Di Marzo et al. 1994). These lipid compounds are the only known endogenous molecules capable of binding to cannabinoid receptors (CB_1 and CB_2) and mimic the pharmacological and behavioral effects of Δ9-THC.

Anandamide and 2-AG posses some features that allow them to be classified as full-fledged neurotransmitters. However, a feature that distinguishes endocannabinoids from many other neuromodulators is that they are not synthesized in advance and not stored in vesicles. Classical neurotransmitters are synthesized in the cytoplasm of neurons and stored in synaptic vesicles, from which they are excreted by exocytosis into the synaptic cleft after excitation of the nerve ending by action potentials (Piomelli et al. 2000; Di Marzo et al. 1994). For endogenous canabinoids, their precursors exist in cell membranes and are cleaved by specific enzymes and released in the synaptic cleft. This form of synthesis is often referred to as "on demand", i.e. when and where necessary.

AEA is the amide formed between arachidonic acid and ethanolamine, from the hydrolysis of N-arachidonoyl-phosphatidylethanolamine. It belongs to the family of the N-acyl-ethanolamines for which the biosynthesis depends on the hydrolysis of the corresponding N-acyl-phosphatidylethanolamines (NAPE) by a phospholipase D-like enzyme (NAPE-PLD) (Di Marzo et al. 1994; Okamoto et al. 2004).

Anandamide levels in the brain are comparable to other neurotransmitters such as dopamine or serotonin. The highest levels correspond to areas of high expression of CB_1 receptors, i.e. the hippocampus, striatum, cerebellum or cortex (Di Marzo et al. 1994; Felder et al. 1996). Anandamide binds preferentially to CB_1 receptors compared to CB_2 receptors (its affinity is four times higher for CB_1).

The enzymatic cascade responsible for the formation of the second messengers inositol (1,4,5)-triphosphate (IP3) and 1,2-diacylglycerol (DAG), is involved in the biosynthesis of 2-AG. Phospholipase C (PLC) hydrolyses phosphatidylinositol (4,5)-biphosphate into DAG, which in turn is converted into 2-AG by DAG lipase (Stella et al. 1997). Two DAG lipase isoenzymes have been cloned so far, enzymatically characterized and proposed to be responsible for the formation of 2-AG (Sugiura et al. 2006). More recently, several studies provided evidence that DAG lipase α is the major isoform involved in the biosynthesis of 2-AG in the brain, using a DAG lipase knock-out mice model (Gao et al. 2010; Tanimura et al. 2010).
The formation of 2-AG has been shown to be traiggered by neuronal activity or the activation of certain receptors (e.g. acetylcholine) (Stella et al. 1997; Mechoulam et al. 1998). After its release, 2-AG can be recaptured by cells via the anandamide transporter (Piomelli et al. 1999) and then hydrolyzed by a monoacylglycerol lipase enzyme (MAGL) or the alpha/beta-Hydrolase 6 (ABDH6) (Marrs et al. 2010). 2-AG equally binds to CB_1 and CB_2 receptors and, in the brain, its levels have been found to be 170 times higher than those of anandamide (Stella et al. 1997).
It is important to remind that classical neurotransmitters are usually inactivated by facilitated re-uptake from neurons and/or astrocytes and subsequent enzymatic degradation. The endocannabinoids, as any other endogenous mediators, need mechanisms for their rapid removal from their molecular targets and subsequent degradation. As lipophilic compounds, they can rapidly and efficiently diffuse through the cell membrane. Once inside the cell, the endocannabinoids are mainly degraded by two enzymes. The fatty acid amide hydrolase (FAAH) catabolises AEA to arachidonic acid and ethanolamine (Cravatt et al. 1996) but it can also degrade 2-AG.
On tho other hand, MAGL, was identified as 2-AG hydrolases and localized in both membrane and cytosolic subcellular fractions (Ben-Shabat et al. 1998). This enzyme is highly expressed in the CNS (Dinh et al. 2002), and converts 2-AG into arachidonic acid and glycerol (Ahn et al. 2008). In hippocampal neurons, MAGL is expressed mainly presynaptically in glutamatergic and GABAergic terminals, in contrast to FAAH, which is mainly postsynaptic (Dinh et al. 2002). MAGL is localized in close proximity to CB_1 receptors to ensure a tight regulation of CB_1 receptor activity by 2-AG (Gulyas et al. 2004). At the subcellular level, MAGL have also been functionally and anatomically identified in the mitochondria (Alger and Tang, 2012; Marsicano and Kuner, 2008).

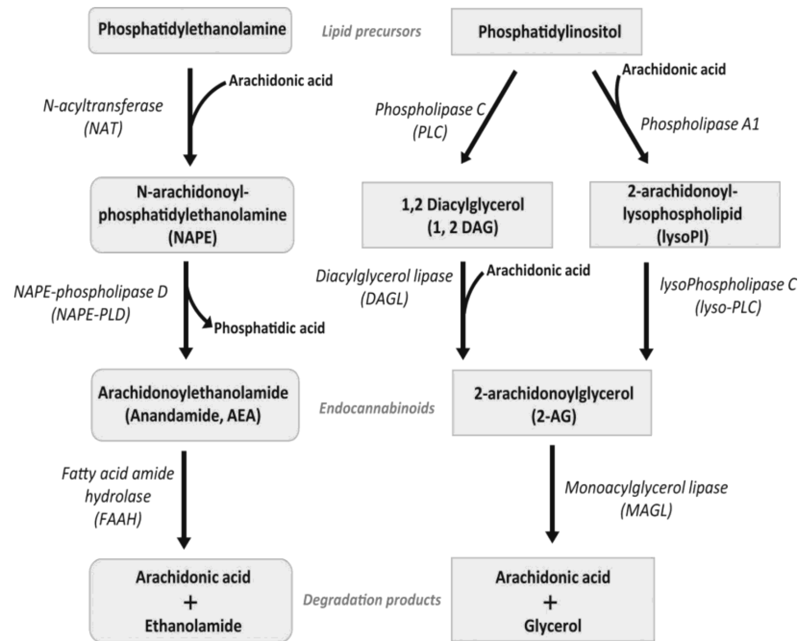

FIGURE 4 – DIFFERENT PATHWAYS INVOLVED IN THE BIOSYNTHESIS AND DEGRADATION OF AEA AND 2-AG
(From Simon and Cota, 2017)

V – Pharmacological and Genetic Tools to study the ECS

Given the wide brain distribution of the CB_1 receptor and the complexity of the endocannabinoid system, it is necessary to describe some of the main pharmacological tools to understand the specific function of CB_1 receptors at cellular, molecular and behavioral levels.

To date, the use of marijuana-derived Δ9-THC and synthetic analogues were key tools in the discovery and characterization of CB_1 receptor.

There are currently different synthetic molecules that can act as complete agonists and partial agonists. Antagonists blocking receptor action and reverse agonists reduce receptor activation below a baseline activity threshold. There are also allosteric modulators which, through binding at the allosteric sites, can modify the function of the CB_1 receptor (Mackie, 2008).

Among the synthetic CB_1 agonists, HU-210 is the most potent synthetic compound. A second group of CB_1 agonists used in pharmacological studies includes analogs of Δ9-THC, such as CP-55,940. A third group of ligands which also exhibit potent agonistic activity at CB_1 receptor includes the aminoalkylindoles, such as WIN-55,212 (Howlett, 2002).

Moreover, ACEA (arachidonoyl-2'-chloroethanolamide), a structural analogue of anandamide, has recently been characterized as a very potent and highly selective CB_1 agonist (Hillard et al. 1999).

Among the synthetic ligands showing antagonistic activity to cannabinoid receptors, SR 141716 (also known as Rimonabant) (Rinaldi-Carmona et al. 1994), AM251 (Gatley et al. 1996; Pertwee et al. 2010) and AM281 (Lan et al. 1999) are specific for CB_1.

Given the huge expression of CB_1 receptors in brain cells (e.g. glutamatergic and GABAergic neurons in the hippocampus) and circuits with apparent functional opposing effects, a valuable tool to study the specific functional role of this receptor at the molecular, cellular and behavioral level was the generation of mouse models which ubiquitously lack CB_1 receptors (Ledent et al. 1999; Marsicano et al. 2002; Zimmer et al. 1999).

However, the constitutive deletion of CB_1 receptor does not allow to study its specific contribution to brain functions (Castillo et al. 2012) but the use of the Cre recombinase (CRE)/loxP technology

allowed us to induce site-specific recombination events and to generate cell type-specific conditional KOs.

Cre recombinase is a 38 kDa protein responsible for intra- and inter-molecular recombination at the loxP recognition sites. LoxP sites are 34-base-pair long recognition sequences generally introduced into the genome by homologous recombination. These sequences do not have impact on the normal animal phenotype. Thus, mice carrying loxP sequences flanking the gene of interest (named floxed mice) are considered as WT animals (Nagy, 2000).

The Cre recombinase catalyzes the site-specific recombination event between two loxP sites (Orban et al. 1992; Sauer and Henderson, 1988), allowing the targeted excision of genes in the genome.

In order to achieve the specific deletion of the gene of interest, "floxed" mice (i.e. with the gene of interest flanked by the LoxP sequences) are crossed with a mouse that expresses the Cre recombinase under the control of a promoter specific for the cell-type to be targeted (Nagy, 2000; Orban et al. 1992). The offspring derived from breeding will express the CRE in the cell-type of interest, allowing to modify the genome by excision of the "floxed" gene and generating a cell-type specific KO mouse (Figure 5) (Nagy, 2000).

However, one of the limits of CRE/LoxP system for genetic recombination lies on the tissue- and developmental- specific activity of the promoter used to drive CRE expression (Malatesta et al. 2003). For example, using the glial fibrillary acidic protein (GFAP, a cytoskeleton protein that is commonly used as a marker for astrocytic identification) promoter to drive CRE for the generation of a conditional KO would generate a mouse with recombination in both neurons and astrocytes (Garcia et al. 2004), thus making cell-type specific functional dissection undetectable (Brenner et al. 1994).

One way to bypass this problem is to generate a system that allows time-dependent inducible gene deletion. In the case of astrocytes, to achieve cell-type specific KO, Hirrlinger and colleagues (2006) developed the tamoxifen-inducible CRE-ERT2/loxP system (Hirrlinger et al. 2006). In this model the CRE is fused to a heat mutated ligand binding domain of the estrogen receptor (ERT). The CRE-ERT2 is expressed in the cells that have GFAP but it is only active after treatment with the selective estrogen ligand tamoxifen. Accordingly, this method allows temporal control of the generation of the tissue specific KO (Hirrlinger et al. 2006) diminishing the risk of having genetic recombination in neurons.

Overall, the use of this method allowed the cell-type specific dissection of the CB_1 receptor function in different brain functions and generate several mouse lines in order to go deeper into the knowledge of molecular function of CB_1 receptor in different cell populations.

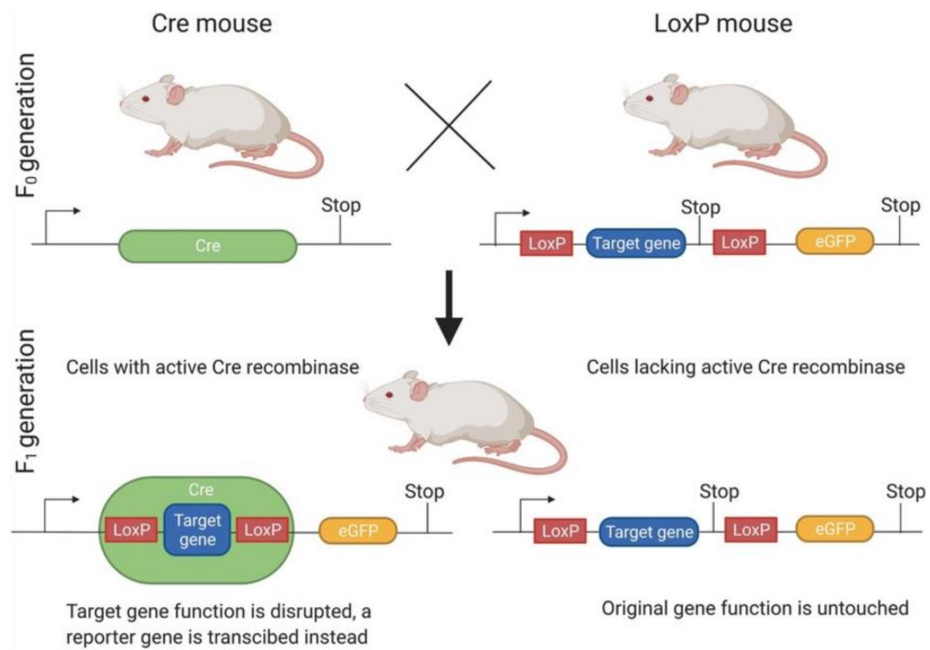

FIGURE 5 – GENERATION OF A CELL-TYPE SPECIFIC KO MOUSE
[from Shi et al. 2021]

VI – Pathological roles of the CB_1 receptor

The first traces of the medicinal use of cannabis derivatives are found in Chinese and Egyptian texts dating back several hundred years BC. Given the widespread distribution of CB_1Rs in the human body and the ubiquity of cannabinoid receptors lends itself to regulate a variety of cellular and physiological processes and, thus, serving essential role in both physiological and pathological conditions (Figure 6) (Reddy et al. 2020).

In recent decades, the endocannabinoid system has attracted considerable attention as a potential therapeutic target in numerous physiological conditions, from regulation of cellular functions, as neuromodulatory, to orchestrating complex metabolic and immune responses. Since the endocannabinoid system is vital for homeostasis of multiple biological processes, several pathological conditions pertaining to cardiovascular, neurological, metabolic and immunological diseases, are often associated with alterations of endocannabinoid system (Haspula and Clark, 2020).

This section describes the roles of cannabinoid receptors in various pathological conditions associated with changes in endocannabinoid tone. This includes some scientific evidence regarding the involvement of CB_1 receptor and the ECS in various aspects of central neural activities and disorders, including energy metabolism and eating disorders, learning and memory, anxiety and depression, pain modulation and addiction, as well as in some of the most important pathological conditions such as Parkinson's disease, Huntington's disease, Alzheimer's disease, and multiple sclerosis which are still being studied (Pacher et al. 2006; Kano et al. 2009; Di Marzo et al. 2015; Iversen, 2003).

I will mention here only some of the best-established examples, to give a general overview of endocannabinoid regulation and function and underscore the significance of understanding and manipulating the endocannabinoid system in a specific pathological condition.

The effects of eCBs on synaptic plasticity and learning and memory will not be mentioned in this section, because the role of CB_1 receptor on hippocampal memory is the main topic of my Thesis and will be discussed in the next sections.

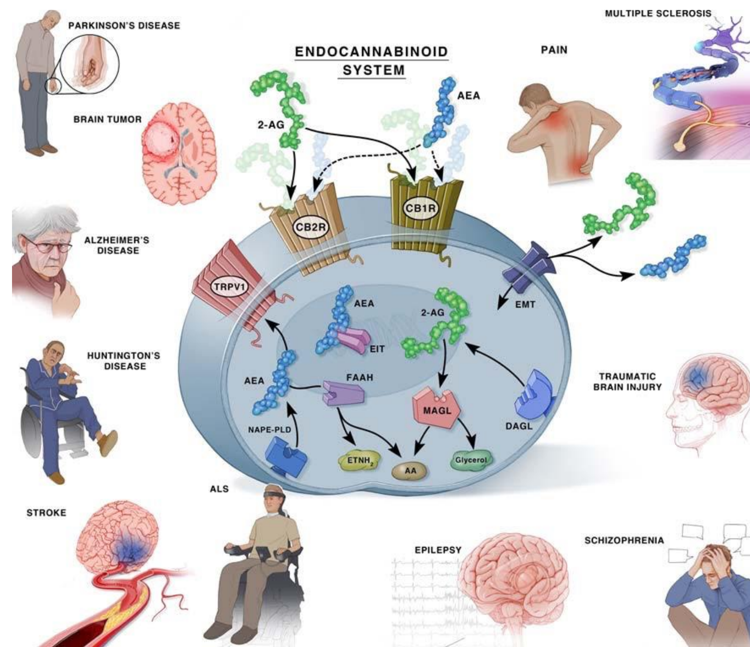

FIGURE 6 – INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN DIFFERENT NEUROPATHOLOGIES
[from Reddy et al., 2020]

VI.A – EATING DISORDERS: OBESITY

The activation of CB_1 receptors in the CNS generally induces an increase in the sensitivity to appetitive properties of food and stimulation of food intake beyond satiety. CB_1 receptor agonists increase the intake of palatable food (Brown et al. 1977; Koch and Matthews, 2001; Higgs et al. 2003; Perio et al. 2001), whereas CB_1 receptor blockade reduces it (Arnone et al. 1997; Freedland et al. 2001; Gallate et al. 1999; Simiand et al. 1998). Genetic studies with mice lacking the CB_1 receptor gene (CB_1-KO) display reduced sucrose operant responding and this phenotype appears to depend on the rewarding properties of sucrose (Sanchis-Segura et al. 2004). Different brain regions appear to be the sites where exogenous cannabinoids increase food intake (Mazier et al. 2015). Among these, the hypothalamus is the region to which researchers have pointed the importance of the ECS in the control of food intake. First, because there is abundant expression of CB_1 receptors in the hypothalamic nuclei controlling food intake (Cota et al. 2003a, 2003b; Fernandez-Ruiz et al. 1997; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999; Mazier et al. 2015). Second, because the hypothalamic endocannabinoid levels are strongly modulated by the feeding status of the animals (Di Marzo et al. 2001; Hanus et al. 2003; Kirkham et al. 2002; Mazier et al. 2015). Accordingly, direct infusion of endocannabinoids or synthetic CB_1 agonists into different

hypothalamic nuclei increases food intake (Jamshidi and Taylor, 2001; Mazier et al. 2015; Williams and Kirkham, 1999; Soria-Gomez et al. 2014a, 2014b).

The hypothalamus plays a key role in the context of exostatic functions, because increased food intake can be interpreted as a behavior bound by the change in the incentive property of external stimuli induced by the activation of the lateral hypothalamus (Trojniar and Wise, 1991; Berridge and Valenstein, 1991). On the other hand, the limbic system, has been linked to the food palatability (Jager and Witkamp, 2014; Silvestri and Di Marzo, 2013). Recently the role of the ECS in olfactory circuits and olfactory bulb, where exogenous and endogenous CB_1 receptor agonists increase perception and attractiveness of both odors and food, has been described role (Soria-Gomez et al. 2014a).

Brain CB_1 receptors can also alter satiety, which normally decrease food intake during feeding. For instance, cannabinoid agonists trigger eating in a CB_1 receptor-dependent manner (Williams and Kirkham, 1999) and their activation overcomes satiety prompts, likely by promoting the incentive value of food (Higgs et al. 2003).

CB_1 receptor antagonism reduces body weight and food intake in free-fed animals (Black, 2004; Chambers et al. 2004; Chen et al. 2004; Colombo et al. 1998; McLaughlin et al. 2003) but stronger effects were observed in obesity animals' models (Hildebrandt et al. 2003; Vickers et al. 2003; Zhou and Shearman, 2004). Accordingly, CB_1-KO mice show slight alteration of food intake under basal conditions, but these effects become much more evident under fasting-induced food intake (Bellocchio et al. 2013; Cota et al. 2003a; Di Marzo et al. 2001).

Increased endocannabinoid levels and CB_1 receptor expression are hallmarks of obesity in rodents and humans, which strongly contribute not only to the development, but also to the maintenance of the pathology (Mazier et al. 2015; Silvestri and Di Marzo, 2013). Thus, the exostatic functions of the ECS are regulated by positive feedback mechanisms by which increased ECS activity leads to higher energy accumulation. This is supported by Osei-Hyiaman and colleagues' study (2005) made in conditional mutant mice lacking hepatic CB_1 receptor. In fact, in WT mice, high-fat diet induces a large increase in liver endocannabinoid levels while it is strongly reduced in mutant animals (Osei-Hyiaman et al. 2005).

Thus, we can argue the ECS plays a physiological role in promoting exostatic processes and energy accumulation, contributing to the development of obesity and metabolic disorders when the system

is activetd beyond physiological levels.

VI.B – MOOD DISORDERS: ANXIETY AND DEPRESSION

Most of the CB_1 agonists and antagonists can produce both anxiogenic- and anxiolytic-like effects, and antidepressant- and prodepressant-like readouts. These effects are dependent on the animal species, its starting emotional state, the behavioral tests used to investigate anxiety- and depression-like behaviors, and the dose of compounds used (Paton and Pertwee, 1973; Ashton et al. 2005; Ashton et al. 1981; Viveros et al. 2005).

It is possible that the reasons for this lie on the biphasic and bidirectional effects of cannabinoids on anxiety, with low doses having anxiolytic, and high doses having anxiogenic-like effects (Chakrabarti et al. 1998; Berrendero and Maldonado, 2002; Onaivi et al. 1990; Genn et al. 2003; Marco et al. 2004; Rodriguez de Fonseca et al. 1996; Giuliani et al. 2000; Arevalo et al. 2001; Marin et al. 2003; Rey et al. 2012). On the other hand, the CB_1 antagonist SR141716A has been largerly reported to induce anxiogenic effects, suggesting that eCB tone contributes to keep anxiety low.

The mechanisms mediating the anxiolytic effects of THC involve CB_1 receptor. For instance, low doses of THC increase the time spent on open arms in elevated plus maze (EPM) in rats, an index of anxiolytic-like effects (Rubino et al. 2007). The response is species-specific because, in mice, THC produces instead a dose-dependent reduction of time spent in the open arm (Patel and Hillard, 2006). These differences could be due to the different responsiveness of GABAergic and glutamatergic neurons to CB_1 activation as well as to different expression/distribution of CB_1 receptors in rats and mice (Haller et al. 2007). Accordingly, in rats, a low dose of THC injected in the prefrontal cortex elicits anxiolytic effects, whereas in the basolateral amygdala it produces an anxiogenic response (Rubino et al. 2008a). High doses usually induce anxiogenic-like responses in rodents (Long et al. 2010; Rubino et al. 2007). Chronic exposure to THC in adolescent rats induces a depressive-like phenotype in adulthood (Realini et al. 2011; Rubino et al. 2008).

CB_1 receptors mediate the extinction of aversive memories as shown by the finding that the pharmacological inhibition or genetic lacking in mice leads to impaired extinction in a fear-conditioning test (Marsicano et al. 2002). Indeed, the administration of THC during extinction learning facilitates extinction by preventing recovery of extinguished fear in rats (Ashton et al. 2005; Grinspoon and Bakalar, 1998).

Several evidence show that the endocannabinoid system may play a role in the aetiology of depression and could represent a new therapeutic target for its treatment. CB_1-KO mice showed altered HPA (hypothalamic–pituitary–adrenal) axis function (Uriguen et al. 2004; Cota et al. 2007) and a higher sensitivity to exhibit depressive-like responses in the chronic unpredictable mild stress procedure, which suggests an increased susceptibility to develop an anhedonic state (Martin et al. 2002). These characteristics together with their heightened anxiety (Haller et al. 2002; Uriguen et al. 2004) and deficits in extinction of aversive memories (Marsicano et al. 2002) have been proposed to be analogous to certain symptoms of depression (Hill and Gorzalka, 2005).

Several cannabinoid compounds have been evaluated in antidepressant-like behavioral tests such as the forced swimming test (FST) and the tail-suspension test (TST) (McArthur and Borsini, 2006). For example, administration of AM404 (endocannabinoid uptake inhibitor) and HU-210, a potent CB_1 receptor agonist, induced decreases in immobility in FST (indicative of antidepressant activity) This effect was blocked by pretreatment with the selective CB_1 receptor antagonist AM251. In turn, the FAAH inhibitor URB597 exerted potent antidepressant-like actions in the mouse TST and the rat FST, and these effects were prevented or attenuated by rimonabant (Gobbi et al. 2005).

During the last years, there has been a huge interest on the involvement of hippocampal neurogenesis in the pathophysiology and therapy of mood disorders. Preclinical and clinical studies indicate that stress, through glucocorticoids-mediated action, and depression lead to atrophy and loss of neurons in the adult hippocampus. On the other hand, chronic antidepressant treatment up-regulates hippocampal neurogenesis which could counteract the stress-induced damage (Elder et al. 2006; Warner-Schmidt and Duman, 2006).

Furthermore, Jiang and colleagues show that both embryonic and adult rat hippocampal neural stem/progenitor cells are immunoreactive for cannabinoid receptor, indicating a possible eCBs implication in the regulation of neurogenesis. Indeed, a chronic treatment with the potent synthetic cannabinoid HU210 promotes neurogenesis in the hippocampal dentate gyrus of adult rats and exertes anxiolytic- and antidepressant-like effects (Jiang et al. 2005). These evidences strongly suggested that cannabinoid agonists might produce anxiolytic- and antidepressant-like effects by promoting hippocampal neurogenesis (Harkany et al. 2007).

In view of the above, it was speculated that the endocannabinoid system is involved in the action of antidepressant drugs as well as in HPA axis. In favor of this hypothesis, chronic administration of

the antidepressant desipramine resulted in a significant increase in the expression of the cannabinoid CB_1 receptor in both hippocampus and hypothalamus as well as in a reduction in swim stress-induced corticosterone secretion. Moreover, acute treatment with the CB_1 receptor antagonist AM251 before exposure to stress blocked the effects of desipramine on corticosterone secretion (Hill et al. 2006).

VI.C – PAIN

Pain is a “sensation” given by a combination of a subjective experience in psychophysics, an objective sensory neurophysiology, as well as an emotional reaction to distressing stimuli (Rainville, 2002). These multifaced “feelings” make it especially difficult to understand and target therapeutically.

One of the earliest uses of cannabis was treating pain as well as its various facets. As there are several modalities of pian modulation, the endocannabinoid system has shown a particular link to a variety of pain pathways (Woodhams et al. 2015; Mallet et al. 2008; Kinsey et al. 2009; Guindon and Beaulieu 2009; Clapper et al. 2010).

Multiple lines of evidence support the important role of the cannabinoid signaling system in the modulation of pain (Guindon and Hohmann, 2009; Piomelli et al. 2014; Piscitelli and Di Marzo, 2012). Moreover, the role of the ECS in pain has been widely tested in animal models of acute, neuropathic and hyperalgesic pain (Pertwee, 2001; Walker and Huang, 2002; Fox and Bevan, 2005) in immune function and inflammation (Klein, 2005; Klein et al. 1998; Klein et al. 2003; Walter and Stella, 2004) and the antinociceptive power of CB_1 agonists has also been observed in humans (Hamann and di Vadi, 1999).

Cannabinoids exert their antinociceptive effects by complex mechanisms involving effects on the central nervous system (Martin et al. 1993; Hohmann and Herkenham, 1999; Richardson et al. 1997; Richardson et al. 1998; Meng et al. 1998; Strangman et al. 1998; Fox et al. 2001; Soria-Gomez et al. 2021). This is consistent with the massive anatomical localization of CB_1 receptors in brain areas relevant to pain, for example in basal ganglia, peripheral sensory ganglia neurons, dorsal horn of the spinal cord and supraspinal brain areas, such as the periaqueductal grey (PAG), rostral ventromedial medulla (RVM), and cortex (Herkenham et al. 1991; Hohmann and Herkenham, 1998; Hohmann and Herkenham, 1999; Sañudo-Peña et al. 1999). Furthermore, using CB_1 and CB_2

knockout (KO) mice it has become clear that that endocannabinoids produce analgesia mostly via cannabinoid receptor-mediated mechanisms (Kinsey et al. 2010).

Endocannabinoids modulate nociception by lowering sensory excitability and regulating the transmission of nociceptive signals to the CNS. Local injection of AEA is able to control the first phases pain (Calignano et al. 1998) showing a CB_1-dependent action, whereas 2-AG antinociceptive effects seem to require activation of both CB_1 and CB_2 receptors (Guindon and Hohmann, 2009).

These effects seem to occur via both presynaptic CB_1 receptors retrograde activation on GABAergic terminals which disinhibit the antinociceptive excitatory neurons of the PAG, and direct TRPV1-mediated activation of these same neurons (Hu et al. 2014; Maione et al. 2007; Starowicz et al. 2007).

In agreement with the important role of the ECS in the regulation of pain and inflammation, several studies highlight the complex anti-inflammatory effects of cannabinoids in the modulation of cytokine (e.g., TNF-α, IL-12, IL-1, IL-6, and IL-10) and chemokine production (e.g., CCL2, CCL5, CXCL8, and CXCL10), the expression of adhesion molecules, and the migration, proliferation, and apoptosis of inflammatory cells (Hampson et al. 2000; Carrier et al. 2006; Klein et al. 1998; Klein et al. 2003; Walter and Stella, 2004; Klein, 2005).

VI.D – ADDICTION

The endocannabinoid system is certainly the primary site of action for the rewarding and pharmacological responses induced by cannabinoids (Ledent et al. 1999; Lichtman and Martin, 2005; Melis and Pistis, 2014; De Vries and Schoffelmeer, 2005; Fattore et al. 2007; Laviolette and Grace, 2006; Maldonado et al. 2006) and was shown to be important for the rewarding effects of most addictive compounds, including nicotine (Cohen et al. 2002; Melis and Pistis, 2014), ethanol (Wang et al. 2003; Lavanco et al. 2018; Brancato et al. 2020), morphine (Ledent et al. 1999), and cocaine (Maldonado et al. 2006).

The ability to activate the mesolimbic dopaminergic reward pathway and increase dopamine levels in the Nucleus Accumbens (NAc) is the common denominator believed to be responsible for addictive properties of different addictive drugs interacting with distinct receptors (Koob, 1992; Wise, 2004) thus, ECS has been shown to play an overall modulatory effect on the reward circuitry. Indeed, endocannabinoids modulate the activity of the mesolimbic dopaminergic pathway by

multisynaptic strengthening of the release of dopamine in the NAc shell. The dopaminergic neurons of the reward circuit are controlled by excitatory and inhibitory inputs that are modulated by CB_1 cannabinoid receptors. Thus, endocannabinoids can be released following depolarization in the NAc (Robbe et al. 2002) and from dopaminergic neurons in the VTA (Melis et al. 2004; Riegel and Lupica, 2004), and they modulate glutamatergic and GABAergic afferents by acting as retrograde messengers on CB_1 receptors. The presence of CB_1 receptors in other structures related to motivation and reward, such as the basolateral amygdala and the hippocampus, also contributes to this function (Katona et al. 2001).

A further indication that endocannabinoids may be involved in mechanisms of drug reward is the evidnce that pharmacological (Cohen et al. 2002) and genetic (Hungund et al. 2003) CB_1 receptor blockade abolishes the increase of extracellular dopamine levels in the NAc shell and the neurochemical and behavioral responses to rewarding as well as addictive substances. Similarly, THC and other agonists have been shown to increases extracellular dopamine levels in the NAc (Chen et al. 1990; Tanda et al. 1999) and boost the activity of dopaminergic neurons in the VTA via activation of CB_1 receptors (Johnson and North, 1992). The latter effect might be due to μ receptor-mediated inhibition of GABA release from the terminals of inhibitory GABAergic interneurons (Cheer et al. 2000). However, cannabinoids also inhibit glutamate release in the VTA, which would have an opposite effect on dopaminergic activity (Melis et al. 2004). Activation of CB_1 receptors on glutamatergic terminals in the NAc was reported to inhibit glutamate release onto GABAergic neurons in the NAc that project to the VTA, which may also result in disinhibition of VTA dopaminergic neurons (Robbe et al. 2001).

THC and related synthetic cannabinoid agonists also fulfill the reward-related behavioral criteria for drugs of abuse: they support conditioned place preference (CPP) (Lepore et al. 1995; Valjent and Maldonado, 2000; Zangen et al. 2006), they are self-administered intravenously or intracerebrally in a CB_1 antagonist-sensitive manner (Martellotta et al. 1998; Ledent et al. 1999; Braida et al. 2001; Zangen et al. 2006), and they reinstate cocaine-or heroine-seeking behavior in rats previously extinguished from self-administration (De Vries et al. 2001).

In a CPP paradigm, nicotine produced a significant rewarding effect in WT mice, but not in CB_1-KO mice (Castane et al. 2002). The CB_1 antagonist SR141716A reduced nicotine self-administration (Cohen et al. 2002) and nicotine-induced CPP (Le Foll and Goldberg, 2004) in rats. In a two-bottle

free-choice paradigm, ethanol preference of WT mice was reduced by SR141716A to the level observed in their CB_1-KO littermates (Wang et al. 2003). Morphine self-administration was abolished in CB_1-KO mice (Ledent et al. 1999).

Effects of CB_1 blockade on rewarding properties of cocaine were different in different paradigms (Maldonado et al. 2006). Cocaine-induced CPP was not modified in CB_1-KO mice. Moreover, cocaine self-administration was neither modified in CB_1-KO mice nor in rats treated with SR141716A. Interestingly, when the effort required to obtain a cocaine infusion was enhanced, however, acquisition of an operant response to self-administrable cocaine was impaired in CB_1-KO mice (Soria et al. 2005). This can be explained as a no-partecipation in the primary reinforcing effects of psychostimulants by endocannabinoid system, but is important for maintaining psychostimulant-seeking behavior.

These findings suggest that endocannabinoid activation of CB_1 receptors in the mesolimbic reward circuit is part of a “common pathway” of drug reward (De Vries and Schoffelmeer, 2005; Maldonado et al. 2006) supporting the idea that CB_1 antagonsits or other drugs interfering with ECs action and release might represent a suitable therapeutic tool to tackle addiction and other drug-related disorders.

PART 2 – SYNAPTIC PLASTICITY AND MEMORY

The circuitry of human brain is composed of a trillion neurons and innumerable synapses, whose connectivity underlies perception, emotion, thought, and behavior (Jorgenson et al. 2015). Several studies have revealed that the complex structure of the nervous system is genetically hard-wired but that neural circuits undergo widespread shaping and remodelling in response to a variety of external or internal stimuli, such as for the storage of information gained through experience (Ho et al. 2011).

This process of experience-dependent changes in synaptic connectivity is called synaptic plasticity, a general mechanism by which stimuli can alter brain neuronal responsiveness and allow changes in the strength and number of synaptic connections between neurons leading to learn new abilities, form new memories and generate new adaptive behaviors (Cheyne and Montgomery, 2020).

In the next sections, I will focus on the hippocampal synaptic plasticity, the most important form of plasticity involved in learning and memory. In addition, I will first attempt to provide a broad overview of the mechanisms of synaptic plasticity emphasize current understanding of the cellular mechanisms and possible functions of phenomena commonly termed long-term potentiation (LTP). Following I will deeply go in the discussion of CB_1 receptor modulation of synaptic transmission and plasticity and the its potential role in the regulation of learning and memory.

I – How to study Synaptic Plasticity?

One of the most versatile approaches to study the role of endocannabinoid system in synaptic plasticity is the electrophysiological investigation of the electrical properties of neuronal networks. Indeed, electrophysiological recordings of synaptic currents have since long been the gold standard for measuring the efficacy of synaptic transmission. Electrophysiology allows the investigation from single cells to populations both in cellular cultures, in vitro slices and in vivo anesthetized, head restrained or freely moving animals. That allows us to understand how neurons communicate and how different cell-types interact, drawning regarding their function and dysfunction in the patho-physiology of the brain.

To investigate neural activity, researchers have developed various types of electrophysiological recording techniques, roughly divided into extracellular and intracellular methods. While extracellular recordings enable us to obtain data on neuronal firing generated by multiple cells surrounding recording electrodes, intracellular recordings allow for the measurement of subthreshold membrane potential dynamics and supra-threshold firing activity at the single-cell level (Noguchi et al. 2021). In vitro electrophysiological techniques in CNS studies exploit ionic conductance of ion-channels and transient modulation of the membrane potential of a neuron. If the membrane potential becomes sufficiently depolarized, an action potential will trigger. Many in vitro electrophysiology techniques have been developed to detect and manipulate ion-channel function and/or action potential generation (Accardi et al. 2016).

The most commonly employed in vitro intracellular electrophysiological technique is the patch-clamp method. In order to attempt to patch-clamp neurons, the researchers press the recording pipette, filled with an artificial intracellular solution onto the cell membrane and tightly seal the membrane with a resistance of >1 GΩ between the pipette and the membrane. This recording mode enables us to capture the dynamics of the membrane currents generated by ions through ion channels on the cell membrane. Once negative pressure is applied and a small hole is made on the cell membrane (“whole-cell”), the net dynamics of currents and voltages ("voltage clamp" and "current clamp" mode) generated through all ion channels expressed on the cell membrane can be measured. Voltage clamp aims at recording the changes in current across the membranes by holding

voltage at a specific value. Current clamp allows the study of the membrane potential when injecting current and give information about the ionic conductance of the membrane (Accardi et al. 2016).
One of the principle limitations of both the patch-clamp and impalement techniques is that they are intracellular recording systems and, as such, are limited to assessment of individual neurons. Thus, extracellular recordings have been developed to study neuronal circuit connectivity, physiology and pathology (Spira and Hai, 2013). Extracellular recording techniques insert electrodes into tissue which, depending on the positioning and tip size, measure the extracellular field potential generated by an action potential discharge from either a single neuron or neuronal population. Therefore, extracellular recordings can be used to understand neuronal communication, information encoding, propagation, processing and computation of neuronal circuits (Obien et al. 2014). These recordings can be carried out either in vivo on anesthetized or awake animals, ex vivo on brain slices or in vitro in cultured embryonic neuronal tissue (Legatt et al. 1980; Gray et al. 1995; Juergens et al. 1999; Erickson et al. 2008).
Focusing on the hippocampus, Hahn et al. first achieved in vivo whole-cell recordings from hippocampal pyramidal cells, dentate granule cells and even hippocampal interneurons of unconscious animals (Epsztein et al. 2006; Hahn et al. 2006, 2007, 2012; Leitner et al. 2016). For instance, the study of LTP can be done by recording the ionic extracellular potentials (from a group of neurons) that are artificially induced by a stimulation electrode in another brain region (e.g. Shaffer collateral to CA1 pathway) (Zhang et al. 2014). In this case, what we call potentiation is purely an increase in the extracellular field excitatory post synaptic potentials (fEPSP) that is a correlation of synaptic changes induced by the stimulation (e.g. increase in receptor concentration, among others) (Zhang et al. 2014).
The study of the endocannabinoid system in the brain improved with the development of advanced techniques for example combining in vivo whole-cell recording methods with optic techniques. This combination allowed to optogenetically manipulate specific neural activities on a restricted time scale and capture individual cellular activity based on fluorescence, uncovering additional characteristics of cellular activity in terms of anatomical connections, genetic properties, and collective activity associated with brain function (Kitamura and Häusser, 2011; Mateo et al. 2011; Lien and Scanziani, 2013; Pala and Petersen, 2015; Reinhold et al. 2015; Valeeva et al. 2016; Zucca et al. 2017; Kato et al. 2017; González-Rueda et al. 2018; Noguchi et al. 2021).

To optogenetically manipulate neurons, researchers first genetically expressed channelrhodopsin-2 (ChR2) in neurons projecting to cells in the brain regions where membrane potentials are recorded. It is a protein serving as sensory photoreceptor that is activated/inactivated in response to photostimulation; it functions as light-gated ion channel that allows ion trafficking (i.e., electric current) through the cell membrane (Deubner et al. 2019; Adamantidis et al. 2014; Deisseroth, 2015; Boyden, 2015; Gautier 2014). This technique enabled to demonstrate the feasibility of empirically evaluating synaptic connectivity between specific neurons in vivo (Mateo et al. 2011; Pala and Petersen, 2015; Valeeva et al. 2016; van Welie et al. 2016; Arlt and Häusser, 2020).

Currently, a new powerful approach was developed: the combination of electrophysiology with imaging techniques. For example, in vivo optical imaging (including voltage-sensitive dye (VSD) imaging (Petersen et al. 2003), two-photon calcium imaging (Kitamura and Häusser, 2011) or stimulated emission depletion (STED) microscopy was simultaneously performed with whole-cell recording to capture neural activity in a wider area than single whole-cell recording alone and allow the imaging of nanoscopic structures in the brain (Takasaki et al. 2013).

These new arrays of techniques have given a fairly broad picture of the endocannabinoid system functionality in different neural circuits, and the advent of new technologies will provide just as much evidence of its contribution to brain physiology.

II – Hippocampal Synaptic Plasticity

A well-studied brain area for investigating synaptic plasticity in the nervous system is the hippocampus (Fig. 7). Critical for memory formation, the anatomy of the hippocampus renders it particularly suitable for electrophysiological investigation (Ho et al. 2011). It is composed of two closely interconnected regions, Ammon's horn and the Dentate Gyrus. Ammon's horn is formed by a layer of principal neurons, the pyramidal neurons, and is subdivided into three regions called CA1, CA2 and CA3. The Dentate Gyrus is formed by a layer of principal neurons called granular cells (Hammond et al. 2015).

The pyramidal cells of CA3 have branched axons. Some of these leave the hippocampus and project to other structures. The other ones are collaterals that form synapses with dendrites of CA1 pyramidal neurons. These collaterals form the Schaffer collateral pathway; and their terminals form asymmetrical synapses with the numerous spines of CA1 dendrites in stratum radiatum and to a lesser extent on the basal dendrites in stratum oriens (Figure 7A) (Hjorth-Simonsen, 1973; Swanson et al. 1978; Laurberg, 1979). These synapses are excitatory and the neurotransmitter is the glutamate.

In the '70 years Bliss and Lomo showed that the Schaffer collateral-commissural projection displays considerable neuronal plasticity (Bliss and Lomo, 1973). Most striking is the LTP produced by brief, high frequency stimulation (HFS) of the pathway (Schwartzkroin and Wester, 1975; Alger and Teyler,

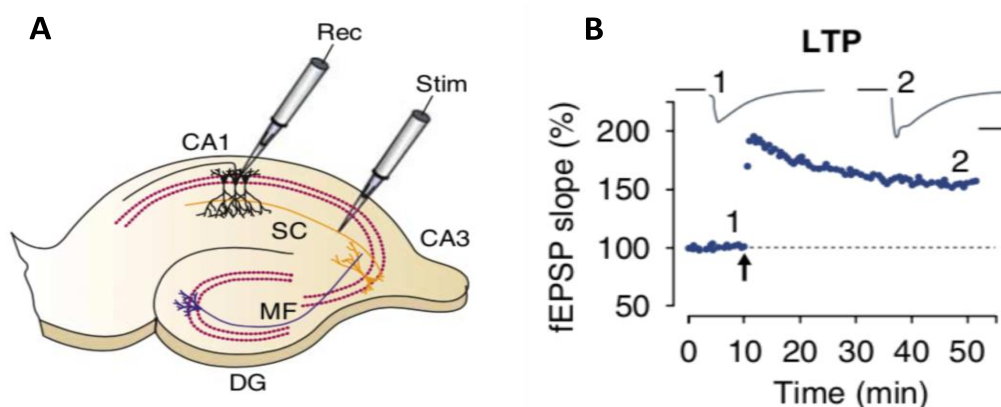

FIGURE 7 – LTP AT HIPPOCAMPAL CA1 SYNAPSES
A. Rapresentation of the CA3-Shaffer collateral to CA1 synaptic pathway in Hippocampus. Axons from the CA3 pyramidal cells (in orange) synapse with the dendrites of the CA1 pyramidal neurons. By electrical stimulation via an electrode in the CA3 axons it is possible to neuronal field or individual responses in the CA1 neurons. **B.** LTP elicited by high-frequency tetanic stimulation. [(A) and (B) from Citri and Malenka, 2008].

1976; Dunwiddie and Lynch, 1978). This potentiation, which in vivo may last for days (Buzsáki, 1980), has been considered as a useful model for memory formation (Cotman et al. 1981).

III – Mechanism of NMDA receptor-dependent LTP

As said previously, it is widely believed that experience of any sort modifies subsequent behavior through activity-dependent, long-lasting modifications of synaptic strength. Experimental support for the existence of such long-lasting, activity-dependent changes in synaptic strength came from Bliss and colleagues (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) which reported that a brief HFS of hippocampal excitatory synapses, typically referred to as a tetanus, produced a rapid and long-lasting increase in the strength of these synapses that could persist for long time (Bliss and Gardner-Medwin, 1973). Over the last three decades, this phenomenon, named long terl potentiation (LTP), has been the object of intense investigation because it is widely believed to be the key to understanding some of the cellular and molecular mechanisms by which memories are shaped (Figure 7B) (Martin et al. 2000; Pastalkova et al. 2006; Whitlock et al. 2006; Citri and Malenka, 2008). It is now clear that hippocampal LTP is only one of several different forms of long-term synaptic plasticity that exist in specific circuits in the mammalian brain.

Others most extensively studied long-term forms of synaptic plasticity observed in the CA1 region of the hippocampus and important in the modulation of neuronal circuits include long-term depression (LTD), spike-timing-dependent plasticity, excitatory postsynaptic potential (EPSP) -spike potentiation and depotentiation (Neves et al. 2008). However, these forms of plasticity won't be discussed in the thesis.

A major advance in the understanding of excitatory synaptic function and hippocampal LTP was the demonstration that two major types of ionotropic glutamate receptors contribute to the postsynaptic response at glutamatergic synapses, a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (Collingridge et al. 1983; Bliss and Collingridge, 1993). Interestingly, Morris and colleagues, by using selective NMDAR antagonists which impaired learning and memory and *in vivo* hippocampal LTP (Morris et al. 1986) provided the first evidence probing the role of LTP and ionotropic glutamatergic transmission in learning and memory (Morris et al. 1986).

LTP can be induced chemically (Stewart et al. 2005), electrically (Bliss and Lomo, 1973; Neves et al. 2008) and optogenetically (Nabavi et al. 2014) both *in vitro* and *in vivo*. These procedures are applied

to mimic neuronal firing able to induce synaptic changes that, depending on the intensity and frequency of the stimulation, potentiate or weaken the synaptic transmission (Nicoll, 2017). LTP is a process that has an early induction phase (early-LTP, lasting around 60 minutes) in which external signals rapidly cause a biochemical cascade that results in a change in synaptic efficacy and a late phase (late-LTP) where these changes are maintained over long periods of time (Figure 8A,B) (Malenka and Bear, 2004). The induction phase requires activation of postsynaptic NMDA receptors by synaptically released glutamate during action potential to the axon terminal. The release of neurotransmitter occurs after the fusion of the vesicles with the membrane from presynaptic terminal (Malenka and Bear, 2004) and binding to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the postsynaptic terminals (Figure 8A) (Malinow and Malenka, 2002). The AMPA receptor has a channel that is permeable to Na+ and K+ and, when the cell is close to its resting membrane potential, its activation provides most of the inward current that generates the excitatory synaptic response. Consequently, AMPA receptor activation quickly depolarizes the membrane of the spine to prime NMDA receptors (Malinow and Malenka, 2002). In contrast, NMDARs have a high Ca2+ permeability relative to AMPA receptors and exhibit a block of its channel at negative membrane potentials by extracellular Mg2+ (Mayer et al. 1984; Nowak et al. 1984; Paoletti et al. 2013). However, when the postsynaptic cell is depolarized, the Mg2+ block is removed and it results in the entry of Ca2+ into the postsynaptic dendrite spine (Nicoll, 2017). The consequent rise in the concentration of intracellular Ca2+ is thought to be a necessary and perhaps sufficient for triggering LTP.

Much of the work on NMDAR-dependent LTP has focused on the mechanisms responsible for its initial 30–60min. Nonetheless, the mechanisms that allow LTP to persist for hours, days, or even longer are of great importance. An elevated number of proteins have been suggested to play a key role in translating the calcium signal that is required to trigger LTP into the long-lasting increase in synaptic strength (Malenka and Bear, 2004). Among these, calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) fulfills these requirements and is a key component of the molecular machinery for LTP. CaMKII undergoes autophosphorylation after the triggering of LTP (Barria et al. 1997), and LTP induction was prevented both in knockout mice lacking a critical CaMKII subunit (Silva et al. 1992), and in knock-in animals in which endogenous CaMKII was replaced with a form lacking the autophosphorylation site (Giese et al. 1998). Furthermore, inhibition of CaMKII activity

by directly loading postsynaptic cells with peptides that impair CaMKII function blocks LTP (Malenka et al. 1989; Malinow et al. 1989), whereas acutely increasing the postsynaptic concentration of active CaMKII increases synaptic strength and occludes LTP (Lledo et al. 1995; Pettit et al. 1994). On the same line, also the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway has also been suggested to be important for LTP, as well as for learning and memory (Sweatt, 2004; Thomas and Huganir, 2004). All these proteins activate key transcription factors that may include cAMP response element-binding protein (CREB) and immediate-early genes such as c-Fos and Zif268/Egr-1 (Thomas and Huganir, 2004). These transcriptional complexes presumably promote expression of effector genes/porteins that are required for maintaining long-lasting LTP. Another compelling possibility for the maintenance mechanism of LTP is the presynaptic structural remodeling of potentiated synapses as well as local dendritic protein synthesis (Figure 8B) (Lüscher and Nicoll, 2000; Reymann and Frey, 2007; Sutton and Schuman, 2006; Zhou et al. 2006).

Given the presynaptic localization of CB_1 receptor, in the next section I will discuss about CB_1-dependent modulation of synaptic transmission and its impact on short- and long-term plasticity as well as the role on learning and memory.

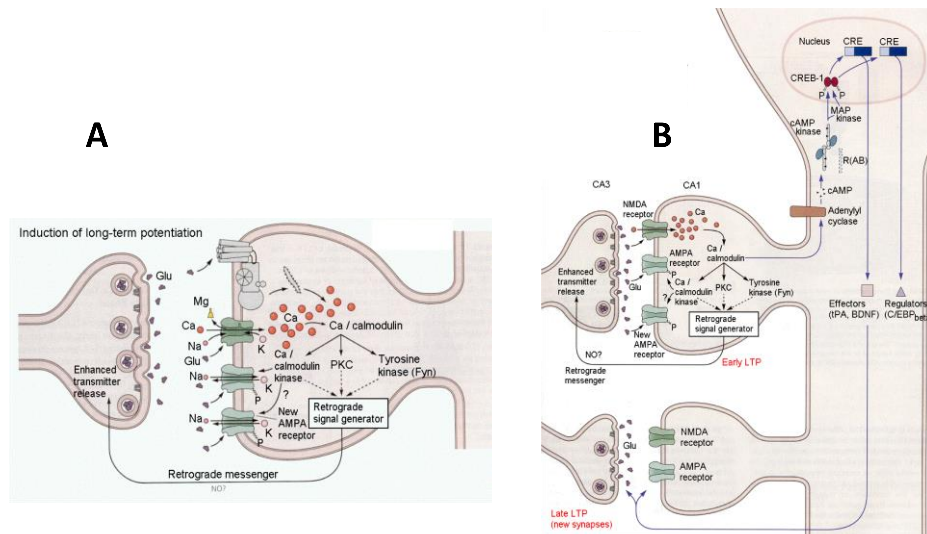

FIGURE 8 – MOLECULAR MECHANISM OF LONG-TERM POTENTIATION
A. Induction of Long-Term Potentiation by high-frequency stimulation. All the events illustrated trigger the Ca2+ entry into the cell and actives Ca-dependent kinases leading to the neurotransmitter increase. **B.** Early and late-phase of LTP. [(A) and (B) from Kandel et al. 2000]

IV – CB_1-mediated Modulation of Synaptic Activity

Some forms of plasticity are initiated and maintained by postsynaptic mechanisms, others by presynaptic mechanisms, and still others by retrograde mechanisms (Malinow et al. 2000; Kemp and Bashir, 2001; Tao and Poo, 2001). This latter expression mechanism garnered significant attention because it required the production of a retrograde messenger that was released by postsynaptic cells and acted on presynaptic terminals altering the neurotransmitter release process (Nicoll, 2003). Post-synaptically on-demand production of endocannabinids (AEA and 2-AG) and release function as such a retrograde signal by activating CB1 receptors, mainly, in the presynaptic terminals in order to decrease neurotransmitter release (GABA and Glutamate) and are critical to the alteration of synaptic efficacy (Alger, 2002; Wilson and Nicoll, 2002).

IV.A – INTRACELLULAR SIGNALING PATHWAY OF CB_1 RECEPTORS

As discussed previously, CB_1 receptors at the presynaptic terminal are intracellularly primarily coupled with the Gi/o subunits of G proteins (Howlett and Fleming, 1984; Howlett et al. 1986).
The activation of CB_1 receptor acts mainly on three intracellular signaling pathways: adenylate cyclase, the mitogen-activated protein kinase (MAPK) pathway and some ion channels (Figure 9). Through Gi/Gs coupling, the activation of CB_1 or CB_2 receptors simultaneously induces an inhibitory signal and an activating signal depending on the cell type (Howlett and Fleming, 1984; Howlett et al. 1986; Glass and Felder, 1997; Navarrete and Araque, 2008).
Adenylate cyclase is the enzyme responsible for the production of cyclic adenosine monophosphate (cAMP), one of the main intracellular second messengers. The inhibition is reversible, dose-dependent and Gi-mediated (Howlett and Fleming, 1984; Howlett et al. 1986); a decrease in cAMP in the cell causes inhibition of PKA and an increase in tyrosine-phosphorylated proteins such as focal adhesion kinase (FAK) (Derkinderen et al. 1996). It has also been shown that CB_1 can be coupled to the Gs protein, causing an increase in cAMP levels and thus activation of PKA (Glass and Felder, 1997). MAP kinases play a key role in the processes of morphological differentiation and neuronal survival (Fukunaga and Miyamoto, 1998). Several members of the MAP kinase family are abundantly present in the brain and are activated during physiological or pathological events (ischemia,

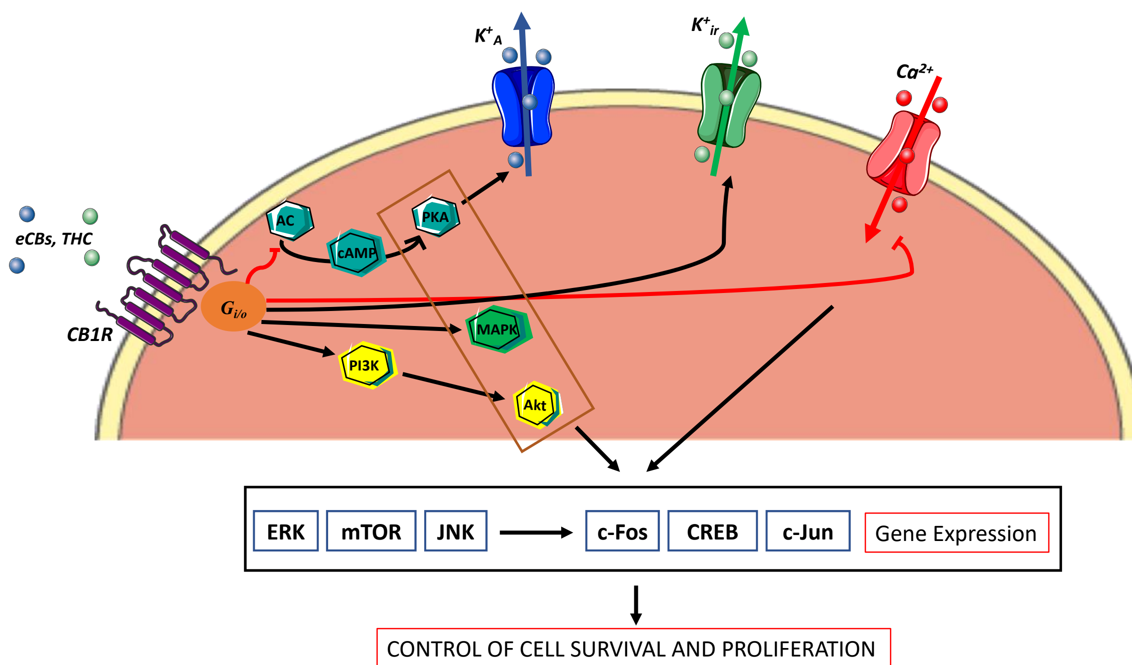

FIGURE 9 – INTRACELLULAR CB1 RECEPTOR SIGNALING PATHWAYS
Stimulation of CB1 receptors lead to multiple cascades of events. A direct modulation of ion channels. Inhibition of Ca2+ channel and activation of K+ channels. Activation of A-type K+ channels can be induced through the inhibition of adenylyl cyclase (AC). Activation of several protein kinases including ERK, JNK and mTOR leads to *de novo* gene expression. [Adapted from Pagotto et al. 2006]

epilepsy). Cannabinoids are able to activate MAP kinase as ERK1/2 (extracellular signal-related protein kinase), involved in the regulation of gene expression and protein synthesis; this effect is dose-dependent and independent inhibition of cAMP production (Bouaboula et al. 1995).

Activation of the CB_1 receptor can also causes inhibition of voltage-sensitive calcium channels (VGCC). These effects are relatively slow, reversible, mediated by a Gi/o type G protein and independent of adenylate cyclase inhibition (Caulfield and Brown, 1992; Mackie and Hille, 1992) and specific for the activation of CB_1 receptor (Ameri, 1999). These calcium channels are preferentially localized at the presynaptic level and are involved in the control of neurotransmitter release.

Cannabinoids cause potassium channel stimulation of incoming rectification (Henry and Chavkin, 1995; Mackie et al. 1995). This effect is mediated by a G-type protein Gi and depends on the state of phosphorylation of the CB_1 receptor by the protein kinase C to the serine of the third intracellular cycle of the receptor (Garcia et al. 1998). Furthermore, activation of CB_1 receptors modulates, in a dose-dependent manner, the voltage sensitivity of rapidly inactivated type A potassium channels

(Deadwyler et al. 1993). This effect is mediated by a Gi-type protein G and depends on the inhibition of adenylate cyclase and the subsequent inhibition of protein kinase A (Hampson et al. 1995; Childers and Deadwyler, 1996).

Other activating signaling pathways are also involved in the binding of cannabinoids to their receptors: Jun (c-Jun N-terminal) kinase (JNK) and p38-kinase, which are involved in the regulation of gene expression and in the processes of cell death apoptosis (Rueda et al. 2000); Akt (or protein kinase B), mainly involved in cell survival, but also in the regulation of glucose metabolism (Gómez del Pulgar et al. 2000). This divergence of signaling pathways could explain why the activation of the endocannabinoid system may exert different biological effects depending on the nature of the tissue/cell type considered.

IV.B – SHORT- AND LONG-TERM FORMS OF ENDOCANNABINOID-MEDIATED PLASTICITY

The first demonstration of retrograde eCB signaling came from the discovery that eCBs mediate forms of short-term synaptic plasticity induced at various types of GABAergic and glutamatergic synapses throughout the brain. They are known as 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). Shortly after it was shown that eCBs also mediate presynaptic forms of long-term depression (eCB-LTD) at both excitatory (Gerdeman et al. 2002; Robbe et al. 2002) and inhibitory synapses (Chevaleyre and Castillo, 2003; Marsicano et al. 2002). Thus, eCBs represent the best characterized retrograde messengers involved in the short- and long-term regulation of synaptic transmission (Wilson and Nicoll,2002; Kreitzer and Regehr, 2002; Doherty and Dingledine, 2003; Regehr et al. 2009; Heifets and Castillo, 2009; Kano et al. 2009; Castillo et al. 2012).

DSI and DSE

Presynaptic inhibition of transmitter release by endocannabinoids may adopt two different forms of short-term synaptic plasticity, depending on the involvement of GABA or glutamate transmission, respectively: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (Figure 10A) (Wilson and Nicoll, 2002; Diana and Marty, 2004).

The contribution of endocannabinoids to these forms of short-term synaptic plasticity remained unknown until the recent work of Wilson and Nicoll: in the hippocampus, inhibition of CB_1 receptors blocks DSI, whereas synthetic CBs lead to DSI occlusion (Wilson and Nicoll, 2002). This phenomenon has been also described in the cerebellum (Diana et al. 2002). Another study with slice preparations demonstrated that DSI could be induced at inhibitory synapses between CCK-positive Schaffer collateral associated interneurons in the stratum radiatum (Ali, 2007), indicating that interneurons can release endocannabinoids. Interestingly, other studies also demonstrated DSI in hippocampal pyramidal neurons can also be triggered by the activation of metabotropic glutamate receptors (mGluRs) (Varma et al. 2001) or muscarinic acetylcholine receptors (Kim et al. 2002), presumably acting on the postsynaptic neuron to stimulate the formation and release of the endocannabinoid.
In parallel, the central role of eCBs was also revealed in DSE in the cerebellum where retrograde endoCB signalling modulates glutamatergic excitatory synapses (Kreitzer and Regehr, 2002; Maejima et al. 2001). This mechanism was also observed in the hippocampal pyramidal cells (Ohno-Shosaku et al. 2002).
Hippocampal and cerebellar DSI and DSE as eCB-mediated modulatory events on synaptic transmission were later reported in other brain areas as well, including the amygdala (Zhu and Lovinger, 2005), the neocortex (Bodor et al. 2005; Trettel et al. 2004), the striatum (Uchigashima et al. 2007) and the hypothalamus (Hentges et al. 2005). Taken together, these results gave further proof of the relevant role endocannabinoid-induced DSI or DSE in physiological processes of memory.

eCB-LTD

Other forms of endocannabinoid modulation of synaptic plasticity involve the LTP and LTD (Lu and Mackie, 2016). Mechanistically diverse forms of endocannabinoid-mediated LTD have also been described, which either involve (Kellogg et al. 2009), or don't involve (Chavez et al. 2010) CB_1 receptors. Notably, endocannabinoids can induce both homosynaptic and heterosynaptic LTD (eLTD). Homosynaptic eLTD is LTD at the synapse being stimulated. It is typically evoked by persistent low frequency stimulation and is prominent at glutamatergic synapse in both dorsal and ventral striatum (Gerdeman et al. 2002; Robbe et al. 2002). Heterosynaptic eLTD occurs at synapses adjacent to the stimulated synapses. For example, stimulation of Schaffer collaterals in hippocampal

CA1 leads to a persistent decrease in GABAergic inhibition of CA1 pyramidal neurons (Chevaleyre and Castillo, 2003). The mechanism of eLTD at hippocampal inhibitory synapses appears to require inhibition of adenylyl cyclase (AC) and the involvement of the presynaptic proteins, RIM1α and RAB3B (Chevaleyre et al. 2007; Tsetsenis et al. 2011).

As discussed previously, much of the mechanistic work on the transient synaptic effects of endocannabinioids was performed in the hippocampus and cerebellum, but the first evidence regarding a form of LTD that required endocannabinoids, eCB-LTD (Figure 10B), was observed in the glutamatergic synapses onto medium spiny neurons (MNS) in the striatum as well as at synapses between layer V pyramidal neurons (Gerdeman et al. 2002; Robbe et al. 2002; Sjöström et al. 2003). In the hippocampus, in contrast, the endocannabinoid messengers regulate a form of LTD that affects inhibitory GABAergic neurons, but not excitatory ones (Chevaleyre and Castillo, 2003).

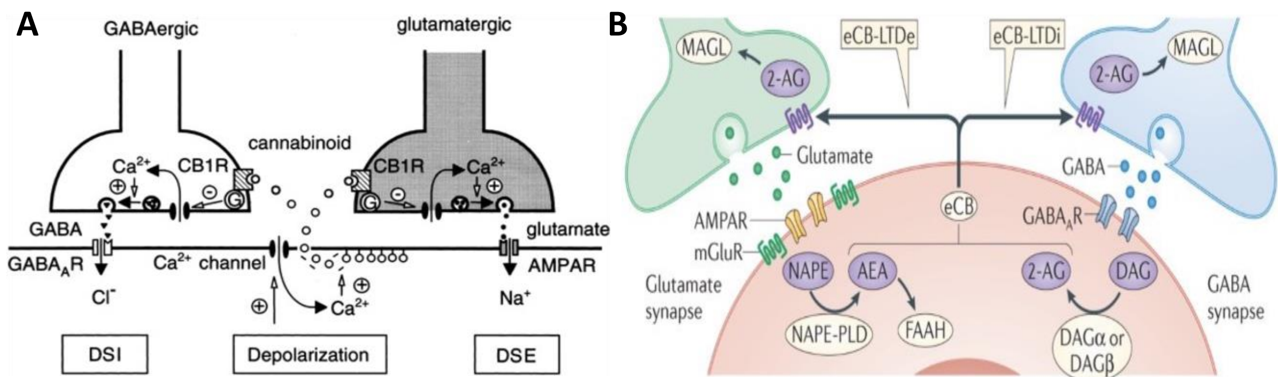

FIGURE 10 – ENDOCANNABINOID-MEDIATED SHORT- AND LONT-TERM SYNAPTIC PLASTICITY MECHANISMS
A. Depolarization of the postsynaptic terminal causes an elevation of intracellular calcium which leads to the production of endocannabinoids that travel to the presynaptic terminal to inhibit neurotransmitters, GABA or Glutamate, release by inhibiting Ca2+ channels. **B.** Description of eCB-LTD of both excitatory (glutamatergic) and inhibitory (GABAergic) afferents. [(A) from Kano et al. 2002 and (B) from Parsons and Hurd, 2015].

In conclusion, CB_1 receptors in the brain can modulate several forms of synaptic plasticity in different cell-types in different brain regions and impacting important physiological processes, such as memory which will be discussed in the next Part.

PART 3 – MEMORY

Memory is defined as the faculty of encoding, consolidating, storing, and retrieving information (Squire et al. 2007; Squire, 2009). The term "encoding" refers to the initial process of recognizing and processing newly learned information. The encoding process is influenced by several factors, including emotional-cognitive-motivational factors. The term "consolidation" corresponds to the process of transforming the learned memory. It requires a change in the structure of nerve circuits with the creation of new synaptic connections between neurons. The term "storage" concerns the mechanisms of maintenance of learned information. Finally, the term "retrieval" is fundamental to recall the stored information to the state of consciousness (Squire et al. 2007; Squire, 2009).

Neuroscientists have divided memory systems into two broad categories, declarative and non-declarative. The declarative memory system is the most familiar system of memory. It is the memory system that has a conscious component and it includes the memories of facts and events.

Non-declarative memory, also called implicit memory, includes the types of memory systems that do not have a conscious component but are nevertheless extremely important. They include the memories for skills and habits (e.g., riding a bicycle, driving a car, playing golf or tennis or a piano) and simple forms of associative learning (e.g., Pavlovian conditioning) (Squire and Zola, 1998).

Tulving (1972) distinguished two types of declarative memory: semantic memory and episodic memory. Semantic memory consists of the set of concepts, knowledge and notions that we have acquired in the course of our life experience. Episodic memory allows us to store specific aspects - in spatio-temporal and situational terms- of events.

Due to its complexity, memory formation involves many neuronal circuits within several brain regions. The hippocampus with its adjacent cortical regions (i.e. entorhinal, perirhinal and parahippocampal cortices), constitutes the medial temporal lobe, which is involved in the formation of declarative memory (Eichenbaum, 2017). Thus, it is a key brain region that has been shown to be important for episodic memory, spatial navigation and time perception (Squire et al. 2007; Howard and Eichenbaum, 2015).

I – Long-term potentiation (LTP): A Synaptic Mechanism for Memory

Activity-dependent synaptic plasticity has since long been proposed to represent the subcellular substrate of learning and memory, one of the most important behavioral processes through which we adapt to our environment (Humeau and Choquet, 2019).
The presumptive causal link between synaptic plasticity and memory has been formalized by Morris and colleagues with the "synaptic plasticity and memory hypothesis" (Martin et al. 2000):

"Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed".

Long-term forms of synaptic plasticity were discovered in the 1970s (Bliss and Lomo, 1970), and have since been extensively studied at various synapse subtypes, with the objective of understanding whether and how they mediate the modifications in brain activity that underlie behavioral adaptation. An enduring form of synaptic plasticity called LTP is believed to be involved in declarative memory. Indeed, LTP was first described in the hippocampus, a structure well established to be critically important for declarative memory (Squire et al. 2004). Much research over the last 20 years aimed at demonstrating a role for hippocampal LTP in encoding new memories (Martin et al. 2000; Morris, 2007). Animal studies revealed that controlled lesions, pharmacological inactivation or molecular knockouts limited to the hippocampus result in either a failure to learn or a loss of spatial memory (Tsien et al. 1996; Martin et al. 2005; Morris et al. 1986; Pastalkova et al. 2006). Electrophysiological recordings and molecular imaging studies in animals, as well as MRI imaging studies in humans, provide correlative evidence that episodic or episodic-like learning and memory involves changes in hippocampal activity (Berger et al. 1983; Vazdarjanova and Guzowski, 2004; Guzowksi et al. 2001; Gabrieli et al. 1997; Maguire, 2001; Henke et al. 1997).
However, linking LTP and memory has always been a difficult task. The first effort to link the two

phenomena was mainly supported by the observation that in vivo NMDA receptors antagonist (which prevent LTP manifestation) induced memory impairments in behavioral tests (Martin et al. 2005; Morris et al. 1986). In addition, Giese and colleagues, generated a knockin mouse containing the mutation of CaMKII. This prevented autophosphorylation and thereby blocked CaM-independent persistent activity of the enzyme. In this mouse, NMDA receptor function was normal, but LTP was absent and learning and memory were strongly impaired (Giese et al. 1998).

Furthermore, during an inhibitory avoidance task, LTP recorded in vivo in a subset of hippocampal CA1 pyramidal cells demonstrated that the patterns of activity generated during a real learning task were sufficient to elicit LTP (Whitlock et al. 2006). Perhaps even more convincing was the demonstration that in vivo infusion of a PKM-delta inhibitor into the hippocampus abolished the maintenance of LTP and simultaneously the storage of a long-lasting spatial memory (Pastalkova et al. 2006). These findings strongly suggest that maintained LTP was required for the engram that stored the spatial information.

Several recent studies have detected LTP-like synaptic changes in the hippocampus in vivo during certain behaviors (e.g. following learning) (Gruart et al. 2006; Whitlock et al. 2006). For instance, learning has been shown to control synapse formation and elimination (Yang et al. 2009) and, on the other hand, ablation of synapses that were formed during the acquisition phase of a certain behavior decreases the performance of that specific behavior (Hayashi-Takagi et al. 2015), according to the synaptic theory of memory storage.

Those concepts have been reinforced by the emergence of sophisticated genetic techniques, optogenetics (Govindarajan et al. 2006; Kim et al. 2017) and chemogenetics (Armbruster et al. 2007; Gomez et al. 2017) that have allowed investigators to manipulate given genes/cell populations and observe their contribution to both memory and synaptic plasticity (Silva, 2003), demonstrating that certain memories can be stored in engrams which can eventually be manipulated and eventually artificially generated (Nabavi et al. 2014; Ramirez et al. 2013).

II – CB_1 Receptors and Memory Process

The high level of expression of CB_1 receptors in the hippocampus, in conjunction with the ability to affect short- and long-term changes in synaptic strengthening, had drawn attention to processes of memory and learning (Scarante et al. 2017; Busquets-Garcia et al. 2015; Hampson and Deadwyler, 1999; Marsicano and Kuner, 2008; Soria-Gomez et al. 2017). It's known since long time that cannabinoids induce learning and memory impairments in both rodents and humans by CB_1 receptors in the brain (Broyd et al. 2016). In the hippocampus, the CB_1 receptor is primarily localized in GABAergic neurons, but it can also be found in glutamatergic neurons and astrocytes as well as in subcellular compartments (Busquets-Garcia et al. 2018a; Gutierrez-Rodrıguez et al. 2018; Hebert-Chatelain et al. 2016; Jimenez-Blasco et al. 2020).

In the last decade, the role of the endocannabinoid system, mainly the CB_1 receptor, in memory processes was studied pharmacologically and genetically through its distinct action in different cell types shaping our current understanding of this relevant and complex system. For instance, Puighermanal and colleagues have demonstrated that the suppression in GABA transmission in the hippocampus is the primary cause of the long-term amnesic effects produced by cannabinoids, as assessed by Novel Object Recognition Task (NOR, which I will discuss in detail in the next paragraph) (Puighermanal et al. 2009). Moreover, they showed that the rescue of this effect was possible by local injection of an NMDA receptors antagonist suggesting that an excess of excitation, most likely due to suppression of GABAergic inhibition, was the substrate for the observed impairment in this memory process indued by cannabinoids. Interestengly, Han and collegueas, by analyzing the acute effect of THC in short-term working memory, confirmed what had previously been suggested by Puighermanal and collegueas, namely that, the glutamate excess can also be responsible for short-term memory impairment (Han et al. 2012).

Conversely, there is evidence that physiological activation of CB_1 receptors in GABAergic neurons is essential for certain types of memory (Busquets-Garcia et al. 2015; Busquets-Garcia et al. 2018b; Oliveira da Cruz et al. 2020). For instance, the inhibition produced by endocannabinoid signaling in hippocampal GABAergic neurons is necessary for complex learning processes such as incidental associations (Busquets-Garcia et al. 2018b). Mice lacking CB_1 receptors from GABAergic cells show

deficits in mediated aversion while maintaining normal expression of direct learning (Busquets-Garcia et al. 2018b). As previously discussed, eCBs modulate synaptic function primarily through their effects on presynaptically expressed CB_1 receptors in both GABAergic and glutamatergic terminals (Castillo et al. 2012; Kano et al. 2009). In the hippocampus, eCBs exert a profound effect on inhibition by reducing GABA release in a transient (Wilson and Nicoll, 2002) or long-lasting manner by triggering long-term depression (LTD) of inhibition (iLTD) (Chevaleyre and Castillo, 2003). Thus, the excitatory-inhibitory balance can be altered and by this means contribute to associative learning (Letzkus et al. 2015). By reducing inhibition, eCBs facilitate the induction of excitatory-LTP at Schaffer collateral (SC) to CA1 pyramidal cell synapse (SC-CA1) (Carlson et al. 2002; Chevaleyre and Castillo, 2004). Changes in the LTP could play an important role in the formation memory (Xu et al. 2014).

Relevant to neuronal plasticity, is the ability of astrocytes to communicate with neurons through mobilization of calcium and neurotransmitters (Fellin, 2009). Endocannabinoids released from neurons could then activate distant neurons by activating the astroglial CB_1R, thereby impacting synaptic plasticity (Navarrete and Araque, 2010; Gómez-Gonzalo et al. 2015) and memory formation (Han et al. 2012). Activation of CB_1 receptors in astrocytes, by regulating synaptic function, may have important consequences on animal behavior. Taking advantage of transgenic mice lacking CB_1 selectively in astrocytes, it has been reported that CB_1 activation in hippocampal astrocytes likely mediates the impairment of spatial working memory and LTD at CA3-CA1 synapses induced by THC (Han et al. 2012). Furthermore, the activation CB_1 receptor through astrocyte-dependent mechanisms can modulate glutamatergic transmission triggering the release of gliotransmitters like glutamate and D-serine and thereby potentiates synaptic transmission (Robin et al. 2018; Perea et al. 2009; Navarrete and Araque, 2010). The biochemical pathway leading to production of D-serine, co-agonist of the NMDAR, takes place primarily in astrocytes (Murtas et al. 2020). Concerning the role of the gliotransmitter D-Serine in memory process, Robin and collegueas have showed recognition memory is dependent on astroglial CB_1 receptor modulation of astrocytic D-Serine production (Robin et al. 2018). Overall, it is known that CB_1 receptors in specific cell populations can modulate several aspects of brain physiology, and regulation of memory processes gives an exquisite example. Thus, dissecting the endogenous and exogenous function of CB_1 receptor will provide important insights on how CB_1 receptor is involved in the pathophysiology of brain function.

III – Novel Object Recognition (NOR) as Tool to investigate Memory

Novel object recognition (NOR) memory task is a commonly used experimental behavioral task aimed at studying different phases of learning and memory in rodents (Ennaceur and Delacour, 1988; Ennaceur, 2010).

The test relies on as few as three sessions: one habituation session, one training session, and one test session. Training simply involves exploration of two identical objects, while the test session involves replacing one of the previously explored objects with a novel object (Figure 11). Because rodents have an innate preference for novelty, a rodent that remembers the familiar object will spend more time exploring the novel object (Berlyne, 1950; Ennaceur and Delacour, 1988; Ennaceur, 2010). Thus, the increased exploration of the novel object is interpreted as indirect evidence that animals acquired a memory of the familiar object.

Finally, the NOR task can be easily modified to interfere with different phases of learning and memory (i.e., acquisition, consolidation, or recall), to assess different types of memory (e.g., spatial memory), or to assess different retention intervals (i.e., short-term vs long-term memory). NOR task is also considered a reliable model to test hippocampal and temporal lobe function, as lesions within these brain regions abolish recognition memory (Winters et al. 2008; Broadbent et al. 2010) and several components of novelty versus familiarity preference are encoded in regions such as the hippocampus (De Lima et al. 2006; Puighermanal et al. 2009, 2013).

The NOR task has been replicated using a variety of environmental designs: in a large open field (Bevins and Besheer, 2006) or in a Y-shaped maze (Winters et al. 2004). In order to overcome some limitations of the open field and Y-maze settings, such as the pro-anxiogenic effects of the environment (Hale et al. 2008) and the short length of the corridors (Winters et al. 2004) respectively, for the aim of this thesis, we used a NOR version in an L- or V-maze (Oliveira da Cruz et al. 2020b) because it has been shown to allow the study of hippocampal-dependent object recognition memory and because it has several technical advantages compared to behavioral tests for memory functions (Puighermanal et al. 2009, 2013; Busquets-Garcia et al. 2011, 2013, 2017,

2018c; Robin et al. 2018; Aso et al. 2012; Hebert-Chatelain et al. 2016; Aloisi et al. 2017).

This version of the maze maximizes the exploration time of the objects relative to the surrounding context and increases the accuracy of the test. The versatility of the NOR task provides a platform for innumerable research applications: pharmacologic and pharmacogenetic studies with agents to either disrupt or enhance memory (Urban and Roth, 2015); varying the time of drug administration before or after training, or prior to testing can lead to disrupted or enhanced memory (Lueptow et al. 2015; Rutten et al. 2007; Prickaerts et al. 2002; Bertaina-Anglade et al. 2006); studies with the optogenetic technology to look at the neural activation/inhibition that contributes to the different phases of learning and memory. The NOR task is also appropriate for assessing differences in transgenic animals or in neurodegenerative models or in aging studies (Li et al. 2008; Frick and Gresack, 2003; Grayson et al. 2015; Tuscher et al. 2015; Balderas et al. 2013; Akkerman et al. 2014). In conclusion, it provides high reproducibility and low variability in the study of memory processes.

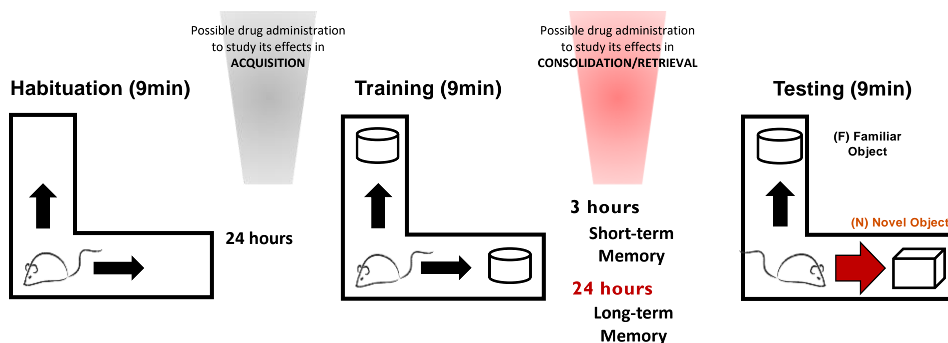

FIGURE 11 – SCHEME OF THE NOVEL OBJECT RECOGNITION TASK
The novel object recognition task consists in 3 sequential daily trials of 9 minutes each. During the habituation session (day 1, Habituation), mice are placed in the center of the maze and allowed to freely explore the arms in the absence of any objects. The acquisition session (day 2, Training) consisted in placing the mice again in the corner of the maze in the presence of two identical objects positioned at the extremities of each arm and left to freely explore the maze and the objects. The test session (day 3, Testing) consisted in replacing one of the previously explored objects with a novel object.

PART 4 – DOPAMINERGIC SYSTEM and the ECS

The dopaminergic system (DS) is involved in a multitude of brain functions: it regulates learning and memory, motivation, mood, movement, and neuroendocrine integration (Undieh, 2010). Dopamine (DA), mainly produced by the VTA, is released after novel (Ljungberg et al. 1992), salient sensory (Ungless, 2004), aversive (Bromberg-Martin et al. 2010), or reinforcement-relevant (reward) stimuli (Schultz et al. 1993). For many decades, the DS role in multiple disorders and brain disease has been intensely studied (Goto and Grace, 2007; Lodge and Grace, 2011; Del Campo et al. 2011; Kumar and Patel, 2007; Jürgensen et al. 2011). The different physiological actions of DA are mediated by two distinct groups of G protein-coupled receptor subtypes. D_1-like receptor subtypes (D_1/D_5) couple to the Gs protein that activate AC pathway. The other receptor subtypes belong to the D_2-like subfamily (D_2/D_3/D_4) and are Gi protein-coupled receptors that inhibit AC pathway and activate K+ channels (Missale et al. 1998; Vallone et al. 2000).

D_1-like receptors show high concentration in the prefrontal cortex, whereas the concentration of D_2-like receptors is elevated in the caudate nucleus, putamen, and nucleus accumbens (Missale et al. 1998; Jaber et al. 1996). Their opposite effect at the molecular level (Verhoeff, 1999; Piccini, 2004) and the expression in different cell populations makes the study of DS relatively difficult (Nagatomo et al. 2017; Romanelli et al. 2010) and, moreover, its complexity lies in its interactions with other modulatory systems, such as the ECS. Indeed, DS and ECS shares an *"intimate relationship"* through which they cooperate in the modulation of several physiological processes.

In this chapter, I will focus on the D_1 receptors, because it has been shown to have an important role in the modulation of learning and memory and synaptic plasticity (Hansen and Manahan-Vaughan, 2014) and I will also go deeper inside crosstalk between CB_1 receptor and D_1 receptor, principal topic in this thesis.

I – D_1 receptors in Hippocampal-dependent Synaptic Plasticity and Memory

DA is highly relevant for the modulation of hippocampus-dependent synaptic plasticity and memory (Lisman and Grace, 2005; Lisman et al. 2011) mostly because of the relevant role of D_1 receptors in such processes (Hansen and Manahan-Vaughan, 2014; Huang and Kandel, 1995; Lemon and Manahan-Vaughan, 2006; Bethus et al. 2010; Clausen et al. 2011; da Silva et al. 2012).

Recently, several studies from the Valjent lab provided evidence regarding the existence of D_1-positive cells in hippocampal sub-regions (Gangarossa et al. 2012; Puighermanal et al. 2017). Among these, D_1-positive cells have been showed in the subiculum, in the granule cells of the dentate gyrus (Fremeau et al. 1991), in glutamatergic pyramidal cells in the CA1-CA3 (Bergson et al. 1995) and very recently immunohistochemical investigations have revealed D_1 receptors to multiple types of inhibitory GABAergic interneurons of CA3/CA1 fields and in stratum radiatum in the mouse hippocampus (Gangarossa et al. 2012; Puighermanal et al. 2017), indicating that DA acting on these interneurons may influence information processing in the hippocampal circuit.

As previously mentioned activity-dependent alterations in synaptic strength encode new information in the brain, with LTP/LTD phenomena as the underlying mechansism of hippocampal learning and the encoding of different elements of a memory representation (Bliss and Collingridge, 1993; Bear, 1996; Kemp and Manahan-Vaughan, 2008). In this scenario, some studies showed that LTP and LTD are under the control of D_1/D_5 receptors, suggesting that these receptors contribute in different forms of synaptic plasticity to memory representations (Huang and Kandel, 1995; Lemon and Manahan-Vaughan, 2006). In particular, D_1/D_5 receptors appear to regulate both early-LTP and late-LTP in the CA1 region of the hippocampus by altering the excitability in the hippocampus (Ito and Schuman, 2007; Hamilton et al. 2010) and therefore influence the thresholds for the induction of synaptic plasticity or memory encoding.

This effect could be due to the engaging of signal cascades triggered by D_1 activation. Indeed, this receptor signaling is coupledc to adenyl cyclase (AC) (Undieh, 2010) which catalyzes the conversion of adenosine triphosphate to the intracellular second messenger cAMP, which in turns activate

protein kinase A (PKA) activity (Vallone et al. 2000; Undieh, 2010). A target of PKA phosphorylation is the DA and cAMP-regulated 32-kDa phosphoprotein (DARPP-32) expressed in the DG of the hippocampus (Undieh, 2010), whose activation results in the potentiation of NMDA receptor function (Cepeda and Levine, 2006). DA-mediated PKA activation also regulates Ca2+ currents (Drolet et al. 1997) and stimulation of nuclear transcription factors such as calcium-response element-binding (CREB) proteins (Undieh, 2010). All this intracellular pathway triggerdd by D_1 receptors activation are well know supporters of long-term synaptic plasticity in the hippocampus (Barco et al. 2002). Furthermore, D_1/D_5 receptors regulate the NMDA receptor directly (Cepeda et al. 1998; Stramiello and Wagner, 2008; Varela et al. 2009) and could affect: a) LTP and LTD induction thresholds (Cummings et al. 1996); b) signaling cascades activated by the D_1/D_5 receptors that lead to the activation of CREB and protein synthesis (Smith et al. 2005; Moncada et al. 2011; Sarantis et al. 2012). Moreover, both *in vitro* early- (Otmakhova and Lisman, 1996) and late-LTP (Frey et al. 1991; Huang and Kandel, 1995) are inhibited by D_1/D_5 blockade (Swanson-Park et al. 1999), whereas agonists of those receptors lead to enhanced early-LTP (Otmakhova and Lisman, 1996), as well as *in vivo* HFS-induced LTP in freely behaving rats (Lemon and Manahan-Vaughan, 2006).

DA is a key component in enabling processing of novel information in the hippocampus. Novelty exploration induces DA release, triggering an up-regulation of the immediate early gene Arc in the CA1 region (Guzowski et al. 1999) and the expression of Zif268 in the DG (Gangarossa et al. 2011). This suggests that DA, via D_1/D_5 receptors, stimulates transcriptional processes leading to consolidate long-term plasticity and memory (Moncada et al. 2011). Learning-facilitated early-LTP and late-LTP by exploration of novel empty space can be prevented by D_1/D_5 receptor blockade in CA1 synapses and the pharmacological activation of D_1/D_5 receptors mimics the spatial novelty-induced facilitation of LTP (Li et al. 2003). Consistent with this observation, local intra-hippocampal injection of D_1/D_5 receptors antagonists blocks late-LTP and prevents place novelty memory (Wang et al. 2010) and optogenetic stimulation of locus coeruleus to dorsal hippocampus terminals induced a D_1-like dependent facilitation of LTP (Takeuchi et al. 2016).

The aforementioned findings suggest that a very tight link exists between the regulation of synaptic plasticity by D_1/D_5 receptors and their role in hippocampus-dependent learning such as spatial and episodic memory. In particular, what drives changes in DA levels in the hippocampus and the relative contribution of D_1/D_5 receptors to synaptic plasticity and memory formation is the response to

novelty.
Hippocampal-mediated long-term episodic-like memory acquisition requires the activation of D_1/D_5 receptors whereas short-term memory does not involve these receptor (Bethus et al. 2010).
In addition, D_1 agonist treatment in rats enhances hippocampus-dependent spatial memory (Bach et al. 1999; da Silva et al. 2012) whereas D_1/D_5 receptor blockade impair short- and long-term spatial memory (Clausen et al. 2011; da Silva et al. 2012). On the same line, D_1/D_5 receptor activation is required during memory encoding to generate a persistent memory trace in the hippocampus (O'Carroll et al. 2006). All these pharmacological observations were further confirmed by studies in transgenic mice poviding further evidence for the essential role of the D_1 receptor for spatial learning (Granado et al. 2008; Xing et al. 2010) and for the encoding of novel environments and hippocampal representations of plasticity (Tran et al. 2008).
Another interesting aspect of dopaminergic signaling in hippocampal memory is that the processing of novelty by the hippocampus may be supported by structures other than the VTA. The hippocampus receives inputs not only from VTA (Lisman and Grace, 2005; Rossato et al. 2009) but also from the substantia nigra pars compacta (Beckstead et al. 1979) and indirectly from NAc (Floresco et al. 2001; Legault and Wise, 2001). In novelty-related context, all these dopaminergic nuclei represent an important source of DA for the hippocampus, which this brai region to integrate and encode this information into a memory engram (Lisman and Grace, 2005). Recently, has been also the locus coeruleus (LC) noradrenergic fibers have been shown to directly release of DA in the CA1 region (Smith and Greene, 2012) thus regulating synaptic plasticity and learning processes mediated by hippocampal D_1 receptors (Kempadoo et al. 2016; Takeuchi et al. 2016; Matta et al. 2017).
Thus, it has become clear that hippocampal neurons express D_1/D_5 receptors and DA, by activating these receptors, play a pivotal role in the enablement of hippocampal information encoding and storage.

II – D_1 and CB_1 receptors: Linking in memory functions

In addition to complex functions in the intracellular signaling, changes in neurotransmitter release and synaptic plasticity, newly genetic and pharmacological studies have demonstrated the importance of CB_1 receptors on the modulation of the dopaminergic system and its participation on the modulation of behavioral responses (Bloomfield et al. 2016; Fernandez-Ruiz et al. 2010). CB_1Rs and the endocannabinoid ligands AEA and 2-AG are abundant in dopaminergic pathways, where they act as a retrograde feedback system on presynaptic glutamatergic and GABA nerve terminals, modulating dopaminergic transmission (Herkenham et al. 1991). *In situ* hybridization (ISH) analysis have showed CB_1 receptor mRNA co-expression with D_1 receptors in several brain regions (Hermann et al. 2002). Among these, functional interaction between the CB_1 and the D_1 receptors have been widely studied in the striatum (Martin et al. 2008) and in the prefrontal cortex (Scheggia et al. 2017), given the high expression of CB_1 and D_1 receptors in this brain area (Paspalas et al. 2005; Marsicano and Kuner, 2008; Freeze et al. 2013). Accordingly, cognitive impairment, which includes executive dysfunction (Hooker and Jones, 1987), working memory impairments (Ranganathan and D'Souza, 2006) and amotivation (McGlothlin, 1968) induced by cannabis use are susceptible to mesocortical dopaminergic manipulation (Goldman-Rakic, 1996), and to prefrontal D_1 receptor blockade (Sawaguchi and Goldman-Rakic, 1991).

Furthermore, mice lacking the CB_1 gene in D_1R+ cells (D_1-CB_1-KO) do not display cannabinoid-induced catalepsy (Monory et al. 2006). Consequently, a recent intriguant article showed that CB_1 receptors in D_1-positive striatonigral neurons are the substrate of both adverse cataleptic and clinically relevant antinociceptive cannabinoid effects (Soria-Gomez et al. 2021).

Interestingly, Terzian and colleagues provided a well clear demonstration of a physiological cross-talk between the cannabinoid CB_1 receptors and the dopamine D_1 receptors examining the anxiety-like profile of conditional CB_1 mutant mice lacking CB_1Rs exclusively in neurons containing D_1Rs receptors (D_1-CB_1-KO mice) by using different tests (Terzian et al. 2011). The authors brought to light the specific role of CB_1 receptors in D_1-positive cells in modulating emotional states under conditions of stress-induced conflict/frustration (grooming behavior) or inescapable situations (social interactions). D_1-CB_1-KO mice showed a behavior reminiscent of a mild anhedonia-like state

(commonly associated with depression) even if this constatation does not support a depressive-like behavioral phenotype, as also suggested by the observation that KO mice performed as WT in the forced swim test. Finally, in the fear-related memory conditioning task, D_1-CB_1-KO mice showed significantly increased auditory-cued and contextual fear responses, which is not surprising when considering the important role of DA receptors in fear adaptation processes (El-Ghundi et al. 2001; de la Mora et al. 2010) and that of CB_1 receptors in fear alleviation (Marsicano et al. 2002; Kamprath et al. 2006).

Although the contribution of dopamine and cannabinoid neurotransmission in brain circuits regulating motivational and emotional neural processing has been well characterized by both animal and clinical studies (LeDoux, 2000; Laviolette and Grace, 2006) the mechanisms by which both D_1 and CB_1 receptor collaborate in mediating their effect in the hippocampus, more specifically in learning and memory, remain largely unknown. Furthermore, altought the precise expression of hippocampal cells expressing D_1Rs was already characterized, suggesting that various classes of GABAergic interneurons expressed D1R (Gangarossa et al. 2012; Wheeler et al. 2015) there is currently no anatomical evidence demonstrating the presence of CB_1 receptors in hippocampal D_1-positive cells.

The study of the potential interaction between D_1 and CB_1 receptorin the hippocampus, and its role in modulating learning and memory could provide a new mechansims for the role of the ECs in cognitive processes and memory-related disorders.

PART 5 – SUBCELLULAR SIGNALING OF CB_1 RECEPTOR: MITOCHONDRIAL CB_1 AND MEMORY

As discussed previously, CB_1 receptors are G-proteins coupled receptors (GPCRs). GPCRs are classically seen as presynaptic plasma membrane proteins in order to convert extracellular signals into intracellular responses (Freund and Hajos, 2003). Until recently CB_1-dependent effects have been exclusively attributed to plasma membrane CB_1 ($pmCB_1$) and its possible presence at intracellular level was disputed mainly for the aspecificity of the anti-CB_1 immunolabelling (Campbell, 2001; Athanasiou et al. 2007). However, the idea that GPCRs targeted by water-soluble ligands were believed to be functional only at plasma membranes has been challenged over the years by some studies, showing that different types of GPCRs can also be located intracellularly, including endosomes, nuclei and mitochondria (Figure 12) (Irannejad et al. 2013, 2017; Jong et al. 2014; Tsvetanova et al. 2015; Jalink and Moolenaar, 2010; Belous et al. 2004; Irannejad and von Zastrow, 2014; Rozenfeld and Devi, 2008; *reviewed in* Busquets Garcia et al. 2016).

As we know, the CB_1 receptor is primarily a lipid receptor and there is now evidence that both eCBs and exogenous cannabinoids reach their binding sites on CB_1 and CB_2Rs via the lipid bilayer (Hurst et al. 2010, 2019; Reggio, 2010; Kimura et al. 2009) and that is also confirmed from a recent analysis of the crystal structure of the CB_1 receptor protein (Hua et al. 2016, 2017; Shao et al. 2016) which suggests that these compounds can easily move either via active or passive mechanisms within cellular membranes (Voelker, 1991). Interestingly, lipid eCBs are produced and degraded within both the plasma membrane and organelle membranes inside cells (Gulyas et al. 2004; Morozov et al. 2004). For example, the FAAH enzyme, which degrades the AEA, and MAGL, the degrading enzyme of 2-AG, are present in intracellular purified brain mitochondria (Morozov et al. 2004; et al. 2007) modulating mitochondrial energetic activity (Benard et al. 2012).

In this context, in 2012, an elegant study from our laboratory established the specific localization of CB_1 receptors within CA1 hippocampal mitochondrial membranes by using electron microscopy and immunohistochemical labelling. Approximately 30% of WT hippocampal mitochondria were observed to contain CB_1 receptors labelling and named $mtCB_1$, whereas only 3% of mitochondria

showed non-specific immunoreactivity in CB_1-KO mice (Benard et al. 2012). Altogether, these observations revealed that the constituent pieces of the ECS are present inside cells and set the stage for more recent studies demonstrating a functional role for intracellular CB_1 receptor signaling in brain functions.

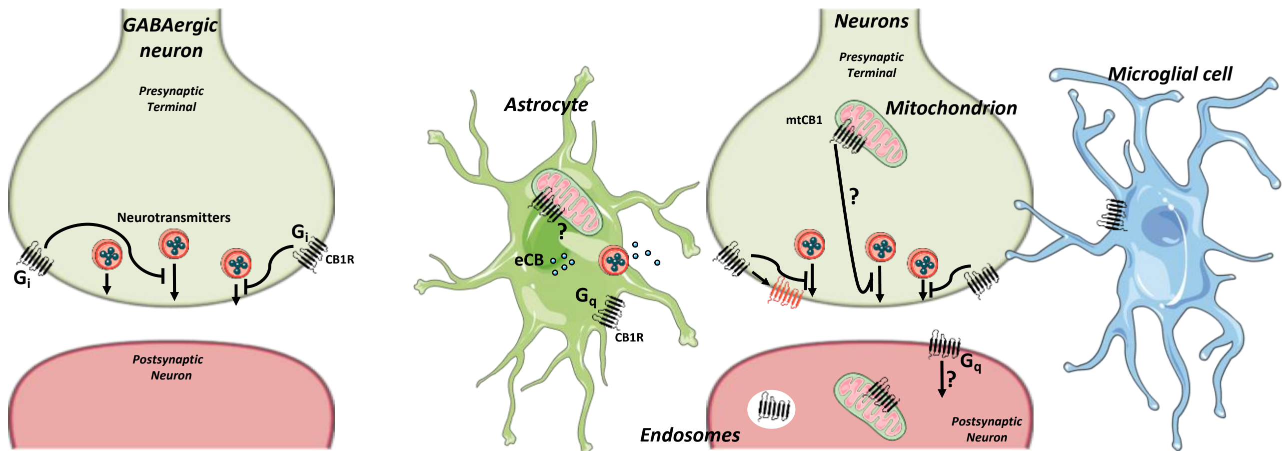

FIGURE 12 – VIEW OF THE CB1 RECEPTOR FUNCTIONAL EXPRESSION: CLASSIC Vs CURRENT
On the left panel the classic distribution of CB1 receptor in the brain where it was thought to be exclusively localized in GABAergic neurons. On the right panel, the current view where it was showed to be expressed in different neuronal and glial cells, astrocytes and microglia. Moreover it was found intracellularly, in the mitochondria and endosomes [Adapted from Busquets Garcia et al. 2016]

I – Mitochondria Functions

According to the first electron microscopy studies (Palade, 1953), mitochondria are organelles resembling to bacteria, composed by a highly specialized double membrane and containing their own DNA (Taanman, 1999). The outer (OM) and inner membrane (IM) have differential properties and functions and create two different mitochondrial compartments: the internal lumen called the matrix, and a much narrow intermembrane space (Alberts et al. 2002). The OM contains large lipid channels which render it permeable to high molecular weight molecules, including adenosine triphosphate (ATP) (Shoshan-Barmatz and Gincel, 2003). Conversely, the IM is largely impermeable, but it contains a variety of carrier proteins that makes it selectively permeable (Alberts et al. 2002). Mitochondria are organelles essential for the survival of our cells and perform various functions. The best known is the production of ATP during oxidative phosphorylation (OXPHOS). That takes place thanks to the succession of a series of redox reactions mediated by a set of four enzyme complexes, which form the respiratory chain, located on the IM of the mitochondrion (Stroud and Ryan, 2013; Brown, 2004). Through this mitochondrial process, the brain, which is one of the most energy demanding organs of the mammalian body (Erecinska and Silver, 2001; Rolfe and Brown, 1997), generates its own high energetic support, which is used to maintain ion flow, neuronal excitability and regulation of synaptic activity (Attwell et al. 2010; Attwell and Laughlin, 2001).
Beyond their central role in energy metabolism, brain mitochondria participate to several other important physiological processes including Ca2+ homeostasis, production of reactive oxygen species (ROS), synthesis and metabolism of neurotransmitters and other signaling molecules, and apoptosis (Turrens, 2003; Ruggiero et al. 2021; Bock and Tait, 2020).
Thus, it is not surprising that mitochondrial malfunctions have been associated to different pathologies affecting the CNS amongst the others (Manji et al. 2012; McInnes, 2013). For example, the insulin-resistance is associated with mitochondrial dysfunctions in myocytes, hepatocytes, adipocytes, and islets b-cells and neurons (Hojlund et al. 2008; Sivitz and Yorek, 2010). In the arcuate nucleus of the hypothalamus, a brain region involved in energy homeostasis, diet-induced obesity tremendously impacts on mitochondrial activity (Schneeberger et al. 2013; Dietrich et al. 2013). Furthermore, recent evidence indicates that events associated with cellular energy homeostasis can

impact synaptic and cognitive function (Suzuki et al. 2011; Wu et al. 2004) highlighting mitochondria as a node for neurotransmission, synaptic plasticity, network activity and behavioral processes (Benard et al. 2012; Kann and Kovacs, 2007; Li-Byarlay et al. 2014; Sun et al. 2013). Therefore, the study of brain mitochondrial activity and regulation is getting an important step to fully understand brain functioning in general and the onset and progression of several neurodegenerative diseases.
Interestingly, the study of different types of G proteins and associated-mitochondria signaling pathways are nowadays on the rise (Kuyznierewicz and Thomson, 2002; Lyssand and Bajjalieh, 2007; Andreeva et al. 2008; Zhang et al. 2010; Beninca et al. 2014) rendering the demonstration that CB_1 receptors are functionally present on brain mitochondrial membranes (Benard et al. 2012; Hebert-Chatelain et al. 2014, 2016; Koch et al. 2015; Jimenez-Blasco et al. 2020; Soria-Gomez et al. 2021) part of a novel field of research.
However, the specific roles of G proteins in the mitochondria and the mechanisms regulating their functions are poorly explored so far and understanding of that will be part of my Thesis.

II – Role of Mitochondrial CB_1 in the Brain

Already in the Seventies, different studies reported the impact of exogenous cannabinoids on mitochondrial processes, including reduction of complex I or V activities of the respiratory chain and changes in mitochondrial structure (Bartova and Birmingham, 1976; Bino et al. 1972; Chari-Bitron and Bino, 1971; Mahoney and Harris, 1972; Schurr and Livne, 1975). With the identification of CB_1 receptors as typical plasma membrane GPCRs, these effects were explained and ascribed to indirect signaling of CB_1 receptor-dependent activation (Campbell, 2001; Athanasiou et al. 2007) or to unspecific alterations of mitochondrial membrane by lipid molecules (Bartova and Birmingham, 1976; Howlett et al. 2002).

One of the first pieces of evidence for the presence of G protein-dependent intracellular cannabinoid signaling came from the observation that cannabinoids can activate CB_1 receptors localized in endosomes and lysosomes (Thibault et al. 2013; Rozenfeld and Devi, 2008). However, the most complete evidence for intracellular functional cannabinoid signaling relies on the study lead by Benard and colleagues on mitochondria (Benard et al. 2012).

This study and subsequently also the one by Hébert-Chatelain (2016), Koch and colleagues (2015) have been demonstrated the importance of mitochondrial CB_1 receptors coupled to the Gi/o protein in physiology and pathophysiology, using experiments with mice lacking CB_1 receptor.

Through electron microscopy of hippocampal CA1 neurons from WT and CB_1-KO mice the authors established that approximately 15.5% of the total amount of CB_1 receptors is located in mitochondria at the OM and only 30% of the mitochondria analysed show CB_1 immunolabelling. On the contrary, only 3% of CA1 mitochondria in CB_1-KO mice shows nonspecific labeling (Benard et al. 2012).

A similar mitochondrial localization also was shown in mouse hypothalamic pro-opiomelacortin (POMC) neurons in which the activation of CB_1 receptors negatively regulates leptin-induced reactive oxygen species formation (Palomba et al. 2015) and increases coupled mitochondria respiration that associates with the generation of reactive oxygen species (Koch et al. 2015). Recent results also indicate the presence of $mtCB_1$ receptors in the periphery, such as in spermatozoa (Aquila et al. 2010) or skeletal and cardiac striated muscle cells (Arrabal et al. 2015; Mendizabal-

Zubiaga et al. 2016) and in astrocytes (Gutiérrez-Rodríguez et al. 2018).

Moreover, Benard and colleagues has shown that activation of mtCB_1, by using different exogenous agonists of CB_1 receptors, negatively regulates the respiratory function of mitochondria decreasing in parallel the activity of complex I of the electron transfer chain (Benard et al. 2012). Indeed, in mitochondria isolated from WT mouse brains, exogenous application of CB_1 agonists decreases mitochondrial oxygen consumption, whereas no change is observed in mitochondria from CB_1-KO mouse brains (Benard et al. 2012). Furthermore, DSI which was previously attributed to presynaptic plasma membrane CB_1 receptors has been shown to be partially dependent on mtCB_1 receptor activation (Benard et al. 2012; Soria-Gomez et al. 2021).

These results suggested a direct link between endocannabinoids, CB_1 receptors, and brain cellular energy homeostasis. Interestingly, brain mitochondrial functions alterations have been recently causally associated to anxiety-related responses in the nucleus accumbens (Hollis et al. 2015), suggesting that brain energetic processes can impact behavior. For instance, a recent elegant study linked astroglial mtCB_1 receptor activation to a disruption of glucose metabolism and lactate production (Jimenez-Blasco et al. 2020) providing the mechanistic link between metabolic changes and cannabinoid-induced social imapirment in mice.

Another interesting study revealed, by immunogold electron microscopy, the existence of mtCB_1 in striatonigral terminals (Soria-Gomez et al. 2021), the output of a well known neuronal circuits in which the activation of CB_1 receptors impairs motor control (Giuffrida and Seillier, 2012; et al. 2007; Freeze et al. 2013). This study further investigated the subcellular-specific CB_1 receptor signaling within striatonigral circuits on behavioral control (Soria-Gomez et al. 2021) and found that the activation of mtCB_1 receptors in striatonigral terminals is responsible for the effects of cannabinoids on cellular respiration, synaptic transmission, and behavioral catalepsy (Soria-Gomez et al. 2021). In detail, the authors elegantly showed that cannabinoid-induced catalepsy involves inhibition of striatonigral mitochondrial soluble AC activity and intra-mitochondrial PKA-dependent cellular respiration (Soria-Gomez et al. 2021).

These results establish a link between mitochondrial activity, synaptic transmission, and motor control, thus by providing further evidence of the importance of mitochondrial functions for the regulation of behavior and the therapeutic potential of selective subcellular manipulations of CB_1 receptors.

III – Mitochondrial CB_1 and Memory

The impact of mitochondrial function or dysfunction in the brain relating to long-term pathological conditions is well known (Cheng et al. 2010; Mattson et al. 2008; Picard, 2015; Raefsky and Mattson, 2017). Conversely, very little is known about the direct impact of physiological regulation of mitochondrial activity on cognitive functions, mainly considering the fact that mitochondrial activity controls a series of processes absolutely necessary for normal synaptic transmission, such as the production of ATP (Rangaraju et al. 2014), the Ca2+ homeostasis (Brini et al. 2014), or the generation of reactive oxygen species (Accardi et al. 2014). Thus, it is very likely that slight alterations of these mitochondrial functions through $mtCB_1$ receptors modulations could have an impact on memory functions and behavior.

With the discovery of the subcellular signaling of CB_1 receptors in the brain (Benard et al. 2012) the molecular mechanisms resulting from the activation of the Gi/0 protein of $mtCB_1$ receptors and the physiological and behavioral effects mediated by $mtCB_1$ receptors started to be deciphered.

In 2016 our lab, by using pharmacological and genetic approaches, characterized the intra-mitchondrial signaling pathway triggerd by $mtCB_1$ receptor activation and its behavioral consequences in a hippocampus-dependent novel object recognition task (Hebert-Chatelain et al. 2016). We showed that $mtCB_1$ receptors signal through intra-mitochondrial Gαi protein activation and consequent inhibition of the activity of the soluble form of adenylate cyclase (sAC) leading to lower amount of intramitochondrial cAMP. This results in a decreades protein kinase A (PKA) activity in the mitochondria and consequently decreases the phosphorylation of proteins involved in OXPHOS, in particular the NDUFS2, specific subunit of complex I of respiratory chain (Busquets-Garcia et al. 2018a; Hebert-Chatelain et al. 2014a, 2014b), leading finally to a reduction of of ATP production (Figure 13). Interestingly, manipulation of sAC activity, intra-mitochondrial PKA signalling or the phosphorylation of the Complex I subunit NDUFS2 completeky abolished both blocked the bioenergetic and amnesic effects of cannabinoids (Hebert-Chatelain et al. 2016) further confirming the role of this intra-mitochondrial pathway. Thus, the Gαi-coupled $mtCB_1$ receptors regulate memory functions via modulation of mitochondrial bioenergy processes (Hebert-Chatelain et al. 2016).

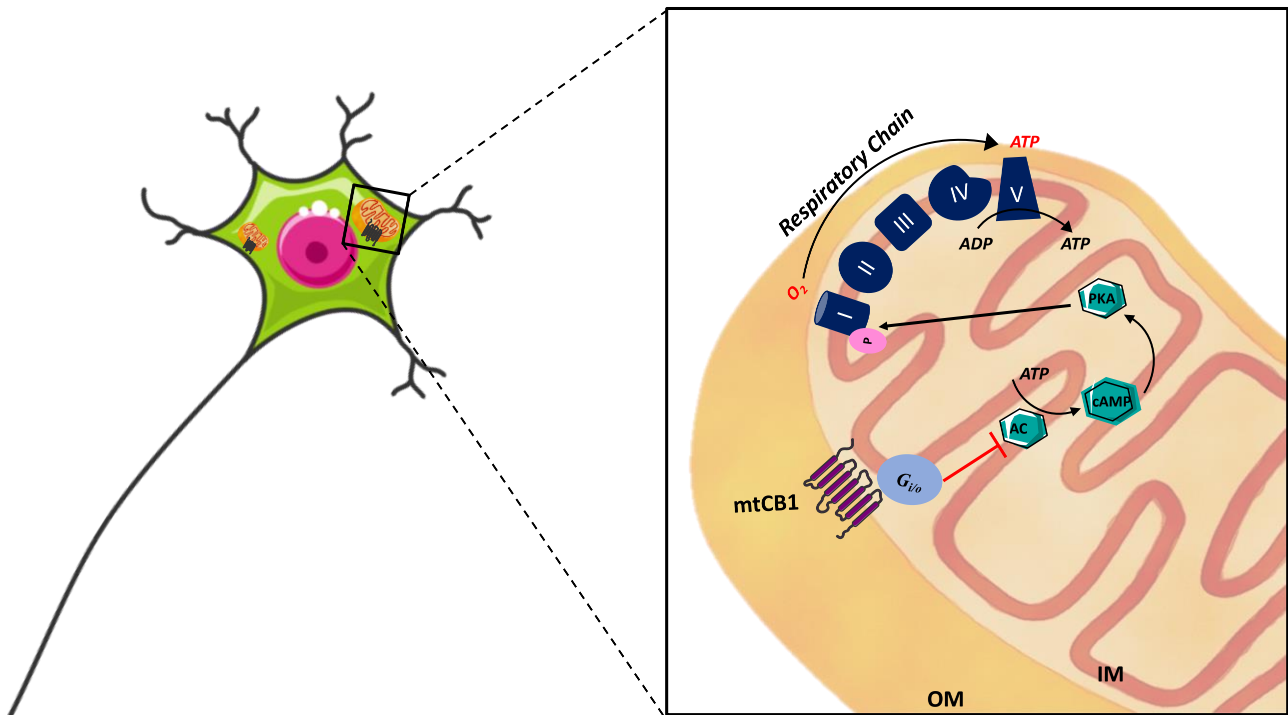

FIGURE 13 – SCHEMATIC VIEW OF THE mtCB1-DEPENDENT SIGNALING PATHWAY
mtCB1 receptors in the brain regulate the respiratory chain and the mitochondrial functions (eg, ATP production). mtCB1 receptors mediate its effects involving intra-mitochondrial Gi/o protein signaling, mitochondrial cAMP synthesis that is catalyzed by adenylyl cyclase (sAC), and the decrease of intra-mitochondrial PKA activity which reduces phosphorylation of respiratory chain complex I, decreasing mitochondrial respiration and affecting important mitochondria bioenergetic processes.
[Adapted from Busquets Garcia et al. 2018]

It is well noted that acute cannabinoid intoxication induces amnesia in humans and animals (Broyd et al. 2016; Marsicano and Lafenêtre, 2009), and the activation of type-1 cannabinoid receptors present at brain mitochondria membranes ($mtCB_1$) can directly alter mitochondrial energetic activity (Bénard et al. 2012; Hebert-Chatelain et al. 2014a; Koch et al. 2015). By directly linking mitochondrial activity to memory formation, Hebert-Chatelain and colleagues reveal that bioenergetic processes are primary regulators of cognitive functions (Hebert-Chatelain et al. 2016). Interestingly, $mtCB_1$ receptors seem not to be required for normal long-term memory formation as the absence of $mtCB_1$ receptors did not impair memory functions in a novel object recognition task, but it accounts for the acute cannabinoid-induced memory impairment as well as the cannabinoid-induced reduction of mitochondrial mobility and synaptic transmission (Hebert-Chatelain et al. 2016). Indeed, although eCB-mediated plasticity was not fully investigated in this study, the activation of the $mtCB_1$ receptor in hippocampal neurons interrupts excitatory synaptic transmission in the CA1-CA3 circuit, resulting in an impairment of long-term memory (Hebert-

Chatelain et al. 2016).

As in neurons also in astrocytes CB_1 receptors are involved in memory processes and CB_1 receptors in hippocampal astroglial mitochondria have recently been described (Gutierrez-Rodriguez et al. 2018). However, their implications on astroglial-dependent memory functions are ongoing studied by our lab. The most auspicious aspect about the study by Hebert-chatelain and colleagues is the demonstration that a mutant version of the CB_1 protein lacking the first 22 amino acids in the N-terminal part of the CB_1 receptor (called DN22-CB_1) is no longer physically and functionally addressed in the mitochondria, but it maintains its functionality at plasma membranes (Hebert-Chatelain et al. 2016). Thus, recently our lab generated a new transgenic mouse model, a knockin mutant mouse line, called DN22-CB_1-KI (Pagano Zottola et al. 2020; Soria-Gomez et al. 2021), in which CB_1 receptor gene is replaced by the coding sequence of the DN22-CB_1 protein, thereby, opening the door to understanding the functionality of the mtCB_1 receptor (McKeon and Mathur, 2021).

SECTION II – RESEARCH OBJECTIVES

Physiological or pharmacological modulation of the endocannabinoid system has been shown to impact the synaptic plasticity and transmission and learning and memory via CB_1 receptor-dependent control of different cell types (i.e. GABAergic, glutamatergic neurons, astrocytes) within the hippocampus (Busquets-Garcia et al., 2017, Soria-Gomez et al., 2017, Robin et al. 2018). This multimodal impact of CB_1 receptors on recognition memory raises the question about the differential cell type-, temporal- and spatial-specific contributions of the endocannabinoid system to memory processes. For instance, CB_1 receptors have been identified in a subclass of hippocampal CCK-positive interneurons which contain dopamine D_1 receptors, thus potentially representing a novel subpopulation of CB_1 expressing hippocampal interneurons (Puighermanal et al., 2017, Gangarossa et al., 2012) with a likely impact in hippocampal mediated functions. As previously mentioned, the consolidation of episodic like memory has been shown to require functional D_1 receptors in the hippocampus (Lisman et al., 2011, Yamasaki and Takeuchi, 2017), where activity-dependent long-term changes in synaptic transmission are considered cellular correlates of learning and memory (Nicoll, 2017, Whitlock et al., 2006). Furthermore, the activation of $D_{1/5}$ receptors mediates LTP induced by the exposure of the animal to hippocampal-dependent behavioral tasks (Frey et al., 1990, Granado et al., 2008, Li et al., 2003, Lemon and Manahan-Vaughan, 2006).
Given the neuromodulatory role of the ECS, this Thesis aims at studing the role of CB_1 receptors in hippocampal D1-positive cells at biochemical, electrophysiological and behavioral levels. The results will provide novel insights regarding the modulation of memory and learning functions, as well as new mechansims for the role of the ECs in cognitive processes and memory-related disorders.

The first objective of the thesis is to identify the cellular and molecular mechanisms by which CB_1 receptors in D_1-positive cells contribute to the physiological modulation of learning and memory.

For this purpose, we used a combination of genetics (constitutive and conditional mutagenesis of CB_1 receptors in mice) and chemogenetics coupled with behavioral, pharmacological and *in vivo* electrophysiological approaches.
During this work we addressed several questions regarding: 1) the role of CB_1 receptors in hippocampal D_1+ cells in the modulation of long-term memory formation, 2) the role of CB_1 receptors in D_1+ cells in the training-induced modulation of hippocampal plasticity, 3) how CB_1

receptors in D_1+ cells modulate LTP and memory.

To address these questions, we used mice lacking CB_1 receptors in D_1+ cells in the hippocampus and we further complemented our study with the use of pharmacological and pharmacogenetics approaches to dissect the cellular and molecular mechanism involved in modulation of memory functions. This first part of this thesis is resumed in the manuscript:

(Published)

Specific hippocampal interneurons shape consolidation of recognition memory

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*: equal contribution, #: equal supervision

My main contribution to this work was to set up and perform in vivo electrophysiology in anesthetized mice and to analyze the data acquired. I addition, I performed experiments in novel object recognition task and participated to the analysis of the behavioral data. I also performed hippocampal extractions from the mouse brain that were subsequently used for amino acid quantification.

In the Seventies, a number of reports demonstrated that cannabinoid drugs interfered with mitochondrial functions (Bartova and Birmingham, 1976; Bino et al, 1972; Chari-Bitron and Bino, 1971; Mahoney and Harris, 1972; Schurr and Livne, 1975). There is now compelling evidence that CB_1 receptors are present in mitochondrial membranes of different tissues (eg, brain, spermatozoa, and skeletal muscles) (Aquila et al, 2010; Benard et al, 2012; Hebert-Chatelain et al, 2014; Koch et al, 2015; Mendizabal-Zubiaga et al, 2016). The study by Hebert-Chatelain and colleagues (2016) showed the behavioral relevance of $mtCB_1$ receptors indicating that the inhibition or genetic exclusion of CB_1 receptors from hippocampal mitochondria prevents the cannabinoid-induced reduction of mitochondrial mobility and synaptic transmission and bioenergetic and amnesic effects of cannabinoids.

Thus, acute cannabinoid-induced memory impairment in mice requires activation of hippocampal $mtCB_1$ receptors. The discovery of this subcellular pool of CB_1 receptors has paved the way to a novel field of research dealing with linking GPCR mitochondrial signaling, cannabinoids and their impact on behavior. These observations fit with the idea that, by regulating innumerous cellular processes beyond ATP production, mitochondria exert a plethora of functions that are particularly crucial for one of the most energy-avid organs of the body, such as the brain (Mattson et al, 2008). However, the direct biochemical and behavioral impact of intra-mitochondrial receptor GPCR signaling modulation is far from being completely understood and further studies are required to clarify this and other issues linked to the discovery of $mtCB_1$ receptors. Thus, it's crucial undertaking efforts directed at identifying and understanding both the specific effects of cannabinoids on mitochondrial functions and the potential impact that GPCR–mitochondrial signaling might have on ECS function.

The second objective of the thesis is extending our knowledge on mitochondrial G-protein activity in order to better understand the intra-mitochondrial signaling and its effects on brain functions.

For this purpose, we developed a new mitochondria-specific chemogenetic tool based on DREADDs (designer receptor exclusively activated by designer drugs) technology and we combined it with in vitro and in vivo approachs. For *in vitro* studies, we used immunoblotting, immunofluorescence and oxigraphy techniques in order to reveal the specific subcellular target location and signaling

mechanism of our tool. Moreover, we were able to show its ability to regulate the intra-mitochondrial functions such as cellular respiration and membrane potential.

For *in vivo* studies, we combined this new tool with genetic and pharmacological approaches in order to investigate how the mitochondrial G-protein signaling modulation can impact bioenergetic processes and memory. The results obtained are presented in a manuscript that is currently in preparation:

Linking Mitochondrial G-protein Signaling to cannabinoids-induced amnesia: A new Mitochondria-specific chemogenetic Strategy

Gianluca Lavanco*, Antonio C. Pagano Zottola*, Yamuna Mariani, Astrid Cannich, Francisca Julio-Kalajzić, Filippo Drago, Giovanni Marsicano[#], Etienne Hebert-Chatelain[#] and Luigi Bellocchio[#]

*: equal contribution, [#]: equal supervision

My main contribution to this work was to perform and analyze data collected from *in vivo* studies. I also performed experiments in novel object recognition task and participated to the analysis of the behavioral data. I participated in the writing of the manuscript.

SECTION III – RESULTS

PART 1 – SPECIFIC HIPPOCAMPAL INTERNEURONS SHAPE CONSOLIDATION OF RECOGNITION MEMORY

Specific Hippocampal Interneurons Shape Consolidation of Recognition Memory

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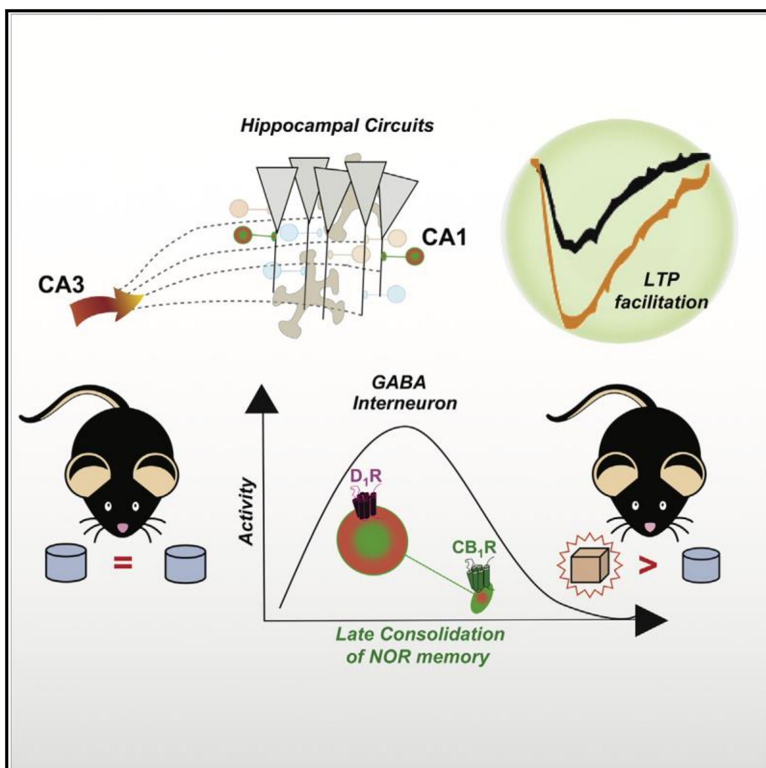
SUMMARY

A complex array of inhibitory interneurons tightly controls hippocampal activity, but how such diversity specifically affects memory processes is not well understood. We find that a small subclass of type 1 cannabinoid receptor (CB_1R)-expressing hippocampal interneurons determines episodic-like memory consolidation by linking dopamine D_1 receptor (D_1R) signaling to GABAergic transmission. Mice lacking CB_1Rs in D_1-positive cells (D_1-CB_1-KO) display impairment in long-term, but not short-term, novel object recognition memory (NOR). Re-expression of CB_1Rs in hippocampal D_1R-positive cells rescues this NOR deficit. Learning induces an enhancement of in vivo hippocampal long-term potentiation (LTP), which is absent in mutant mice. CB_1R-mediated NOR and the associated LTP facilitation involve local control of GABAergic inhibition in a D_1-dependent manner.

This study reveals that hippocampal CB_1R-/D_1R-expressing interneurons control NOR memory, identifying a mechanism linking the diversity of hippocampal interneurons to specific behavioral outcomes.

Graphical Abstract



INTRODUCTION

Formation of episodic memory is a multistep brain process that requires activity of the medial temporal lobe (Squire et al., 2007). The hippocampus in particular participates in long-term storage of recently acquired events. Hippocampal circuits are regulated by a large variety of local inhibitory interneurons that are controlled by neuromodulatory systems ensuring their coordinated function to shape behavioral responses (Klausberger and Somogyi, 2008); the identities and functions of the interneurons are under intense scrutiny (Harris et al., 2018; Pelkey et al., 2017; Parra et al., 1998).

The endocannabinoid system is a brain-modulatory signaling hub formed mainly by type 1 cannabinoid receptors (CB_1Rs), their endogenous ligands (endocannabinoids), and enzymes for their synthesis and degradation. In the hippocampus, CB_1Rs are present in principal neurons and astroglial cells (Busquets-Garcia et al., 2015; Oliveira da Cruz et al., 2016). However, the largest expression of CB_1Rs resides in GABAergic interneurons (Marsicano and Kuner, 2008; Katona and Freund, 2012),

where they modulate local inhibition of hippocampal circuits. Particularly, the largest amount of CB_1Rs is expressed in cholecystokinin (CCK)-positive interneurons, which are characterized by asynchronous neurotransmitter release (Harris et al., 2018; Katona et al., 1999; Marsicano and Lutz, 1999).

Hippocampal CB_1Rs control episodic-like memory processes and synaptic plasticity (Robin et al., 2018; Hebert-Chatelain et al., 2016; Puighermanal et al., 2009). However, the specific locations where these receptors participate in the mechanisms underlying hippocampus-dependent memory are only partially known.

Activity-dependent long-term changes in hippocampal synaptic transmission are considered cellular correlates of memory consolidation (Nicoll, 2017; Whitlock et al., 2006), which involves local dopamine D_1 receptor (D_1R) signaling (Lisman et al., 2011; Yamasaki and Takeuchi, 2017). Exposure to hippocampus-dependent behavioral tasks induces changes in long-term potentiation (LTP) of synaptic transmission that require activation of D_1-like receptors (Frey et al., 1990; Granado et al., 2008; Li et al., 2003; Lemon and Manahan-Vaughan, 2006). A novel subpopulation of hippocampal CB_1R/CCK-positive interneurons containing D_1R was recently described (Puighermanal et al., 2017; Gangarossa et al., 2012). However, the potential interactions between D_1Rs and CB_1Rs in regulating

learning-induced plasticity, activity of hippocampal circuits, and memory processes remain unexplored.

Here we assessed the role of D1R/CB_1R-positive cells in regulation of episodic-like novel object recognition (NOR) memory. We found that conditional deletion of the CB_1R gene in hippocampal D1R-positive cells impairs long- but not short-term NOR memory and learning-induced LTP enhancement involving local control of GABAergic transmission. These intriguing results suggest that CB_1R signaling provides a functional link between hippocampal dopaminergic and GABAergic control of synaptic plasticity and memory consolidation.

RESULTS

CB_1Rs in Hippocampal D_1R-Positive Neurons Are Necessary for Consolidation of NOR Memory

Mutant mice bearing a deletion of the CB_1R gene in cells expressing D_1R (D_1-CB_1-KO mice; Monory et al., 2007) displayed no phenotype in the short-term version (3 h post-training) of a NOR task (Figures 1A and 1B; Puighermanal et al., 2009; Busquets-Garcia et al., 2011; Robin et al., 2018). Conversely, they showed strong impairment in long-term (24 h) memory compared with their wild-type (WT) littermates (Figure 1C), with no changes in total exploration time (Figures S1A–S1D).

The majority of CB_1Rs in D_1R-positive neurons have been characterized previously in striatonigral circuits (Monory et al., 2007). Considering the involvement of these circuits in NOR memory (Darvas and Palmiter, 2009), we tested the role of striatal CB_1Rs. We infused an adeno-associated virus carrying a Cre-dependent expression of CB_1Rs (pAAV-CAG-DIO-CB1) into the striatum of D_1-CB_1-KO mice to obtain re-expression (RS) of CB_1Rs in cells where Cre is present (hereafter called D_1R-positive) in this brain region (striatum [STR]-CB_1-RS mice; Figures 1D and 1E), as revealed by immunodetection of a myc-tagged version of CB_1Rs (CB1R-myc; STAR Methods; Figure 1E). This re-expression was not sufficient to rescue the phenotype of D_1-CB_1-KO mice in long-term NOR (Figures 1F, S1E, and S1F), suggesting that CB_1Rs in striatal D_1R-positive cells do not participate in this type of memory. Anatomical data indicate that a subset of hippocampal neurons contain D_1Rs (Gangarossa et al., 2012), likely co-expressing CB_1R protein (Puighermanal et al., 2017). Thus, we re-express the CB_1R gene in the hippocampus of D_1-CB_1-KO mice to obtain hippocampus (HPC)-CB_1-RS

mice (Figures 1D and 1G). This manipulation fully rescued the phenotype of the mutant mice (Figure 1F, S1E, and S1F), indicating that hippocampal CB_1Rs expressed in D_1R-positive cells are required for NOR memory.

We recently reported that deletion of CB_1Rs in hippocampal glial acidic fibrillary protein (GFAP)-positive cells (i.e., mainly astrocytes, GFAP-CB_1-KO mice) also impaired NOR memory (Robin et al., 2018). Indeed, GFAP-CB_1-KO mice were impaired in NOR (Figures S1G–S1I; Robin et al., 2018), but, in contrast to D_1-CB_1-KO mice, this phenotype extended to short-term NOR memory (Figures S1J–S1L). This difference suggests that CB_1Rs expressed in hippocampal astrocytes or D_1R-positive cells might control distinct phases of NOR memory consolidation.

The primary function of CB_1R activation in neurons is to decrease neurotransmitter release (Castillo et al., 2012; Busquets-Garcia et al., 2017). Accordingly, deletion of CB_1Rs from neurons often results in excessive neurotransmission. Thus, we reasoned that inhibition of hippocampal D_1R-positive neurons during NOR consolidation should be able to rescue the memory impairment of D_1-CB_1-KO mice. Viral vectors carrying Cre-dependent expression of an inhibitory designer receptor exclusively activated by designer drugs (DIO-hM4DGi, Gi-DREADD; Robinson and Adelman, 2015) or control mCherry protein were infused into the hippocampi of D_1-CB_1-KO mice and WT littermates (Figure 1H). Post-training clozapine N-oxide (CNO) injections did not affect the NOR performance of D_1-CB_1-KO and WT mice injected with Gi-DREADD or mCherry, indicating that the drug or its metabolites had no effect per se (Gomez et al., 2017; Figures 1I, S1M, and S1N). Conversely, post-acquisition CNO treatment fully rescued the NOR impairment of D_1-CB_1-KO mice expressing Gi-DREADD (Figures 1I, S1M, and S1N). This strongly suggests that excessive activity of D_1R-positive neurons during the consolidation process is responsible for the memory impairment observed in D_1-CB_1-KO mice.

CB_1Rs in Hippocampal D_1R-Positive Neurons Control Learning-Induced Changes of LTP In Vivo

Cellular and molecular mechanisms underlying activity-dependent changes in synaptic plasticity are proposed to underlie long-term memory (Aggleton and Morris, 2018). Previous studies showed that conditional and global deletion of CB_1Rs in neuronal and glial cell populations induces deficits in learning and associated synaptic plasticity (Busquets-Garcia et al., 2017; Robin et al., 2018). To address the role of CB_1Rs in hippocampal D_1R-positive neurons in modulation of synaptic plasticity, we recorded in-vivo-evoked field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA3-CA1 pathway of anesthetized mice. HFS induced similar long-lasting LTP of synaptic fEPSPs in

D_1-CB_1-KO and WT littermates (Figures 2A and 2B), indicating that hippocampal D_1R/CB_1R-positive neurons are dispensable for expression of LTP in naive animals.

HPC-dependent memory-related processes such as LTP are sensitive to pharmacological and genetic modulation of hippocampal D_1Rs, particularly after learning (Li et al., 2003; Lemon and Manahan-Vaughan, 2006; Takeuchi et al., 2016; Yamasaki and Takeuchi, 2017). Thus, we hypothesized that CB_1Rs in D_1R-positive neurons may modulate learning-dependent hippocampal synaptic plasticity. To explore whether acquisition of the NOR task modulates in vivo LTP, we recorded fEPSPs from C57Bl6/NRj mice after a NOR task (Figure 2C). HFS induced stronger LTP in animals exposed to NOR acquisition than in control mice (Figures 2D and 2E), showing that the training modulates hippocampal synaptic plasticity. Strikingly, D_1-CB_1-KO mice lacked this learning-induced enhancement of LTP (Figures 2F and 2G). Thus, physiological activation of CB_1Rs in hippocampal D_1R-positive neurons is required for learning-dependent facilitation of LTP.

CB_1R in Hippocampal D_1R-Positive Neurons Modulate NOR Memory Consolidation through a GABA-Dependent Mechanism

D_1Rs are expressed in different hippocampal cells, including subsets of GABAergic and glutamatergic neurons (Gangarossa et al., 2012). Considering that CB_1R signaling decreases the activity of hippocampal neurons (Busquets-Garcia et al., 2017; Castillo et al., 2012), we asked whether excessive glutamatergic or GABAergic neurotransmission might underlie the phenotype of D_1-CB_1-KO mice. Thus, we injected non-amnesic doses (Puighermanal et al., 2009) of the NMDA receptor blocker MK-801, the AMPA/kainate receptor antagonist NBQX (Figure S2A), or the GABAA receptor antagonist bicuculline into D_1-CB_1-KO and WT littermates immediately after NOR training. MK-801 and NBQX did not alter memory performance in WT mice, nor did it rescue the amnesic phenotype of D_1-CB_1-KO littermates (Figures 3A, S2B, and S2C). Conversely, bicuculline completely reversed the memory impairment of D_1-CB_1-KO mice when injected immediately after training or 1 h later without affecting WT littermates' performance (Figures 3A, S2B, and S2C).

These data indicate that excessive GABAergic but not glutamatergic ionotropic receptor activity is involved in the phenotype of D_1-CB_1-KO mice. A large proportion of GABAergic hippocampal interneurons contain CB_1R mRNA, which is expressed at different levels (high CB_1R- and low CB_1R-

expressing cells; Marsicano and Lutz, 1999). Conversely, D_1R mRNA is expressed at very low levels in the HPC (http://mouse.brain-map.org/ experiment/show/35; data not shown), which makes it difficult to accurately quantify its expression above background. Therefore, to pinpoint which CB_1R-positive interneurons in the HPC contain D_1R, we combined fluorescence ISH for CB_1R mRNA in D_1-Cre and D_1-CB_1-KO mice carrying viral Cre-dependent expression of mCherry (STAR Methods; Figure 3B). As described (Marsicano and Lutz, 1999), detectable levels of CB_1R mRNA were present throughout the HPC in pyramidal neurons and in GABAergic interneurons (Figure S2D). The distribution of mCherry-tagged D_1-positive neurons in the dorsal CA1 region of D_1-Cre mice was similar to previous findings (Puighermanal et al., 2017; Gangarossa et al., 2012). Double staining revealed that virtually no high CB_1R-expressing interneurons in the *strata oriens, pyramidale, radiatum,* or *lacunosum moleculare* contain D_1Rs (Figures 3C–3F and S2D). Conversely, D_1Rs are present in a small subpopulation of low CB_1R-expressing interneurons along the different hippocampal layers (Figures 3C and 3F). Importantly, this co-expression was virtually abolished in hippocampi of D_1-CB_1-KO mice (Figures 3C, 3D, and 3F).
Altogether, these data indicate that CB_1R-dependent modulation of a small subpopulation of D_1R-positive GABAergic interneurons is required during NOR memory consolidation.

Synaptic Mechanisms Underlying NOR Memory Consolidation and Associated Hippocampal Plasticity

The data collected so far show that reduction of GABAergic signaling prevents the deficits in D_1-CB_1-KO mice of NOR consolidation. Therefore, we tested whether inhibition of GABAA receptors could rescue the lack of learning-induced LTP enhancement observed in D_1-CB_1-KO mice. Trained mice received bicuculline or vehicle (VEH) before testing LTP induction in hippocampal circuits. In vehicle-treated animals, D_1-CB_1-KO mice showed no training-induced LTP enhancement (Figures 4A–4C). Strikingly, although bicuculline did not affect LTP in WT animals, it rescued the training-induced LTP of D_1-CB_1-KO mice (Figures 4A–4C).
Recent data suggest that hippocampal D_1R-like receptors participate in memory formation, but little is known concerning the cell types involved (Lisman et al., 2011; Yamasaki and Takeuchi, 2017). Our data indicate that CB_1R-dependent control of GABAergic transmission from a low number of

hippocampal interneurons expressing D_1R is required to guarantee late consolidation of NOR memory. Therefore, it is possible that endocannabinoid actions are secondary to activation of D_1Rs in these cells. To address this issue, we first reasoned that partial inhibition of D_1Rs should ''replace'' the lack of CB_1R-dependent control of neurotransmission in D_1-CB_1-KO mice. Thus, we administered a sub-effective dose of the $D_{1/5}R$ antagonist SCH-23390 (Figures S3A–S3C) to D_1-CB_1-KO mice and WT littermates after NOR acquisition and analyzed the training-induced enhancement of in vivo LTP. This treatment slightly reduced the late phase of LTP in WT animals (Figures 4A–4C). However, the antagonist abolished the differences between D_1-CB_1-KO mice and WT littermates (Figures 4A–4C), indicating that reducing D_1R activity counteracts the absence of CB_1Rs in the mutants. If LTP is mechanistically linked to NOR consolidation, then the same treatment should rescue the memory impairment of D_1-CB_1-KO mice. Administration of SCH-23390 did not alter the behavior of WT mice (Figures 4D, S3D, and S3E), but, strikingly, it fully rescued the memory impairment of D_1-CB_1-KO littermates (Figures 4D, S3D, and S3E).
Altogether, these results indicate that endocannabinoid-dependent regulation of hippocampal D_1R-positive interneurons is a necessary step in dopaminergic control of NOR memory consolidation and associated synaptic plasticity.

DISCUSSION

The present study reveals that a specific subpopulation of hippocampal D_1R/CB_1R-positive neurons controls late consolidation of NOR memory and associated synaptic plasticity by moderating local inhibitory GABAergic activity in the HPC. Specifically, CB_1Rs expressed in D_1R-positive interneurons participate in learning-induced facilitation of in vivo LTP and are required for consolidation of NOR memory. Moreover, CB_1Rs in D_1R-positive neurons are necessary for physiological D_1R-dependent modulation of memory processes, suggesting that cannabinoid signaling is part of a complex modulatory circuit regulated by dopamine transmission in the HPC. By determining cellular and behavioral functions of a specific CB_1R-expressing interneuron subpopulation, these data uncover an unforeseen role of CB_1Rs in the D_1R-dependent control of long-term memory.
The endocannabinoid system regulates episodic-like recognition memory processes via CB_1R-dependent control of different cell types in the HPC (Busquets-Garcia et al., 2017; Soria-Gomez et

al., 2017; Busquets Garcia et al., 2016; Puighermanal et al., 2009; Robin et al., 2018). In the present study, we observed that the transition from short- to long-term memory processes is controlled by a functional interaction between D_1Rs and CB_1Rs in a specific subpopulation of hippocampal interneurons. In contrast, CB_1R deletion from all body cells or in all forebrain GABAergic neurons does not reproduce the phenotype of D_1-CB_1-KO mice (Puighermanal et al., 2009; Hebert-Chatelain et al., 2016). These apparently contrasting observations can be explained by different possibilities. Long- term deletion of the CB_1R gene starting from early developmental stages in CB_1-KO and GABA-CB_1-KO mice might induce compensatory mechanisms (El-Brolosy et al., 2019; El-Brolosy and Stainier, 2017), masking the functional role of the CB_1R in NOR memory. An alternative or complementary explanation might point to the presence of different subpopulations of brain cells expressing CB_1Rs and exerting opposite effects on memory processes. For instance, endocannabinoid signaling might promote or inhibit memory formation when acting at D_1R-positive cells or at other neuronal subpopulations, respectively. We have shown previously that astroglial CB_1Rs are necessary for consolidation of NOR memory by allowing D-serine availability at glutamatergic synapses (Robin et al., 2018). We cannot fully exclude that deletion of CB_1Rs in D_1R-positive cells does not also involve astrocytes (Nagatomo et al., 2017). However, so far, no conclusive anatomical evidence has been presented for expression of D_1Rs in hippocampal astrocytes (Chai et al., 2017; Zhang et al., 2014; but see Jennings et al., 2017 for $D_{1/5}$R pharmacological experiments). Moreover, our current and past results suggest that endocannabinoid control of astrocytes is likely involved in the initial phases of memory formation, whereas CB_1R-dependent inhibition of D_1R-positive hippocampal interneurons determines later phases of NOR memory consolidation. The time-course effects of pharmacological treatments indicate that D-serine can rescue memory performance of GFAP-CB_1-KO mice only when administered immediately after learning (Robin et al., 2018). This idea is reinforced by the fact that these mutants do not express in vivo LTP even under basal ‘‘home cage’’ conditions (Robin et al., 2018), whereas D_1-CB_1-KO mice only lack the specific facilitation of LTP induced by learning. Altogether, these observations allow speculation that at least two distinct temporal windows exist in CB_1R-dependent control of NOR. First, astroglial CB_1R are necessary for the plastic processes to initiate the memory. Later, endocannabinoid-dependent regulation of D_1R-positive interneurons is required to maintain the memory trace for longer periods.

Hippocampal D_1R have been shown previously to be mainly on GABAergic interneurons, but lower levels were also detected on glutamatergic neurons (Gangarossa et al., 2012; Puighermanal et al., 2017; http://celltypes.brain-map.org/rnaseq/ mouse_ctx-hip_smart-seq). Our data show that the D_1-Cre mouse line used in the present study (Lemberger et al., 2007) induces recombination in a small sub-fraction of hippocampal interneurons containing low levels of CB_1R mRNA but also in pyramidal neurons and mossy cells. Therefore, we cannot fully exclude that cell types other than hippocampal interneurons might participate in D_1R/CB_1R-dependent control of memory consolidation. However, our data show that partial blockade of GABAA receptors, but not of AMPA/kainate or NMDA glutamatergic ones, reverse the memory impairment of D_1-CB_1-KO mice. Therefore, our findings strongly suggest that CB_1R control of GABA release from D_1R-positive interneurons regulates late consolidation of NOR memory. However, recent data using emerging technologies suggest that hippocampal cells are more diverse and functionally segregated than previously thought (Harris et al., 2018; Soltesz and Losonczy, 2018). By identifying specific markers, future studies will extend our genetic and pharmacological evidence that a specific subpopulation of D_1R/CB_1R-positive hippocampal interneurons regulates consolidation of NOR memory.
LTP at the CA3-CA1 pathway is a potential molecular and cellular mechanism underlying behavioral expression of episodic-like memory processes (Morris, 2013). Interestingly, although deletion of CB_1Rs from D_1R-positive cells impairs NOR memory, the same manipulation does not impair in vivo LTP of hippocampal synaptic transmission in naive animals. In agreement with previous evidence under other experimental conditions (Li et al., 2003; Lemon and Manahan-Vaughan, 2006), WT mice exposed to the NOR learning task display facilitation of in vivo LTP compared with animals exposed to the same environment without any learning. Importantly, this facilitation is absent in D_1-CB_1-KO mice, suggesting that endocannabinoid control of D_1R-positive hippocampal interneurons is recruited only after learning. The facilitation might be due to ‘‘real’’ stronger synaptic transmission after learning or a decrease in baseline synaptic activity (Lisman, 2017), which might be occluded in D_1-CB_1-KO mice. The fact that partial blockade of GABAA receptors in trained WT mice does not alter LTP facilitation suggests that this phenomenon is due to a genuine increase in LTP. In addition, our data indicate that reducing GABAergic transmission in D_1R-positive neurons is required for this form of learning-induced synaptic plasticity. These results reinforce the idea that, to reveal relevant mechanisms, investigations of synaptic plasticity associated with memory processes should include

not only naive animals but also behaviorally challenged ones (Lisman et al., 2011).

D_1R activity in the HPC is necessary for long-term memory, synaptic plasticity, and network dynamics (Lisman et al., 2011; Yamasaki and Takeuchi, 2017; Kaufman et al., 2020; Bethus et al., 2010). Consistently, our results show that high doses of the $D_{1/5}R$ antagonist SCH-23390 impair memory performance in the NOR task. In addition, our data suggest that D_1R/CB_1R-positive hippocampal interneurons are one of the targets of dopaminergic control of learning and memory processes. Interestingly, it has been shown that parvalbumin (PV)-expressing interneurons require D_1R activity for late phases of memory consolidation through coordinated control of the activity of hippocampal pyramidal neurons (Karunakaran et al., 2016). Particularly, the authors describe that this D_1R activity modulates hippocampal network oscillations (i.e., sharp-wave ripples), which is a proposed correlate for synaptic plasticity and memory consolidation (Buzsáki, 2015). In addition, previous studies have shown that PV/CB_1R-negative and CCK/CB_1R-positive interneurons have complementary roles in ensuring such high oscillatory ripple events with consequent capacity to modulate synaptic plasticity (Klausberger et al., 2005; Buzsáki, 2015). Therefore, we speculate that the subpopulation of D_1R/CB_1R-positive interneurons described in our work could play a complementary role in maintaining a proper excitation/inhibition balance in the hippocampal network activity required for memory consolidation.

Although complete elucidation of the complex microcircuitry requires further characterization, our findings support the hypothesis that D_1R/CB_1R-positive hippocampal interneurons belong to a broader circuit participating in dopaminergic control of memory (Yamasaki and Takeuchi, 2017). Our data are compatible with a scenario where D_1R activation during the learning/consolidation process potentiates GABAergic transmission. However, this D_1R-dependent increase in inhibition is kept within adequate limits by activation of CB_1Rs, allowing proper flow of information. In this sense, in the absence of CB_1R-dependent control of D_1R/CB_1R-positive interneurons (i.e., D_1-CB_1-KO mice), partial inhibition of D_1-like or GABAA receptors rescues the phenotype. In other words, although activation of D_1Rs in interneurons seems to be necessary for the memory process, their abnormally high activity (e.g. in the absence of CB_1Rs) impairs such functions. In this context, an interesting question relates to the functional link between endogenous activation of D_1Rs and CB_1Rs. Our results allow speculation about two potential scenarios based on autocrine or paracrine modes of action of endocannabinoid signaling (Busquets-Garcia et al., 2017). (1) General D_1R-dependent

dopaminergic signaling in the HPC might activate pyramidal neurons (Roggenhofer et al., 2013; Shivarama Shetty et al., 2016) targeted by D_1R/CB_1R-positive interneurons. This depolarization of pyramidal neurons would, in turn, induce canonical endocannabinoid-dependent retrograde inhibition of GABAergic release (Castillo et al., 2012), moderating, among others, activation of D_1R/CB_1R-positive interneurons. (2) Following D_1R activation and consequent interneuron depolarization (Anastasiades et al., 2019; Gorelova et al., 2002), endocannabinoids might be mobilized locally and act in an autocrine manner to decrease the membrane potential and thereby moderate the activity of the neuron (Bacci et al., 2004). These two possibilities are not mutually exclusive, and they might reflect the effect of the mechanisms described on general network activity and/or on specific plastic cellular processes, respectively. Future studies will investigate these intriguing scenarios using adapted experimental approaches.

Altogether, these data reveal that functionally distinct cell types are present in the general population of hippocampal GABAergic interneurons expressing CB_1Rs. In particular, D_1R/CB_1R-positive interneurons provide specific behavioral and hippocampal synaptic mechanisms sustaining the fine-tuned regulation of memory processes. The close interaction of CB_1Rs and D_1Rs in modulating recognition memory might provide novel therapeutic frame-works for treatment of cognitive diseases characterized by alterations of endocannabinoid or dopaminergic systems or both.

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AUTHOR CONTRIBUTIONS

J.F.O.d.C., A.B.-G., G.M., and E.S.-G. conceived and supervised the whole project. J.F.O.d.C. performed and analyzed in vivo electrophysiology and behavioral experiments. A.B.-G. and E.S.-G. performed and analyzed behavioral experiments. L.B. and A.C. contributed to experiments using viral vectors. L.R. and G.L. contributed to behavioral experiments. M.V., F.J.-K., T.L.-L., and M.M. performed cytochemical experiments. Z.Z. and M.V. helped with analysis of the data. J.F.O.d.C., A.B.-G., G.M., and E.S.-G. wrote the manuscript. F.D. contributed to writing. All authors edited and approved the manuscript.

Oliveira da Cruz et al. Figure 1

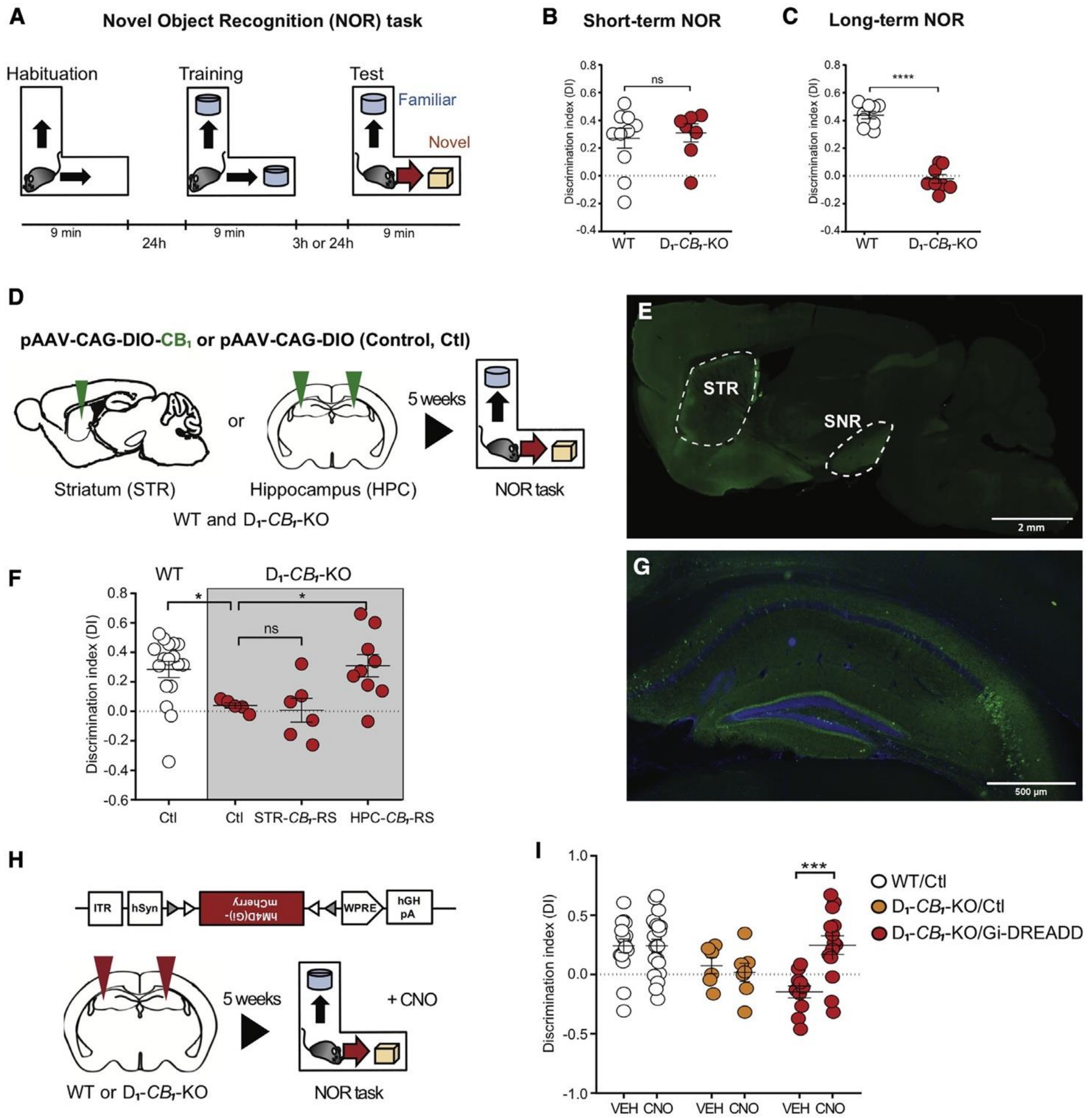

Figure 1. Hippocampal CB_1Rs in D_1R-Positive Cells Are Necessary for Late but Not Early Consolidation of NOR (A) Schematic representation of the NOR memory task. (B) Short-term (3 h) NOR memory performance of D_1-CB_1-WT mice (n = 10) and D_1-CB_1-KO littermates (n = 7). (C) Long-term NOR (24 h) memory performance of D_1-CB_1-WT mice (n = 9) and D_1-CB_1-KO littermates (n = 8). (D) Schematic representation of the experiment using viral re-expression of the CB1R gene in the striatum (STR) or the hippocampus (HPC) of D_1-CB_1-WT mice and D_1-CB_1-KO littermates. (E) Representative images of Cre-expressing D_1-CB_1-KO mice injected with CB_1R-myc in the STR using the same procedure as described in (D) (STAR Methods). Scale bar, 2 mm. (F) NOR memory performance of mice with re-expression of the CB_1R gene in the STR or HPC. Control, n (D_1-CB_1-WT) = 17 and n (D_1-CB_1-KO) = 5; STR-CB1-RS, n (D_1-CB_1-KO) = 6; HPC-CB_1-RS, n (D_1-CB_1-KO) = 9. (G) Immunofluorescence of cells expressing CB_1R-myc in the HPC. Scale bar, 500 mm. (H) Schematic representation of the experiment using viral expression of the Gi-DREADDs or mCherry in the HPC of D_1-CB_1-WT mice and D_1-CB_1-KO littermates. Clozapine N-oxide (CNO; 2 mg/kg) injections take place after the training phase of the NOR task. (I) NOR memory performance of D_1-CB_1-WT mice injected intra-hippocampally with hM4D(Gi) virus or mCherry (n VEH = 16, n CNO = 21), D_1-CB_1-KO mice injected with mCherry (n VEH = 6, n CNO = 7), and D_1-CB_1-KO mice injected intra-hippocampally with hM4D(Gi) (n VEH = 11, n CNO = 14). Data, mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant. See also Figure S1 and Table S1.

Oliveira da Cruz et al. Figure 2

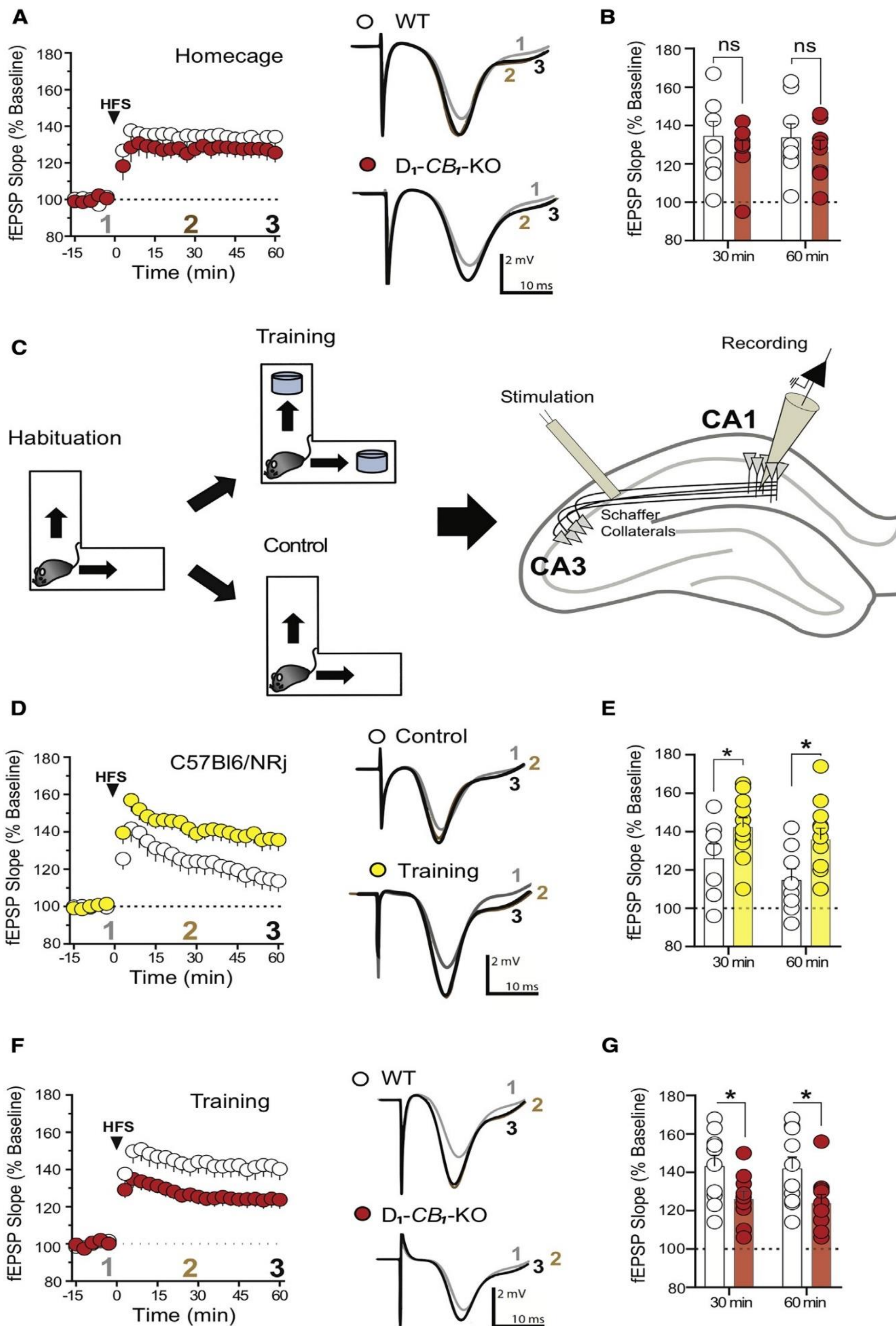

Figure 2. Learning-Induced Facilitation of In Vivo Hippocampal LTP Requires CB_1Rs at D_1R-Positive Neurons (A and B) HFS in the dorsal hippocampal CA3 Schaffer collateral pathway induces in vivo LTP in the dorsal CA1 stratum radiatum. (A) Summary plots of recorded evoked fEPSPs in anesthetized D_1-CB_1-WT (n = 8) and D_1-CB_1-KO (n = 8) mice. (B) Bar histograms of normalized fEPSPs from (A), representing 30 and 60 min after HFS. (C) Schematic representation of the experimental setup (STAR Methods). (D and E) Learning modulates in vivo LTP. (D) Summary plots of recorded evoked fEPSPs from mice exposed to control (n = 8) and NOR training (n = 11) conditions.

(E) Bar histograms of normalized of evoked fEPSPs from (D), representing 30 and 60 min after HFS. (F and G) Learning-induced modulation of in vivo LTP is impaired in D_1-CB_1-KO mice. (F) Summary plots of recorded fEPSPs in anesthetized D_1-CB_1-WT (n = 10) and D_1-CB_1-KO (n = 10) mice. (G) Bar histograms of normalized of evoked fEPSPs from (F), representing 30 and 60 min after HFS. Traces on the right side of the summary plots represent 150 superimposed evoked fEPSPs before HFS (1, gray) and 30 min (2, brown) and 60 min (3, black) after HFS. Data, mean ± SEM. *$p < 0.05$. See also Table S1.

Oliveira da Cruz et al. Figure 3

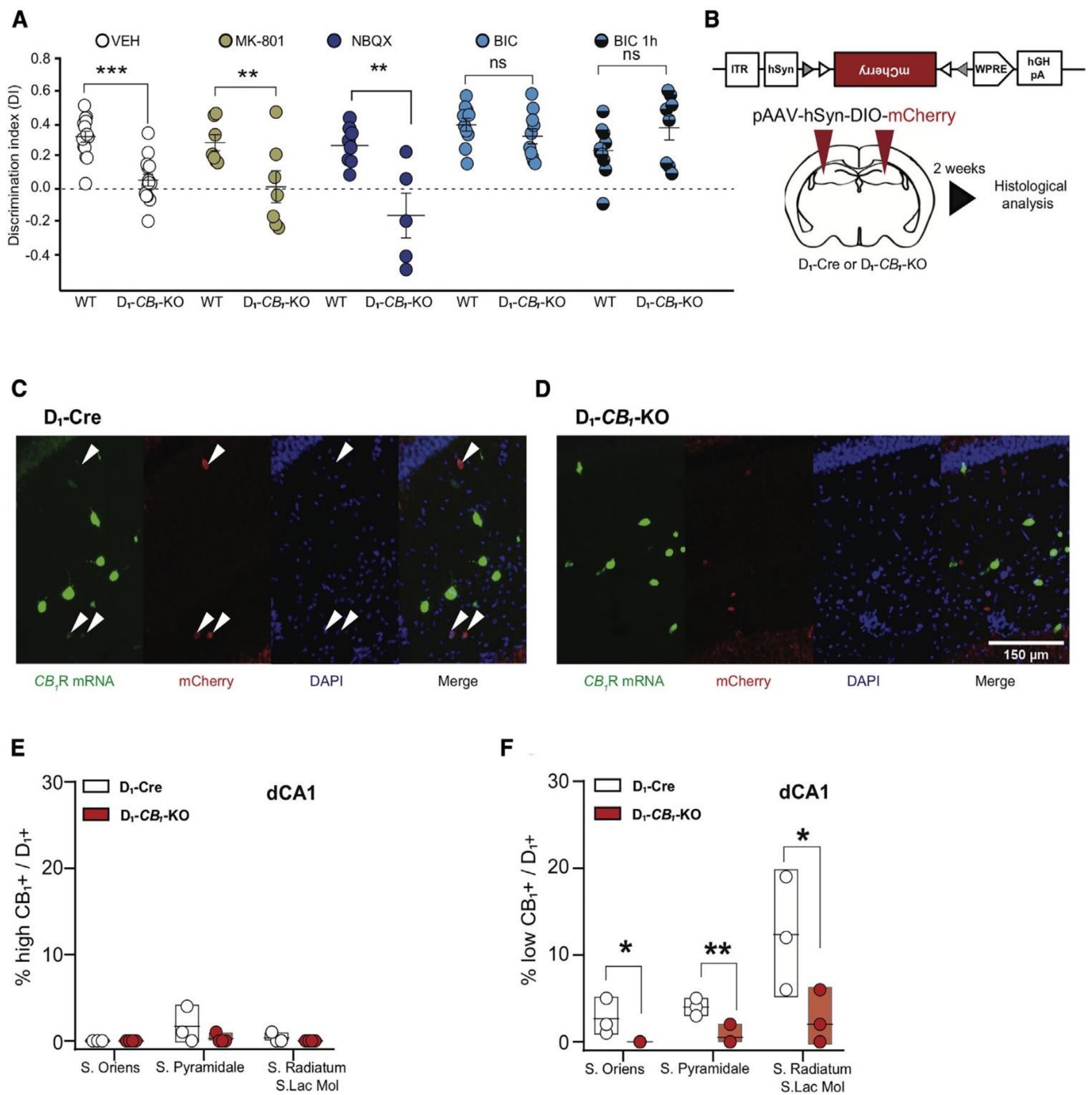

Figure 3. Hippocampal CB_1R/D_1R-Positive Interneurons Modulate Synaptic GABAergic Transmission (A) NOR memory performance of mutant mice administered vehicle (n D_1-CB_1-WT = 14, n D_1-CB_1-KO = 14), MK-801 (0.1 mg/kg, intraperitoneally [i.p.]; n D_1-CB_1- WT = 7, n D_1-CB_1-KO = 7), NBQX (5 mg/kg, i.p.; n D_1-CB_1-WT = 8, n D_1-CB_1-KO = 5), or bicuculline immediately after (n D_1-CB_1-WT = 10, n D_1-CB_1-KO = 10) or 1 h after the training phase (n D_1-CB_1-WT = 10, n D_1-CB_1-KO = 8). (B) Schematic representation of the experimental procedure to detect CB_1R mRNA in D_1R-positive cells. (C and D) Representative images of CB_1R mRNA (green) and mCherry protein (red) labeling in the hippocampal CA1 region of D_1-Cre (C) and D_1-CB_1-KO (D) mice. White arrows indicate colocalization of CB_1R-positive and D_1R-positive cell bodies. Scale bar, 150 mm. (E and F) Layer-specific distribution of the percentage of cell bodies expressing high (E) and low amounts (F) of CB_1Rs, which colocalize with mCherry-positive (i.e., D_1R-positive) in D_1-Cre (n = 3) and D_1-CB_1-KO (n = 3). Data, mean ± SEM. *$p < 0.05$, **$p < 0.01$. See also Figure S2 and Table S1.

Oliveira da Cruz et al. Figure 4

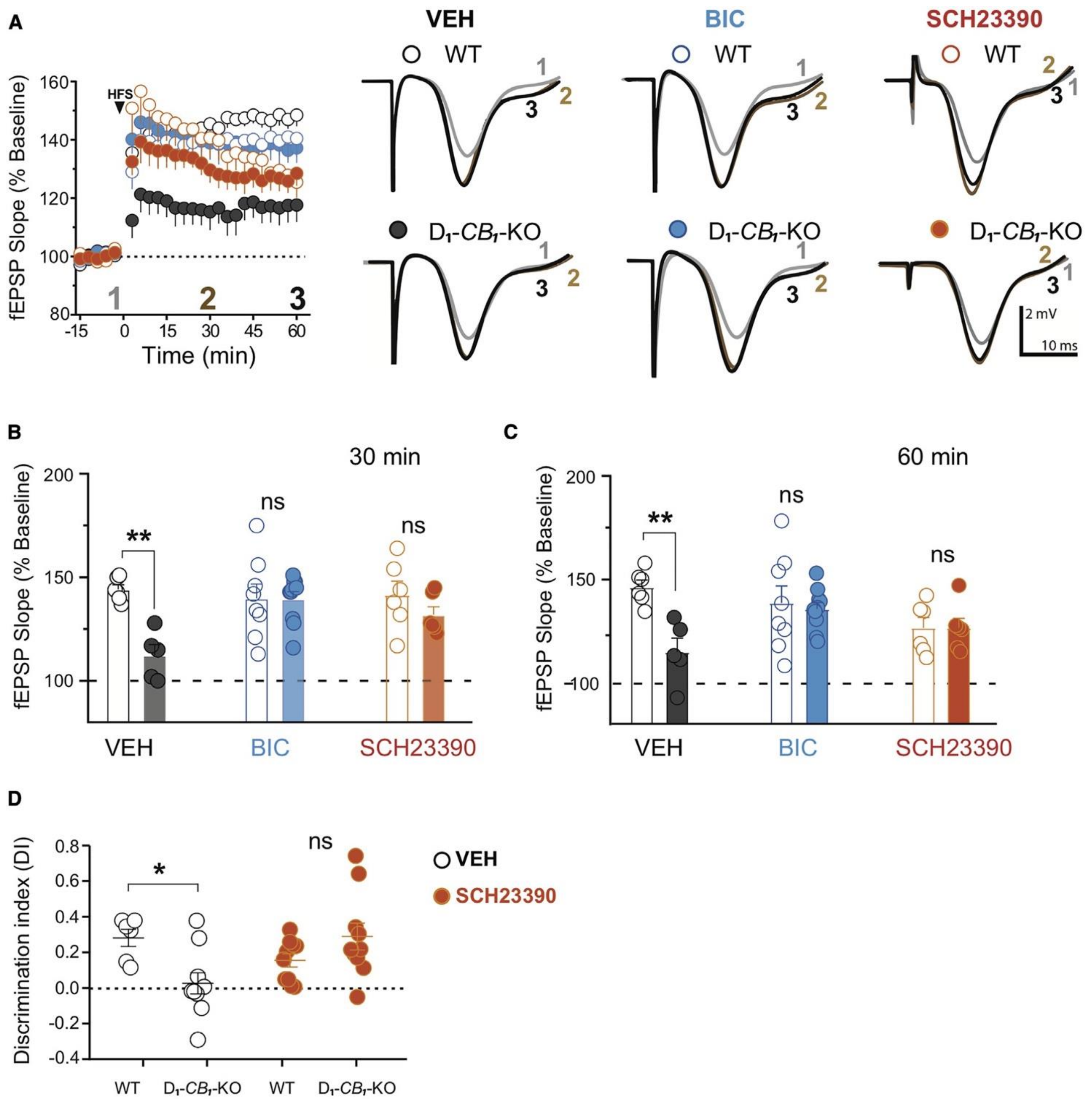

Figure 4. Cellular Mechanisms Linking D_1R Signaling with GABAergic Activity during Learning-Induced Facilitation of In Vivo LTP and Memory Consolidation (A) Effects of the GABAA receptor antagonist bicuculline and the $D_{1/5}R$ antagonist SCH-23390 on learning-induced modulation of in vivo LTP in D_1-CB_1-WT and D_1-CB_1-KO mice. Shown are summary plots of recorded evoked fEPSPs in vehicle (n D_1-CB_1-WT = 6, n D_1-CB_1-KO = 8), bicuculline (0.5 mg/kg, i.p.; n D_1-CB_1-WT = 9, n D_1-CB_1-KO = 11), and SCH-23390 (0.3 mg/kg, i.p.; n D_1-CB_1-WT = 6, n D_1-CB_1-KO = 6). (B and C) Bar histograms of (A), representing normalized fEPSPs from 30 (B) and 60 (C) min after HFS. (D) Memory performance D_1-CB_1-WT and D_1-CB_1-KO mice after being injected with vehicle (n D_1-CB_1-WT = 6, n D_1-CB_1-KO = 10) or SCH-23390 (0.3 mg/kg, i.p.; n D_1-CB_1-WT = 10, n D_1-CB_1-KO = 10). Traces on the right side of the summary plot (A) represent 150 superimposed evoked fEPSPs before HFS (1, gray) and 30 min (2, brown) and 60 min (3, black) after HFS. Data, mean ± SEM. $*p < 0.05$, $**p < 0.01$. See also Figure S3 and Table S1.

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Supplemental Material for

Specific Hippocampal Interneurons Shape Consolidation of Recognition Memory

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Content:

Material and Methods

Figures S1-S3 (relative to main figures)

Table S1

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Model

All experimental procedures were approved by the ethical committee of the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#18111). Maximal efforts were made to reduce the suffering of the animals. Male mice were used in this study.

D_1-CB_1-KO mice were generated as previously described (Monory et al., 2007; Terzian et al., 2011). Briefly, CB1 floxed mice (Marsicano et al., 2003) were crossed with D_1-Cre line (Lemberger et al., 2007), in which the Cre recombinase was placed under the control of the D1 gene (Drd1a) regulatory sequences using transgenesis with modified bacterial artificial chromosomes. The pattern of Cre expression recapitulated the expression pattern of the endogenous Drd1a (Lemberger et al., 2007). Breeding was performed by mating male Cre-positive D_1-CB_1-KO mice with homozygous CB_1-flox female mice deriving from a separate colony. In order to detect possible germline or ectopic recombination events, genotyping of tail samples from pups (PD10) was performed by genomic PCR using primers suited to identify WT, ''floxed'' and ''recombined'' bands. No germline or ectopic recombination was detected. Eight to 14 weeks-old naive male D_1-CB_1-KO and WT littermates were used. 8-14 weeks old male C57BL/6NRj mice purchased from Janvier (France). 8-12 weeks-old D_1-Cre mice breed in the animal facilities of the U1215 we also used. Animals were housed collectively under standard conditions of temperature and humidity in a day/night cycle of 12/12 hours (light on at 7 am). Animals that underwent surgery were kept in individual cages after the procedures to avoid conflict with their littermates. Food and water were provided ad libitum. All the experiments were performed during the light phase. Behavioral experiments were performed from 9 am to 3 pm. Electrophysiology experiments were performed from 8 am to 7 pm.

METHOD DETAILS

Drug preparation and administration

Bicuculline, MK-801, NBQX and SCH-23390 were purchased from Merck (formerly Sigma-Aldrich, France) and were dissolved to their final concentration in physiological saline (NaCl 0.9%). The exogenous DREADD ligand clozapine-N-oxide (CNO, 2 mg/kg) was purchased from Tocris Bioscience (Bristol, UK) and dissolved in saline after gently mixing with a vortex. All drugs were injected intraperitoneally in a volume of 10 ml/kg. Vehicle in all the conditions was composed of physiological saline (NaCl 0.9%) injections.

Novel object recognition memory

We used the novel object recognition (NOR) memory task in an L-maze (Busquets-Garcia et al., 2011, 2013; Hebert-Chatelain et al., 2016; Puighermanal et al., 2009, 2013; Robin et al., 2018).
The task took place in a L-shaped maze made of dark gray polyvinyl chloride made by two identical perpendicular arms (35 cm and 30 cm long respectively for external and internal L walls, 4.5cm wide and 15 cm high walls) placed on a white background (Busquets-Garcia et al., 2011; Puighermanal et al., 2009). The task occurred in a room adjacent to the animal house with a light intensity fixed at 50 lux. The maze was overhung by a video camera allowing the detection and offline scoring of animal's behavior. The task consisted in 3 sequential daily trials of 9 minutes each. During the habituation phase (day 1), mice were placed in the center of the maze and allowed to freely explore the arms in the absence of any objects. The training phase (day 2) consisted in placing the mice again in the corner of the maze in the presence of two identical objects positioned at the extremities of each arm and left to freely explore the maze and the objects. The testing phase occurred 24 hours later (day 3): one of the familiar objects was replaced by a novel object different in its shape, color and texture and mice were left to explore both objects. The position of the novel object and the associations of novel and familiar were randomized. All objects were previously tested to avoid biased preference. Memory performance was assessed by the discrimination index (DI). The DI was calculated as the difference between the time spent exploring the novel (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): DI = [TN-TF]/[TN+TF]. Memory was also evaluated by directly comparing the exploration time of novel and familiar objects, respectively. Object exploration was defined as the orientation of the nose to the object at less than 2 cm. Experienced investigators evaluating the exploration were blind of treatment and/or genotype of the animals. Pharmacological treatments were immediately administered after the training phase.

In vivo electrophysiology in anesthetized mice

Experiments were performed as described in Robin et al. (2018). Mice were anesthetized in a box containing 5% Isoflurane (Virbac, France) before being placed in a stereotaxic frame (Model 900, Kopf instruments, CA, USA) in which 1.0% to 1.5% of Isoflurane was continuously supplied via an anesthetic mask during the whole duration of the experiment. The body temperature was maintained at ± 36.5°C using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA) and the state of anesthesia was assessed by mild tail pinch. Before surgery, 100 mL of the local anesthetic lurocaine (vetoquinol, France) was injected in the scalp region. Surgical procedure started with a longitudinal incision of 1.5 cm in length aimed to expose Bregma and Lambda. After ensuring the correct alignment of the head, two holes were drilled in the skull for electrode placement. Glass recording electrodes were inserted in the CA1 stratum radiatum, and a concentric stimulating bipolar electrode (Model CBARC50, FHC, ME, USA) placed in the CA3 region. Coordinates were as follows: CA1 stratum radiatum: A/P 1.5, M/L 1.0, DV 1.20; CA3: A/P 2.2, M/L 2.8, D/V 1.3 (20° insertion angle). The recording electrode (tip diameter = 1–2 mm, 2-4 MU) was filled with a 2% pontamine sky blue solution in 0.5M sodium acetate. At first the recording electrode was placed by hand until it reached the surface of the brain and then to the final depth using a hydraulic micro-positioner (Model 2650, KOPF instruments, CA, USA). The stimulation electrode was placed in the correct area using a standard manipulator. Both electrodes were adjusted to find the area with maximum response. In vivo recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 1000 times and filtered (low-pass at 1Hz and high-pass 3000Hz) by a DAGAN 2400A amplifier (DAGAN Corporation, MN, USA). fEPSPs were digitized and collected on-line using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design, Cambridge, UK). Test pulses were generated through an Isolated Constant Current Stimulator (DS3, Digitimer, Hertfordshire, UK) triggered by the SPIKE 2 output sequencer via CED 1401 and collected every 2 s at a 10 kHz sampling frequency and then averaged every 180 s. Test pulse intensities were typically between 40-250 mA with a duration of 50 ms. Basal stimulation intensity was adjusted to 30%–50% of the current intensity that evoked a maximum field response. All responses were expressed as

percent from the average responses recorded during the 15 min before HFS. HFS was induced by applying 3 trains of 100 Hz (1 s each), separated by 20 s interval. fEPSP were then recorded for a period of 60 min. C57BL6/NRj mice underwent this in vivo electrophysiology procedure after the training phase of NOR task. Also, where specified, D_1-CB_1-KO and D_1-CB_1-WT received an injection of Bicuculine (0.5 mg/kg, intraperitoneal) or SCH 23390 (0.3 mg/kg, intraperitoneal) or vehicle immediately after undergoing training in NORT and before being subjected to the in vivo electrophysiology procedure. At the end the experiment, the position of the electrodes was marked (recording area: iontophoretic infusion of the recording solution during 180 s at 20mA; stimulation area: continuous current discharge over 20 s at +20mA) and histological verification was performed ex vivo.

Surgery and viral administration

Mice were anesthetized in a box containing 5% Isoflurane (Virbac, France) before being placed in a stereotaxic frame (Model 900, Kopf instruments, CA, USA) in which 1.0% to 1.5% of Isoflurane was continuously supplied via an anesthetic mask during the whole duration of the experiment. For viral intra-HPC AAV delivery, mice were submitted to stereotaxic surgery (as above) and AAV vectors were injected with the help of a microsyringe (0.25 mL Hamilton syringe with a 30-gauge beveled needle) attached to a pump (UMP3-1, World Precision Instruments, FL, USA). Where specified, D_1-CB_1-WT and D_1-CB_1-KO mice were injected directly into the hippocampus (HPC) or striatum (STR) (0.5 ml per injection site at a rate of 0.5 ml per min), with the following coordinates: HPC, AP -1.8; ML ± 1; DV -2.0 and -1.5; Striatum: AP -1.34; ML ± 2.8; DV -1.84. Following virus delivery, the syringe was left in place for 1 minute before being slowly withdrawn from the brain. CB_1 floxed mice were injected with rAAV-CAG-DIO (empty control vector), AAV-CAG-DIO-CB1 or AAV-CAG-DIO-CB1-myc to induce re-expression of the CB_1 receptor gene in hippocampal or striatal D_1-positive cells. To generate the aforementioned rAAVs, mouse CB_1 receptor coding sequence (either native or fused to myc-tag at the C term) was cloned in rAAV-CAG-DIO vector using standard molecular cloning technology. The coding sequence was cloned inverted in orientation to allow Cre-dependent expression of CB_1 receptors (Atasoy et al., 2008). In another experiment, and using the same procedure as described as above, D_1-CB_1-WT and D_1-CB_1-KO mice were injected intra hippocampally (AP -1.8; ML ± 1; DV -2.0 and -1.5), with pAAV-hSyn-DIO-hM4D(Gi)-mCherry or pAAV-hSyn-DIO-

mCherry (addgene, USA). For anatomical experiments and using the same procedure as above, D_1-Cre and D_1-CB_1-KO were injected intra hippocampally with pAAV-hSyn- DIO-mCherry. In this specific experiment, expression was allowed to take place for 2 weeks. For the remaining experiments, animals were used around 4-5 weeks after local infusions. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments.

Immunohistochemistry on free-floating sections

Mice were anesthetized with pentobarbital (Exagon, Axience SAS, 400 mg/kg body weight), transcardially perfused with phosphate-buffered solution (PBS 0.1M, pH 7.4) before being fixed with 4% formaldehyde (Sigma-Aldrich). The brains were extracted and incubated overnight at 4C in the same fixative, then embedded with sucrose 30% for 3 days and finally frozen in 2-methylbutane (Sigma-Aldrich) at -80°C. Free-floating frozen coronal sections (40 mm) were cut out with a cryostat (Microm HM 500M Microm Microtech), collected collected in an antifreeze solution and conserved at -20°C. Sections were permeabilized in a blocking solution (in PBS: 10% donkey serum, 0.3% Triton X-100) for 1 hour at room temperature (RT). Then, sections were incubated with a rabbit primary antibody against the C-myc epitope tag (1:1000, BioLegend) overnight at 4°C. After several washes with PBS, slices were incubated for 2 hours with a secondary antibody goat anti-rabbit Alexa Fluor 488 (1:500, Fisher Scientific) and then washed in PBS at RT. Finally, sections were incubated with 4’,6-diamidino-2-phenylindole (DAPI 1:20000, Fisher Scientific) diluted in PBS for 5 minutes to visualize cell nuclei and then were washed, mounted and coverslipped. All the antibodies were diluted in blocking solution. The sections were imagedimaged with a slides scanner Hamamatsu Nanozoomer 2.0 HT.

Combined Fluorescent in situ hybridization (FISH)/ Immunohistochemistry (IHC) on free-floating frozen sections

Mice were anesthetized with pentobarbital (Exagon, Axience SAS, 400 mg/kg body weight), transcardially perfused with PBS (0.1M, pH 7.4) before being fixed with 4% formaldehyde (Sigma-Aldrich). The brains were extracted and incubated overnight at 4°C in the same fixative, then embedded with sucrose 30% for 3 days and finally frozen in 2-methylbutane (Sigma-Aldrich) at -

80°C. Free-floating frozen coronal sections were cut out with a cryostat (30 mm, Microm HM 500M Microm Microtech) and collected in an anti-freeze solution and conserved at -20°C.

Section were washed several times with PBS with diethyl pyrocarbonate (PBS-DEPC) to wash out the antifreeze solution. The endogenous peroxidases were inactivated by incubating the free-floating sections with 3% H2O2 in PBS-DEPC for 30 minutes. All endogenous biotin, biotin receptors, and avidin binding sites present in the tissue were blocked by using the Avidin/Biotin Blocking Kit (Vector Labs, USA). Then, the slices were incubated overnight at RT with a rabbit polyclonal primary antibody against DsRed (1:1000, Takara Bio) diluted in a blocking solution (0.3% Triton X-100 diluted in PBS-DEPC). The following day, after several washes, the sections were incubated with a secondary antibody goat anti-rabbit conjugated to a horseradish peroxidase (HRP) (1:500, Cell Signaling Technology) during 2 hours at RT followed by TSA Biotin System (Biotin TSA 1:100, PerkinElmer) for 10 minutes at RT. After several washes, the slices were fixed with 4% of formaldehyde (Sigma Aldrich) for 10 minutes and blocked with 0.2M HCl for 20 minutes at RT. Then, the section was acetylated in 0.1 M Triethanolamine, 0.25% Acetic Anhydride for 10 minutes. This step was performed to reduce non-specific probe binding. Sections were hybridized overnight at 60°C with Digoxigenin (DIG)-labeled riboprobe against mouse CB_1 receptor (1:1000, prepared as described in Marsicano and Lutz, 1999). After hybridization, the slices were washed with different stringency wash buffers at 65°C. Then, the sections were incubated with 3% of H2O2 for 30 minutes at RT and blocked 1 hour with NEN blocking buffer prepared according to the manufacturer's protocol (PerkinElmer). Anti-DIG antibody conjugated to HRP (1:2000, Roche) was applied for 2 hours at RT. The signal of CB_1 receptor hybridization was revealed by a TSA reaction using fluorescein isothiocyanate (FITC)-labeled tyramide (1:80 for 12 minutes, Perkin Elmer). After several washes, the free-floating slices were incubated overnight at 4°C with Streptavidin-Texas Red (1:400, PerkinElmer). Finally, the slices were incubated with DAPI (1:20000; Fisher Scientific) diluted in PBS, following by several washes, to finally be mounted, coverslipped and imaged with an epifluorescence Leica DM 6000 microscope (Leica, Germany).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data collection

No statistical methods were used to pre-determine sample sizes, but they are similar to those reported in previous publications. All data collection and/or analysis were performed blind to the conditions of the experimenter except for the in vivo electrophysiological experiments. All mice were assigned randomly to the different experimental conditions.

Fluorescence quantifications

Cells expressing mRNAs were quantified in the different layers (stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum moleculare) of the dorsal hippocampus. CB_1 receptor positive cells were classified according to the level of transcript visualized by the intensity of fluorescence (Marsicano and Lutz, 1999; Terral et al., 2019). ''High-CB_1'' cells were considered to be round-shaped and intense staining covering the entire nucleus whereas ''Low-CB_1'' cells were defined with discontinuous shape and lowest intensity of fluorescence allowing the discrimination of grains of staining.

Statistical analyses

Data were expressed as mean ± SEM or single data points and were analyzed with Prism 6.0 (Graphpad Software), using two-tails t test (paired, unpaired) or one-way ANOVA (Dunnett's), two-way ANOVA (sidak's). Sample sizes and p values can be found in figure legends and Table S1.

Figure S1

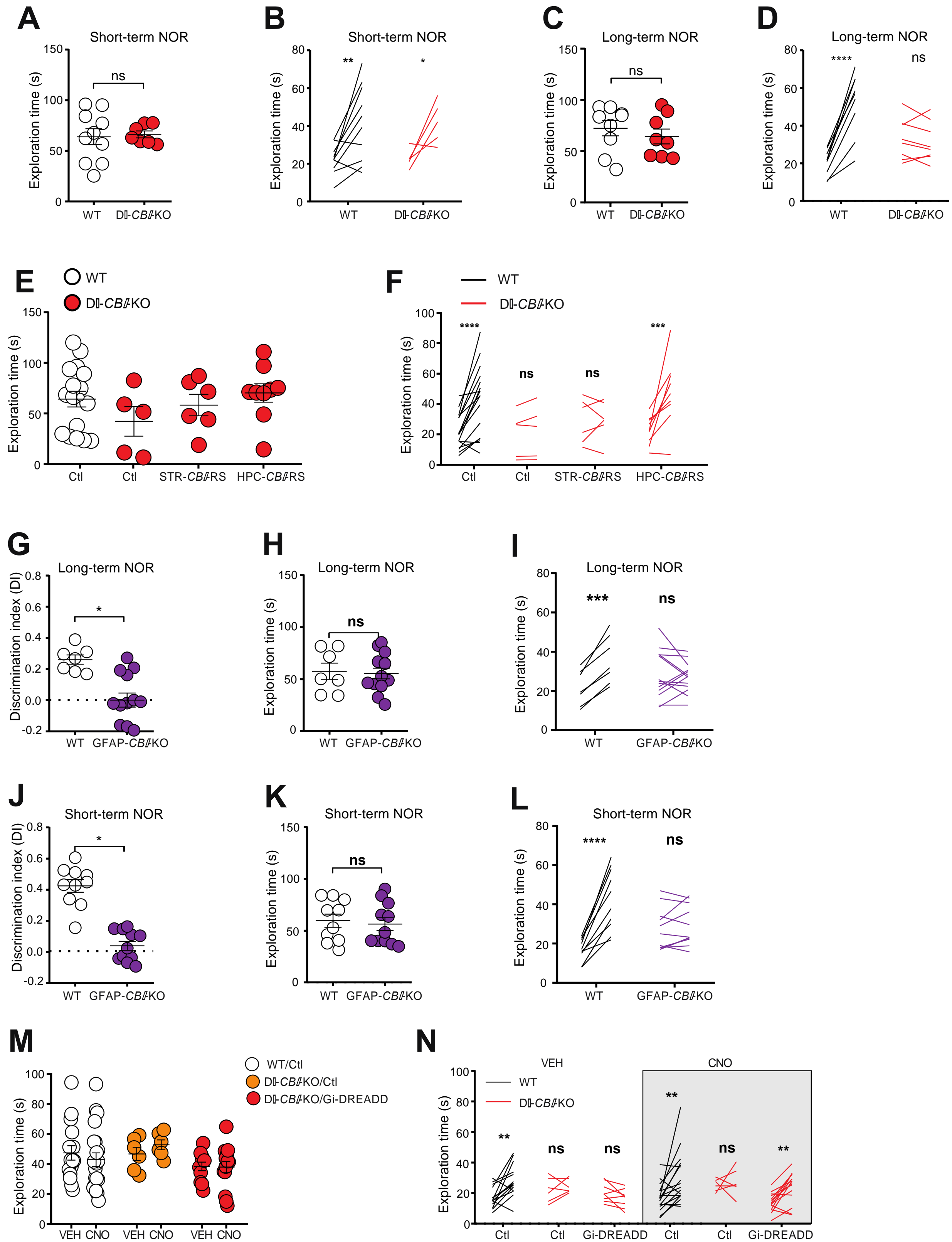

FIGURE S1. RELATED TO FIGURE 1.

(A) Total exploration time, and (B) exploration time of the familiar versus the novel object of D_1-CB_1-KO mice and WT littermates in the Short-term NOR task. (C) Total exploration time, and (D) exploration time of the familiar versus the novel object of D_1-CB_1-KO mice and WT littermates in the Long-term NOR task. (E) Total exploration time, and (F) exploration time of the familiar versus the novel object of STR-CB_1-RS, HPC-CB_1-RS and control mice in the Long-term NOR task. (G) Memory performance, (H) total exploration, and (I) exploration time of the familiar versus the novel object of D_1-CB_1-KO mice and WT littermates in the Long-term NOR task. (J) Memory performance, (K) total exploration, and (L) exploration time of the familiar versus the novel object of D_1-CB_1-KO mice and WT littermates in the Short-term NOR task. (M) Total exploration time, and (N) exploration time of the familiar versus the novel object of D_1-CB_1-KO and WT littermates intra-hippocampally injected with hM4D(Gi) virus or mCherry, with VEH or CNO in the Long-term NOR task. See also Table S1.

Figure S2

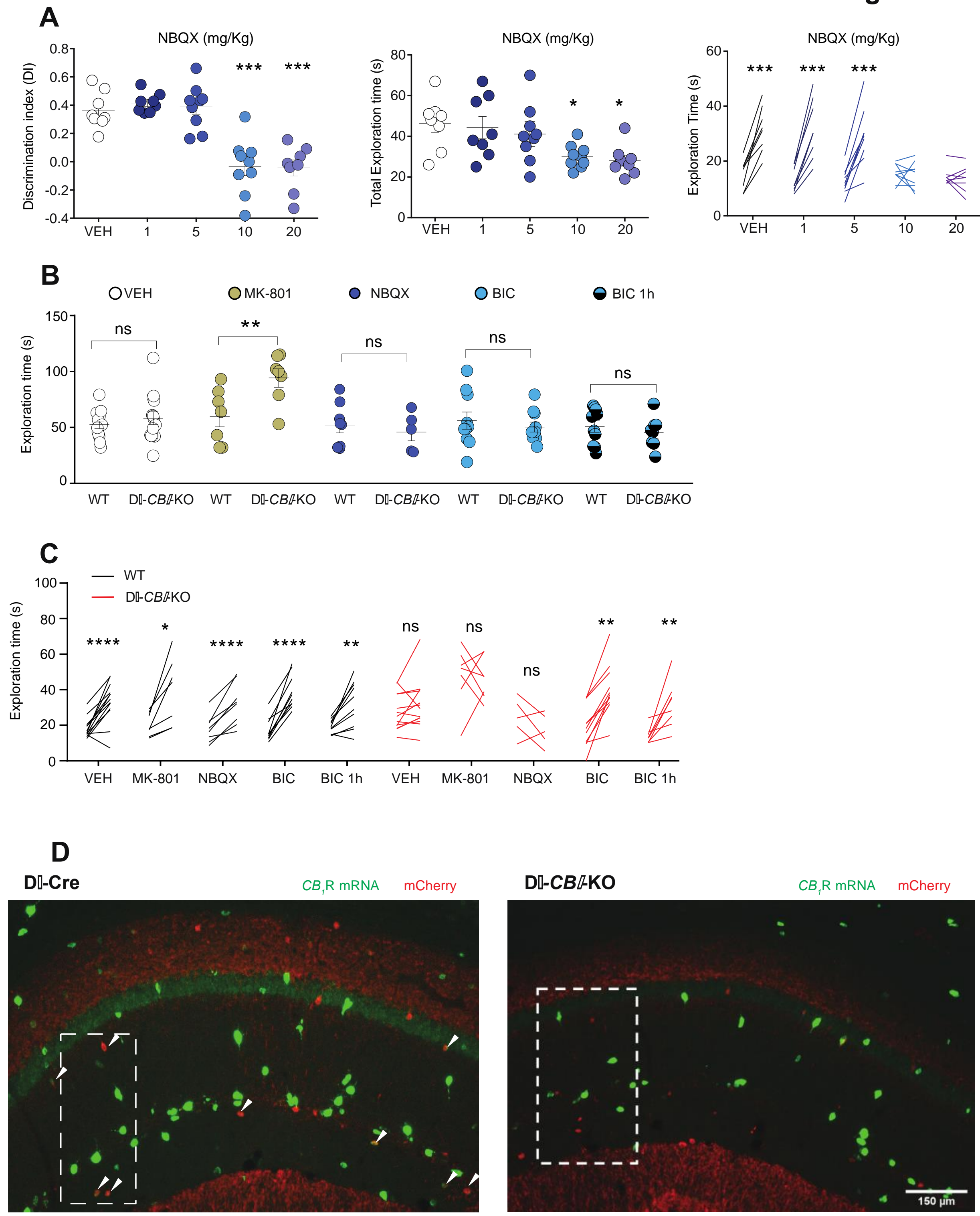

FIGURE S2. RELATED TO FIGURE 3

(A) Memory performance, total exploration time, and exploration time of the familiar versus the novel object of C57BL6/NRj mice injected systemically with different doses of NBQX (in mg/kg, IP). (B) Total exploration, and (C) exploration time of the familiar versus the novel object time of D_1-CB_1-KO mice and WT littermates with systemic injections of VEH, MK-801, NBQX and Bicuculine (BIC). (D) Representative micrographs of the dorsal hippocampus of D_1-Cre and D_1-CB_1-KO mice showing the co-expression of CB_1R mRNA and mCherry (D_1R-positive cells). The dotted white square is the area showed in main Figure 3C. Scale bar = 150 µm. See also Table S1.

Figure S3

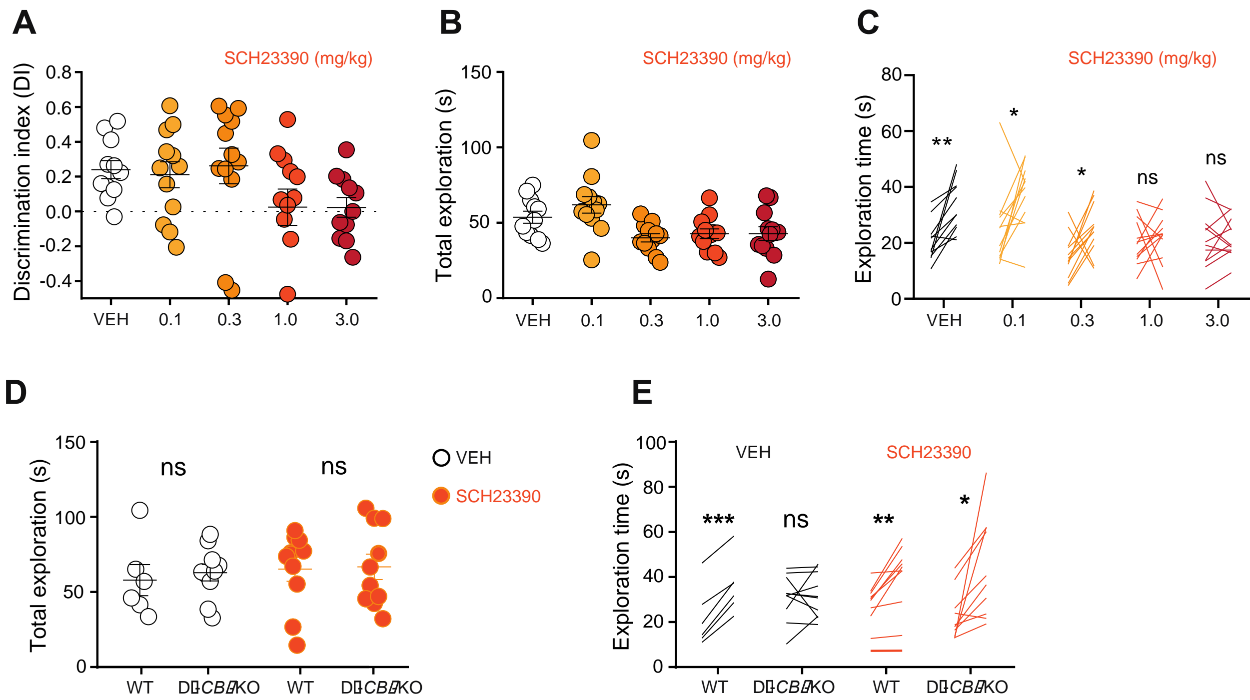

FIGURE S3. RELATED TO FIGURE 4

(A) Memory performance, (B) Total exploration time, and (C) Exploration time of the familiar versus the novel object of C57BL6/NRj mice injected with different doses of SCH23390 (in mg/kg, IP). (D) Total exploration, and (E) exploration time of the familiar versus the novel object time of D_1-CB_1-KO mice and WT littermates with systemic injections of SCH23390 (0.3 mg/kg, IP). See also Table S1.

Table S1

Figure	Panel	Conditions	"n"	Analysis (post-hoc test reported)	Factors analyzed	F-ratios	P values
1	B	D1-CB1-WT vs D1-CB1-KO	10, 7	Unpaired t-test	-	-	0,708
	C	D1-CB1-WT vs D1-CB1-KO	9, 8	Unpaired t-test	-	-	< 0,0001
	E	D1-CB1-WT(Control) vs D1-CB1-KO (Control)	17, 5	Unpaired t-test	-	-	0,0271
	E	D1-CB1-KO: (Control) vs (R-CB1-STR) vs (R-CB1-HPC)	5, 6, 9	One-way ANOVA (Dunnet)	-	-	0,0133
	I	" VEH vs CNO": "Control", D1-CB1-KO-DREADD-Gi", " D1-CB1-KO-mCherry"	(16, 14) (11, 14) (6, 7)	2-WAY ANOVA (Sidak)	Treatment vs Genotype	Interaction F (2, 62) = 5,107	0,0088
						Genotype F (2, 62) = 7,266	0,0015
						Treatment F (1, 62) = 4,587	0,0362
2	B	D1-CB1-WT vs D1-CB1-KO - 30 min	8, 8	Unpaired t-test	-	-	0,9444
		D1-CB1-WT vs D1-CB1-KO - 60 min	8, 8	Unpaired t-test	-	-	0,4546
	E	D1-CB1-WT vs D1-CB1-KO - 30 min	8, 11	Unpaired t-test	-	-	0,0455
		D1-CB1-WT vs D1-CB1-KO - 60 min	8, 11	Unpaired t-test	-	-	0,0253
	G	D1-CB1-WT vs D1-CB1-KO - 30 min	10, 10	Unpaired t-test	-	-	0,0266
		D1-CB1-WT vs D1-CB1-KO - 60 min	10, 10	Unpaired t-test	-	-	0,0306
3	A	" D1-CB1-WT vs D1-CB1-KO": "VEH" / "MK-801" /"NBQX"/ "BIC" / "BIC 1h"	(14, 14) (7, 7)	2-WAY ANOVA (Sidak)	Treatment vs Genotype	Interaction F (4, 83) = 2,811	0,0003
			(8, 5)			Treatment F (4, 83) = 6,787	0,0001
			(10, 10)			Genotype F (1, 83) = 10,17	0,0021
			(10, 8)				
	F	S Oriens: D1-CRE vs D1-CB1-KO	(3, 4)	Unpaired t-test	-	-	0,0453
		S Pyramidale: D1-CRE vs D1-CB1-KO	(3, 4)	Unpaired t-test	-	-	0,0059
		S Radiatum / L Moleculare: D1-CRE vs D1-CB1-KO	(3, 4)	Unpaired t-test	-	-	0,0337
4	B	" D1-CB1-WT vs D1-CB1-KO": "VEH" / "Bicuculline" / "SCH 23390" at 30 min	(6, 5)	2-WAY ANOVA (Sidak)	Genotype vs Treatment	Interaction F (2, 35) = 4,670	0,0159
			(8, 10)			Genotype F (1, 35) = 10,30	0,0028
			(6, 6)			Treatment F (2, 35) = 2,492	0,0973
	C	" D1-CB1-WT vs D1-CB1-KO": "VEH" / "Bicuculline" / "SCH 23390" at 60 min	(6, 5)	2-WAY ANOVA (Sidak)	Genotype vs Treatment	Interaction F (2, 35) = 4,232	0,0226
			(8, 10)			Treatment F (1, 35) = 6,108	0,0185
			(6, 6)			Genotype F (2, 35) = 2,014	0,1487
	D	" D1-CB1-WT vs D1-CB1-KO": "VEH"/"SCH 23390"	(6, 10)		Genotype vs Treatment	Interaction F (1, 32) = 10,15	0,0032
						Treatment F (1, 32) = 0,9844	0,3285
			(10, 10)			Genotype F (1, 32) = 1,236	0,2746

TABLE S1. STATISTICAL ANALYSIS RELATED TO MAIN AND SUPPLEMENTARY FIGURES

PART 2 – LINKING MITOCHONDRIAL G-PROTEIN SIGNALING TO CANNABINOIDS-INDUCED AMNESIA: A NEW MITOCHONDRIA-SPECIFIC CHEMOGENETIC STRATEGY

Linking Mitochondrial G-protein Signaling to cannabinoids-induced amnesia: A new Mitochondria-specific chemogenetic Strategy

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*: share first authorship

#: share senior authorship

Until recently physiological and behavioral CB_1R-dependent effects had been exclusively attributed to canonical activation of plasma membrane CB_1Rs (Campbell, 2001; Athanasiou et al. 2007). With the identification of intracellular mitochondrial CB_1Rs in different tissues, in particular in the brain (Aquila et al, 2010; Benard et al, 2012; Hebert-Chatelain et al, 2014; Koch et al, 2015; Mendizabal-Zubiaga et al, 2016), this idea has been progressively changing.
Considering the brain as a high demander of energy support (Erecinska and Silver, 2001; Rolfe and Brown, 1997), the study of the impact of mitochondrial CB_1 receptor and its G-protein intra-mitochondrial signaling on cellular energy metabolism is becoming a highly interesting research field. So far, studies on the role of mitochondria on brain functions have led to understand their involvement in neuronal excitability and regulation of synaptic activity, beyond their central role in the ATP production, Ca2+ homeostasis, production of ROS, synthesis and metabolism of neurotransmitters and apoptosis (Turrens, 2003; Ruggiero et al. 2021; Bock and Tait, 2019). However, the consequences of brain mitochondrial modulation via factivation of the intra-mitochondrial G-protein signaling and its physiological and behavioral effects are far from being completely understood.
In the last years, some studies have shown that slight alterations of mitochondrial functions through mtCB_1 receptors modulations could have an impact on energy metabolism and memory functions and behavior (Benard et al. 2012; Hebert-Chatelain et al. 2016; Jimenez-Blasco et al. 2020; Soria-Gomez et al. 2021). Indeed, the particular activation of this subcellular pool of the receptor results in mitochondrial inhibition and decrease of synaptic transmission, providing the physiological bases for cannabinoid-induced behavioral alterations such as memory and motor impairment (Hebert-Chatelein et al. 2016; Soria-Gomez et al. 2021).
Thus, our work aims at investigating how the mitochondrial G-protein signaling may represent a regulator of cellular functions and thus analyzing the effects of its modulation. Indeed, we aim at understanding how playing with mitochondrial G-protein signaling can affect the bioenergetic processes and behavior, in particular focusing on hippocampal neurons and the amnesic effects of cannabinoids.

RESULTS

As shown in Oliveira da Cruz et al. (2020), mutant mice bearing a deletion of the CB_1R gene in cells expressing D_1R (D_1-CB_1-knockout [KO] mice) displayed an impaired phenotype in long-term memory (24h) in NOR which was shown to be hippocampus-dependent.

To understand whether and how $mtCB_1$ receptor in D_1-positive cells can contribute to memory impairment we infused a viral vector leading to the Cre-dependent re-expression of the CB_1 receptor (AAV-CAG-Flexx-CB_1) or the mutant form DN22-CB_1 (which is excluded from mitochondria without losing functionality [Hebert-Chatelain et al. 2016], AAV-CAG-Flexx-DN22-CB_1) or control (AAV-CAG-Flexx) in the hippocampus of mutant mice lacking CB_1 receptors in D_1-positive cells (D_1-CB_1-KO mice) in order to obtain a re-expression of total CB_1Rs or $pmCB_1$ respectively, exclusively in cells where Cre is present (hereafter called D_1R-positive) in this brain region **(Fig. 1A,B)**. As expected, D_1-CB_1-KO mice injected with AAV-CAG-Flexx showed strong memory impairment in long-term memory performance as compared to their wild-type littermates **(Fig. 1C)**, with no changes in total exploration time **(Fig. 1D)**. Re-expression of CB_1R in D_1-CB_1-KO mice was able to rescue the memory performance of the mutant mice **(Fig. 1C)** and strikingly, also the infusion of AAV-CAG-Flexx-DN22-CB_1 in the hippocampus of D_1-CB_1-KO mice fully rescued the NOR memory impairment **(Fig. 1C)**, indicating that $mtCB_1$ receptors in D_1-positive cells is not required for normal long-term memory formation as well as the absence of $mtCB_1$ receptors in the same cells did not impair memory functions in NOR task **(Fig. 1C)**.

As previously showed not only from our team, THC treatment after training induced strong memory impairment in the NOR test via CB_1 receptor. Rescue of CB_1 receptor in D_1-CB_1-KO mice resulted in amnesia when mice were treated with THC **(Fig. 1C)**, as also shown for control wild type mice **(Fig. 1C)**. Interestingly, rescue of DN22-CB_1 was not able to produce amnesia after THC treatment despite recovery of normal memory performance **(Fig. 1C)**.

Therefore, our results showed that appropriate memory performance in the NOR require CB_1 receptors in D_1R-positive hippocampal neurons. Amongst these receptors the one associated to mitochondria-are the ones responsible for THC induced memory impairment, highlighting the difference between endogenous system activation and pharmacological stimulation.

The primary function of CB_1R activation in mitochondria by exogenous cannabinoids is to decrease

mitochondrial oxygen consumption with negative effects on cellular respiration, synaptic transmission, and behavior (Benard et al. 2012; Hebert-Chatelain et al. 2016; Soria-Gomez et al. 2021). Furthermore, we showed that the activation of the Giα protein of $mtCB_1$ receptors is relevant for amnesic effects of cannabinoids presumably through inhibition of sAC and intra-mitochondrial PKA signaling (Hebert-Chatelain et al. 2016).

Thus, we reasoned that the activation of mitochondrial Gs-protein signalling, possibly by increasing mitochondrial respiration with a mechanism that counteract $mtCB_1$ activation, should be able to rescue the THC-induced memory impairment during NOR consolidation in D_1R-positive neurons.

For this purpose, we took advantage of the DREADDs technology (Robinson and Adelman, 2015) and developed a new mitochondria-specific Gs-coupled designer receptor. Indeed, we created a mito-DREADD by fusing the Gs-coupled designer receptor exclusively activated by designer drugs (DREADD-Gs receptor; CNO, clozapine N-oxide, ligand) to a mitochondria leader sequence (MLS) (MLS-DREADD) **(Fig. 2A)**. In this way we aimed at obtaining a mutant Gs coupled receptor which can be targeted to mitochondria, expressed in given cell types and, when activated by a specific ligand (CNO), triggering Gs signaling in mitochondria.

Immunodetection of HA-tag and mitochondrial protein TOM20, in HEK293 cells expressing control vector, native DREADD (used as control) or MLS-DREADD, by confocal microscopy revealed that DREADD spreads throughout the cell, whereas MLS-DREADD-HA is specifically addressed to mitochondria **(Fig. 2B,C)** indicating the succesful targeting of DREADD to this intracellular compartment.

One useful readout of Gs activation at plasma membrane level is the phosphorylation of extracellular signal regulated kinases (ERKs) (Goldsmith and Dhanasekaran, 2007). The activation of Gs-protein by CNO was able to strongly activate ERK phosphorylation in DREADD-transfected cells. Interstingly MLS-DREADD-transfected cells did not increase the phosphorylation of ERK upon CNO treatment, further suggesting the intracellular (mitochondrial) targeting of MLS-DREADD. **(Fig. 2D,E)**.

The functional impact of DREADD and MLS-DREADD on mitochondrial activity was studied by mitochondrial respiration assays. MLS-DREADD activation was able to increase mitochondrial oxygen consumption rate both whole HEK293 cells and isolated mitochondrias from the same cell type. This effect was strongly specific to mitochondrial Gs activation since not present in control or

DREADD-Gs transfected cells **(Fig. 3A,B)**. Similarly, when MLS-DREADD-Gs and DREADD-Gs were virally expressed in mouse hippocampi the mitochondrial respiration was increased by CNO only in hippocampal homogenates from MLS-DREADD-Gs injected mice **(Fig.3D)**.

Moreover, the mitochondrial membrane potential, an important parameter of OXPHOS status, was increased by CNO administration in HEK293 cells expressing MLS-DREADD (and not native DREADD-Gs) suggesting that a higher activation of OXPHOS can be induced via specific modification of mitochondrial G-proteins activity **(Fig. 3C)** together with increased mitochondrial activity.

To understand how the modulation of the mitochondrial signaling can be specifically involved in cannabinoids-induced memory impairment, we expressed DREADD-Gs or MLS-DREADD-Gs in the hippocampal D_1-positive cells, by using CRE-dependent viral vector in D_1-Cre mice. Post-training clozapine N-oxide (CNO) injections did not affect the NOR performance of C57Bl/6N and does not rescue the amnesic effect induced by THC, indicating that the drug or its metabolites have no effect per se (Gomez et al., 2017; **Fig. 4A-C**). Similarly, post-training CNO treatment does not rescue the NOR impairment induced by THC of D1-Cre mice expressing DREADD-Gs **(Fig. 5A-D)**, indicating that plasma membrane Gs activation in D_1-positive neurons is not sufficient for memory rescue. Conversely, post-training CNO treatment fully rescue the NOR impairment induced by THC in D_1-Cre mice expressing MLS-DREADD-Gs, revealing that the specific activation of mitochondrial signaling in D_1-positive neurons is necessary for rescue of THC-induced amnesia during the consolidation process in NOR **(Fig. 6A-D)**.

Altogheter, the results obtained *in vivo* by using MLS-DREADD, confirm an impairment of mitochondrial activity in D_1-positive hippocampal neurons as the leading mechanism for THC-induced amnesia, further strengthening the link between brain bio-energetic status and behavioral processes.

AUTHOR CONTRIBUTIONS

GL performed and analyzed *in vivo* characterization and behavioral experiments and participated to the manuscript writing. ACPZ performed and analyzed *in vitro* characterization experiments and participated to the manuscript writing. GM, LB and EH-C conceived and supervised the whole project and wrote the manuscript. All authors edited and approved the manuscript.

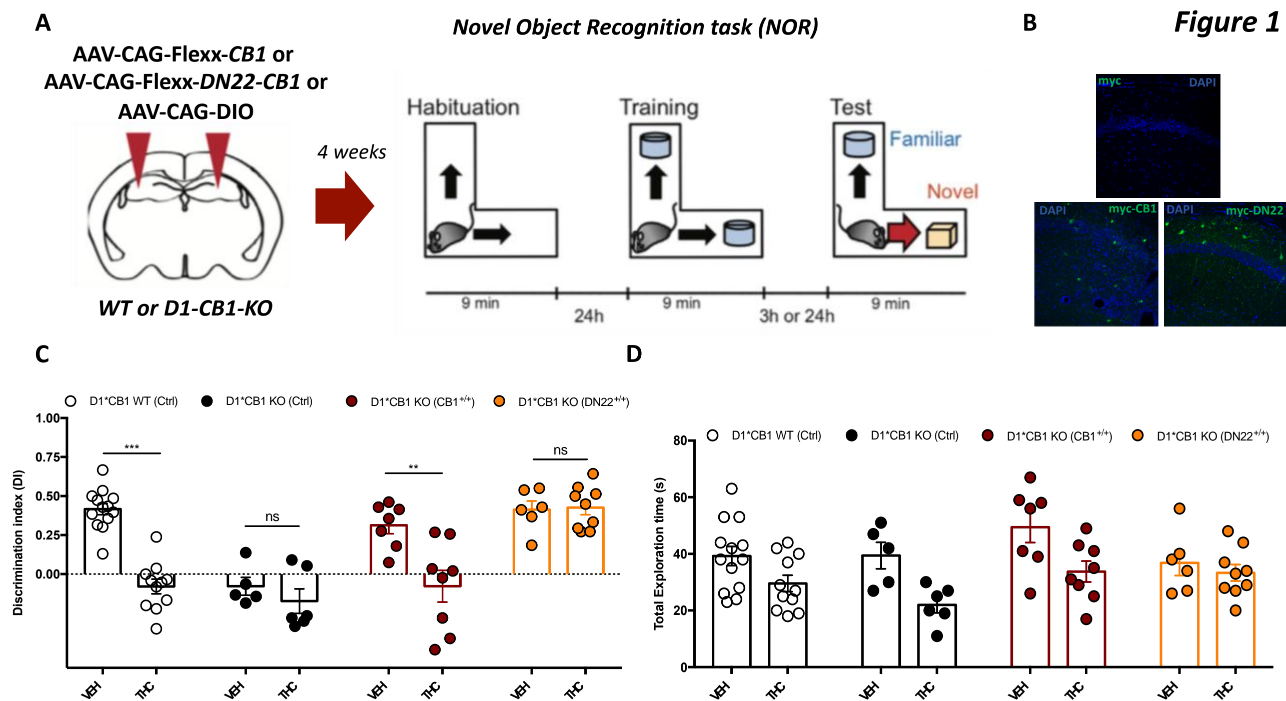

FIGURE 1. HIPPOCAMPAL mtCB$_1$RS IN D1R-POSITIVE CELLS ARE REQUIRED FOR THC-INDUCED MEMORY IMPAIRMENT OF NOR

(A) Schematic representation of the NOR memory task. Schematic representation of the experiment using viral re-expression of the CB$_1$R or DN22-CB$_1$ in the hippocampus of D$_1$-CB$_1$-WT mice and D$_1$-CB$_1$-KO littermates. VEH or THC (5 mg/kg) injections take place after the training phase of the NOR task. (B) Immunofluorescence for myc-tag in order to detect CB$_1$ or DN22-CB$_1$. (C) NOR memory performance of D$_1$-CB$_1$-WT (n VEH = 13, n THC = 11) and D$_1$-CB$_1$-KO mice injected intra-hippocampally with control virus (n VEH = 5, n THC = 6), virus re-expressing CB$_1$Rs (n VEH = 7, n THC = 8), virus re-expressing DN22-CB$_1$ (n VEH = 6, n THC = 9). (D) Total object exploration during the testing phase for all experimental groups. Data, mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant.

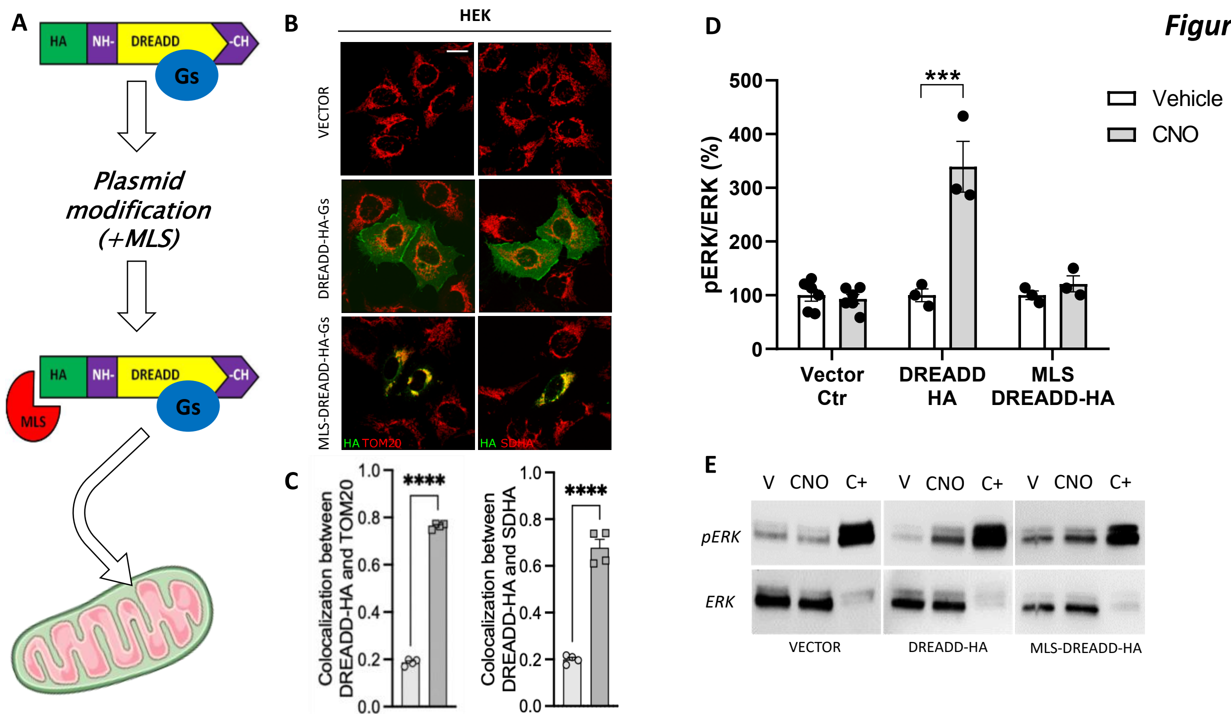

FIGURE 2. CREATION AND CHARACTERIZATION OF MITOCHONDRIA LEADER SEQUENCE DREADD-HA (MLS-DREADD-HA). (A) Cloning strategy to express DREADD-HA at mitochondria. (B) Representative immunofluorescence of HEK cells transfected with empty vector or DREADD-HA or MLS–DREADD-HA expressing plasmid. Green, HA tag staining; red, mitochondrial staining TOM20 or SDHA, succinate dehydrogenase A. (C) Quantification of (MLS-)DREADD-HA expressing plasmids and TOM20 or SDHA colocalization. (D) Effect of CNO (50 uM) on ERK phosphorylation in HEK293 cells transfected with empty vector, DREADD-HA or MLS-DREADD-HA-expressing plasmids FBS was used as positive control (C+). (E) Representative immunoblot showing the effect of CNO (50 uM) on pERK and ERK levels in HEK293 cells transfected with with plasmids expressing empty vector, DREADD-Gs or MLS-DREADD-Gs.

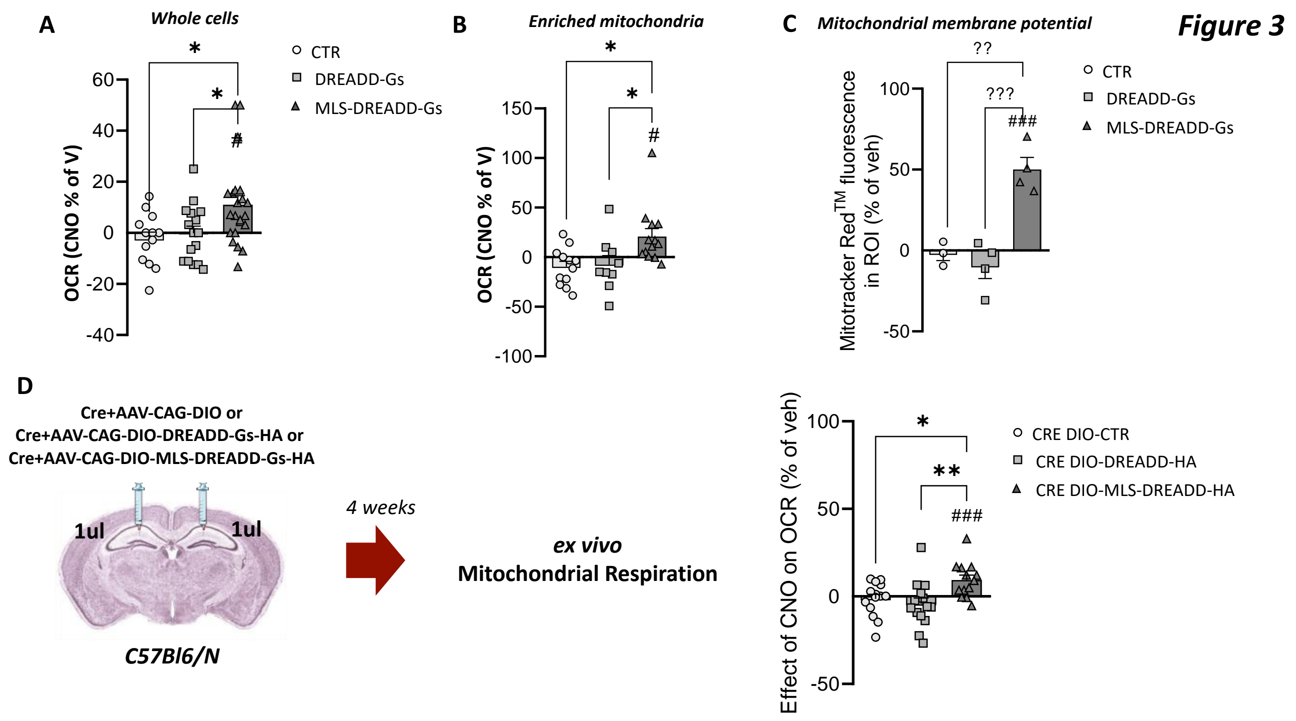

FIGURE 3. THE ACTIVATION OF THE MITOCHONDRIAL G-PROTEIN SIGNALING via MLS-DREADD-HA INCREASES MITOCHONDRIAL RESPIRATION AND MEMBRANE POTENTIAL. (A-B) Effect of CNO (50 uM) on oxygen consumption rate (OCR) in HEK293 whole cells (A) or enriched mitochondrial fraction (B) transfected with control (Ctr) or DREADD-HA or MLS-DREADD-HA plasmid. (C) MitoTracker RedTM fluorescence in HEK293 cells expressing empty vector (CTR), DREADD-HA or MLS-DREADD-HA. (D) The effect of CNO (50 uM) on mitochondrial respiration of homogenized hippocampi from C57Bl/6N mice that received intrahippocampal injection of AAV-Cre and Cre-dependent AAV-DIO-DREADD-HA or AAV-MLS-DREADD-HA or AAV-DIO-CTRL.

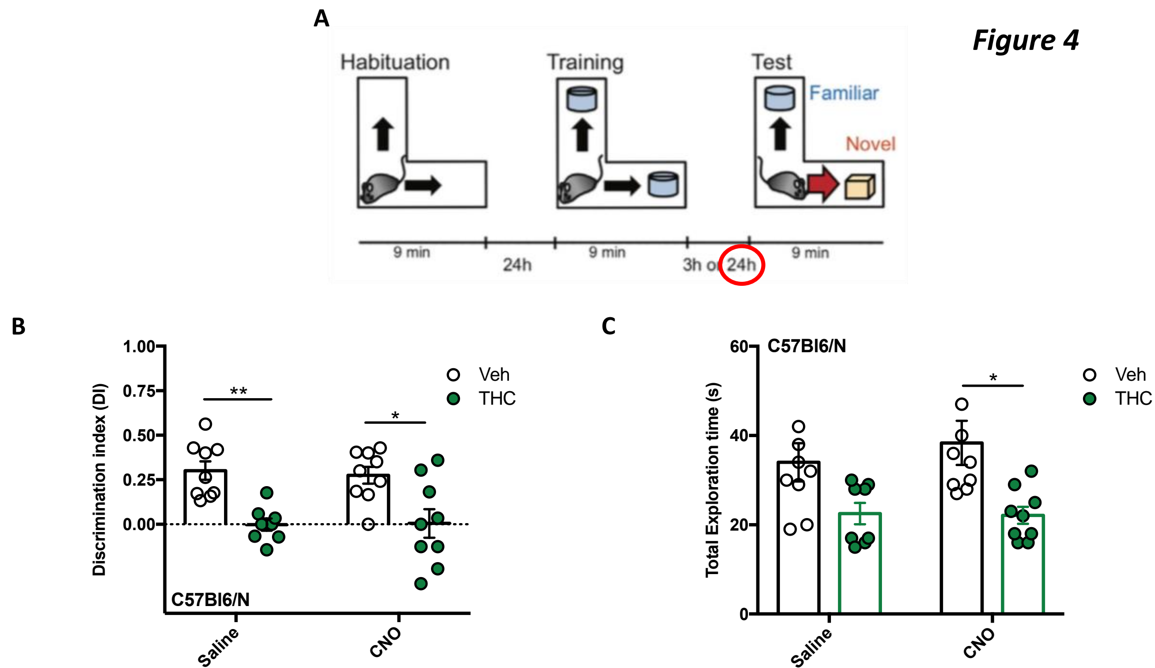

FIGURE 4. NO ALTERATIONS ON MEMORY PERFORMANCE AND THC-INDUCED AMENSIC EFFECT BY CLOZAPINE N-OXIDE (CNO)

(A) Schematic representation of the NOR memory task with treatment after training. (B) NOR memory performance of C57Bl/6N mice. Clozapine N-oxide (CNO; 5 mg/kg) injections take place after the training phase of the NOR task and THC (5 mg/kg) 15 minutes after CNO injection. (B) NOR memory performance of C57Bl/6N mice (n VEH/VEH = 9, n VEH/THC = 8, n CNO/VEH = 9, n CNO/THC = 9). (C) Total object exploration during the testing phase for all treatment groups.

Data, mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. ns, not significant.

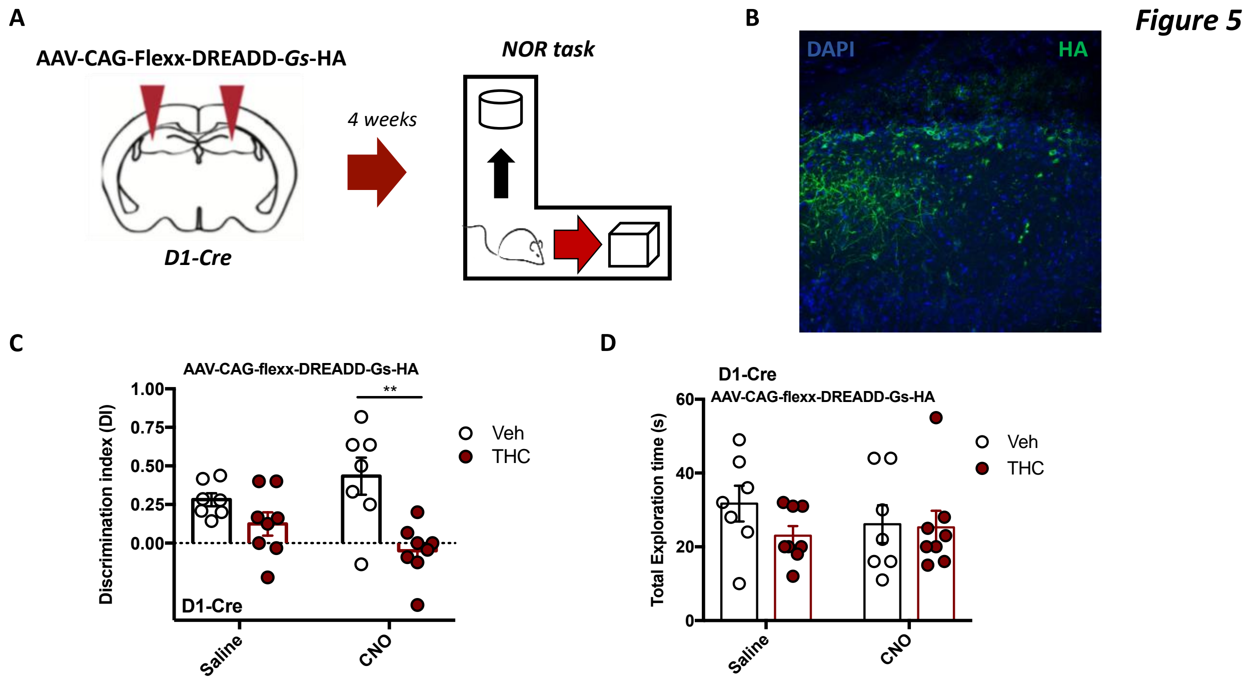

FIGURE 5. THE ACTIVATION OF Gs PROTEIN IN D_1-POSITIVE NEURONS IS NOT ABLE TO RESCUE THE THC-INDUCED AMNESIC EFFECT

(A) Schematic representation of the experiment using viral expression of the DREADD-Gs in the hippocampus of D_1-Cre mice. Clozapine N-oxide (CNO; 5 mg/kg) injections take place after the training phase of the NOR task and THC (5 mg/kg) 15 minutes after CNO injection. (B) Immunofluorescence for HA-tag in order to detect DREADD-Gs. (C) NOR memory performance of D_1-Cre mice injected intra-hippocampally with DREADD-Gs virus (n VEH/VEH = 8, n VEH/THC = 8, n CNO/VEH = 7, n CNO/THC = 8). (D) Total object exploration during the testing phase for all treatment groups. Data, mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant.

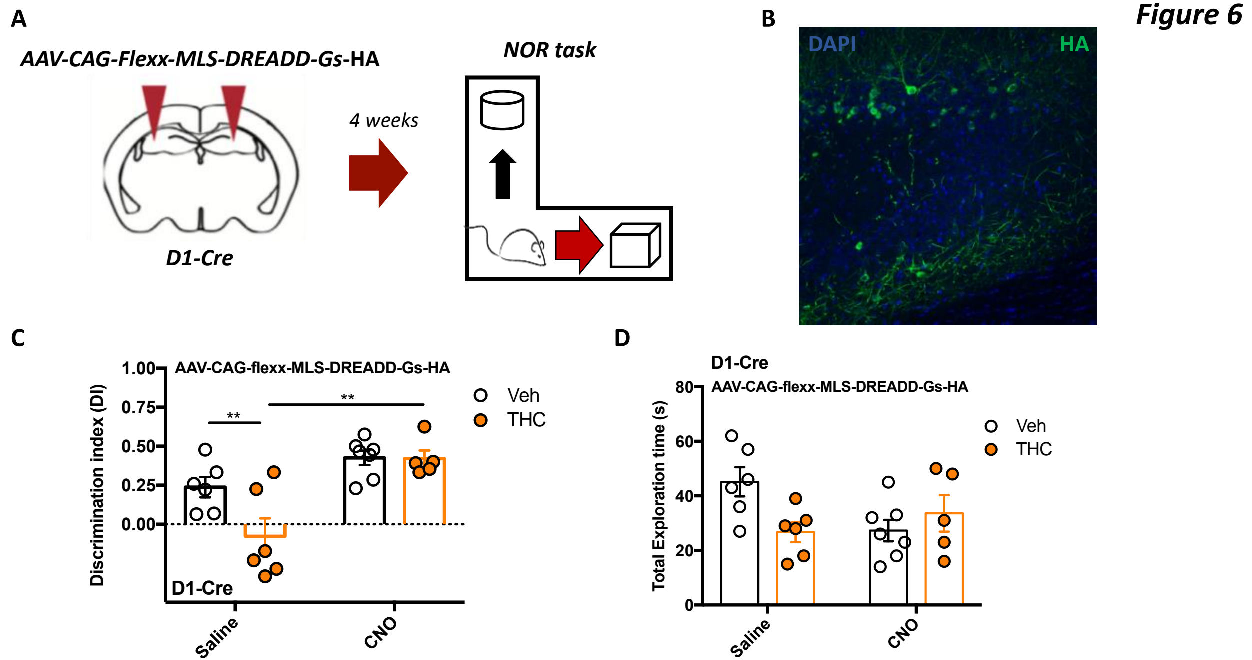

FIGURE 6. THE SPECIFIC ACTIVATION OF Gs PROTEIN IN MITOCHONDRIA OF D_1-POSITIVE NEURONS IS ABLE TO RESCUE THE THC-INDUCED AMNESIC EFFECT

(A) Schematic representation of the experiment using viral expression of the MLS-DREADD-Gs in the hippocampus of D_1-Cre mice. Clozapine N-oxide (CNO; 5 mg/kg) injections take place after the training phase of the NOR task and THC (5 mg/kg) 15 minutes after CNO injection. (B) Immunofluorescence for HA-tag in order to detect MLS-DREADD-Gs. (C) NOR memory performance of D_1-Cre mice injected intra-hippocampally with MLS-DREADD-Gs virus (n VEH/VEH = 6, n VEH/THC = 6, n CNO/VEH = 7, n CNO/THC = 5). (D) Total object exploration during the testing phase for all treatment groups. Data, mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001. ns, not significant.

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SUPPLEMENTARY METHODS

Mice

All experimental procedures were approved by the ethical committee of the French Ministry of Higher Education, Research and Innovation. Maximal efforts were made to reduce the suffering of the animals. Male mice were used in this study.

Animals were housed collectively under standard conditions of temperature and humidity in a day/night cycle of 12/12 hours (light on at 7 am). Food and water were provided ad libitum. All the experiments were performed during the light phase. Behavioral experiments were performed from 9 am to 3 pm. 8-14 weeks old male C57BL/6NRj mice purchased from Janvier (France). D_1-CB_1-KO mice were generated as previously described (Monory et al., 2007; Terzian et al., 2011). Briefly, CB_1 floxed mice (Marsicano et al., 2003) were crossed with D_1-Cre line (Lemberger et al., 2007), in which the Cre recombinase was placed under the control of the D1 gene (Drd1a) regulatory sequences using transgenesis with modified bacterial artificial chromosomes. The pattern of Cre expression recapitulated the expression pattern of the endogenous Drd1a (Lemberger et al., 2007). Breeding was performed by mating male Cre-positive D_1-CB_1-KO mice with homozygous CB_1-flox female mice deriving from a separate colony. In order to detect possible germline or ectopic recombination events, genotyping of tail samples from pups (PD10) was performed by genomic PCR using primers suited to identify WT, ''floxed'' and ''recombined'' bands. No germline or ectopic recombination was detected. 8-14 weeks-old naive male D_1-CB_1-KO and WT littermates were used. 8-12 weeks-old D_1-Cre mice breed in the animal facilities of the U1215 we also used. Animals which underwent surgery were kept in individual cages after the procedures to avoid conflict with their littermates.

Plasmids

pcDNA-DREADD-Gs (fused to HA-tag) was kindly provided by Brian Roth (UNC). pcDNA-MLS-DREADD-Gs (fused to HA-tag) was obtained by cloning the coding sequence of DREADD-Gs in frame with 4× repetition of the mitochondrial leader sequence (aa. 1-30) of human COX-VIII (Di Benedetto et al. 2013). PcDNA based vectors were used for HEK293T expression as well as donor inserts for AAV generation (see after). HA tag was kept in both constructs for in vitro and in vivo immune-detection.

Cell lines

Certified HEK293T (ATCC, CRL-3216TM, lot 62729596) cells were cultured in DMEM Glutamax, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/ mL streptomycin (expect for Oxygen consumption measurements on cells), nonessential amino acids (0.1 mM of Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic Acid, L-Proline, and L-Serine), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA) and maintained at 37C in the presence of 5% CO2. 24H after the seeding, cells were transfected with control plasmid or (MLS-) DREADD-HA-Gs plasmid using polyethylenimine (Polysciences, Warrington, PA).

Drugs

THC was obtained from THC Pharm GmbH (Frankfurt, Germany). For in vivo administration, THC (5 mg/Kg) was prepared freshly before the experiments and was dissolved in a mixture of 5% ethanol, 4% cremophor and saline. Corresponding vehicle solutions were used in control experiments. The exogenous DREADD ligand clozapine-N-oxide (CNO, 5 mg/kg) was purchased from Tocris Bioscience (Bristol, UK) and dissolved in saline after gently mixing with a vortex. For in vitro experiment, CNO was dissolved in DMSO. All drugs were injected intraperitoneally in a volume of 10 ml/kg. Vehicle in all the conditions was composed of physiological saline (NaCl 0.9%) injections. Doses and concentrations of the different drugs were chosen on the basis of previous published data or preliminary experiments.

Viral vectors

To generate AAV-DIO-CB1 and AAV-DIO-DN22, AAV-Flexx-MLS-DREADD and AAV-FLexx-DREADD the coding sequences for the 4 proteins (Hebert-Chatelain et al., 2016) were subcloned in pAAV-CAG-flex plasmid (kindly gifted by Matthias Klugmann, UNSW, Australia) by using standard molecular cloning techniques. The same pAAV-CAG-flex plasmid was used as empty control (AAV-DIO-ctr). AAVs were generated by PEI transfection of HEK293T cells and purified by iodixanol-gradient ultracentrifugation as previously described (Hebert-Chatelain et al., 2016).
Virus titers were between 10^{10} and 10^{11}, expressed as genomic copies (GC) x ml.

Oxygen consumption measurements on tissue homogenates and enriched mitochondria

Hippocampus extracts: Preparation of hippocampus extracts was done as previously described (Hebert-Chatelain et al., 2016) with some modification. Briefly, C57Bl/6N mice that received intrahippocampal injection of AAV-Cre and Cre-dependent AAV-DIO-DREADD-HA or AAV-MLS-DREADD-HA or AAV-DIO-CTRL were sacrified by cervical dislocation, the mouse brain was extracted and the hippocampus was rapidly dissected. Hippocampus was homogenized in 450 uL of Miro5 buffer without taurine supplementation (Makrecka-Kuka et al., 2015) using a Politron homogenizer (11.000 rpm 3-5 s). After brief centrifugation at 4 C the supernatant was treated with saponin at a final concentration of 12.5 mg/ml.

Mitochondrial enrichment from HEK cells: HEK cells expressing DREADD-GS or MLS-DREADD-Gs or transfected with control plasmid were collected in cold Isolation buffer (70mM Sucrose, 210mM Mannitol,1mM EDTA, 50mM Tris base, pH 7.4, supplemented with protease inhibitor (Sigma-aldrich, France)) and lysed by passing the cell suspension through a 25G needle. After a first centrifugation step at 1000g (5 min, 4C), the supernatant was collected and a second centrifugation at 6900g was carried out for 10 min at 4C. The pellet containing the mitochondrial fraction was resuspended in fresh isolation buffer and used for mitochondrial respiration

Respiration analyses were carried out using a 2K Oroboros device (Makrecka-Kuka et al., 2015). 30 ul of sample were put in each chamber and complex I-dependent respiration was triggered by adding malate (2mM), pyruvate (5mM) and glutamate (10 mM) (MPG) (Makrecka-Kuka et al., 2015). Vehicle or CNO were added at final concentration of 50 uM and 12 minutes after 6.25 uM ADP was injected. Each measure of oxygen consumption rate (OCR) was expressed as ratio of ADP/MPG stimulation and the effect of CNO was calculated as percentage of vehicle conditions. Only samples for which the ratio of ADP/MPG in the vehicle was equal or superior to 1.5 were retained for the analyses.

Oxygen consumption measurements on cells

HEK cells were transfected with control plasmid or expressing DREADDs-Gs or MLS-DREADDs-Gs. Each cell suspension was divided in two and transferred into Oroboros chambers (Makrecka-Kuka et al., 2015) Routine respiration in culture medium was recorded until a stable signal was reached. CNO (50uM) or Vehicle were added directly into the chamber after 20 minutes, Rotenone (0.5µM)

and Antimycin A (2.5µM) were injected allowing to correct the mitochondrial respiration for residual oxygen consumption (Makrecka-Kuka et al., 2015).

Oxygen consumption rate (OCR) after CNO or Veh treatment was normalized on the values recorded before the injection. CNO effect on OCS was expressed as % of vehicle condition.

ERK phosphorylation assays

After the transfection with empty vector (CTRL), DREADD-HA or MLS-DREADD-HA plasmid, HEK cells were allowed to recover for 48 h. Cells were then starved overnight in serum-free DMEM before treatment with CNO (50uM) or vehicle or FBS (10% of final volume) for 30 min. The medium was rapidly aspirated and the samples were snap-frozen in liquid nitrogen and stored at −80 °C before preparation for western blotting.

Protein extraction and western immunoblotting

For ERK-phosphorylation assays, lysis buffer (1 mM EGTA, 50 mM Tris pH 7.5, 0.1% triton X-100, 30 mM 2-mercaptoethanol) supplemented with protease and phosphatase inhibitors (Sigma-aldrich, France) was added, cells were collected by scraping and pelleted by centrifugation at 12,500g (4 °C) for 5 min to remove cell debris. Protein concentrations were measured using the Roti- Nanoquant protein quantification assay, following manufacturer's instruction (Carl Roth, Karlsruhe, Germany), mixed with Laemmli buffer and kept at −20 °C.

For western blotting, the proteins were separated on Tris-glycine 10% acrylamide gels and transferred to PVDF membranes. Membranes were soaked in 5% BSA in TBS-T (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20). Antibodies against pERK (phospho-p44/42 MAPK, #4370, Cell Signaling Technology, Danvers, MA) and ERK (p44/42 MAPK, #9102, Cell Signaling Technology Danvers, MA, USA) were used. Bound primary antibodies were detected with HRP-linked antibodies (Cell Signaling Technology, Danvers, MA, USA). HRP signal was revealed with Clarity Western ECL Substrate using ChemiDoc MP System (Bio-Rad, Hercules, CA, USA). Image processing was performed in ImageLab software. pERK levels were normalized to the amount of total ERK. CNO effect was expressed as percentage of vehicle effect.

Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using Mitotracker Red™ (Life Technologies, CA, USA). Briefly, cells were incubated with 150 nM Mitotracker Red™ during 30 min at 37 °C in 5% CO_2 and 95% humidity.

After incubation with probes, cells were rinsed and imaging was performed. Mitotracker Red™ fluorescence were examined using the EVOS FL Auto 2 imaging system (Thermo Fisher Scientific, MA, USA) with a ×40 objective (LPLAN 40×, 0.65NA, EVOS). For each independent experiment, total fluorescence intensity was quantified for 50 cells using ImageJ (NIH, MD, USA).

Novel object-recognition memory task

We used the novel object recognition (NOR) memory task in an L-maze (Busquets-Garcia et al., 2011, 2013; Hebert-Chatelain et al., 2016; Puighermanal et al., 2009, 2013; Robin et al., 2018) as described in detail in Oliveira da Cruz et al. (2020).

The task consisted in 3 sequential daily trials of 9 minutes each. During the habituation phase (day 1), mice were placed in the center of the maze and allowed to freely explore the arms in the absence of any objects. The training phase (day 2) consisted in placing the mice again in the corner of the maze in the presence of two identical objects positioned at the extremities of each arm and left to freely explore the maze and the objects. The testing phase occurred 24 hours later (day 3): one of the familiar objects was replaced by a novel object different in its shape, color and texture and mice were left to explore both objects. The position of the novel object and the associations of novel and familiar were randomized. All objects were previously tested to avoid biased preference. Memory performance was assessed by the discrimination index (DI). The DI was calculated as the difference between the time spent exploring the novel (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): DI = [TN-TF]/[TN+TF]. Memory was also evaluated by directly comparing the exploration time of novel and familiar objects, respectively. Object exploration was defined as the orientation of the nose to the object at less than 2 cm.

Pharmacological treatments were immediately administered after the training phase.

Surgery and virus administration

Mice were anesthetized in a box containing 5% Isoflurane (Virbac, France) before being placed in a stereotaxic frame (Model 900, Kopf instruments, CA, USA) in which 1.5% to 2.0% of Isoflurane was continuously supplied via an anesthetic mask during the whole duration of the experiment. For viral intra-hippocampal AAV delivery, mice were submitted to stereotaxic surgery and AAV vectors were injected with the help of a microsyringe (0.25 mL Hamilton syringe with a 30-gauge beveled needle) attached to a pump (UMP3-1, World Precision Instruments, FL, USA). Where specified, D_1-CB_1-WT and D_1-CB_1-KO mice were injected directly into the hippocampus (HPC) (0.5 μl per injection site at a rate of 0.5 μl per min), with the following coordinates: HPC, AP -1.8; ML ± 1; DV -2.0 and -1.5. Following virus delivery, the syringe was left in place for 1 minute before being slowly withdrawn from the brain. D_1-CB_1-WT mice were injected with rAAV-CAG-DIO (empty control vector) and D_1-CB_1-KO with rAAV-CAG-DIO (empty control vector), AAV-CAG-DIO-CB1 or AAV-CAG-DIO-DN22-CB1-myc to induce re-expression of the CB_1 receptor gene or DN22-CB_1 respectevely in hippocampal D_1-positive cells. The aforementioned rAAVs were generated as described in Hebert-Chatelain et al. (2016). In another experiment, and using the same procedure as described as above, D_1-Cre mice were injected intra hippocampally (AP -1.8; ML ± 1; DV -2.0 and -1.5), with pAAV-CAG-flexx-Gs-HA or pAAV-CAG-flexx-4MLS-Gs-HA (1 μl per injection site at a rate of 0.5 μl per min). For these experiments, animals were used around 4-5 weeks after local infusions. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments. Mice that underwent behavioral experiments were fixed by transcardial perfusion of 4% pfa and their brain were processed for immunofluorescence (Hebert-Chatelain et al., 2016), with primary antibodies directed against myc-tag (#2276; Cell Signaling Technology), to detect CB1 or DN22-CB1 rescue or against HA-tag (#26183, Thermo Fisher Scientific, France) to confirm overexpression of DREADD-Gs-HA and MLS-DREADD-Gs-HA. Mice that did not fulfill histological positive criteria were excluded from the study.

Statistical analyses

Data were expressed as mean ± SEM or single data points and were analyzed with Prism 8.0 (Graphpad Software), using t-test (unpaired), one-way ANOVA (post-hoc dunnett's test) or 2-way ANOVA (post-hoc Bonferroni’s test).

SECTION IV – GENERAL DISCUSSION

PART 1 – SPECIFIC HIPPOCAMPAL INTERNEURONS SHAPE CONSOLIDATION OF RECOGNITION MEMORY

It is well known that the endocannabinoid system is present in different cell types in the brain where it posses an active role in the modulation of synaptic plasticity and in learning and memory processes. There is evidence that memory formation does not rely solely on hippocampal function but it could require also other components directly or indirectly. Among these, it is known that striatal D_1-positive MSNs express functional CB_1 receptors (Monory et al., 2007) and the dopaminergic cells in the striatum can indirectly modulate the hippocampal activity (Goldfarb et al. 2016; Sales-Carbonell et al. 2013; Tort et al. 2008) but there are not currently known direct connections between striatal structures and the hippocampus which can prove the involvement in memory formation.

Simultaneously, it is known that D_1Rs are expressed mainly on GABAergic interneurons in the hippocampus (Gangarossa et al., 2012; Puighermanal et al., 2017), in which CB_1 receptors are mainly expressed in GABAergic cells (Marsicano and Lutz, 1999, Marsicano and Kuner, 2008).

In line with this evidence, the first aim of this thesis was to investigate the physiological role of CB_1 receptors in hippocampal D_1+positive cells in the modulation of learning and memory processes.

We showed that specific deletion of CB_1 receptors from D_1-positive cells impairs long-term, but not short-term, object recognition memory formation. Although it is quite well clear the role of dopamine in the modulation of consolidation and storage of new memories (Lisman et al., 2011), the mechanism of this modulation remains poorly explored.

Given the higher expression of CB_1R in the striatum, our results show that the local re-expression of CB_1 receptors in the hippocampus but not the striatum of D1-CB_1-KO mice is able to rescue the memory impairment displayed by these animals. Thus, we provide evidence that hippocampal CB_1 receptors in D_1-positive cells are necessary and sufficient for the consolidation of object recognition memory. Since the endocannabinoid/dopamine crosstalk has been shown to be important in aversive memory (in particular mice lacking CB_1 receptors in D_1-positive cells have impaired extinction of freezing in fear conditioning tasks (Micale et al., 2017)) and for spatial memory formation (Xing et al., 2010; Riedel and Davies, 2005), we can speculate that CB_1 receptors in D_1-

positive cells might be also involved in this episodic-like memory. Supporting this idea, LTP phenomenon is widely accepted as a cellular model of memory consolidation and dopamine, via D_1 receptors, is highly relevant for the modulation of hippocampus-dependent synaptic plasticity and memory (Lisman and Grace, 2005; Lisman et al. 2011; Hansen and Manahan-Vaughan, 2014; Huang and Kandel, 1995; Lemon and Manahan-Vaughan, 2006; Bethus et al. 2010; Clausen et al. 2011; da Silva et al. 2012). Accordingly, LTP at the CA3-CA1 schaffer collater pathway is a potential molecular and cellular mechanism underlying behavioral expression of episodic-like memory processes (Morris, 2013). Our results showed that deletion of CB_1 from D_1-positive cells in homecage conditions do not alter in vivo LTP. However, when the animals are exposed to the objects in the training phase of NORT, LTP is impaired in D_1-CB_1-KO mice as compared with their WT littermates. These results demonstrate, in accordance with the literature, that the presentation of a novel stimulus (learning) induces hippocampal dopamine release that modulates consolidation of memory and enhances the LTP by acting on D_1 receptors in the hippocampus (Kempadoo et al., 2016, Takeuchi et al., 2016) which cannot be done in the absence of CB_1 receptors in the D_1-positive cells, suggesting that endocannabinoid control of D_1R-positive hippocampal interneurons is recruited only after learning and is responsible for the consolidation of memory and LTP enhancement. Unfortunately, we cannot fully exclude that cell types other than hippocampal interneurons might participate in D_1R/CB_1R-dependent control of memory consolidation. Indeed, our data show that the D_1-Cre mouse line used in the present study (Lemberger et al. 2007) induces recombination in a small sub-fraction of hippocampal interneurons containing low levels of CB_1R mRNA but also in pyramidal neurons and mossy cells. Thus, it could represent most likely a subclass within CB_1-expressing GABAergic interneurons.

However, our data show that partial blockade of GABAA receptors, but not of AMPA/kainate or NMDA glutamatergic ones, reverse the memory impairment of D_1-CB_1-KO mice. Therefore, our findings indicate that excessive GABAergic activity is involved in the phenotype of D_1-CB_1-KO mice and CB_1R control of GABA release from D_1R-positive interneurons regulates the consolidation of NOR memory. Moreover, partial blockade of GABAA receptors in trained WT mice does not alter LTP facilitation suggests that this phenomenon is due to a genuine increase in LTP. In addition, our data indicate that reducing GABAergic transmission in D_1R-positive neurons is required for this form of learning-induced synaptic plasticity.

D_1R activity in the HPC is necessary for long-term memory and synaptic plasticity (Lisman et al., 2011; Yamasaki and Takeuchi, 2017; Kaufman et al. 2020; Bethus et al., 2010). Consistently, our results show that high doses of the $D_{1/5}R$ antagonist SCH-23390 impair memory performance in the NOR task. Although activation of D_1Rs in interneurons seems to be necessary for the memory process, their abnormally high activity (e.g. in the absence of CB_1Rs) impairs such functions.

Thus, our results show that partial reducing D_1R activity counteracts the absence of CB_1Rs in D_1-CB_1-KO mice by abolishing the differences in LTP enhancement and rescuing the NOR memory consolidation impairment. Altogether, our results suggest an important role of this small subpopulation of D_1R/CB_1R-positive interneurons in maintaining a proper excitation/inhibition balance in the hippocampal network activity required for memory consolidation.

Taking advantage of a study from our team, the astroglial CB_1Rs are also necessary for consolidation of NOR does memory by allowing D-serine availability at glutamatergic synapse (Robin et al., 2018). Thus, we cannot fully exclude that deletion of CB_1Rs in D_1R-positive cells does not also involve astrocytes. In this study, D-serine rescues memory performance of GFAP-CB_1-KO mice only when administered immediately after training. Moreover, GFAP-CB_1-KO mice do not express in vivo LTP even in basal ‘’home cage’’ conditions (Robin et al., 2018). Conversely, D_1-CB_1-KO mice only lack the specific facilitation of LTP induced by learning. Comparing the current and past results, we could speculate that at least two distinct temporal windows exist and CB_1R in hippocampal astrocytes is likely involved in the starting memory formation, whereas CB_1R in D_1R-positive hippocampal interneurons is required in the maintaining the memory for longer periods.

In conclusion, although it is very complex elucidating the neuro-biological and physiological mechanism of hippocampal D_1R/CB_1R microcircuit, our data provide a novel link between CB_1 receptor-mediated activity and D_1-positive cells in learning-dependent facilitation of hippocampal LTP and in the consolidation of object recognition memory, which might provide novel therapeutic strategies for treatment of cognitive diseases.

PART 2 – LINKING MITOCHONDRIAL G-PROTEIN SIGNALING TO CANNABINOIDS-INDUCED AMNESIA: A NEW MITOCHONDRIA-SPECIFIC CHEMOGENETIC STRATEGY

Our brain consumes up to 80% of body energy in order to maintain a series of active bioenergetic processes. Our brain cellular activity depends on the high energetic support provided by mitochondria, the cell organelles which process the energy sources and transform them into ATP (MacAskill and Kittler, 2010; Mattson et al., 2008; Rangaraju et al., 2014). In this regard, memory formation and storage are among the most important brain functions which requires an intense consume of energy (Watts et al. 2018). Moreover, events associated with perturbation in cellular energy homeostasis can dramatically impact on synaptic and cognitive function (Suzuki et al. 2011; Wu et al. 2004). In this *scenario*, mitochondrial alterations (e.g. interruptions of the ATP production) have been showed to alter memory processes (Lin et al. 2013; Roubertoux et al. 2003).

Cannabinoid drugs and CB_1R activation have a multitude of therapeutic potentials (Pacher et al. 2006) but the amnesic side effect is amongst the well known side effects of these compounds (Broyd et al. 2016; Marsicano and Lafenêtre, 2009). With the discovery of the functional presence of GPCRs on mitochondrial membranes (Beninca et al., 2014) and the mitochondrial CB_1 receptors in the brain (Benard et al. 2012), the hypothesis that the effects induced by exogenous cannabinoids on mitochondrial functions are due to an indirect signaling of plasma membrane CB_1 receptor-dependent activation (Campbell, 2001; Athanasiou et al. 2007) is now remote and, the molecular signaling pathways mechanisms resulting from the activation of the $mtCB_1$ G-protein and the physiological and behavioral effects mediated by $mtCB_1$ receptors started to emerge (Hebert-Chatelain et al. 2014, 2016; Koch et al. 2015; Gutiérrez-Rodríguez et al. 2018; Jimenez-Blasco et al. 2020; Soria-Gomez et al. 2021).

Accordingly, direct bioenergetic effects of cannabinoids play a central role on brain mitochondrial activity, memory and motor regulation. The activation of intra-mitochondrial $G\alpha i$ proteins by exogenous CB_1 receptor agonists decreases the respiratory function of mitochondria, in particular the oxygen consumption and ATP production, through a sAC- and PKA- dependent mechanism.

Overall, this chain of events leads to a decrease of brain mitochondrial activity, which is responsible for cannabinoids-induced amnesia (Hebert-Chatelain et al. 2016). Conversely, mice lacking hippocampal $mtCB_1R$ lose this effect of cannabinoids during NOR consolidation task, indicating that the $mtCB_1$ activation is required for cannabinoid-induced amnesia via the modulation of bioenergetics processes.

The first part of thesis demonstrates that a small subpopulation of hippocampal D_1R/CB_1R interneurons is necessary for NOR memory consolidation (Oliveira da Cruz et al. 2020a). Taking advantage of these findings, we supposed that $mtCB_1Rs$ in hippocampal D_1-positive interneurons can play a key role in THC-induced memory impairment during NOR consolidation. Accordingly, our data show that THC impairs the NOR performance of D_1-CB_1-WT mice without altering total exploration and confirm the impaired NOR memory phenotype of D_1-CB_1-KO mice. The amnesic effect of THC is fully rescued in D_1-CB_1-KO re-expressing CB_1 but not in re-expressing DN22-CB_1 ones (mutant version of CB_1 receptor not targeted to mitochondria, [Hebert-Chatelain et al. 2016]) suggesting that $mtCB_1$ in hippocampal D_1-positive neurons is not necessary for memory formation per se but its activation is involved in memory consolidation impairment induced by THC during NOR task.

Thus, the second challenge of this thesis was to investigate how the modulation of mitochondrial G-protein signaling can affect the mitochondrial bioenergetic processes. To this aim, we adopted a novel chemogenetic tool. We developed a new adeno-associated virus expressing a Cre-dependent Gs-coupled DREADD specifically targeted to mitochondrial membrane. To this aim, we first fused a DREADD-Gs construct to a mitochondrial leading sequence (MLS) in order to induce the specific mitochondrial localization of the designer receptor mutant construct (MLS-DREADD-Gs).

MLS-DREADD-Gs was unable to activate the ERK phosphorylation, but its activation by CNO resulted in an increased mitochondrial respiration and membrane potential, representing the ideal tool to manipulate mitochondrial Gs activity and counteract the inhibitory effects of CB_1 activation on mitochondrial respiration.

Thus, given the relevance of mitochondrial G-protein signaling and bioenergetic processes in behavioral cognitive aspects, we evaluated how in vivo MLS-DREADD-Gs activation can interfere with the effects of cannabinoids on memory consolidation.

The activation of DREADD-Gs in D_1-positive hippocampal cells during NOR memory consolidation did not block the THC amnesic effect, suggesting that the excessive cytoplasmic Gs-cAMP-PKA signaling in hippocampal D_1-positive neurons is not required for this effect. Conversely, over-activation mitochondrial respiration by MLS-DREADD-Gs in hippocampal D_1 neurons inhibited the amnesic effect of THC, indicating the direct control of mitochondrial activity as the mechanism for cannabinoid-induced memory impairment.

In summary, this study extends our knowledge on direct link between the impact of cannabinoids on brain mitochondrial signaling and some of their most important intracellular and behavioral effects, such as cellular respiration and amnesia respectively. In particular, it introduces an innovative genetic tool able to modify the intra-mitochondrial GPCRs signaling and regulate mitochondrial bioenergetics processes and relevant brain functions as memory. The importance of mitochondrial-dependent effects opens new hopes for the development of innovative therapeutical strategies against several brain disorders which can specifically take advantage of the functional and energetic potentials of these intracellular organelles, possibly avoiding/limiting undesired side effects.

SECTION V – REFERENCES

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