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Cell-type specific CB1 receptor modulation of hippocampal synaptic plasticity and memory

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International PhD Program in Neuroscience – XXIX Cycle

Par José Fernando OLIVEIRA DA CRUZ

Contrôles distincts de la plasticité synaptique de l'hippocampe et de la mémoire par différentes populations de récepteurs CB1

Sous la direction de Giovanni MARSICANO

et de Filippo DRAGO

Soutenue le 18 Décembre 2017

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SUBMITTED

(**1**) Astroglial CB1 receptors determine synaptic D-serine availability to enable recognition memory

Laurie M. Robin*, Jose F. Oliveira da Cruz*, Valentin C. Langlais*, Mario Martin-Fernandez, Mathilde Metna-Laurent, Arnau Busquets-Garcia, Luigi Bellocchio, Edgar Soria-Gomez, Thomas Papouin, Ilaria Belluomo, Isabel Matias, Barbara Bosier, Filippo Drago, Ann Van Eeckhaut, Ilse Smolders, Francois Georges, Alfonso Araque, Aude Panatier, Stéphane H.R. Oliet[#] and Giovanni Marsicano[#]

*: equal contribution, #: equal supervision

IN PREPARATION

(**2**) Deletion of CB1 receptors in hippocampal D1-positive cells impairs object recognition memory and associated synaptic plasticity

Jose F. Oliveira da Cruz[*], Arnau Busquets-Garcia[*], Luigi Bellocchio, Zhe Zhao, Filippo Drago, Marjorie Varilh, Giovanni Marsicano[#], Edgar Soria-Gomez[#]

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LIST OF COMMUNICATIONS

Poster communications

2017 – NEUROFRANCE 2017 (BORDEAUX, FRANCE)

Deletion of CB1 receptors in D1-positive cells impairs object recognition memory
Jose F. Oliveira da Cruz, Arnau Busquets-Garcia, Luigi Bellocchio, Zhe Zhao, Marjorie Varilh, Giovanni Marsicano, Edgar Soria-Gomez

2016 – INTERNATIONAL NEUROSCIENCE CONFERENCE 2016 (MARSEILLE, FRANCE)

Astroglial CB1 receptors control memory via D-serine
Jose F. Oliveira da Cruz, Laurie M. Robin, Valentin C. Langlais, Mathilde Metna-Laurent, Arnau Busquets-Garcia, Luigi Bellocchio, Edgar Soria-Gomez[1,2], Thomas Papouin, Barbara Bosier, Filippo Drago, Ann Van Eeckhaut, Ilse Smolders, Francois Georges, Aude Panatier, Stephane H.R. Oliet, Giovanni Marsicano

2015 – CANNABINOID CONFERENCE (SESTRI LEVANTE, ITALY)

Astroglial CB1 receptors are required for *in vivo* hippocampal long-term potentiation of synaptic transmission
José F. Oliveira da Cruz, Laurie Robin, Valentin C. Langlais, Arnau Busquets-Garcia, Edgar Soria-Gomez, Filippo Drago, Aude Panatier, François Georges, Mathilde Metna-Laurent, Stéphane Oliet, Giovanni Marsicano

2015 – 13TH SUMMER SCHOOL OF NEUROSCIENCE (CATANIA, ITALY)

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Astroglial type-1 cannabinoid receptors (CB1) are required for memory formation
José F. Oliveira da Cruz, Laurie Robin, Valentin C. Langlais, Arnau Busquets-Garcia, Edgar Soria-Gomez, Filippo Drago, Aude Panatier, François Georges, Mathilde Metna-Laurent, Stéphane Oliet, Giovanni Marsicano

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Cannabinoid receptor 1 in hippocampal astrocytes is crucial for memory formation
José F. Oliveira da Cruz, Laurie Robin, Valentin C. Langlais, Arnau Busquets-Garcia, Edgar Soria-Gomez, Filippo Drago, Aude Panatier, François Georges, Mathilde Metna-Laurent, Stéphane Oliet, Giovanni Marsicano

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Astroglial type-1 cannabinoid receptors (CB1) are required for memory formation
José F. Oliveira da Cruz, Laurie Robin, Valentin C. Langlais, Arnau Busquets-Garcia, Edgar Soria-Gomez, Filippo Drago, Aude Panatier, François Georges, Mathilde Metna-Laurent, Stéphane Oliet, Giovanni Marsicano

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The role of Astroglial Type-1 Cannabinoid Receptor in Memory Functions
Jose F. Oliveira da Cruz, Mathilde Metna-Laurent, Laurie Robin, Valentin C. Langlais, Arnau Busquets-Garcia, Thierry Leste-Lasserre, Thomas Papouin, Stéphane Oliet, François Georges, Giovanni Marsicano

ABSTRACT

The endocannabinoid system is a major brain modulatory system that controls memory and learning mainly via the cannabinoid receptor type 1 (CB1)-dependent regulation of neuronal and glial activity. In the hippocampus, bidirectional communication between neurons and astrocytes shapes synaptic plasticity and behavior. CB1 receptors have been shown to be present in the astrocytes and to mediate the disruptive effects of cannabinoids in synaptic plasticity and working memory. Yet, it is not currently known the role of this receptor in the physiological modulation of memory processes. Also, previous studies have shown that CB1 receptors expressed in dopamine D1 receptor-expressing cells are involved in the modulation of hippocampal-dependent aversive memories. However, their involvement in the modulation of non-aversive long-term memory formation and synaptic plasticity is presently unknown. In this thesis, I aimed at identifying the cellular and molecular mechanisms by which specific CB1 receptors in distinct brain neuronal and glial populations contribute to the physiological modulation of synaptic plasticity and learning and memory. For this aim we used conditional genetic mutant mice lacking CB1 receptors specifically in astrocytes or in D1-positive cells. By coupling these genetic mouse models with behavioral, pharmacological, and *in vitro* and *in vivo* electrophysiological approaches, we dissected the role of these CB1 receptors in the formation of memory. First, we show that astroglial CB1 receptors in the hippocampus control long-term potentiation (LTP) of CA3-CA1 synaptic transmission and long-term recognition memory. By allowing physiological availability of D-serine at NMDA receptors via gliotransmission, astrocytes are important elements controlling glia-neuron interactions that underlie synaptic plasticity and memory functions. The data show that astroglial CB1 receptors control plasticity and memory by regulating the synaptic availability of D-serine at NMDA receptors. Second, we show that CB1 receptors D1-positive cells control the consolidation, but not acquisition, of new memories and the enhancement of LTP induced by learning, showing that specific subpopulations CB1 receptor-expressing cells differentially modulate these processes.

Overall, by showing that the endocannabinoid system in astrocytes is an important modulator of learning and memory and by suggesting that CB1 receptors in D1-positive cells are important for specific components of memory formation, we provide functional evidence for the complex cell type-dependent regulation of long-term recognition memory by the CB1 receptors.

Key words: CB1 receptors, D1 receptors, astrocytes, D-serine, LTP, Memory.

RÉSUMÉ

Le système endocannabinoïde est un système neuromodulateur majeur du cerveau. Ainsi, il contrôle la mémoire et l'apprentissage, et ce, principalement par l'intermédiaire des récepteurs aux cannabinoïdes de type 1 (CB1) qui régulent de manière fine les activités neuronales et gliales. Dans l'hippocampe, une communication bidirectionnelle entre neurones et astrocytes modèle la plasticité synaptique et le comportement. Il a été rapporté que les effets disruptifs des cannabinoïdes sur la plasticité synaptique et la mémoire de travail sont dépendants de récepteurs CB1 présents dans les astrocytes. Cependant, le rôle de ce récepteur dans la modulation physiologique des processus mnésiques n'est pas encore connu. De précédentes études ont également montré que les récepteurs CB1 exprimés dans les cellules hébergeant le récepteur dopaminergique D1 sont impliqués dans la modulation hippocampique de la mémoire associée aux évènements aversifs. Toutefois, leur implication dans la modulation de la formation de la mémoire associée à des évènements non aversifs ainsi que dans la plasticité synaptique sous-jacente reste encore inconnue. Dans cette thèse, mon objectif était d'identifier les mécanismes cellulaires et moléculaires par lesquels des populations distinctes de récepteurs CB1 dans des populations gliales et des régions cérébrales bien définies contribuent à la modulation physiologique de la plasticité synaptique, de l'apprentissage et de la mémoire. Pour ce faire, nous avons utilisé des souris mutantes conditionnelles dans lesquelles le récepteur CB1 a été rendu silencieux sélectivement dans les astrocytes ou dans les cellules exprimant le récepteur D1. En couplant ces modèles génétiques murins avec des approches comportementales, pharmacologiques et électrophysiologiques *in vitro* et *in vivo*, nous avons disséqué le rôle de ces populations de récepteurs CB1 dans la formation de la mémoire. Tout d'abord, nous avons montré que les récepteurs CB1 astrogliaux dans l'hippocampe contrôlaient la potentialisation à long terme (PLT) de la transmission synaptique CA3-CA1 et la mémoire de reconnaissance à long terme. En contrôlant, via la gliotransmission, la disponibilité effective de D-sérine aux récepteurs NMDA, les astrocytes sont des éléments importants contrôlant les interactions glie-neurones qui sous-tendent la plasticité synaptique et les fonctions mnésiques. Les données obtenues montrent que les récepteurs CB1 astrogliaux contrôlent la plasticité et la mémoire en régulant la disponibilité synaptique de la D-sérine aux récepteurs NMDA. Deuxièmement, nous avons montré que les récepteurs CB1 dans les cellules exprimant le récepteur D1 contrôlaient la consolidation, mais pas l'acquisition, de nouveau souvenirs et l'augmentation de la PLT induite par l'apprentissage. Ces résultats indiquent que des populations spécifiques de cellules exprimant le récepteur CB1 modulent ces processus de manière différentielle.

En conclusion, ces travaux démontrent que le système endocannabinoïde dans les astrocytes est un important modulateur de l'apprentissage et de la mémoire alors que les récepteurs CB1 dans les cellules exprimant le récepteur D1 semblent importants pour des composantes spécifiques de la formation de la mémoire. Prise dans son ensemble, cette thèse apporte des preuves fonctionnelles quant à la régulation complexe de la mémoire de reconnaissance à long-terme par des populations distinctes de récepteurs CB1.

Mot clés: Récepteurs CB1, récepteurs D1, astrocytes, D-sérine, PLT, Mémoire.

LONG RÉSUMÉ

Le système endocannabinoïde est un modulateur majeur du système nerveux central et des tissus périphériques contrôlant et régulant d'important processus physiologiques. Les cannabinoïdes exogènes et endogènes agissent principalement via le récepteur cannabinoïde de type 1 (CB1) qui régule des fonctions cérébrales clés telles que la consommation alimentaire, le métabolisme énergétique, les réponses immunitaires, les réponses de stress, les performances motrices, la perception de la douleur et la mémoire. Dans l'hippocampe, une région cérébrale importante pour l'apprentissage et la mémoire, la présence des récepteurs CB1 (principalement présynaptiques) sur les neurones GABAergiques et glutamatergiques modulent l'activité neuronale en diminuant la libération de neurotransmetteurs. En combinant des approches génétiques, de mutagénèses conditionnelles, et pharmacologiques, il a été montré que des récepteurs CB1 de populations neuronales bien distinctes sont responsables des altérations des processus mnésiques induites par les cannabinoïdes. Toutefois, il reste de nombreux points à explorer concernant le rôle physiologique des récepteurs CB1 dans ces fonctions. De plus, les récepteurs CB1 peuvent être présents dans d'autres types cellulaires que les neurones, e.g. les astrocytes, et le rôle physiologique de ces récepteurs dans l'apprentissage et la mémoire reste inconnu.

Il a été montré que les astrocytes de l'hippocampe expriment des récepteurs CB1 fonctionnels. Les astrocytes constituent le type de cellules gliales le plus abondant dans le soutien au réseau neuronal en fournissant les substrats métaboliques nécessaires au fonctionnement optimal du cerveau. Toutefois, il a été démontré au cours des dernières décennies que les astrocytes étaient impliqués dans d'autres fonctions importantes telles que la modulation directe de l'activité et la plasticité synaptique via une communication bidirectionnelle avec les structures synaptiques neuronales (les terminaisons présynaptiques et les épines postsynaptiques). Ce concept, communément désigné sous les termes de "synapse tripartite", définit ces synapses comme étant composées de trois éléments : les terminaisons pré- et post-synaptiques ainsi que les processus fins astrocytiques les entourant. Cette localisation permet aux astrocytes de percevoir les signaux neuronaux et de libérer des molécules modulant leur activité. Les modifications fonctionnelles et/ou structurelles à court- et long-terme de la transmission synaptique, qui constituent la plasticité synaptique, ont été proposées comme étant des mécanismes cellulaires clés sous-jacents à la formation de nouveaux souvenirs. Plus précisément, l'induction de la potentialisation à long terme (PLT) dépendante des récepteurs NMDA a été montrée au niveau des synapses de l'hippocampe pendant l'apprentissage. Il est alors intéressant de noter que la libération astrocytaire de D-

sérine, le co-agoniste principal du récepteur NMDA, considéré comme un gliotransmetteur, est nécessaire à l'induction de la PLT dans cette région cérébrale particulière. De plus, les récepteurs CB1 astrogliaux sont les médiateurs des effets perturbateurs des cannabinoïdes synthétiques et naturels sur la plasticité synaptique et la mémoire de travail. Actuellement, le rôle physiologique des récepteurs CB1 astrogliaux dans la modulation des fonctions mnésiques et synaptiques n'est pas connu.

Comme mentionné précédemment, le ciblage d'un gène spécifique couplé à des méthodes technologiques avancées ont permis l'identification des récepteurs CB1 (i) dans des localisations intracellulaires jusqu'alors inconnues (mitochondrie) et (ii) au sein de nouvelles populations cellulaires cérébrales. Ainsi, les récepteurs CB1 ont été récemment localisés au sein de cellules exprimant le récepteur dopaminergique de type 1 (D1). Néanmoins, le nombre de cellules exprimant D1 dans l'hippocampe étant relativement faible, il est difficile de les identifier anatomiquement. Bien que la nature de ces cellules demeure insaisissable (neuronale ou gliale), des preuves fonctionnelles pointent vers l'existence de ces cellules au sein des structures de l'hippocampe avec un impact probable sur les fonctions médiées par celui-ci. Bien que ces cellules représentent une petite fraction du nombre total de cellules exprimant CB1, elles représentent une sous-population neuronale ou gliale exprimant le récepteur CB1. Ainsi, il est nécessaire d'étudier le rôle de ces nouvelles sous-populations de cellules exprimant le récepteur CB1 et d'identifier le mécanisme moléculaire et les conséquences comportementales qui sont liées à leur activité.

Le principal objectif de ma thèse a été d'identifier les mécanismes moléculaires et cellulaires par lesquels les récepteurs CB1 au sein de populations cellulaires spécifiques, qu'elles soient neuronales (par exemple les cellules dopaminergiques) ou gliales (par exemple les astrocytes) contribuent à la modulation physiologique de l'apprentissage et de la mémoire. Ceci est important non seulement pour la compréhension des fonctions cérébrales mais également pour l'appréhension des mécanismes par lesquels certaines dérégulations peuvent conduire à des états pathologiques.

Afin d'atteindre l'objectif principal de cette thèse, nous avons utilisé une combinaison d'outils génétiques (mutagénèse constitutive et conditionnelle du récepteur CB1 chez la souris) et pharmacologiques (agonistes et antagonistes du récepteur CB1) couplés à des paradigmes comportementaux précédemment conçus et validés pour étudier la formation de la mémoire chez la souris. De plus, en combinant ces précédentes approches à de l'électrophysiologie *in vitro* et *in vivo*, l'objectif était de disséquer les mécanismes impliqués dans la modulation de la formation de la mémoire dépendant spécifiquement du récepteur CB1.

Spécifiquement, le premier but était d'étudier les mécanismes cellulaires impliqués dans la modulation physiologique de la mémoire à long-terme par les récepteurs CB1 astrogliaux. Au cours de ces travaux, nous nous sommes intéressés à plusieurs questions concernant (i) le rôle de ces récepteurs dans la modulation de la formation des mémoires à court- et long-terme, (ii) le rôle des récepteurs CB1 astrogliaux dans la modulation de la PLT, (iii) les mécanismes par lesquels les récepteurs CB1 contrôlent les fonctions astrogliales afin de permettre la gliotransmission et iv) la caractérisation du mécanisme sous-tendant le contrôle des fonctions mnésiques par le récepteur CB1.

La délétion spécifique des récepteurs CB1 astrogliaux chez la souris (GFAP-CB1-KO) altère la tâche de mémoire de reconnaissance d'un nouvel objet, montrant que ces récepteurs sont nécessaires pour la formation de la mémoire d'objet nouveau. De plus, nous avons montré que la transmission du récepteur NMDA dans l'hippocampe était nécessaire à la formation de ce type de mémoire. Ensuite, par l'enregistrement *in vivo* du potentiel de champ postsynaptique excitateur (fEPSP) dans les voies CA3-CA1 de l'hippocampe chez des souris sauvages ou mutantes anesthésiées, nous avons montré que les souris GFAP-CB1-KO avait une altération *in vitro* et *in vivo* de la PLT dépendante du récepteur NMDA dans l'hippocampe. Pour étudier le mécanisme cellulaire impliqué dans le phénotype présenté par les souris GFAP-CB1-KO, nous avons exploré la relation entre les astrocytes et leurs homologues neuronaux au sein des synapses tripartites de l'hippocampe. En montrant qu'un agoniste du récepteur CB1 pouvait induire une augmentation des niveaux intracellulaires de calcium au sein des astrocytes des souris sauvages mais pas des souris GFAP-CB1-KO, nous avons révélé que les récepteurs CB1 astrogliaux pouvaient contrôler les niveaux de calcium intracellulaire. Considérant que les niveaux de calcium intracellulaires sont les mécanismes astrocytiques potentiels impliqués dans la sécrétion de gliotransmetteurs, nous avons étudié si plusieurs gliotransmetteurs supposément libérés par les astrocytes d'une manière dépendante du calcium étaient également affectés par la modulation du récepteur CB1. Nous avons observé que la D-sérine, un important co-agoniste du récepteur NMDA, était modulée par le récepteur CB1, fournissant un bon candidat pour le phénotype observé chez les souris GFAP-CB1-KO. De plus, nous avons montré que les récepteurs CB1 astrogliaux étaient nécessaires au maintien de concentrations appropriées de D-sérine au sein de la fente synaptique. Ces concentrations assuraient un niveau adapté d'occupation du site de liaison du co-agoniste sur le récepteur NMDA. Ensuite, par analyse du rôle potentiel de la transmission de D-sérine chez les souris GFAP-CB1-KO, nous avons montré que les récepteurs CB1 astrogliaux régulaient les niveaux synaptiques de D-sérine, un élément nécessaire pour la PLT dépendante du récepteur NMDA aussi bien *in vitro* qu'*in vivo*. Nous avons également montré par la modulation des niveaux de D-sérine (réalisée par administration exogène de D-sérine ou par augmentation endogène de cette dernière via l'inhibition de sa dégradation) que les récepteurs CB1 astrogliaux étaient requis pour les

performances de mémoire dans le test de reconnaissance de nouvel objet. Ce contrôle s'effectue par l'intermédiaire de la signalisation de la D-sérine pendant les phases initiales de la consolidation de la mémoire. De manière générale, les résultats présentés dans la première partie de cette Thèse montrent que les récepteurs CB1 astrogliaux sont nécessaires pour la formation de la mémoire de reconnaissance d'objet et pour l'induction de la PLT hippocampique via la modulation du gliotransmetteur D-sérine, illustrant un mécanisme physiologique inattendu sous-tendant la plasticité synaptique et la formation de la mémoire.

Dans une seconde partie de la Thèse, nous avions pour objectif d'explorer le rôle des récepteurs CB1 dans les cellules D1-positives dans la modulation des fonctions de mémoire de reconnaissance d'objet à court- et long-terme, et ce afin de comprendre quelle région cérébrale était responsable de ce phénotype afin d'étudier le rôle de ces récepteurs dans la modulation de la PLT.

Bien qu'il n'y ait actuellement aucune preuve anatomique de la présence des récepteurs CB1 sur les cellules D1-positives, des preuves fonctionnelles ont suggéré qu'ils pourraient être présents dans l'hippocampe. La délétion spécifique des récepteurs CB1 des cellules D1-positives chez la souris (D1-CB1-KO) altère spécifiquement la formation de la mémoire de reconnaissance de nouvel objet à long-terme mais pas à court-terme indiquant que les récepteurs CB1 dans ce type de cellules en particulier étaient nécessaires à la formation de la mémoire à long-terme. Il est intéressant de noter que l'expression dépendante de la CRE recombinase du récepteur CB1 dans le striatum des souris D1-CB1-KO n'a pas permis de restaurer les performances de mémoire des souris mutantes alors que la même manipulation dans l'hippocampe a permis de rétablir totalement les performances de mémoire des souris D1-CB1-KO. Ceci indique donc que les récepteurs CB1 de l'hippocampe dans les neurones D1-positifs sont nécessaires à la consolidation de la mémoire à long-terme. Nous avons montré précédemment que la PLT dans l'hippocampe était dépendante de la fonction du récepteur CB1 dans cette région cérébrale. Par l'enregistrement *in vivo* du fEPSP des souris mutantes anesthésiées, nous avons étudié le mécanisme de la PLT dans l'hippocampe des souris D1-CB1-KO. Il est intéressant de noter que nous avons trouvé que l'exposition à un entraînement avant la stimulation à haute fréquence induisait une PLT chez les animaux sauvages qui est altérée chez leurs frères D1-CB1-KO, montrant que dans ces conditions les récepteurs CB1 dans les cellules exprimant le récepteur D1 sont nécessaires pour une expression correcte de la PLT. Considérés dans leur ensemble, ces résultats apportent une nouvelle preuve fonctionnelle que les récepteurs CB1 dans les cellules exprimant le récepteur D1 dans l'hippocampe contrôlent la consolidation mais pas l'acquisition des mémoires à long-terme et la plasticité synaptique associée.

De manière générale, en montrant que le système endocannabinoïde dans les astrocytes est un important modulateur de l'apprentissage et de la mémoire, et en suggérant que les

récepteurs CB1 dans les cellules D1-positives sont importants pour certains composants spécifiques de la formation de la mémoire, nous avons apporté une preuve fonctionnelle d'une régulation de la mémoire de reconnaissance à long-terme qui est dépendante du type cellulaire exprimant les récepteurs CB1.

LIST OF ABREVIATIONS

2-AG	arachidonoylglycerol
5-HT1B	5-hydroxytryptamine type-1B
5-HT3	5-Hydroxytryptamine type-3
ACEA	arachidonyl-2'-chloroethylamine
AEA	arachidonoyl ethanolamide
Aldh1L1	aldehyde dehydrogenase 1 family member L1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
CaER	calcium-dependent release
CaMKII	ca2+/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate)
CaN	calcium sensitive phosphatase calcineurin
CB1	cannabinoid type-1
CB2	cannabinoid type-2
CB3	cannabinoid type-3
CB-LTD	cannabinoid-induced LTD
CCK	cholecystokinin
CNS	central nervous system
COMT	catechol-O-methyltransferase
COX-2	cyclooxygenase-2
CRE	CRE recombinase
CREB	cAMP responsive element binding protein
D1	dopamine type-1
D2	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

FLAT	FAAH-1-like Anandamide transporter
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
GLT-1	glutamate transporter 1
GPCR	G protein coupled receptor
HCN	hyperpolarization-activated cyclic nucleotide–gated
HFS	high frequency stimulation
Ih	hyperpolarization-activated cationic depolarizing current
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
LTS	low threshold spiking
M1	muscarinic type-1
M3	muscarinic type-3
MAGL	monoacylglycerol Lipase
MAPK	mitogen-activated protein kinases
mGLu1	metabotropic type-one glutamate
mGLu5	metabotropic type-one glutamate 5
mRNA	messenger ribonucleic acid
MSN	medium spiny neurons
mtCB1	mitochondrial CB1
mTOR	mechanistic target of rapamycin
NAPE	N-arachidonoyl phosphatidyl ethanol
NAT	N-acetyltransferase
NMDA	N-methyl-D-aspartate receptor
NORT	novel object recognition task
OXPHOS	oxidative phosphorylation
PIP2	phosphatidyl inositol bisphosphate
PKA	protein Kinase A
PLC	phospholipase C
PLD	phospholipase D
PTPN22	protein tyrosine phosphatase non-receptor type-22
PV	parvalbumin
RER	regulated endocannabinoid release
S100β	S100 calcium-binding protein β
SPW-R	sharp wave ripples
SR	serine racemase
SSI	slow self-inhibition

STED	stimulated emission depletion
THC	Δ9-tetrahydrocanabinol
t-LTD	spike-timing-dependent LTD
TRPV-1	transient receptor potential vanilloid 1
VGCC	voltage-gated calcium channel
VTA	ventral tegmental area
WT	wild-type

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SECTION I – GENERAL INTRODUCTION

PART 1 – THE ENDOCANNABINOID SYSTEM IN THE BRAIN

I – Introduction

The discovery of the endogenous cannabinoid system (ECS) as a major modulatory system involved in health and disease started with the interest in understating how *Cannabis sativa*, commonly known as marijuana or simply cannabis, could induce a plethora of effects after consumption in both humans and animals. Notably, early societies in China, India and Assyria were already aware about the properties of cannabis millennia ago, using it not only as a medicine but also recreationally, to experience states of euphoria or higher emotional awareness (Curran et al., 2016, Mechoulam et al., 2014).

The use of cannabis became known by European societies around the 19th century when the Napoleon Armies, returning from their campaign in Egypt and Syria, brought the plant with them (Mechoulam and Parker, 2013). The modern therapeutic use of cannabis was initiated by the Irish physician William O'Shaughnessy (1809–1889), medical officer of the British Army who was stationary in Calcutta (India) in the beginning of the 19th century. During this period, O'Shaughnessy had the opportunity to observe the medical use of cannabis by the Indian people and introduced its therapeutic use in Europe. Another important figure in the application of medical cannabis was the French psychiatrist Jacques-Joseph Moreau (1804-1884). In his book "*Du hachisch et de l'aliénation mentale*" (Moreau, 1845), Moreau provided important medical data on the effects of cannabis consumption in humans. For instance, he described effects from cannabis consumption such as the feeling of happiness and excitement, illusions and hallucinations, troubles in navigation or enhancement of perception.

Scientific research aiming at characterizing the mechanisms behind the effects of *Cannabis sativa* in the brain started mainly in the 20th century. With the progressive availability of chemical methodologies to analyze the extracts of *Cannabis sativa*, it was possible to investigate which compounds were responsible for its psychoactive effects.

After a period of successive isolation and identification of several phytomolecules of cannabis, Δ9-tetrahydrocanabinol (THC), the main active compound of cannabis, was finally identified by Roger Adams (Adams, 1942) and further isolated and characterized (Gaoni and Mechoulam, 1964). Interestingly, although more than 60 cannabinoids have been currently identified in the cannabis plant, THC remains the main psychoactive compound (Mechoulam and Parker, 2013). This key discovery allowed further development of synthetic analogs of THC

that provided valuable tools to identify the putative endogenous target of these molecules (Piomelli, 2003).

The discovery of THC improved greatly the understanding of the major effects of cannabinoid consumption. For instance, THC administration in animal models was able to mimic certain phenotypes that can be correlated to human consumption-related effects such as hypolocomotion, hypothermia, increased analgesia, catalepsy, stress reactivity, among others (Mechoulam et al., 2014).

Around 25 years after the definitive chemical characterization of THC, the identification of the first cannabinoid receptor (CB1) (Devane et al., 1988, Matsuda et al., 1990) not only provided evidence that cannabinoids act *via* a specific endogenous receptor but also unraveled the pathway to uncover a new modulatory system. The further identification of a second cannabinoid receptor (CB2) (Munro et al., 1993), the discovery and characterization the endogenous cannabinoid ligands (endocannabinoid(s), eCB(s)) of CB1/CB2 receptor (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995), together with the identification of corresponding metabolic pathways (Di Marzo, 2009), provided the main components of the ECS (Piomelli, 2003).

The aim of this thesis is to understand how the ECS modulates two of the most important adaptive functions of an organism: learning and memory. In order to understand the importance of this system and its broad modulatory action, in the following sections I will review some important past and current findings that shed light on the ECS. In particular, I aim at describing the gaps in the current knowledge and how this work might contribute to improve our understanding of this key modulatory system.

II – Cannabinoid receptors in the brain

The discovery of THC and the generation of highly selective and potent synthetic analogs allowed the identification of the brain target for these molecules. The discovery of the CB1 receptor was accomplished by the group of Allyn Howlett in the early 90s. By using the specific radio-labeled cannabinoid analogue [3H]CP55940, they identified and characterized a novel cannabinoid receptor (named CB1) from rat brain membranes and synaptosomes that was bound to a Gi protein subunits (Devane et al., 1988). Later, the receptor was cloned (Matsuda et al., 1990) and more recently crystalized (Hua et al., 2016, Hua et al., 2017, Shao et al., 2016). The CB1 receptor is a 7 transmembrane G protein coupled receptor (GPCR), widely expressed in the central nervous system (CNS), and arguably the most abundant GPCR in the brain (Herkenham et al., 1990).

Following the discovery of the CB1 receptor, a second cannabinoid receptor (CB2) was cloned from the macrophages in the human spleen (Munro et al., 1993). Since CB2 was initially characterized in the spleen, and not in the CNS, it was mainly thought to be present in the periphery rather than in the CNS. However, recent evidence have demonstrated that not only CB2 receptors can be expressed in the CNS, both in neurons and in glial cells (Marsicano and Kuner, 2008), but also that CB2 receptors can modulate neuronal and glial activity (Atwood and Mackie, 2010, Li and Kim, 2015, Stempel et al., 2016).

CB1 and CB2 receptors share 48% of amino acid sequence and are similarly sensitive to the endogenous agonists (Mechoulam and Parker, 2013). Dimerization between receptors of different and same class GPCRs has been reported (Mackie, 2005). For instance, CB1 receptors can be found in the monomeric, homomeric (Wager-Miller et al., 2002) or heteromeric forms with CB2 receptors (Callen et al., 2012), D2 or opioid receptors (Mackie, 2005). Although these dimers were anatomically identified, the functional relevance remains poorly understood (Turu and Hunyady, 2010).

Other metabotropic and ionotropic receptors have been reported to respond to the endogenous agonists of classical cannabinoid receptors. Transient receptor potential vanilloid 1 (TRPV-1) is a nonselective cation channel with high calcium permeability that belongs to the transient receptor potential superfamily (Caterina et al., 1997). They are involved in the transduction of signals such as temperature, electrical charge, light, olfactive and taste stimuli and endogenous lipids (Pertwee et al., 2010b). Although TRPV-1 channels in the digestive track are most known for the mediation of the burning sensation elicited by the molecule capsaicin (present in “chili peppers”) (Caterina et al., 1997), they can also be present in the brain (Cristino et al., 2006, Menigoz and Boudes, 2011, Toth et al., 2005) where they mediate

endocannabinoid-mediated forms of synaptic plasticity (Chavez et al., 2010, Marsch et al., 2007).

Another example of proteins that might act as potential cannabinoid receptors includes the deorphanized GPCRs GPR55 and GPR119. GPR55 was initially isolated from the human striatum (Sawzdargo et al., 1999) and it has been reported to respond to 2-AG and Anandamide (Sharir and Abood, 2010). GPR119 is found predominantly in the pancreas and gastrointestinal tract (Fredriksson et al., 2003) and it can respond to the endocannabinoid oleoylethanolamide (OEA) (Overton et al., 2006). Although both receptors can be expressed in the brain, their function and potential action as cannabinoid receptor 3 (CB3) remains mostly unknown (Godlewski et al., 2009). Pertwee and colleagues (2010) established a range of criteria to classify a potential candidate protein as CB3 receptor. These include: 1) the candidate receptor should be activated by CB1/CB2 agonist at the orthosteric site with similar potency, 2) endogenous ligands at physiological conditions should elicit a response via this receptor, 3) it should display an amino acid similarity with CB1/CB2, 4) it should have specific functions elicited by classical agonist and 5) it should not be a receptor with other already identified functions (Pertwee et al., 2010b). The evidence of potential CB1 receptor-independent targets of endocannabinoids in the brain underlines the importance of the use of specific pharmacological methods together with genetic knock-out (KO) strategies to understand specific functions of CB1 receptors.

Because CB1 receptors are known to mediate the majority of the cannabinoid-induced psychotropic effects, studying the role of CB1 receptors in brain physiology and pathology is a major topic in cannabinoid research. In accordance with the aim of this thesis, I will thereby concentrate in the next section of the introduction on CB1 receptor and how they modulate brain functions, how their biology is strategically involved in behavior and what are the currently important unsolved questions regarding the role of this receptor in brain function.

III – Distribution of CB1 receptors in the brain

CB1 receptors are widely, but not exclusively, expressed in the CNS (Hu and Mackie, 2015, Marsicano and Kuner, 2008). Being likely the most abundant GPCR in the brain (Herkenham et al., 1990, Howlett et al., 1990), CB1 receptors have been extensively described in regions involved in key brain functions such as learning and memory, pain perception, reward, motor coordination and energy and metabolism (Di Marzo et al., 2004, Piazza et al., 2017). Consequently, CB1 receptors are present in important parts of the CNS such as the retina, the neocortex, the olfactory system, the amygdala, the hippocampus, the striatum, the cerebellum, the thalamus, the substantia nigra, the ventral tegmental area (VTA), the periaqueductal gray and the spinal cord (Busquets Garcia et al., 2016, Hu and Mackie, 2015, Marsicano and Kuner, 2008, Soria-Gomez et al., 2017). As the characterization of CB1 receptors is crucial to understand where and how they modulate the diverse brain functions, I will describe the cellular and subcellular localization of CB1 receptors in the hippocampus as it is the main region of interest of this thesis.

III.A – CB1 RECEPTOR DISTRIBUTION IN THE HIPPOCAMPUS

The hippocampus, a key brain region for learning and memory, has one of the broadest and highest expressions of CB1 receptors in the brain (Figure 1) (Herkenham et al., 1990, Marsicano and Kuner, 2008).

Among the different cell-types that compose this region, CB1 receptors have been initially characterized in hippocampal interneurons, more precisely in the terminals of cholecystokinin (CCK)-positive cells. It is mostly absent in parvalbumin(PV)-positive GABAergic basket cells in the pyramidal cell layer, the molecular layer and also the granule cell layer of the dentate gyrus (Katona et al., 1999, Marsicano and Lutz, 1999, Marsicano and Kuner, 2008, Tsou et al., 1999). This mutual exclusion has been consistently observed and fosters hypotheses on how PV- and CCK-positive interneurons might interact in the modulation of hippocampal network activity (Klausberger et al., 2005, Klausberger and Somogyi, 2008). Besides its presence in gamma-aminobutyric acid (GABA)-ergic neurons, CB1 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). Mossy cells of the dentate gyrus, which are glutamatergic neurons, also express high levels of CB1 receptor in their terminals

(Kawamura et al., 2006, Monory et al., 2006). Expression of CB1 receptors have also been reported in glial cells such as astrocytes (further reviewed in the part 3 – V). Additionally, CB1 receptors have been shown to be present in hippocampal cells expressing acetylcholine (Degroot et al., 2006) and dopamine type-2 (D2) receptor, 5-hydroxytryptamine type-1B(5-HT1B) and 5-Hydroxytryptamine type-3(5-HT3) receptors (Hermann et al., 2002) suggesting functional crosstalk between the ECS and the cholinergic, the dopaminergic and the serotonergic systems (Marsicano and Kuner, 2008).

III.B – SUBCELLULAR DISTRIBUTION OF CB1 RECEPTORS

The lipidic nature of the (endo)cannabinoids suggests that they can act on intracellular targets. Notably, besides the classical distribution at the cellular membrane (Dudok et al., 2015, Katona et al., 1999), CB1 receptors have been recently described in intracellular compartments, such as endosomes (Dudok et al., 2015) and brain mitochondria (mtCB1) (Figure 1F) (Benard et al., 2012, Hebert-Chatelain et al., 2014, Hebert-Chatelain et al., 2016, Koch et al., 2015). Mitochondria are highly dynamic organelles that act as powerhouses of eukaryotic cells by generating adenosine triphosphate (ATP), the universal cellular energy substrate, using a process called oxidative phosphorylation (OXPHOS) (Yin and Cadenas, 2015). In neurons, besides mitochondrial contribution to energy supply via generation of ATP to support intracellular processes (*e.g.* active transport, endocytosis and neurotransmitter production), they can contribute to phospholipids synthesis, production of intermediate metabolites and intracellular signaling molecules (Picard and McEwen, 2014). Furthermore, the apoptotic function of mitochondria, which normally leads to programmed cell death, can be responsible at synaptic level for the physiological induction of Long-term Depression (LTD) of synaptic transmission in hippocampal neurons (Li et al., 2010) showing that traditional functions of these organelles can previously unknown roles under certain conditions. Recently it has been shown that chronic THC administration (10 mg *per* kg, twice a day for 6.5 days) decreases overall CB1 receptor content in GABAergic axon terminal, with increased CB1 receptor internalization (Dudok et al., 2015). Although the authors suggest that CB1 receptors are possibly internalized in endosomes, one cannot exclude that these intracellular CB1 receptors might rather be in the mitochondria. Further examination will elucidate how CB1 receptors in intracellular compartments can impact the synaptic function and how they are functionally related to plasma membrane CB1 receptors.

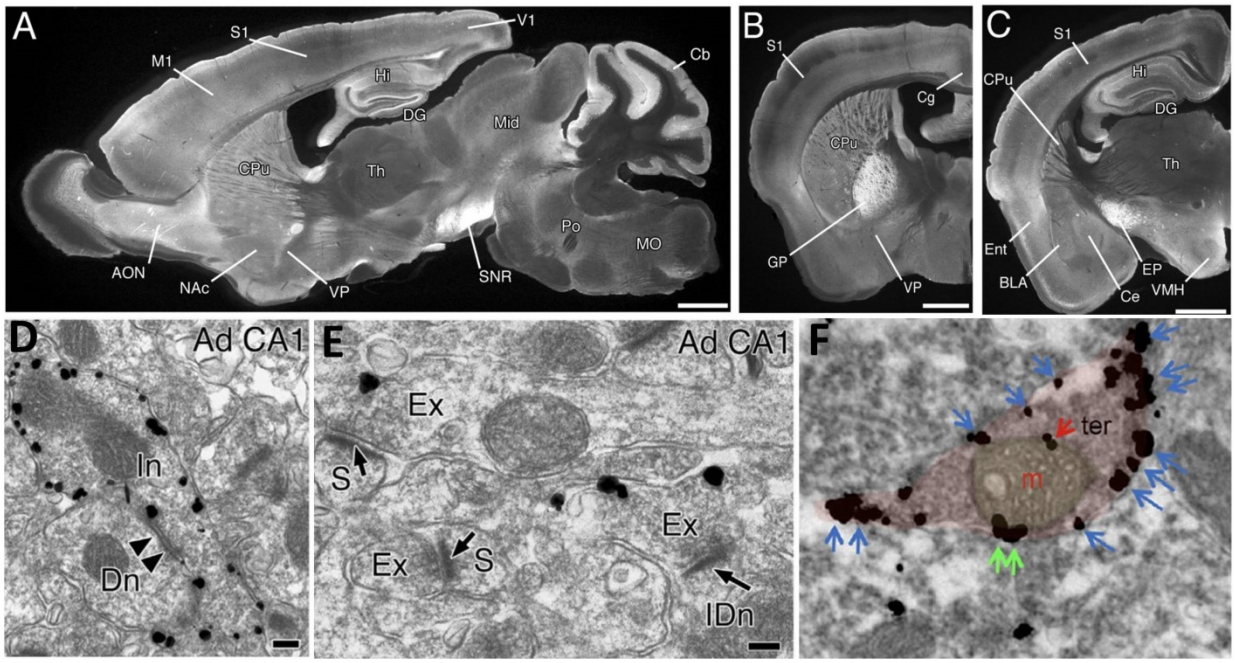

FIGURE 1 – DISTRIBUTION OF CB1 RECEPTORS IN THE ADULT MOUSE BRAIN

A-C. CB1 receptor protein distribution in the brain shows high immunoreactivity in the structures of the temporal lobe: hippocampus (Hi), dentate gyrus (DG) and entorhinal cortex (Ent). Other regions of high CB1 receptor expression include the anterior olfactory nucleus (AON), neocortex, caudate putamen (CPu), thalamus (Th) basolateral (BLA) and central (Ce) amygdaloid nuclei (C), cerebellum (Cb). **D-E.** In the hippocampus, CB1 receptors are mainly present in the presynaptic terminal (Ad CA1: Adult CA1; Dn, dendrite; Ex, Excitatory terminal; IDn, interneuronal dendrite; In, Inhibitory terminal; S, Synapse). **F.** CB1 receptors are also found in intracellular organelles such as mitochondria (m) present in the presynaptic terminals (ter). M1, primary motor cortex; S1, primary somatosensory cortex; V1, primary visual cortex; Cg, cingulate cortex; Ent, entorhinal cortex; DG, dentate gyrus; NAc, nucleus accumbens, GP, globus pallidus; VP, ventral pallidum; Mid, midbrain; SNR, substantia nigra pars reticulata; PO, pons; MO, medulla oblongata; EP, entopedoncular nucleus; VMH, ventromedial hypothalamus; DH, dorsal horn; DLF, dorsolateral funiculus. Bars: 1 mm (A-E), 100 nm (D-E), 0.5 µm (F) [(A- E) Adapted from(Kano et al., 2009); (F) Adapted from (Hebert-Chatelain et al., 2014).

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IV – Metabolism of Endocannabinoids

The discovery of the cannabinoid receptors prompted the search for endogenous ligands that could act as agonists (Mechoulam and Parker, 2013). These endocannabinoids are lipidic signaling molecules that act as endogenous agonists of CB1 and CB2 receptors (Lu and Mackie, 2016). Classically, electrically charged signaling molecules can be actively stored in synaptic vesicles, which are transported and docked near the synaptic terminals and released during neuronal activity. In the case of endocannabinoids, their lipophilic nature makes a similar scenario not plausible. Rather than being classically stored in synaptic vesicles to posterior release, endocannabinoids are thought to be produced "on demand", a process controlled by a tight regulation of synthesis and degradation via specific enzymes (Piomelli, 2003).

In the following section, I will briefly discuss the metabolic pathways for the synthesis and degradation of the major endocannabinoids and some background on how the enzymatic machinery location might modulate the endocannabinoid signaling.

IV.A – The major endocannabinoids, Anandamide and 2-AG

The first endocannabinoid to be identified was the lipid molecule of arachidonoyl ethanolamide (AEA), named Anandamide from the Sanskrit word "Ananda" which means "bliss" (Devane et al., 1992). Anandamide is a derivative of arachidonic acid that acts as a partial agonist for CB1 and CB2 receptors in the brain as well as in the periphery (Pertwee et al., 2010a). Soon after the discovery of Anandamide, a second endogenous lipid ligand called 2-arachidonoylglycerol (2-AG) was isolated, from the canine gut (Mechoulam et al., 1995) and from the brain (Sugiura et al., 1995). 2-AG, also a derivative of arachidonic acid, was found to be a full agonist of the CB1 receptors with high potency and selectivity (Pertwee, 2008).

Besides these two well-characterized endocannabinoids, there are other molecules that act as endogenous cannabinoid modulators with different selectivity and potency for CB1 receptors (Figure 2). These include: noladin ether, virodhamine and N-arachidonoyldopamine (Pertwee, 2008). The functional relevance of these molecules is not yet well characterized (Pertwee, 2008) and therefore I will focus only on the main two eCBs: 2-AG and Anandamide.

Apart from the endogenous CB1 receptor ligands, endogenous allosteric modulators that can modify the CB1 receptor activity have also been identified (Morales et al., 2016). The currently known allosteric modulators of CB1 receptors are: the anti-inflammatory lipid lipoxin

A4 (Pamplona et al., 2012), the neurosteroid pregnenolone (Vallee et al., 2014) and the hemopressin-like polipeptide pepcan-12 (Hofer et al., 2015).

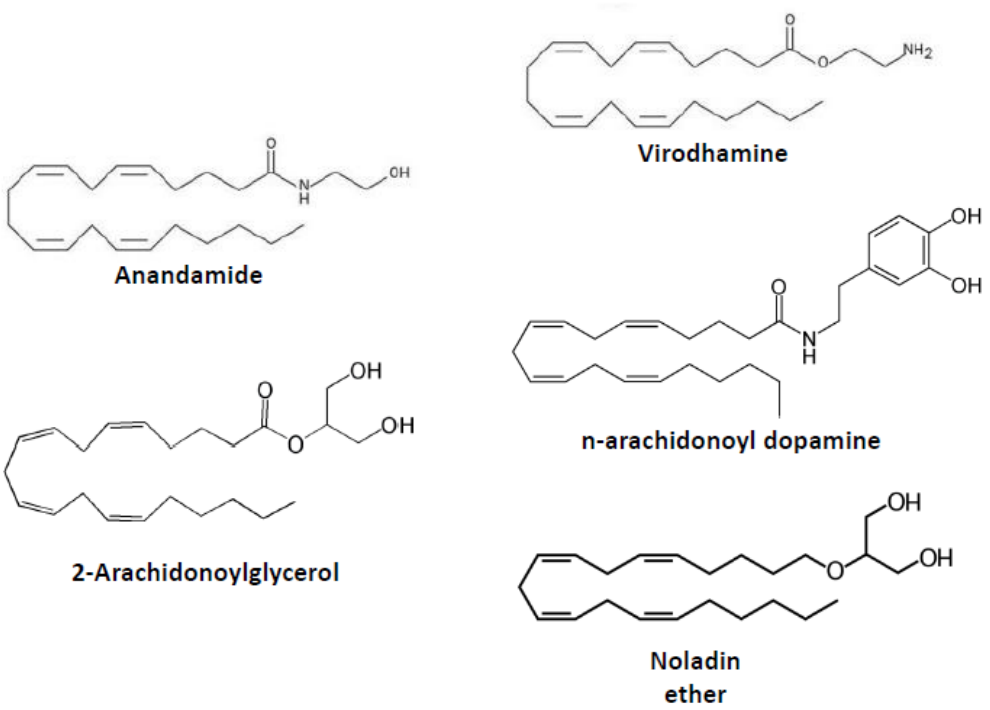

FIGURE 2 – CHEMICAL STRUCTURES OF ENDOGENOUS MOLECULES THAT BIND TO THE CANNABINOID RECEPTORS

[Adapted from (Piomelli, 2003)]

IV.B – Synthesis, Transport and Degradation of the 2-AG and Anandamide

Anandamide

Anandamide synthesis starts with the conversion of the phosphatidylethanolamines by N-acetyltransferase (NAT) into the precursor N-arachidonoyl phosphatidyl ethanol (NAPE) (Piomelli, 2003). Currently, there is evidence of multiple synthesis pathways for the production of Anandamide which, most likely, depend on several factors such brain region, local circuitry or local neuronal and/or glial activity (Ahn et al., 2008, Lu and Mackie, 2016). Two of the most well characterized are NAPE-phospholipase D (PLD) (Di Marzo et al., 1994), the first to be discovered, and the NAPE-phospholipase D pathway. In the first case, NAPE is converted to Anandamide by the action of NAPE-phospholipase D (Lu and Mackie, 2016). This pathway has been extensively studied and is present in the CNS (Lu and Mackie, 2016). In the second pathway, NAPE is first converted to phosphoanandamide via Phospholipase C (PLC) and then dephosphorylated by a protein tyrosine phosphatase non-receptor type-22 (PTPN22) to produce Anandamide (Figure 3A)(Liu et al., 2006).

After release to the extracellular space most likely by passive diffusion, Anandamide exerts its effects by retrograde signaling at CB1 receptors located at the presynaptic terminals (Piomelli, 2003). After the activation of CB1 receptors, Anandamide is cleared from the extracellular space and quickly degraded by the enzyme fatty acid amid hydrolase (FAAH) (Figure 3C) (Cravatt et al., 1996). In the hippocampus, FAAH has been shown to be integral membrane bound protein found in the soma and dendrites of pyramidal cell that are innervated by CB1 receptor-positive axon terminals, most likely from CCK-positive interneurons (Gulyas et al., 2004, Hu and Mackie, 2015). Interestingly, intracellular membrane systems such as mitochondria and the endoplasmic reticulum are highly enriched in FAAH (Ahn et al., 2008, Gulyas et al., 2004). A catalytically silent variant of FAAH, the FAAH-1 (named FLAT by the authors) has been described (Fu et al., 2011). This membrane-bound protein, which lacks catalytic activity due to alternative splicing, has high affinity to Anandamide and has been shown to lead to an accumulation of Anandamide in the cytosol thus being suggested to act as an endocannabinoid transporter (Lu and Mackie, 2016).

2-ARACHIDONOYLGLYCEROL (2-AG)

The endocannabinoid 2-AG is synthetized mainly by two principal mechanisms: 1) a calcium-dependent release (CaER) mechanism and 2) calcium-assisted receptor regulated endocannabinoid release (RER) (Figure 3B) (Ahn et al., 2008). The calcium-dependent release is likely initiated by the activation of postsynaptic metabotropic type-one glutamate (mGlu1) receptors (Maejima et al., 2005) and also muscarinic type-1 (M1), type-3 (M3) receptors (Ohno-Shosaku et al., 2003) or orexin receptors (Kukkonen and Leonard, 2014). Following activation of these receptors, which induces PLC activity, will produce inositol trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidyl inositol bisphosphate (PIP2). Next, DAG is converted by a DAG lipase, isoform α and β, into 2-AG (Di Marzo, 2008). It is currently believed that isoform DAGα is the one responsible for the synthesis of the majority of 2-AG necessary for the retrograde suppression of neurotransmitter release in the cerebellum, the hippocampus and the striatum (Tanimura et al., 2010). The second main mechanism found to regulate 2-AG production is *via* the generation of the intermediate molecule phosphatidyl inositol by the action of phospholipase A, which is then converted into 2-AG by the enzyme lyso-PLC (Lu and Mackie, 2016).

2-AG, similarly to Anandamide, is thought to be transported by facilitated transport across the membranes to the extracellular space. However, such transport protein has not yet been identified (Di Marzo, 2008).

The degradation of 2-AG is mainly processed by the enzyme monoacylglycerol lipase (MAGL) (Figure 3D) (Dinh et al., 2002b). This enzyme belongs to the family of the serine hydrolase, highly expressed in the CNS (Dinh et al., 2002a), and it 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., 2002a). MAGL is localized in close proximity to CB1 receptors to ensure a tight regulation of CB1 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).

The idea that ECS is tightly regulated, together with recent characterization of CB1 receptors in intracellular compartments (*i.e.* mitochondria), raises questions regarding the functional relevance of the presence of both the degradation enzymes and the receptors at the same locations.

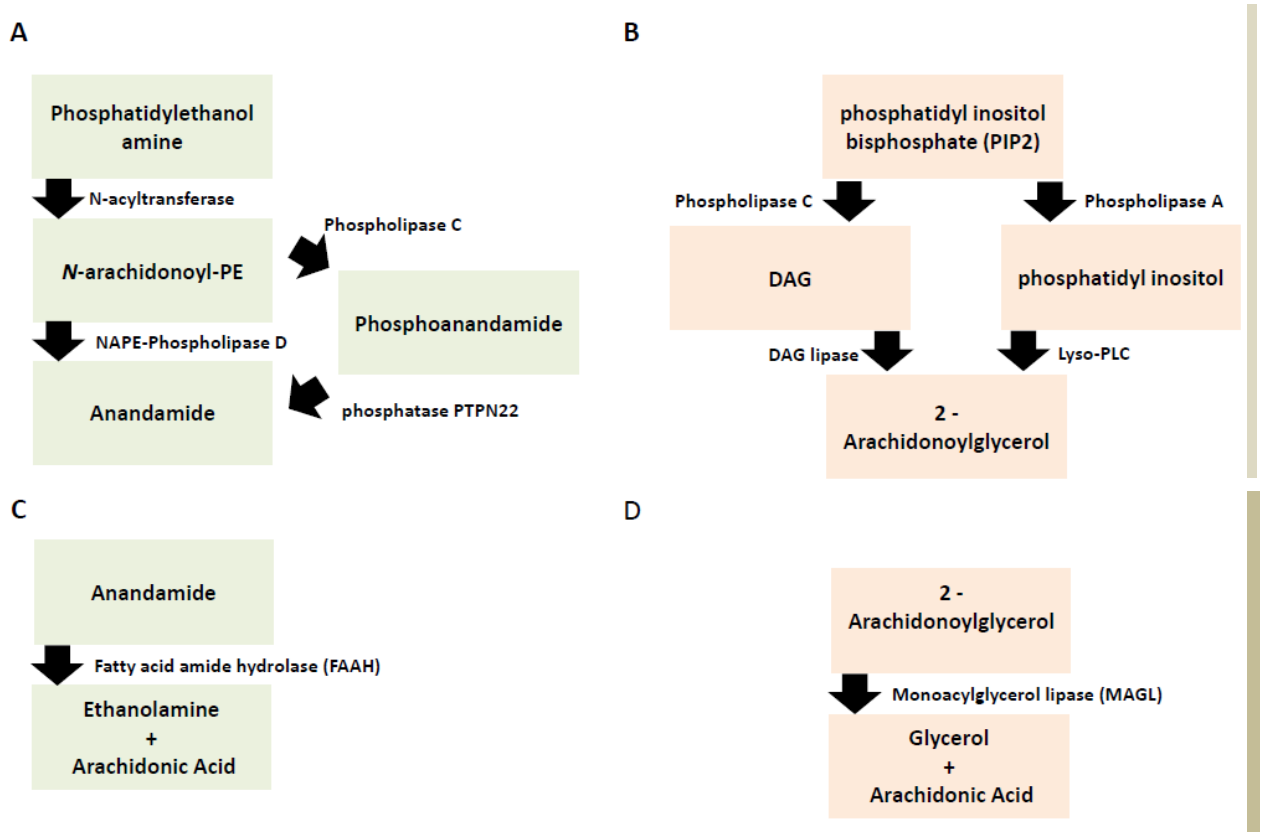

FIGURE 3 – MAIN MECHANISMS OF SYNTHESIS AND DEGRADATION OF ANANDAMIDE AND 2-AG

V – Methodologies to dissect the function of CB1 receptors

The broad distribution of CB1 receptor in several brain regions, circuits and close associated cells illustrates the complexity of this system. In order to understand the specific contribution of CB1 receptors to the modulation of synaptic plasticity and behavior both in physiology and pathology, a combination of genetic and pharmacological approaches is needed. Advanced methods such as cutting edge microscopy and/or electrophysiological approaches will allow further dissection of the specific role of the CB1 receptor in brain function. In the following section, I will review some of the current main pharmacological and genetic tools used to dissect the role of CB1 receptors in brain function.

V.A – PHARMACOLOGICAL TOOLS

The use of pharmacological tools that are based on the structure of natural exogenous and endogenous cannabinoid molecules is very important to identify and dissect CB1 receptor specific functions from the molecular to the behavioral level. There are currently several synthetic molecules that can act as full agonists with high activity and partial agonists with mild affinity of the receptor. Antagonists that block the action of the receptor and inverse agonists decrease the activation of the receptor below a threshold of basal activity. There are also allosteric modulators that through binding in allosteric rather than orthosteric sites can modify the function of the receptor (Mackie, 2008).

Besides the natural agonists (*e.g.* THC), there are several synthetic ligands that are currently used to address specific functions of CB1 receptors (Figure 4). So far, the best characterized are: the agonist HU-210, with high affinity and potency; CP55940, a potent agonist with high affinity (though inferior to HU-210), WIN 55212-2 and arachidonyl-2'-chloroethylamine (ACEA), both highly selective and potent agonists (Pertwee et al., 2010b) . As selective CB1 receptor antagonists, the best characterized are SR141716A (also known as Rimonabant) and AM-251 (Pertwee et al., 2010b). As many known CB1 receptor agonists have also high affinity for CB2 receptors (*e.g.* HU-210), before claiming that an effect is CB1 receptor-dependent it is important to demonstrate that it can be blocked by specific antagonists of CB1 receptors (*e.g.* Rimonabant) or that the phenotype of interest is absent in full CB1 receptor KO models.

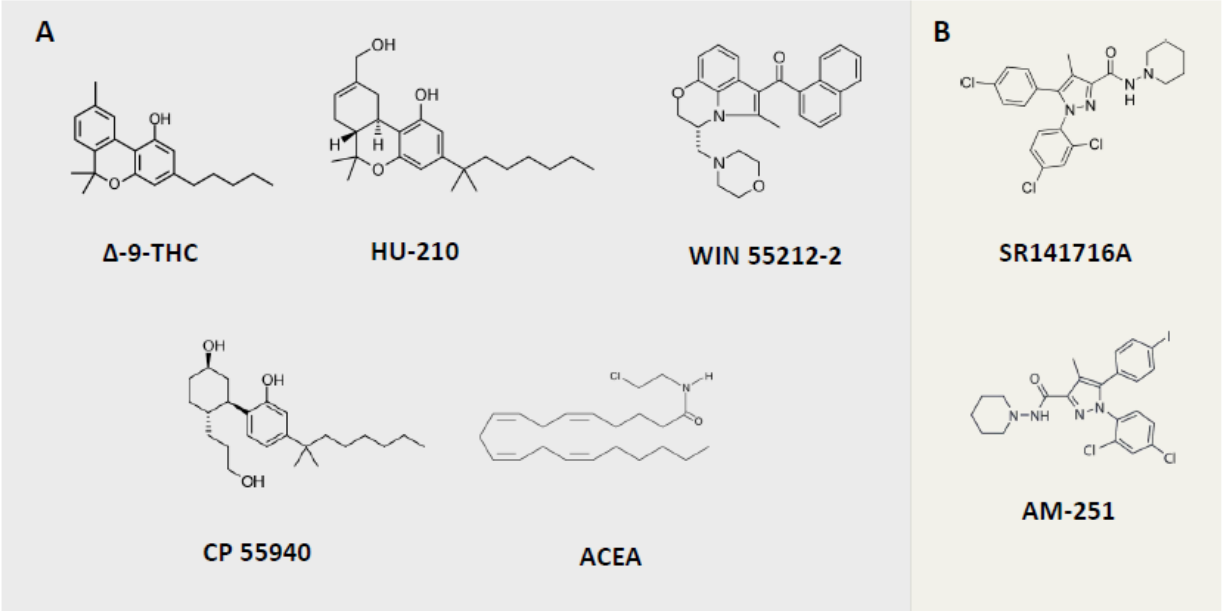

FIGURE 4 – CHEMICAL STRUCTURES OF EXOGENOUS NATURAL AND SYNTHETIC MOLECULES THAT BIND TO THE CANNABINOID RECEPTORS

A. Agonists **B.** Antagonists [Adapted from (Piomelli, 2003)]

V.B – GENETIC TOOLS TO STUDY THE ECS

The generation of mouse models ubiquitously lacking CB1 receptors provided a major step to study the specific functional of these receptors at the molecular, cellular and behavioral level (Ledent et al., 1999, Marsicano et al., 2002, Zimmer et al., 1999). However, as CB1 receptors are expressed in brain cells and circuits with apparent functional opposing effects (*e.g.* glutamatergic and GABAergic neurons in the hippocampus) the constitutive deletion of CB1 receptor does not allow to study its specific contribution to brain functions (Castillo et al., 2012).

In order to dissect the role of CB1 receptors in specific neuronal and glial cells, the use of the Cre recombinase(CRE)/loxP system of genetic recombination to generate cell type-specific conditional KOs provided a valuable tool. CRE is a protein that allows the targeted excision of genes in the genome that are located between two artificially introduced 34-bp sequences, known as loxP (Orban et al., 1992, Sauer and Henderson, 1988). The loxP sequences (generally introduced into the genome by homologous recombination) are very small and 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).

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). Once the breeding is done, the offspring will express the CRE in the cell-type of interest, allowing it to modify the genome by excision of the “floxed” gene, thereby generating a cell-type specific KO mouse (Figure 5)(Nagy, 2000).

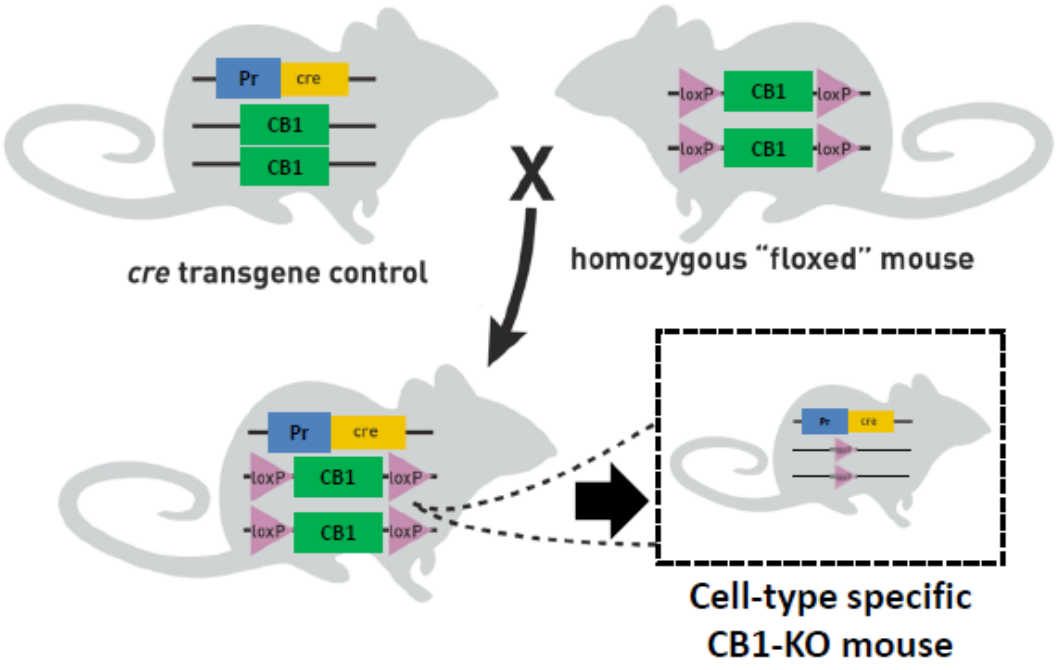

FIGURE 5 – GENERATION OF A CELL-TYPE SPECIFIC CB1 RECEPTOR KO MOUSE

[Adapted from The Jackson Laboratory online resources (https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding-for-dummies, last access 12/11/2017)]

Conditional deletion of CB1 receptors is a powerful tool to examine the role in brain circuits. However, compensatory mechanisms often emerge during development that can hinder the relevance of CB1 receptor deletion to some important functions. Moreover, 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 instance, it is well known that precursor cells can differentiate into neurons or astrocytes during development and in adulthood in regions that have conserved neurogenesis (*e.g.* the dentate gyrus or the olfactory bulb) (Garcia et al., 2004). In this specific case, constitutive deletion of a gene at early developmental stages can cause unspecific recombination in both neurons and astrocytes. For example, glial fibrillary acidic protein (GFAP) is a cytoskeleton protein that is commonly used as a marker for astrocytic identification (Brenner et al., 1994) (further explained in the Part 3-I). This marker is also present in neuronal and glial precursor cells during development (Garcia et al., 2004). Consequently, using the GFAP promoter to drive CRE for the generation of a conditional KO would generate a mouse with recombination in both neurons and astrocytes, thus making cell-type specific functional dissection undoable.

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), diminishes the risk of having genetic recombination in neurons and is a powerful tool to dissect specific astroglial function in the adult brain.

In the specific case of CB1 receptor research, several mouse lines were generated using this method allowing the cell-type specific dissection of CB1 receptor function in different brain functions.

PART 2 – SYNAPTIC PLASTICITY

Activity-dependent changes in synaptic connectivity are currently thought to be the cellular bases of higher brain functions such as learning and memory (Ho et al., 2011)(Further discussed in Part 4 – I). Synaptic plasticity, which can vary between short- and long-term forms depending on the duration of the modifications induced, can be characterized in terms of changes in synaptic release of neurotransmitters, structural modifications in synaptic organization, receptor trafficking, cell-adhesion properties and gene expression (Luscher and Malenka, 2012). Thus synaptic plasticity will imprint on the synapse an activity-dependent state that modulates neuronal and glial activity with important consequences at circuit and behavioral level.

In the next section, I will first review an important form of long-term synaptic plasticity in the hippocampus, a key brain region involved in learning and memory, as well as specify the involvement of N-methyl-D-aspartate (NMDA) receptor transmission in the modulation of these important functions both at circuit and behavioral level. This description will provide the ground to discuss the potential molecular mechanism involved in the CB1 receptor-dependent regulation of learning and memory.

I – NMDA receptors

NMDA receptors are glutamate-gated ion channels important for neuronal transmission and plasticity. One of the key functions of NMDA receptor transmission is the modulation of synaptic plasticity that underlies the cellular and molecular basis of learning and memory (Paoletti et al., 2013). NMDA receptor-dependent long-term potentiation (LTP) of synaptic transmission is one of the best characterized forms of synaptic plasticity and the modulation of NMDA receptor activity by genetic deletion or pharmacological blockade has shown that these receptors are vital components for proper of brain function (Nicoll, 2017). The modulation of NMDA receptor-dependent glutamatergic transmission by the endocannabinoid system is thus an interesting topic of research with far reaching consequences to understand important physiological brain functions.

NMDA receptors are heteromeric complexes with a diverse subunit composition and distribution throughout the brain. They can form di- or tri-heteromeric complexes (with subunits ranging from the GluN1, GluN2A–D, and GluN3A and B) and they are mainly distributed at postsynaptic structures, both at synaptic and extrasynaptic domains (Figure 6) (Paoletti et al., 2013).

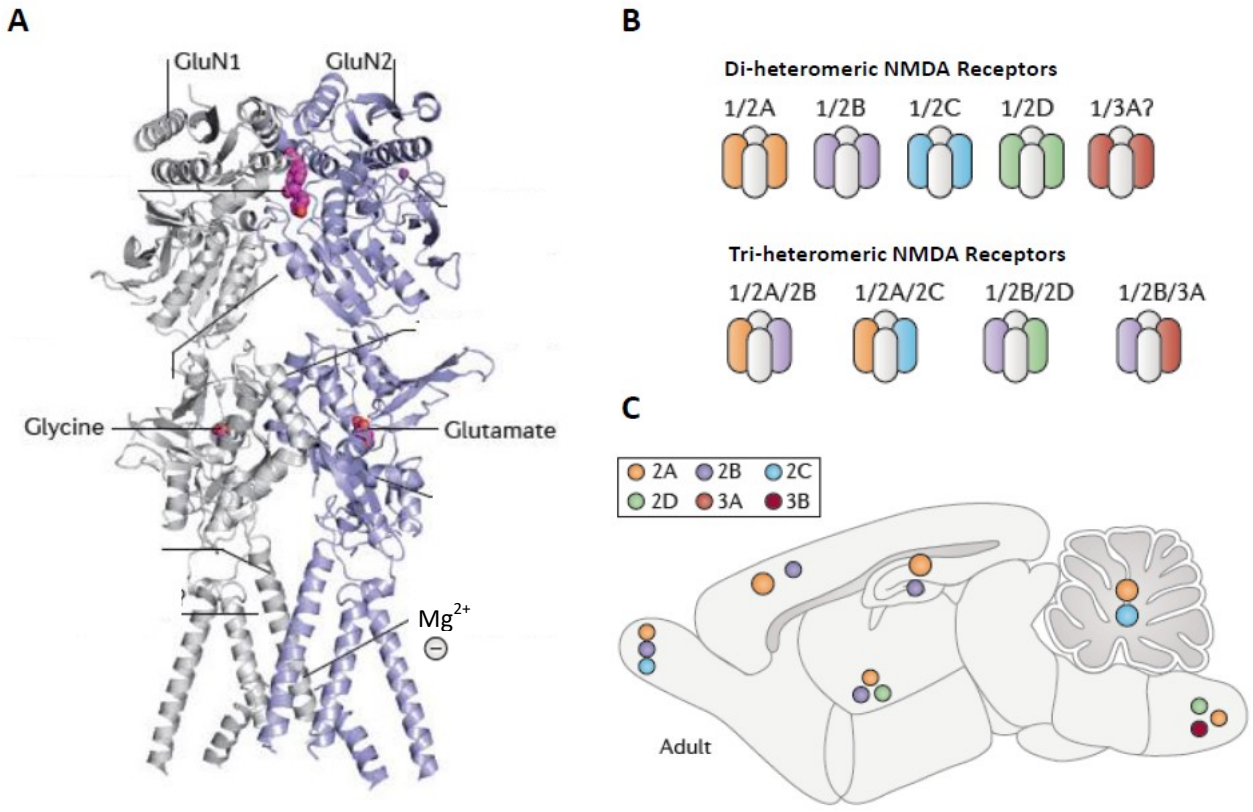

FIGURE 6 – NMDA RECEPTORS

A. Crystal structure of an NMDA receptor composed by the subunit GluN1 and GluN2. **B.** Subunit composition of different types of NMDA receptors. **C.** Distribution of NMDA receptors in the adult mouse brain. [Adapted from (Paoletti et al., 2013)]

Postsynaptic NMDA receptors display specific properties: in order to be activated they require both the binding of glutamate and a coagonist, and the removal of the magnesium block by membrane depolarization (Paoletti et al., 2013). The subunit GluN1 ubiquitously expressed in NMDA receptors contains the binding site of the coagonist. Together with GluN1 another subunit responsible for the binding of glutamate will determine the structure of the receptor and the affinity of the coagonist that is required for the NMDA receptor activation (Paoletti et al., 2013). Interestingly, there are some subunit configurations that generate NMDA receptors

that do not have a magnesium blocker. However, the well-studied forms of plasticity in key brain regions for learning and memory involve receptors that have this interesting feature.

NMDA receptors require glycine or D-serine as main coagonist (Figure 7A) (Papouin et al., 2012). Glycine was the first to be identified (Kleckner and Dingledine, 1988) and for a long time it was considered to be the main coagonist. However, S-serine was later described to be the main NMDA receptor coagonist at synaptic level (Mothet et al., 2000, Papouin et al., 2012). The discovery of S-serine as NMDA receptor co-agonist was a surprise mostly because of the D-structure of the amino acid. Until D-serine was found in large quantities in the brain (Wolosker et al., 2008), D-structure amino acids were thought to be absent in mammalian organisms (Hashimoto and Oka, 1997). Thus, the discovery of D-serine and serine racemase (SR) in the brain (Wolosker et al., 1999) led to a better understanding of NMDA receptor physiology. D-Serine is produced from the amino acid L-Serine in a reaction catalyzed by SR (Figure 7B) (Wolosker et al., 1999). In the hippocampus, D-Serine is the main coagonist of NMDA receptors. Interestingly, it has been observed the subunit type-2 present in the NMDA receptor determines their localization in either the synaptic cleft (GluN1 + GluN2A) or extrasynaptic cleft (GluN1 + GluN2B) (Papouin et al., 2012). Furthermore, it has been shown that D-serine has higher affinity for synaptic NMDA whereas glycine shows higher affinity to extrasynaptic receptors (Papouin et al., 2012). At the cellular level D-Serine is thought to be mainly synthetized in the astrocytes and further released by astrocytes to the synaptic cleft during synaptic function (Henneberger et al., 2010, Papouin et al., 2017b) however the origin of D-serine in the brain remains highly controversial (Wolosker et al., 2016, Wolosker et al., 2017).

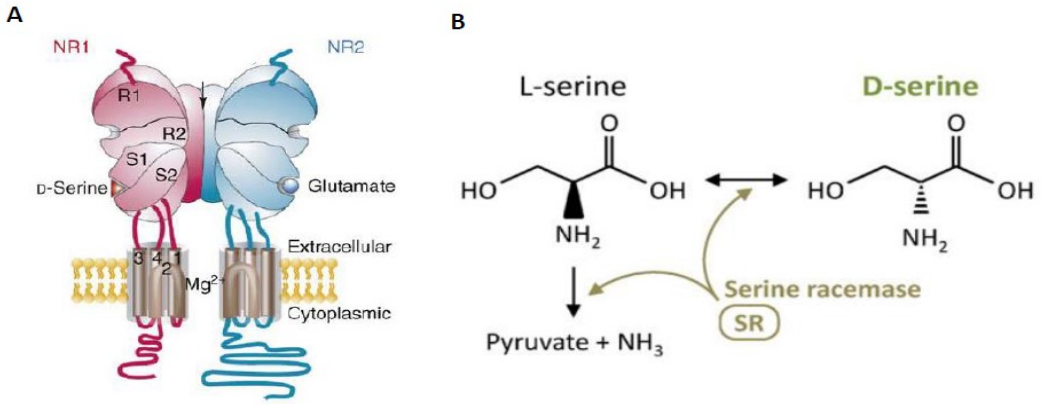

FIGURE 7 – SIGNALLING AND METABOLISM OF D-SERINE

A. D-serine binds to the subunit NR1 of the NMDA receptor. **B.** L-serine can be converted into D-serine or pyruvate by the action of the enzyme serine racemase. [(A)Adapted from (Martineau et al., 2006); (B) Adapted from (Henneberger et al., 2012)]

II – NMDA receptor-dependent LTP

The hippocampus is an important brain region important for learning, memory and spatial navigation (Eichenbaum, 2017). Thanks to its laminar structure with well-defined circuits that transfer information from within defined hippocampal areas to other brain regions, extracellular and intracellular electrophysiological investigations have provided important insights into understanding the role of neuronal and astrocytic activity in this kind of structure. One of the circuits within the hippocampus that is best characterized in terms of synaptic communication is the one between the CA3 pyramidal neuronal axons that synapse at the CA1 dendrites, also known as the Schaffer collateral to CA1 pathway (Figure 8). The best-characterized form of synaptic plasticity in the hippocampus is the LTP of synaptic transmission (Luscher and Malenka, 2012). Other types of long-term forms of synaptic plasticity currently known in the hippocampus include the LTD, spike-timing-dependent plasticity, excitatory postsynaptic potential (EPSP) -spike potentiation and depotentiation (Neves et al., 2008). Despite their putative importance in the modulation of neuronal circuits, these forms of plasticity fall outside the scope of this thesis and won't be further discussed.

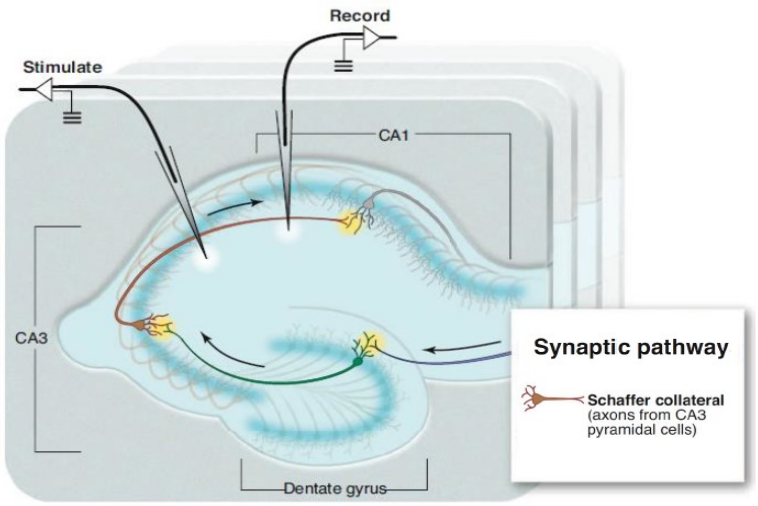

FIGURE 8 – REPRESENTATION OF THE CA3-SCHAFFER COLLATERAL TO CA1 SYNAPTIC PATHWAY IN A TRANSVERSE SLICE OF THE MOUSE HIPPOCAMPUS

Axons from the CA3 pyramidal cells (in red) 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 [Adapted from (Ho et al., 2011)]

The discovery of LTP happened around 40 years ago in the perforate pathway of the rabbit hippocampus and constitutes one of the major breakthroughs in the understanding of molecular and cellular mechanisms of brain function (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973). After this key finding, another major discovery was that hippocampal LTP induced by high frequency stimulation (HFS) depends on NMDA receptor transmission (Collingridge et al., 1983). Since LTP is artificially induced by evoked electrical activity, a causal-relation to corroborate its role in behavioral function remained highly theoretical. Morris and colleagues (1986) provided a key study aiming at probing the role of LTP and NMDA receptor transmission in learning and memory. The authors showed that infusion of an NMDA receptor antagonist in the brain can impair learning and memory and *in vivo* hippocampal LTP in rodents (Morris et al., 1986). More recently, it has been demonstrated that learning can induce LTP-like changes in hippocampal neuronal circuits (Gruart et al., 2006, Whitlock et al., 2006). Since the key events in the identification of NMDA receptor-dependent LTP and their physiological significance, multiple research groups elucidated the molecular, cellular and circuit modifications induced by LTP as well as behavioral functions that share similar mechanisms (Ho et al., 2011, Luscher and Malenka, 2012, Neves et al., 2008).

III – Mechanisms of LTP

LTP is characterized by an increase of the efficacy of synaptic transmission following certain cellular "experience". It 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* preparations. These procedures are intended to mimic neuronal firing capable of inducing 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 can be essentially divided in two main phases: 1) early-LTP, lasting around 60 minutes and 2) late-LTP, lasting from hours to days (Figure 9A, B) (Malenka and Bear, 2004).

During the induction phase of early-LTP, the arrival of an action potential to the axon terminal can induce an increase in intracellular calcium levels, mainly via voltage-gated calcium channels (VGCC)s, leading to the fusion of synaptic vesicles filled with neurotransmitters into the active zone of the terminal (Malenka and Bear, 2004). After the fusion of the vesicle with the membrane, neurotransmitters will diffuse and act on the postsynaptic terminals where they bind to specific receptors that are docked in specific dendritic structures called spines (Figure 9C). These structures represent compartmental units that are filled with multiple proteins that allow activity dependent changes in their structure (Segal, 2005). In glutamatergic synapses, glutamate, the most abundant excitatory neurotransmitter in the brain, released from the presynaptic terminal binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the postsynaptic terminals (Malinow and Malenka, 2002). AMPA receptors are ionotropic glutamatergic receptors permeable to cations (*e.g.* calcium or sodium) that mediate fast synaptic transmission (Malinow and Malenka, 2002). AMPA receptor activation quickly depolarizes the membrane of the spine to prime NMDA receptors (Malinow and Malenka, 2002). NMDA receptors are ionotropic glutamate receptors permeable to calcium that are, at resting membrane potential, blocked by a magnesium (Paoletti et al., 2013). The release of the blocker, together with the binding of glutamate and the coagonist to the NMDA receptors, induce its permeability to calcium from the extracellular space to the postsynaptic terminal (Nicoll, 2017). Intracellular calcium will then bind to calmodulin that will trigger Ca2+/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation. Next, an intercellular signaling cascade will deliver intracellular AMPA receptors, present in endosomes, to the membrane to mediate the induction of LTP of transmission (Malinow and Malenka, 2002).

The transition from early-LTP to a more stable form (*i.e.* maintenance phase) requires, besides AMPA receptor mobilization to the synaptic cleft, structural modification in specific loci

of the synapse that will allow the stabilization of the changes over time (Figure 9D). One of the outcomes of the induction of LTP is the activation of activity-dependent gene expression, which leads to protein synthesis to support LTP maintenance (Govindarajan et al., 2006). Another important outcome of LTP induction are the morphological changes induced. For instance, active dendritic spines will increase their numbers and size compared with pre-LTP (Engert and Bonhoeffer, 1999). Cytoskeleton components such as actin will be rearranged to allow spine growth and increased postsynaptic density area where several key proteins will maintain proper synaptic function (Malenka and Bear, 2004). Another interesting aspect of LTP is that the presynaptic/postsynaptic expression locus of LTP can be circuit-dependent. For instance, whereas LTP in the Schaffer collaterals to CA1 synapses is mainly expressed postsynaptically in a NMDA receptor-dependent way, at the synapses between mossy fiber axons and CA3 dendrites it is mostly expressed presynaptically in a NMDA receptor-independent way (Granger and Nicoll, 2014).

The changes induced by LTP will allow the stabilization of inputs that are intended to strengthen the connections. These changes are currently thought to be the cellular basis of learning and memory. The modulation of these changes by other systems will impact on the way synapses communicate, leading to consequences at a behavioral level. Interestingly, the CB1 receptor localization at the presynaptic terminals confers to this receptor a strategic point for the modulation of synaptic transmission and, consequently, synaptic plasticity. In the next sections, I will describe how the CB1 receptor modulates synaptic transmission and how it can impact short- and long-term forms of plasticity. These insights are intended to illustrate what is currently known about the CB1 receptor modulation of synaptic function and clarify the potential role of the ECS in the molecular and cellular mechanisms of learning and memory.

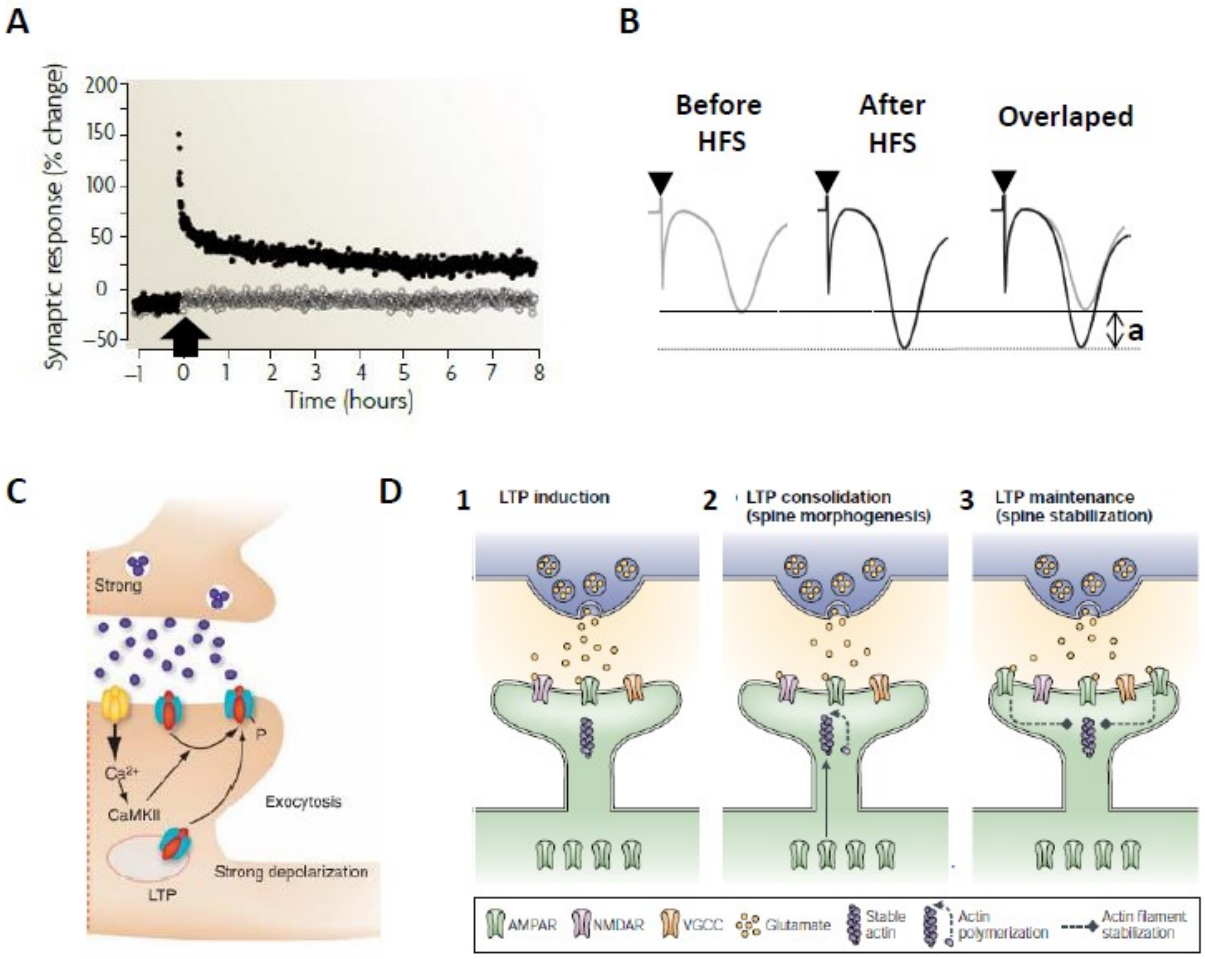

FIGURE 9 – LTP AT THE HIPPOCAMPAL CA1 EXCITATORY SYNAPSES

A. By applying specific stimulation patterns (black arrow) such as high frequency stimulation (HFS) protocols to the CA3 axons that synapse at the CA1 region, it is possible to induce a stable long-term potentiation of synaptic transmission. **B.** Superimposed trances comparing evoked excitatory postsynaptic field responses before and after HFS and the potentiation induced by HFS (**a**). **C.** Postsynaptic depolarization induced by HFS in the CA1 pyramidal neurons leads to intracellular calcium increase, activation of CaMKII and further mobilization of AMPA receptors to the membrane. **D 1-3.** HFS leads to an increase of the active spine by activity-induced morphogenesis and protein synthesis. [(A) Adapted from (Lamprecht and LeDoux, 2004); (C) Adapted from (Luscher and Malenka, 2012); (D) Adapted from (Neves et al., 2008)]

IV – CB1 receptor modulation of synaptic transmission and plasticity

The CB1 receptor, which controls eCB-mediated retrograde signaling, is an important feedback mechanism that modulates synaptic transmission (Figure 10) (Freund et al., 2003, Piomelli, 2003). As previously described, the postsynaptic on-demand production of Anandamide and 2-AG activates CB1 receptors, mainly, in the presynaptic terminals to quickly decrease neurotransmitter release (Soltesz et al., 2015). Besides the classical mechanisms that were initially described, it has been recently shown that endocannabinoids can also modulate synaptic transmission and plasticity by acting non-retrogradely on TRPV-1 receptors in the postsynaptic terminals or in CB1 receptors located in adjacent astrocytes (Figure 10C, D). In the following section, I will describe currently characterized neuronal intracellular pathway underlying classical CB1 receptor activation as well as the result of this activation in the modulation of synaptic transmission and plasticity.

IV.A – CANONICAL CB1 RECEPTOR-MEDIATED INTRACELLULAR SIGNALING PATHWAY

CB1 receptors at the presynaptic terminal are intracellularly primarily coupled with the Gi/o subunits of G proteins (Figure 10A, B) (Howlett et al., 1986, Howlett and Fleming, 1984). The activation of CB1 receptors by endocannabinoids inhibits adenylate cyclase and decreases cyclic adenosine monophosphate (cAMP) levels. Hence, downregulation of cAMP levels will inhibit the cAMP-dependent protein kinase A (PKA) (Davis et al., 2003). CB1 receptor activation also leads to the inhibition of N-type (Guo and Ikeda, 2004), L-type (Straiker et al., 1999) and P/Q-type of VGCC (Fisyunov et al., 2006, Mackie et al., 1995) and the modulation of inwardly-rectifying potassium channels (Guo and Ikeda, 2004, Mackie et al., 1995). These effects overall contribute to a hyperpolarization of the presynaptic terminal and a decrease of neurotransmitter release into the synaptic cleft (Di Marzo, 2009). Furthermore, CB1 receptor activation by different ligands modulates several intracellular cascades, such as mitogen-activated protein kinases (MAPK) pathway. This pathway is responsible for long-lasting changes in neuronal function and is involved in cell proliferation, cell differentiation, cell mobility and apoptosis (Turu and Hunyady, 2010). The activation of this pathway leads to the activation of extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), p38 MAPK or ERK5 proteins (Turu and Hunyady, 2010).

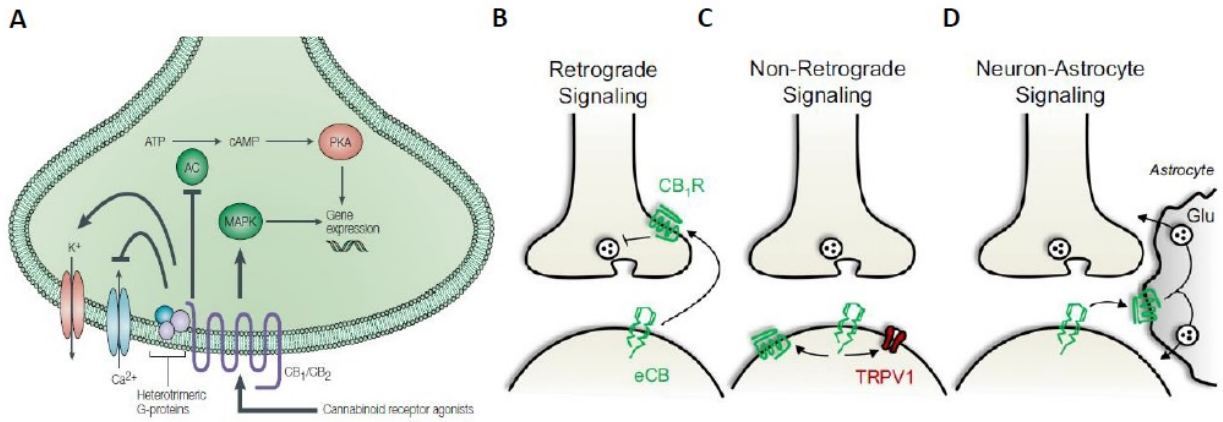

FIGURE 10 – CB1 RECEPTOR INTRACELLULAR SIGNALLING AND MODULATION OF SYNAPTIC FUNCTION

A. Presynaptic intracellular cascades induced by CB1 receptor activation. **B.** Postsynaptic production of endocannabinoids activates CB1 receptors in the presynaptic terminal or **C.** postsynaptic targets. **D.** Endocannabinoids have been shown to modulate neurotransmission by interacting with astroglial CB1 receptors and promoting gliotransmission. [(A) Adapted from (Di Marzo et al., 2004); (B-D) Adapted from (Castillo et al., 2012)].

Besides the previously described mechanism of action, it is important to keep in mind that CB1 receptor-dependent selectivity for a certain intracellular pathway depends on the conformation of the receptor following the binding by different ligands (Turu and Hunyady, 2010). This feature induces biased ligand signal transduction for a certain pathway depending on the availability and recruitment of different G proteins subunits (Turu and Hunyady, 2010). For instance, it has been reported that in certain conditions, CB1 receptors can recruit Gs (Glass and Felder, 1997) or Gq (Lauckner et al., 2005, Navarrete and Araque, 2008) rather than Gi/o proteins (Turu and Hunyady, 2010). It has been shown that different agonists can induce different effects via the same receptors. The highly potent CB1 receptor agonist WIN 55212-2 can induce Gq-dependent calcium increase in cultured hippocampal neurons whereas HU-210, another highly potent CB1 receptor agonist, does not (Lauckner et al., 2005). Functional implications of these findings might explain why different agonists might not induce the same effect, though all of them are specific to CB1 receptors. It would be interesting to understand whether certain agonists *in vivo* can have more affinity for the receptors that are in a certain configuration or location in terms of cellular specificity (*e.g.* neuronal vs astrocytic) or intracellular vs membrane localization (*e.g.* mtCB1 receptors). Activation of CB1 receptors can also prompt an intracellular interaction with recruited β-Arrestins to promote CB1 receptor internalization, a mechanism involved in the desensitization of the receptors (Breivogel et al., 2008, Jin et al., 1999).

IV.B –CB1 RECEPTOR-MEDIATED SHORT- AND LONG-TERM SYNAPTIC PLASTICITY

The overall signaling cascade mediated by CB1 receptors under certain conditions can lead to short- or long-term changes in synaptic function. Depending on the stimulation condition and the circuit studied, CB1 receptor-mediated plasticity can act as a powerful means to modulate synaptic function. Several lines of research have shown that activation of CB1 receptors either endogenously (*i.e.* by endocannabinoids) (Stella et al., 1997) or exogenously (*i.e.* by natural and synthetic cannabinoids) (Hoffman et al., 2007) in different preparations and paradigms can be involved in both the modulation of short- and long-term form of synaptic plasticity (Castillo et al., 2012). In the following section, I will describe currently known forms of synaptic modulation mediated by CB1 receptors.

DSI AND DSE

The discovery of CB1 receptors in GABAergic presynaptic terminals closely associated with the synaptic cleft raised questions regarding their function in the modulation of synaptic activity. In 2001, several works demonstrated that CB1 receptors can mediate a retrograde suppression of synaptic activity lasting from tens of seconds up to 1 min (Gerdeman, 2008). The mechanism, first observed in inhibitory connections between hippocampal neurons, demonstrated a CB1 receptor-dependent decrease of presynaptic GABA release and consequent suppression of inhibitory currents (named depolarization induced suppression of Inhibition: DSI) (Figure 11A) (Ohno-Shosaku et al., 2001, Wilson and Nicoll, 2001). Interestingly not only GABAergic transmission can be modulated by CB1 receptors. In 2001, Kreitzer and Regehr described a CB1 receptor-dependent decrease of presynaptic glutamate release in the cerebellar neurons that causes a suppression of postsynaptic excitatory currents (Kreitzer and Regehr, 2001a, Kreitzer and Regehr, 2001b). This mechanism, named depolarization induced suppression of excitation (DSE), was later also observed in the hippocampal pyramidal cells (Ohno-Shosaku et al., 2002). Besides the cerebellum and the hippocampus, DSI and DSE have been characterized in several other brain regions such as 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).

LONG-TERM FORMS OF ENDOCANNABINOID-MEDIATED PLASTICITY

Besides the role of presynaptic CB1 receptors in the modulation of transient changes in synaptic transmission, CB1 receptor can also mediate long-term forms of synaptic plasticity (Figure 11B) (Castillo et al., 2012). First evidence regarding endocannabinoid modulation of long-term synaptic plasticity was reported in the glutamatergic synapses of nucleus accumbens where endocannabinoids can induce a specific form of LTD (eCB-LTD) (Gerdeman et al., 2002, Robbe et al., 2002). Within this region, eCB-LTD required metabotropic glutamate receptor activation, postsynaptic calcium increase and an "on demand" production and release of Anandamide (Gerdeman et al., 2002, Robbe et al., 2002). eCB-LTD was also described in inhibitory synapses both in the amygdala (Azad et al., 2004, Marsicano et al., 2002) and in the hippocampus (Chevaleyre and Castillo, 2003). More specifically, in the hippocampus, 2-AG besides inducing an eCB-LTD in the presynaptic excitatory terminals has been shown to promote a heterosynaptic inhibitory-LTD (I-LTD) (Chevaleyre and Castillo, 2003). In these synapses, CB1 receptor activation during the induction of the I-LTD decreased PKA activity via downregulation of cAMP and calcium sensitive phosphatase calcineurin (CaN). RIM1α and Rab3B in the active zone were also identified as necessary for the I-LTD (Chevaleyre et al., 2007). More recently, it has been found that I-LTD is dependent on protein synthesis in the axons but not in the soma of interneurons, a process that is enhanced by CB1 receptor-dependent mechanistic target of rapamycin (mTOR) activity (Younts et al., 2016).

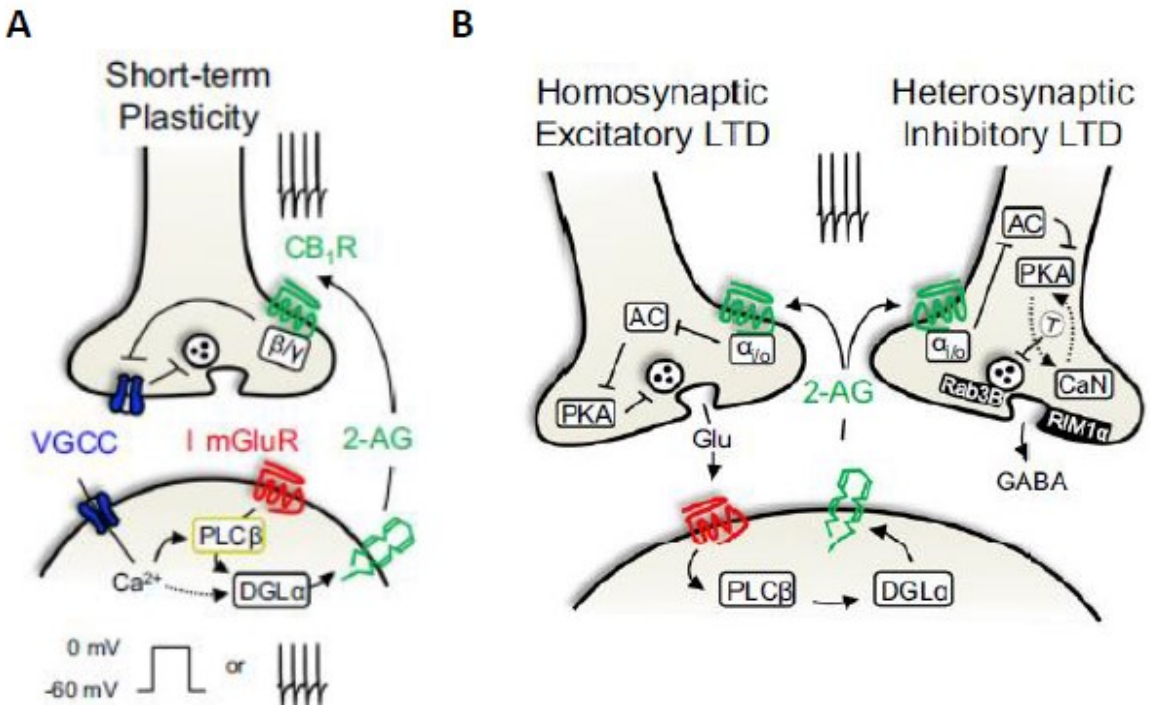

FIGURE 11 – ESTABLISHED MECHANISMS OF ENDOCANNABINOID-MEDIATED SHORT- AND LONT-TERM SYNAPTIC PLASTICITY

A. Brief bursts of activity or depolarization of the postsynaptic terminal lead to the production of endocannabinoids that travel to the presynaptic terminal to inhibit neurotransmitter release. **B.** Sustained pattern of activity can induce a long-term depression (LTD) of excitatory or inhibitory terminals via presynaptic CB1 receptors by postsynaptically produced endocannabinoids. [(A, B) Adapted from (Castillo et al., 2012)]

IV.C – NON-CANONICAL ENDOCANNABINOID-MEDIATED MODULATION OF SYNAPTIC ACTIVITY

Besides the classical presynaptic retrograde activity mediated by CB1 receptors, a non-retrograde CB1 receptor-dependent postsynaptic mechanism has also been described. Autaptic transmission in the fast spiking inhibitory neurons of the neocortex has been shown to be an important way to modulate neuronal self-activity. Interestingly, in another class of inhibitory neurons, the cholecystokinin- or somatostatin-expressing low threshold spiking (LTS) interneurons, the self-modulation was induced via a CB1 receptor-dependent mechanism (Bacci et al., 2004). Slow self-inhibition (SSI), a hyperpolarization achieved by an increased conductance via somatodendritic potassium channels that could last for minutes, was dependent on intracellular calcium increase in the extracellular space and CB1 receptor

activation by 2-AG (Bacci et al., 2004, Marinelli et al., 2008). This mechanism of endocannabinoid-mediated self-modulation was shown not to be exclusive to inhibitory neurons and can was also found in the layer 2/3 glutamatergic pyramidal neurons of the neocortex (Marinelli et al., 2009).

Another non-canonical, postsynaptic form of plasticity dependent on CB1 receptors has been recently described in the hippocampus. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are voltage-gated ion channels permeable to Na+ and K+ that mediate a neuronal hyperpolarization-activated cationic depolarizing current (Ih) in distal synapses and in the soma (Maroso et al., 2016). HCN receptors have been shown to modulate spike firing and dendritic integration by modulating membrane properties (*e.g.* membrane potential, membrane resistance) and to modulate synaptic plasticity and hippocampal-dependent spatial memory (Voglis and Tavernarakis, 2006). In 2016, Maroso and colleagues have shown a novel mechanism of modulation of dendritic excitability mediated by CB1 receptor-HCN channels in superficial pyramidal neurons of CA1 region (Maroso et al., 2016). By studying Ih currents mediated by HCN channels, they reported that activation of the CB1 receptor-HCN pathway decreased dendritic excitability, impaired LTP and long-term memory formation (Maroso et al., 2016). By using specific viral approaches the authors have shown that postsynaptic, rather than presynaptic, CB1 receptors in the pyramidal neurons were responsible for this interaction. Although it is not currently known the location of these receptors, mitochondria can be a possible candidate location for this pool of these postsynaptic CB1 receptors.

IV.D – CB2 RECEPTOR-DEPENDENT MODULATION OF NEURONAL ACTIVITY

Although several groups reported the presence of CB2 receptors in the CNS (Gong et al., 2006, Morgan et al., 2009, Onaivi et al., 2006) the functional relevance of CB2 receptors in neuronal, rather than glial, cellular populations remains largely unknown (Quraishi and Paladini, 2016). In 2012, it has been reported that postsynaptic intracellular CB2 receptors control the neuronal excitability of the layer 2/3 pyramidal neurons in the mouse prefrontal cortex (den Boon et al., 2012). The activation of these receptors at an autocrine has been shown to induce a self-inhibition of neuronal excitability (similarly to SSI) through a mechanism based on IP3-dependent modulation of calcium-activated chloride channels (den Boon et al., 2012). More recently, it has been shown that CB2 receptors expressed in CA2/3, but not CA1, pyramidal neurons of the hippocampus, were responsible for a self-inhibition of excitation (Stempel et al., 2016). In this study, the authors reported postsynaptic mechanism in which CB2 receptors modulate a sodium-bicarbonate co-transporter that underlies a hyperpolarization of the

neuron. This effect, which was shown to be mediated by the endocannabinoid 2-AG, was absent in mice lacking CB2 receptors. The overall effect was a reduced spike probability of CA3 pyramidal cells and an alteration in gamma oscillations *in vivo* (Stempel et al., 2016).

IV.E – TRPV-1 RECEPTOR-DEPENDENT MODULATION OF NEURONAL ACTIVITY

TRPV-1 receptors, being sensitive to the endocannabinoids, are potential players in the endocannabinoid modulation of synaptic transmission and plasticity (Castillo et al., 2012, Di Marzo and De Petrocellis, 2010). As previously discussed, TRPV-1 receptors are fully activated by the endocannabinoid Anandamide and they mediate a long-term form of synaptic plasticity (TRPV1-LTD). Initially described in the hippocampal neurons, this form of plasticity is induced by the activation of TRPV-1 receptors in interneurons, but not in neighboring pyramidal neurons, by capsaicin (exogenous ligand) or endogenous eicosanoid 12-S-HPETE (Gibson et al., 2008). In the dentate gyrus, synaptic TRPV-1 are reported to induce a LTD mediated by the endocannabinoid Anandamide which is independent of CB1 receptors (Chavez et al., 2010) and requires internalization of AMPA receptors (Grueter et al., 2010).

To conclude, CB1 receptors in the brain can modulate several forms of synaptic plasticity between different cell-types in different brain regions. Furthermore, exogenous cannabinoids or dysfunctional endocannabinoid signaling, by acting on CB1 receptors, can disrupt normal synaptic function. Thus, understanding how the modulation of CB1 receptors can control synaptic transmission is key to understand pathological consequences resulting from unbalanced CB1 receptor function at the synapse.

V – Methodologies to investigate synaptic function

The study of the endocannabinoid system in the brain improved with the development of advanced techniques to study neuronal circuits at the micro-, meso- and macro-scales. One of the most versatile approaches is the electrophysiological investigation of the electrical properties of neuronal networks. Electrophysiology thus 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. By understanding how neurons communicate and how different cell-types interact, important insights can be drawn regarding their function and dysfunction in the patho-physiology of the brain.

Electrophysiological investigations allow the measurement of diverse components of the electrical activity in the brain by studying the movement of ions through cellular membranes (Zhang et al., 2014). The major ions that modulate membrane potential of neurons and are involved in their communication are the sodium, potassium, chloride and calcium ions (Accardi et al., 2016). As the cellular membranes are lipid systems with a hydrophobic nature, they will not allow the free flow of these ions. Through active and passive transport (mediated by transporters and channels), these ions will be kept in different concentrations between the outside and the inside of the neurons, creating a negative membrane potential which is around -60 to -70 mV (Accardi et al., 2016). Changes in the neuronal membrane potential by certain inputs (electrically or sensory) can promote depolarization and hyperpolarization events. If a certain input depolarizes the membrane potential above a certain threshold it can induce an action potential responsible for neuronal communication (Booker et al., 2014). From the several methodologies available, the whole-cell patch clamp and the extracellular field recordings are the most used techniques to assess network function.

Intracellular recording from neurons are obtained by introducing a glass sharp microelectrode (±1 µm tip) filled with a conductive intracellular solution in the inside of the cell of interest. In the patch clamp technique, instead of impaling the cell, the tip of the glass electrode will touch the membrane (thus “patch”) and then, by applying a mild suction to remove the membrane, will allow the creation of a continuum between the cytosol and the solution inside the recording electrode (*i.e.* whole cell patch clamp) (Accardi et al., 2016). In whole cell patch clamp it is possible to be in voltage clamp and current clamp mode. Voltage clamp aims at measuring the changes in current across the membranes. For this it is needed that the amplifier used to record the signal holds the membrane voltage at a certain value. It is a feedback mechanism that measures the membrane potential and alters the current to maintain the previously set value. On the other hand, current clamp allows the study of the membrane

potential when injecting current and give information about the ionic conductance of the membrane. The use of whole-cell recordings in the endocannabinoid field proved very useful as it allowed to identify intracellular mechanisms of CB1 receptor-mediated signaling and how the CB1 receptors modulates short- (*e.g.* DSI and DSE) and long-term forms of synaptic plasticity (*e.g.* I-LTD or eCB-LTD) (Castillo et al., 2012, Gerdeman, 2008).

Whereas the use of whole-cell patch clamp can give precious insight of single cell activity, the investigation of extracellular field recordings allows the study of extracellular changes in ion concentrations due to the activity of large populations of neurons (Zhang et al., 2014). 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). Field recordings also allow the identification of single action potentials in the surroundings of the recording electrodes. By using analytic methods (*e.g.* spike sorting) that check for the properties of these action potentials, it is possible to identify specific populations of neurons that are firing in certain conditions (Buzsaki, 2006). In the endocannabinoid field, the study *in vitro* or *in vivo* extracellular field potentials have allowed the dissection of the role of CB1 receptors in brain oscillations and spike activity (Robbe and Buzsaki, 2009, Robbe et al., 2006), LTP (Stella et al., 1997), LTD (Han et al., 2012), among several other forms of synaptic plasticity (Araque et al., 2017, Castillo et al., 2012).

Currently, the electrophysiological techniques described above are being complemented with new powerful approaches in order to answer more complex questions. One example is the combination of electrophysiology with advance imaging techniques such as stimulated emission depletion (STED) microscopy. STED is a super-resolution technique that allows the imaging of nanoscopic structures in the brain (*e.g.* single synapses, single receptors or intracellular organelles) (Takasaki et al., 2013). This allows the imaging of single synapses and small astrocytic processes that are otherwise too small for normal imaging techniques. One of the advantages of this technique is that it can be performed in *vitro* or *in vivo* tissue, thus allowing the exploration of electrical properties of the neurons by electrophysiology at the same time. This is particularly interesting for the study of the endocannabinoid system as it is both very dynamic and its function depends on the model used (*in vitro* vs *in vivo*). Optogenetics is a technique that involves the expression and control of light-inducible proteins in specific cell populations in the brain with very high temporal and spatial precision (Boyden et al., 2005). The use of optogenetics can allow the dissection of specific neuronal and glial circuits within certain networks that are recorded using classical electrophysiology. Thus, advanced genetic techniques

could allow the expression of optogenetic proteins that modulate Gi/Gα or Gq proteins in cells with specific deletion of CB1 receptors, thus allowing the modulation (positive or negative) of the effect of CB1 receptors.

Overall, tools with higher specificity, temporal and spatial resolution to assess the role of the CB1 receptors in neuronal circuits will provide important insights as they allow more complex questions to be addressed, thus revealing the contribution of the ECS to brain physiology.

PART 3 – ASTROCYTES IN THE BRAIN

Astrocytes are complex glial cells that are widely distributed in the CNS and Peripheral Nervous System and are well conserved throughout evolution (Haim and Rowitch, 2017). Whereas simpler invertebrate animals possess simpler astrocytes, more complex mammals have increasingly complex astrocytes, not only in terms of morphology but also in terms of functions (Allen and Barres, 2009). Astrocytes can modulate a broad range of functions ranging from the support of neurotransmission, the homeostasis of the extracellular ionic content or the metabolic support of neuronal networks, to the more recently investigated role in synaptogenesis and in the bidirectional communication with their neuronal counterparts (Allen and Barres, 2009). Astrocytic dysfunction is implicated in pathological conditions as reactive glia and neuronal inflammation are thought to underlie glial scar tissues and, in some cases, the development of astrocytic tumors (*i.e.* gliomas) (Allen and Barres, 2009). More recently, it has be argued that astrocytes can prompt pathology through dysregulation of the neuro-glial signaling (Chung et al., 2015). Contrary to neurons, astrocytes are not-electrically excitable. This property led wrongly to the conclusion that astrocytes were not very active and they would just assist neuronal function rather than having an intrinsic role in the process of information processing (Verkhratsky et al., 2012a).

After briefly introducing the biology of astrocytes and their main functions, I will focus on the synaptic role of astroglial cells and explain how astrocytes can modulate neuronal and glial activity in physiological conditions.

I – Astrocytes: morphology, distribution, physiology and function

Astrocytes are the most abundant class of glial cells in the brain (Volterra and Meldolesi, 2005). They derive from the same neuroepithelial cells that generate neurons and oligodendrocytes (Eroglu and Barres, 2010) and their development starts soon after the initial development of neurons where they become key elements of the development and maturation of neuronal circuits (Eroglu and Barres, 2010).

Astrocytes in the mouse brain can be divided into two main categories: the protoplasmatic and the fibrous astrocytes. Protoplasmatic astrocytes are highly ramified, form a bridge between blood vessels and other astrocytes, are part of the blood-brain barrier, are capable of unsheathing the synapses (Eroglu and Barres, 2010) and are found in the gray matter (Allen and Barres, 2009). On the other hand, the fibrous astrocytes, which have similar functions as the protoplasmatic astrocytes, are mainly present in the white matter associated with axons (Allen and Barres, 2009). Human astrocytes are known to be increasingly more complex than simpler mammalian astrocytes (Herculano-Houzel, 2014). For instance, when compared with rodents, human protoplasmatic astrocytes are larger, have increased synaptic coverage and increased branching and process domains (Oberheim et al., 2009).

Besides their morphological features, astrocytes are characterized by several molecular markers: the GFAP, glutamate-aspartate transporter (GLAST), S100 calcium-binding protein β (S100β), glutamate transporter 1 (GLT-1), glutamine synthetase and the aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (Khakh and Sofroniew, 2015, Srinivasan et al., 2016). GFAP is a structural intermediate filament protein that is commonly used as a marker of astrocytes in the brain (Khakh and Sofroniew, 2015). It is expressed at modest levels in the main branches of the astrocytes but it is not detectable in the fine processes that surround the synapses. Although it is commonly used as an astrocytic marker, such has two main caveats that must be kept in consideration: it is expressed in neuronal progenitor cells and there are astrocytes that do not express GFAP. Nevertheless, GFAP-expressing astrocytes are commonly found in the hippocampus and are confirmed as such by using other astrocytic markers such as S100β (Khakh and Sofroniew, 2015).

Astrocytes can be ubiquitously found in the brain. Major regions where astrocytes can be found include the cortex, the hippocampus, the striatum, the retina, the cerebellum and olfactory bulb (Khakh and Sofroniew, 2015). Interestingly, circuit distribution of astrocytes varies from region to region (John Lin et al., 2017). For instance, both in the hippocampus and in

the striatum, astrocytes occupy single non-overlapping domains, with striatal astrocytes displaying larger territories coverage compared with the hippocampal astrocytes (Bushong et al., 2002, Chai et al., 2017). Furthermore, hippocampal astrocytes target more excitatory synapses while astrocytes in the striatum interact much more directly with the neuronal somata (Chai et al., 2017).

Although commonly denominated as a single class of cells, astrocytes are increasingly viewed as diverse populations. Besides the two main classes of protoplasmatic and fibrous astrocytes, there is increasing evidence pointing to region-specific astrocytes with possible intra-region circuit-specificity (Ben Haim and Rowitch, 2017). Specific astrocytic markers can identify different astrocytes in the different brain regions. For instance, whereas GLT-1 can identify astrocytes in the hippocampus, the lateral septum, the cerebral cortex, and the striatum, it is expressed at lower levels in the cerebellum (Lehre et al., 1995). Conversely, the specific astrocytic markers GLAST identifies many more astrocytes in cerebellum than in cortex or hippocampus (Lehre et al., 1995). Furthermore, there are studies that demonstrate that different astrocytes possess functional and morphological differences that further point to distinct populations within brain regions and most likely within close associated circuits (Chai et al., 2017). Another issue that is important to keep in mind is that targeting astrocytes with a specific markers (*e.g.* GFAP) might not yield the same results as targeting with another specific marker (*e.g.* GLAST), as these markers might represent to some extent independent populations of astrocytes. For instance, in a recent study where astrocytes from the dorsal lateral striatum and the hippocampus were characterized, it was shown that although there were many similarities between the two regions, astrocytes differ in terms of function, morphology and molecular characterization (Chai et al., 2017). Another important aspect is that astrocytes during development can have different levels of proteins expression. For instance, mGlu5 receptors are downregulated throughout aging, with a peak expression in young animals and low expression in adult animals (Sun et al., 2013).

Astrocytes are major determinants of homeostasis in the CNS (Verkhratsky et al., 2012a). They are responsible for the metabolic support to the brain by retrieving nutrients from the blood-brain barrier (Verkhratsky et al., 2012a). They are regulators of neurogenesis and synaptogenesis during synaptic pruning (Eroglu and Barres, 2010). Astrocytes also control axon guidance during development, neurotransmitter clearance and the removal of other types of ions from the extracellular space (*e.g.* potassium, sodium). They protect the brain against insults that might damage the brain and they regulate the synaptic function and plasticity (Verkhratsky et al., 2012b, Eroglu and Barres, 2010). Moreover, astrocytes control blood flow to augment the delivery of oxygen and nutrients to regions undergoing high activity (Giaume et al., 2010). Also, in response to brain injury and disease astrocytes can transform themselves into reactive astrocytes in a process called astrogliosis (Sofroniew, 2014). Reactive astrocytes are

characterized by modifications in gene expression coupled with cellular changes (Sofroniew and Vinters, 2010). For instance, brain damage leads to the upregulation of the expression of GFAP with hypertrophy of the cell body and processes. Depending on the severity of the insult, reactive astrocytes can increase proliferation, overlap with each other and lead to densely packed cell agglomerates called glial scars (Sofroniew and Vinters, 2010). Although reactive astrocyte form in response to brain damage, the extent from which reactive astrocytes are beneficial or prejudicial to brain recover is yet to be clarified. Although some studies have demonstrated that glial scar formed by reactive astrocytes can support axon recover and regeneration (Anderson et al., 2016), others have described that they can inhibit neuronal recovery (Silver and Miller, 2004). Interestingly, in a recent study it has been shown that different classes of reactive astrocytes are formed during brain damage. In 2017, Liddelow and colleagues have shown that activated microglia (another glial cell-type involved mainly in active immune responses in the CNS) could induce a specific class of reactive astrocytes (named by the authors A1 reactive astrocytes) that proved to be neurotoxic by losing their ability to control key recovery functions to support neuronal survival, outgrowth, synaptogenesis and phagocytosis (Liddelow et al., 2017). Nevertheless, the role of reactive glia remains poorly studied and more research is needed to clarify their influence in CNS injury and disease.

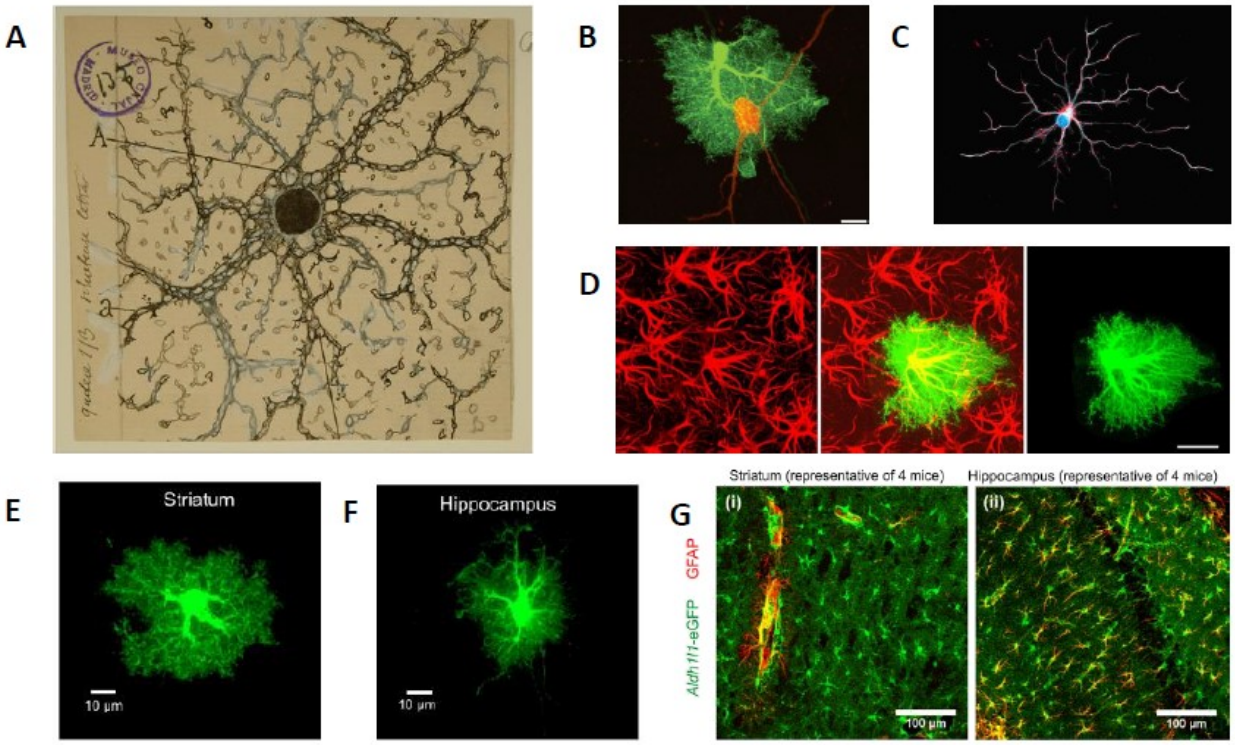

FIGURE 12 - ASTROCYTES IN THE BRAIN

A. Sketch representation of a brain protoplasmatic astrocyte drawn by Santiago Ramon and Cajal. **B.** Example of protoplasmatic astrocyte (green) in close associated with neuronal cell body and processes (red) **C.** Example of Fibrous astrocyte. **E-F.** Representative pictures of a protoplasmatic astrocyte from the mouse striatum and hippocampus, respectively. **G.** Individual astrocytes in both striatum and hippocampus occupy specific non-overlapping domains. Presence of GFAP-positive astrocytes is much lower in striatum as compared with hippocampus. On the other side, presence of ALDH1L1-positive astrocytes is equally distributed in both regions. [(A) Adapted from (Navarrete and Araque, 2014); (B) Adapted from (Allen and Barres, 2009); (C) Adapted from https://fhs.mcmaster.ca/fxar/astrocytes_gallery.html, last access 20/10/2017; (D) Adapted from (Pekny and Pekna, 2014); (E-G) Adapted from (Chai et al., 2017)]

II – The tripartite synapse and the neuroglial interactions

The non-electrically excitable nature of the astrocytes led to the incorrect idea that astrocytes did not participate in the active modulation of neuronal function (Bazargani and Attwell, 2016). However, a growing body of evidence has shown that astrocytes are responsive to neuronal signals and consequently release molecules (*i.e.* gliotransmitters) that can directly impact neuronal function thus linking astrocytic activity and synaptic function (Araque et al., 1999, Araque et al., 2014). This association, named the tripartite synapse, reviews the traditional model of a bi-component synapse (*i.e.* presynaptic and postsynaptic neuronal elements only) and includes the astrocyte as a third participant (Figure 13A, B) (Araque et al., 1999). This configuration allows the modulation of synaptic activity by astrocytes both indirectly, by clearing excess of neurotransmitters, remodeling of the extracellular space, provisioning metabolic intermediates, and directly, by releasing synaptic active molecules to modulate neuronal activity (Araque et al., 2014).

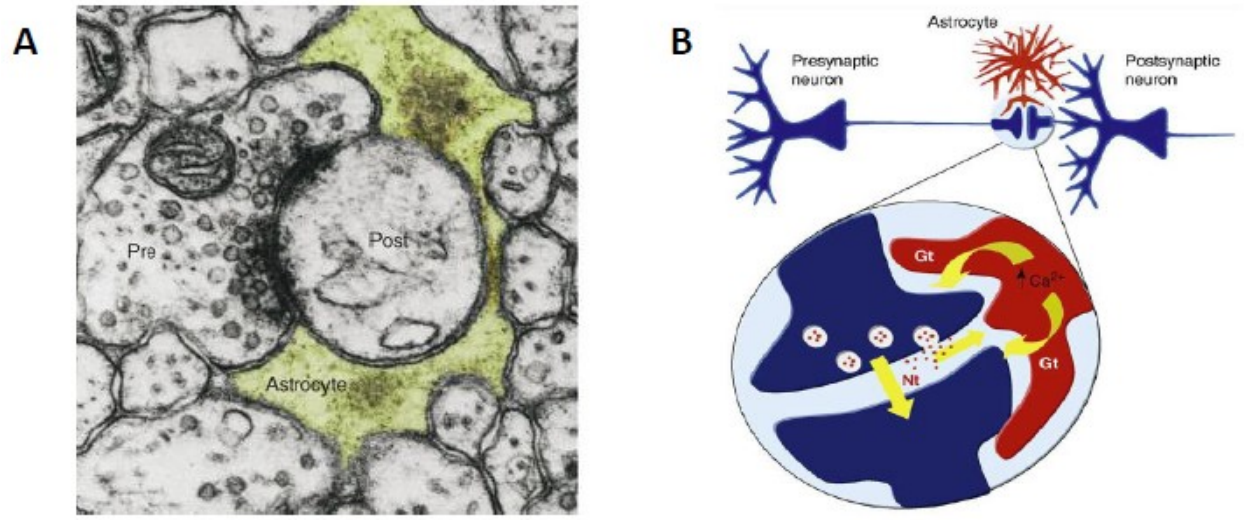

FIGURE 13 – THE TRIPARTITE SYNAPSE

A. Electron microscopy image showing an astrocyte process (yellow) involving the pre-(Pre) and the postsynaptic (Post) terminal. **B.** Schematic representation of the signalling mechanism in the tripartite synapse. Neurotransmitter release from the presynaptic terminal can act in the receptors or postsynaptic targets but also in the astrocytic process. This will lead to an astrocytic intracellular calcium increase, triggering gliotransmission that in turn will modulate neuronal transmission. [(A) Adapted from (Halassa et al., 2007a); (B) Adapted from (Perea et al., 2009)]

III – Network properties of astrocytes

Contrary to previous belief, brain astrocytes are not isolated cell types that only carry local supportive functions. Indeed, evidence indicates that astrocytes can sense neuronal activity locally to then modulate synapses both locally and globally (Fields et al., 2015). Astrocytes are connected with other astrocytes in a syncytium that allows the flow of molecules through cellular contacts at processes called gap junctions (Figure 14A) (Giaume et al., 2010). The junctions, formed of subunits of connexin, allow the flow of molecules that can range from gliotransmitters to metabolic intermediates, which are transferred from astrocyte to astrocyte depending on the needs of the network (Figure 14B) (Giaume et al., 2010). Interestingly, one single astrocyte can contact thousands of synapses in their non-overlapping domains (Bushong et al., 2002, Halassa et al., 2007b, Ogata and Kosaka, 2002).

One form of communication that is quite well established between astrocytes is maintained through calcium signaling (Bazargani and Attwell, 2016). Calcium rises in the astrocytes have been reported to produce local, regional and global changes in astrocytic networks that depending on the mechanism from which they are triggered (Figure 14C) (Araque et al., 2014). One example of this long-range modulation of astrocytic activity was reported in the cerebellum. In 2009 the group of Mark Schnitzer showed that Bergmann glial cells exhibited calcium activity during locomotor behavior that could recruit hundreds of Bergmann glial cells to an extension of at least several hundred microns (Nimmerjahn et al., 2009).

Calcium sources in the mitochondria can vary depending on the type of receptor that is activated during astrocytic activity. Currently, the main source of intracellular calcium is thought to be provided by the endoplasmic reticulum (Bazargani and Attwell, 2016). Another less known source of calcium in astrocytes are mitochondria (Agarwal et al., 2017). Although it was previously thought that fine astrocytic processes did not contain mitochondria (Khakh and Sofroniew, 2015), it has been recently found that not only fine astrocytic processes have functional mitochondria, but also that they can modulate synaptic activity via mitochondrial-dependent calcium release (Agarwal et al., 2017). It would be interesting to investigate whether similarly to neurons, astrocytic mitochondria have CB1 receptors and if they could control gliotransmission. Another interesting function regarding astrocytic calcium signaling is the regulation of extracellular calcium. It has been recently reported that astrocytes can modulate neuronal firing pattern activity by providing calcium to the extracellular space (Morquette et al., 2015). It is well known that NMDA receptors allow the influx of calcium into the postsynaptic terminal as to induce and modulate long-term changes in synaptic activity. However, whether astrocytes can control NMDA receptors not only by providing glutamate and other

gliotransmitters but also by increasing the availability of extracellular calcium levels remains to be addressed.

Intracellular calcium rises in brain astrocytes are partially mediated by GPCR activity (Araque et al., 2014) and have been observed *in vitro* (Araque and Navarrete, 2010, Gomez-Gonzalo et al., 2015) and *in vivo* in rodents models (Kuga et al., 2011, Nimmerjahn et al., 2009) and *in vitro* from human postmortem preparations (Navarrete et al., 2013). One of the main drivers of astrocytic calcium activity is glutamate acting on metabotropic glutamate receptors coupled to GPCR Gq (Figure 14D). This activation of GPCRs generates IP3, which by acting in the IP3 receptors in the endoplasmic reticulum, induces the increase of intracellular calcium in the astrocytes (Santello et al., 2012). Although there is controversy about the extent to which IP3 receptor-mediated gliotransmission modulates circuit activity (Agulhon et al., 2012), it is now well established that the fine processes can also exhibit IP3 receptor-independent calcium activity with functional relevance for synaptic function (Chai et al., 2017, Srinivasan et al., 2015, Srinivasan et al., 2016).

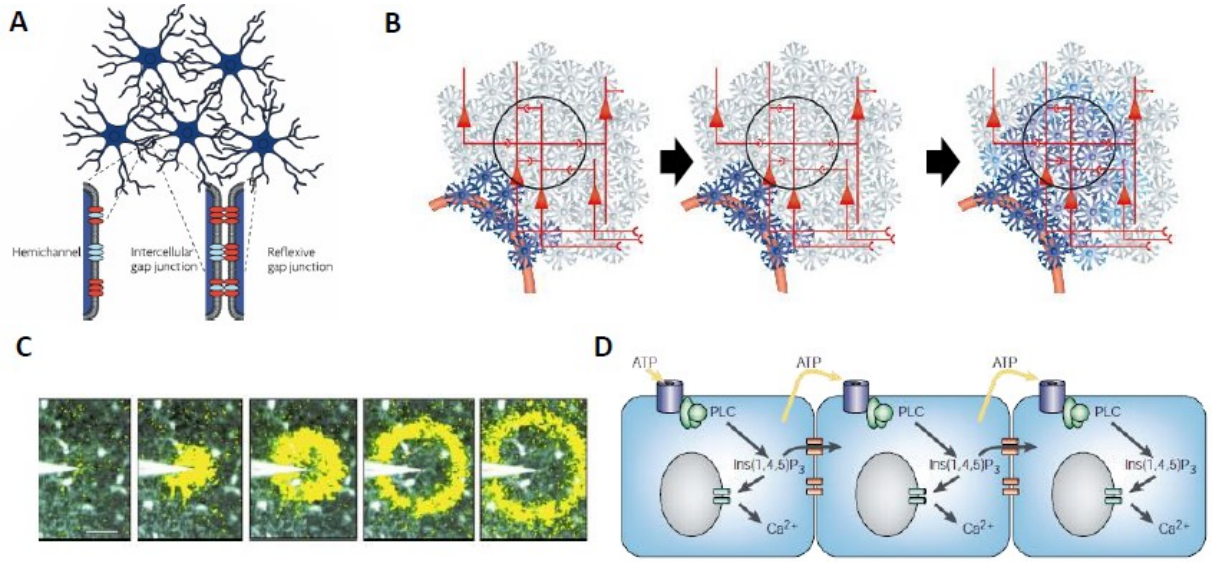

FIGURE 14 – NETWORK PROPERTIES OF THE ASTROCYTES

A. Astrocytes are connected among themselves by gap junctions allowing the flow of several molecules between wide ranges of astrocytic networks. **B.** The occurrence of high intensity neuronal activity (black circle) in certain parts of the astrocytic syncytium associated with the neuronal network will trigger the mobilization of molecules (*e.g.* metabolic substrates, gliotransmitters) from the astrocytes close to the blood vessels to the astrocytes in contact within the active zone. **C.** The mechanical stimulation of an individual astrocytes lead to the increase of calcium in the same astrocytes and progressively in the astrocytes closely associated with it. **D.** Schematic representation of the main intracellular mechanism responsible for the intracellular increases in astrocytes. [(A, B) Adapted from (Giaume et al., 2010); (C, D) Adapted from (Haydon, 2001)]

IV – Gliotransmission

The process of gliotransmission involves the release of neuroactive molecules from astrocytes which, by acting on neurons, can modulate either positively or negatively the transfer and processing of information (Araque et al., 2001). This concept of gliotransmission is a keystone of the tripartite synapse as it provides a feedback mechanism from astrocytes to neurons (and vice-versa) that modulates overall synaptic activity (Allen and Barres, 2009, Araque et al., 2014). Gliotransmitters are a wide category that include excitatory and inhibitory amino acids, ATP and related nucleotides, lipidic molecules (including endocannabinoids), neurotrophic factors and cytokines, among others (Santello et al., 2012).

Gliotransmitters can be released by multiple ways such as by ion channel (Woo et al., 2012), reverse reuptake transporters (Grewer et al., 2008) and by secretory vesicles in calcium-dependent and independent ways (Santello et al., 2012). Although functional and anatomical evidence of vesicle-dependent gliotransmission is present (Lee et al., 2014, Navarrete et al., 2013, Zorec et al., 2012), it remains controversial as several groups are unable to find appropriate machinery to support such a mechanism (Bazargani and Attwell, 2016). For instance, Chai and colleagues have found no evidence of glutamate release from astrocytes to astrocytes or to neurons (Chai et al., 2017). As lack of evidence is not evidence of a lack, more research into this field is needed to address these issues. Another interesting mechanism of gliotransmission recently described relies on the mitochondrial calcium buffering properties. By using 2-photon microscopy *in vitro* and *in vivo*, Agarwal and colleagues (2017) have shown that mitochondria in fine astrocytic processes were responsible for spontaneous calcium activity, this occurring in an IP-3 independent way (Agarwal et al., 2017). Even after inhibition of the main intracellular calcium sources (*i.e.* endoplasmic reticulum), there was still calcium activity that could be blocked by intracellular application of calcium chelators. The discovery that mitochondria are possible calcium sources in these conditions might explain how metabotropic receptors (also potentially CB1 receptors) can induce IP3-independent calcium changes (Agarwal et al., 2017, Srinivasan et al., 2015).

As previously described, one of the most determinant functions of the tripartite synapse is the bidirectional communication between neurons and astrocytes. The discovery of gliotransmission and calcium excitability helped to understand how astrocytes regulate synaptic activity. Astrocytes can modulate both short-term (Navarrete and Araque, 2008, Navarrete and Araque, 2010) and long-term synaptic plasticity (Henneberger et al., 2010) through gliotransmission (De Pitta et al., 2016). Hippocampal NMDA receptor-dependent LTP has been classically described in respect to an exclusive interaction between pre- and postsynaptic

activities (Nicoll, 2017). However, a landmark study demonstrated that astrocytes could control the induction of LTP by releasing, in a calcium-dependent manner, the major endogenous NMDA receptor co-agonist D-serine at hippocampal synapses (Henneberger et al., 2010). Another way astrocytes can influence the synaptic plasticity is through the availability of metabolic molecules that are shuttled from astrocytes to neurons. For instance, lactate has been shown to be released from astrocyte to neurons to support LTP and memory (Suzuki et al., 2011).

Overall, evidence from the past two decades pinpoints the importance of the release of active signalling molecules and metabolites by astrocytes to control synaptic function, expression of long-term synaptic plasticity and hence behavior (Oliveira et al., 2015). As gliotransmission is important for brain function, understanding the functional crosstalk between astrocytes and the ECS is necessary to understand how CB1 receptor activity modulates behavior.

V – Astrocytes, CB1 receptors and gliotransmission

Besides the characterization of CB1 receptors in the neuronal terminals, it has been shown in the past few years that CB1 receptors are also present in astrocytes (Metna-Laurent and Marsicano, 2015, Oliveira da Cruz et al., 2016, Scheller and Kirchhoff, 2016). As previously described, astrocytes play a broad role in regulating synaptic physiology and high brain functions, functions most often associated with endocannabinoid modulation.

The presence of CB1 receptors on astrocytes has been controversial due to the inability to visualize both the protein and its mRNA (Kano et al., 2009, Metna-Laurent and Marsicano, 2015, Stella, 2010). Nevertheless, functional and anatomical studies (*e.g.* CB1 receptor immunolabeling coupled with electron microscopy) have evidenced CB1 receptors on astrocytes in the hippocampus (Han et al., 2012, Navarrete and Araque, 2008), the hypothalamus (Bosier et al., 2013), the striatum (Martin et al., 2015, Rodríguez et al., 2001), the neocortex (Min and Nevian, 2012), the amygdala (Moldrich and Wenger, 2000) and the spinal cord (Salio et al., 2002). Further evidence reinforcing the crosstalk between CB1 receptors and astrocytes comes from evidence suggesting that astrocytes can participate in the full metabolism of the main endocannabinoids: 2-AG and Anandamide (Metna-Laurent and Marsicano, 2015). For instance, the use of KO mouse models lacking CB1 receptors in astrocytes and specific neuronal populations has indicated that astroglial CB1 receptors are involved in the turnover of endocannabinoids in the brain (Belluomo et al., 2015). Another interesting aspect is the intracellular signaling machinery used by astroglial CB1 receptors (Metna-Laurent and Marsicano, 2015). In neurons, as previously discussed, presynaptic CB1 receptor activation recruits mainly Gi/o proteins that negatively modulate cAMP levels and inhibit neuronal transmission (Piomelli, 2003). However, it has been reported that CB1 receptor activation in astrocytes recruits Gq proteins instead of Gi/o that mediate an intracellular calcium increase via IP3-receptor modulation (Navarrete and Araque, 2008).

V.A – MODULATION OF SYNAPTIC PLASTICITY BY CB1 RECEPTORS IN ASTROCYTES

The close association between fine astrocytic processes and neuronal synapses and the evidence supporting the tripartite synapse, confer to the astroglial CB1 receptors a potential key role in the regulation of synaptic activity (Oliveira da Cruz et al., 2016).

The first evidence for the astroglial CB1 receptor modulation of synaptic activity comes from a functional study suggesting that a CB1 receptor-dependent mechanism in astrocytes, rather than in neurons, was responsible for the modulation of specific forms of synaptic transmission in the hippocampus (Navarrete and Araque, 2008). In this study, Navarre and Araque (2008) reported that the activity-dependent postsynaptic release of endocannabinoids by pyramidal neurons could induce a calcium increase in astrocytes that was mediated by a CB1 receptor-dependent mechanism (Navarrete and Araque, 2008). As a consequence, astrocytes release the gliotransmitter glutamate that, by stimulating postsynaptic NMDA receptors, modulates slow inward currents in neurons (Navarrete and Araque, 2008). As this effect was dependent on PLC inhibition, it suggested that astrocytes act via a Gq-dependent mechanism instead of classical pertussis toxin-sensitive Gi/o proteins (Navarrete and Araque, 2008). In another study from the same group, the authors reported that astrocytes could also modulate synaptic activity both at the homosynaptic and heterosynaptic level (Navarrete and Araque, 2010). To reach this conclusion, the authors explored the anatomical feature of the independent, non-overlapped, astrocytic domains (Bushong et al., 2002). Using an elegant approach, they observed that activity-dependent endocannabinoid production, presumably by the postsynaptic neuron, besides inducing a classical retrograde DSE, could also induce a heterosynaptic short-term facilitation of synaptic transmission via astrocytic calcium activity (Navarrete and Araque, 2010). The authors showed that endocannabinoids by acting through a CB1 receptors in astrocytes elicit a somatic calcium increase that potentiates the release of the putative gliotransmitter glutamate. Then, by acting on the presynaptic mGlu1 receptors, glutamate induces a short-term facilitation of synaptic transmission (Navarrete and Araque, 2010). In another study from the same group, the authors demonstrated that in addition to the lateral heterosynaptic facilitation, astroglial CB1 receptor activation could also induce a LTP in single neurons (Gomez-Gonzalo et al., 2014).

The modulation of synaptic plasticity by endo- and exogenous cannabinoids has been widely described (Hoffman and Lupica, 2013, Stella et al., 1997). Yet, in most of the cases, it is not currently known which specific neuronal or glial population of CB1 receptor-positive cells mediates such effect. The modulation of CB1 receptor-dependent synaptic plasticity by exogenous agonists such as THC is thought to underlie the behavioral effects of cannabinoids (Castillo et al., 2012). In 2012, Han and colleagues, by using a novel genetic mouse model lacking

CB1 receptors in astrocytes, reported that this receptor is responsible for a cannabinoid(THC)-induced LTD (CB-LTD) in the hippocampus (Han et al., 2012). The authors demonstrated that following THC administration, CB1 receptor activation in astrocytes induced the putative release of glutamate which, by acting on postsynaptic NMDA receptors, induced a CB-mediated LTD via the internalization of AMPA receptors (Han et al., 2012). Furthermore by using electron microscopy, the authors were able to demonstrate anatomically for the first time that astrocytes in the hippocampus expressed functional CB1receptors (Han et al., 2012).

Another example of astroglial CB1 receptor modulation of synaptic plasticity was described in the somatosensory neocortex. In this region, astroglial CB1 receptor has been shown to modulate spike-timing-dependent LTD (t-LTD) (Min and Nevian, 2012). In this study, the authors report that t-LTD in the excitatory synapses from the L4 to L2/3 synapses, depends on astroglial CB1 receptor activation. The intracellular calcium increase induced by astroglial CB1 receptor activation leads to the release of glutamate which, by acting on neuronal NMDA receptors, induces a t-LTD (Min and Nevian, 2012).

Another interesting example of astroglial CB1 receptor modulation of synaptic function was described in the striatum. This region, composed of the caudate and the putamen, is involved in several important functions like critical motivation, adaptive motor control and procedural learning (Kreitzer and Malenka, 2008). By receiving direct excitatory afferent inputs from the cortex and thalamus, these regions modulate motor control via two distinct pathways: the direct and indirect pathways. The striatum is composed mostly of GABAergic medium spiny neurons (MSNs) which can be divided in two main classes: the striatonigral MSNs that have high expression of dopamine D1 receptors and project directly to the basal ganglia, internal globus pallidus and substantia nigra pars raticulata and the striatopallidal MSNs which have high expression of dopamine D2 receptors and project to the external globus pallidus (Kreitzer and Malenka, 2008). Although functionally and molecularly different, MSNs occupy the same entangled and highly packed space, suggesting that astrocytes might be responsible for how specific circuits are modulated in such as restricted space. In 2015, Martin and colleagues demonstrated that distinct astrocytes functionally modulate specifically homotypic (D1-D1 or D2-D2 MSNs) but not heterotypic MSNs (D1-D2 MSNs) (Martin et al., 2015). Furthermore, astroglial CB1 receptor modulation of intracellular astrocytic calcium was necessary for glutamate release to the synapse. Glutamate then led to the activation of NMDA receptors in the same, but not opposite, class of MSN (Martin et al., 2015). The suggestion that anatomically different astrocytic domains allow different functions raises the question of the means to identify these specific populations and whether similar populations can be found in other brain regions such as the cortex and the hippocampus.

PART 4 – MEMORY

Memory is often defined as the ability to retain and recall on demand previously encountered experiences based on processes of learning, retention and retrieval (Squire et al., 2007). Learning and memory provide one of the most important biological functions that allow the survival and adaptation of organisms.

Memory can be divided into main broad categories: the declarative and non-declarative (often referred as procedural) memories (Cohen and Squire, 1980). Declarative memory has been defined as the capacity to remember past experiences (Squire and Zola, 1996). Non-declarative memory, which can be divided into motor, perceptual and cognitive memory is acquired by trial and error experiences (Squire and Zola, 1996). For instance, the learning of procedural tasks that requires repetition to increase experience, such as the learning to play an instrument or playing darts.

Declarative memory can be divided into two main sub-categories: semantic memory and episodic memory (Tulving, 1972). Whereas semantic memory refers to general knowledge that an individual acquired during his life (*e.g.* history, science, geography), the episodic memory refers to the capacity to learn and recollect memories that are self-generated (*e.g.* places we visit, specific events that happened) (Tulving, 1972).

Memory formation is a complex task that involves many neuronal circuits within several brain regions. Due to its complexity, untangling specific contributions to the several components of individual memories requires the examination of the role of each of these brain regions in relation with specific components of the task studied. The hippocampus is a key brain region that has been shown to be important for episodic memory, spatial navigation, time perception (Howard and Eichenbaum, 2015, Squire et al., 2007). Together with its adjacent cortical regions (*i.e.* entorhinal, perirhinal and parahippocampal cortices), it constitutes the medial temporal lobe, which is involved in the formation of declarative memory (Eichenbaum, 2017).

Episodic memory formation has been described as a series of events that result in the stabilization of a previously acquired experience (Tulving, 1972). At the beginning of the process, there is an event leading to the acquisition of the information and its encoding within specific brain circuits. In order to maintain this memory for the long-term, a consolidation phase takes place in which the experiences are stabilized and the memory is stored from days to years (Dudai, 2012). In the past decades another phase called reconsolidation has been proposed, in which the retrieval of a previously stored memory renders it unstable and prone for a process

called reconsolidation (Dudai, 2012). This process of reconsolidation will disrupt the original memory with small details producing a memory that is not the same as the one originally generated. For instance, if we learn a story and we re-tell the same story over the years, at some point we will add and/or exclude details. The story will end up to being modified, even if we do not intend to do so and we are unaware of the changes. Because different types of memories can be stored in independent brain regions, I will consider only hippocampal dependent episodic memories as their study falls in the scope of this thesis.

I – Plasticity and memory: cellular and molecular mechanisms of memory formation

How memories are stored, preserved and retrieved from the brain is a complex question that fascinates scientists. One of the most interesting theories behind the formation of memory was introduced by Richard Semon: the theory of memory traces (Semon, 1921). Semon introduced the concept of an engram which is a group of neurons that becomes active during the acquisition of a new memory. The connectivity between these cells is preserved most likely by the means of synaptic plasticity, thereby allowing the memory to be physically stored in specific circuits (Dudai, 2012). The hypothesis of synaptic plasticity being the key cellular and molecular mechanism underlying memory formation came from the Canadian psychologist Donald Hebb (Hebb, 1949). In a landmark work, Hebb proposed that when two cells are connected, upon the activation of the first cell (*e.g.* presynaptic terminal) the second gets activated (postsynaptic terminals), and the connection between both will be strengthened (Hebb, 1949). However it took 20 years to demonstrate the existence of this possible "Hebbian plasticity". The discovery of LTP (Bliss and Lomo, 1973) and the further identification of its molecular pathways, lead to demonstration how connections between neurons can be reinforced or weakened for days or years (Bliss and Collingridge, 1993). Interestingly, NMDA receptor dependent-LTP is biological phenomenon that confirms the hypothesis of Hebb. Thus, in glutamatergic synapses neurotransmitter release from the presynaptic terminal, bind to the postsynaptic AMPA receptors that will act as coincident detectors and allow the activation of NMDA receptors (Collingridge et al., 1983). The latter produces an LTP or LTD of synaptic transmission that strengthens or weakens the connection, respectively (Nicoll, 2017). It has been widely described that LTP can lead to spine growth. This postulate has not only been observed under artificial stimulation conditions but also *in vivo* during certain behaviors. For instance, learning can control synapse formation and elimination (Yang et al., 2009). Indeed, spine dynamics can correlate the improvement after learning further demonstrating that events can be stored in synapses (Yang et al., 2009). On the other hand, according to the synaptic theory of memory storage, 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). As for the cell assemblies, the way they interact and they are established is currently a hot topic in neuroscience. The development of joint sophisticated genetic tagging to modulate active neurons during specific tasks, optogenetics (Govindarajan et al., 2006, Kim et al., 2017) and chemogenetics (Armbruster et al., 2007, Gomez et al., 2017) allowed the demonstration 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).

The biology of memory formation is far from being understood. There are several levels of complexity from the molecular, cellular and systems that ultimately impact on the behavioral expression of memory. I will focus in the further sections on how to study episodic memory in rodent models and how CB1 receptors impact the formation of this type of memory.

II – Novel object recognition memory

The cellular and molecular basis of episodic memory can be studied using specific behavioral paradigms in animal models that allow the manipulation of specific components of memory formation, storage and retrieval.

One of the most well-studied forms of episodic memory is recognition memory which can be investigated in humans, monkeys and rodents (Squire et al., 2007). Novel object recognition memory is an experimental strategy aimed at studying learning and memory functions (Ennaceur, 2010). The main postulate behind this test is that, in the presence of novel and familiar object, rodents increase their exploration towards the novel one (Ennaceur and Delacour, 1988). The increased exploration of the novel object is interpreted as indirect evidence that animals acquired a memory of the familiar object. It is important to verify that mice, when presented with two different objects for the first time, do not show an intrinsic preference for any of them. One variation of novel object recognition task (NORT) is performed in an L-maze (Figure 15). This test has been extensively used to access short- and long-term dependent memory performance in rodents. Several components of novelty versus familiarity preference are encoded in regions such as the hippocampus (de Lima et al., 2006, Puighermanal et al., 2009, Puighermanal et al., 2013), prefrontal cortex (Banks et al., 2012) or hippocampus adjacent cortices (*e.g.* perirhinal cortex) (Albasser et al., 2010, Wan et al., 1999).

Several studies reported that the hippocampus is involved in the object recognition memory while other studies reported that hippocampal manipulations (*e.g.* lesions) do not impair this type of memory. It is important to note that several properties of the task might explain such results. For instance, the shape of the maze (*e.g.* an open maze has anxiogenic properties), the duration of the exposure or the animal model. Nevertheless, current evidence suggests that depending on the experimental design proposed, NORT might recruit or not the hippocampal region (Brown et al., 2010, Brown and Aggleton, 2001). In addition, novelty as assessed by exploration might also include several other factors that can contribute to the overall expression of memory. For instance, attention and motivation to explore are important for novel detection and exploration (Ennaceur, 2010). Another possible explanation is that the hippocampal contribution might occur in distinct temporal domains, meaning that manipulations performed outside this window (*e.g.* immediately after acquisition vs 4 hours post acquisition) might wrongly suggest the of lack of necessity of the hippocampus (de Lima et al., 2006).

For the aim of this thesis, we used a NORT version in an L-maze 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 (Busquets-Garcia et al., 2011, Hebert-Chatelain et al., 2016, Puighermanal et al., 2009). First, as the acquisition in NORT happens in a single session, it allows to study the different phases of memory formation. Pre- or post-training pharmacological treatments (or even pharmacogenetic manipulations such as Designer Receptor Exclusively Activated by Designer Drugs (DREADD)s (Urban and Roth, 2015)) allow acute manipulation of the acquisition and consolidation phase, respectively. Furthermore, pharmacological manipulations during the pre-test session, allow the study of retrieval. Overall, NORT provides high reproducibility and low variability in the study of episodic memory formation.

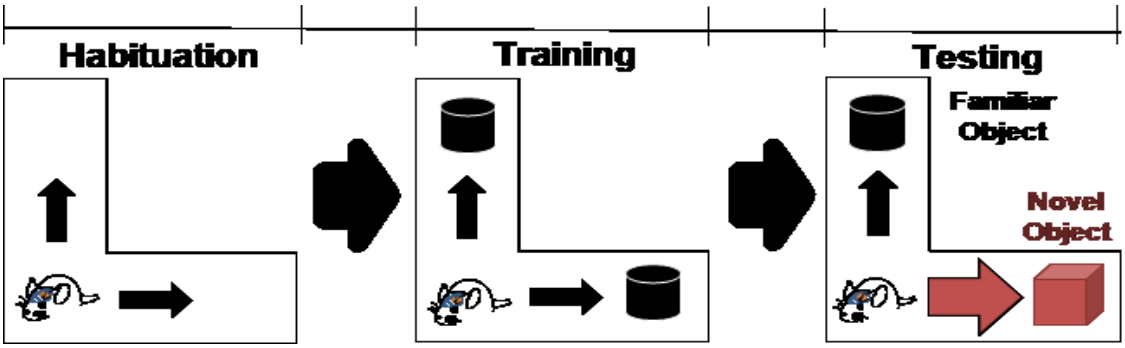

FIGURE 15 – NOVEL OBJECT RECOGNITION MEMORY TASK

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. For long-term memory evaluation, test phase occurs 24 hours later (day 3, testing).

III – Memory and CB1 Receptors

The presence of CB1 receptors in brain regions that modulate memory functions and their role in the modulation of short- and long-term forms of synaptic plasticity, provides a solid framework supporting the role of the ECS in the modulation of learning and memory (Soria-Gomez et al., 2017).

Cannabinoid intoxication has been shown to induce learning and memory impairments in both rodents and humans (Broyd et al., 2016). Also, it leads to deficits in attention, poorer cognitive performances, impairment in working memory and impairment in long-term memory formation (Broyd et al., 2016). Interestingly, the majority of these effects have been shown to be mediated by direct activation of CB1 receptors in the CNS. Among several brain regions modulating memory, the hippocampus plays an important role and shows high levels of the CB1 receptor protein (Marsicano and Kuner, 2008, Soria-Gomez et al., 2017). By combining genetic, targeted pharmacological and electrophysiological approaches, successive studies have shown that CB1 receptor modulation in specific cell populations within the hippocampus can modulate synaptic plasticity and consequently learning and memory (Busquets Garcia et al., 2016).

Cannabinoids and endocannabinoids, via CB1 receptors, can modulate both short-term (Busquets-Garcia et al., 2017b, Han et al., 2012) and long-term memory formation (Puighermanal et al., 2009). For instance, local hippocampal treatment with CB1 receptor agonists has been shown to impair hippocampal memory whereas CB1 receptor antagonists can block those effects (Barna et al., 2007, Wise et al., 2009). Although in the past decades studies have demonstrated that CB1 receptors mediate the memory-disruptive effects of cannabinoids, only recently, with the generation of conditional KO models lacking CB1 receptors in specific brain cells, did we began to understand how specific neuronal/glial populations are involved in these effects. It has been demonstrated that cannabinoids can disrupt long-term episodic memory, as assessed by NORT (Puighermanal et al., 2009). This effect is dependent on CB1 receptors expressed in GABAergic but not in glutamatergic neurons (Puighermanal et al., 2009) suggesting that it could be due to the THC-induced stimulation of CB1 receptors in hippocampal GABAergic terminals (Laaris et al., 2010). Interestingly, the authors of this study were able to rescue the memory impairment by local application of an NMDA receptors antagonist, suggesting that excess of excitation, most likely due to suppression of GABAergic inhibition, impaired the neuronal network (Puighermanal et al., 2009). Besides long-term memory, acute THC administration has been reported to impair short-term working memory in mice (Han et al., 2012). This impairment could be reversed by application of an NMDA receptor antagonist, suggesting that also in the short-term level, glutamate excess can be responsible for memory

impairments (Han et al., 2012). An indirect finding from Puighermanal and colleagues (2009) showed that genetic ablation of CB1 receptors in glutamatergic or GABAergic cells did not impair NORT, suggesting that these cells do not contribute to object recognition memory (Puighermanal et al., 2009). Similarly, in the study from Han and colleagues (2012) genetic deletion of CB1 receptors in glutamatergic and GABAergic neurons did not impair working memory, suggesting that they are also not required for this kind of memory (Han et al., 2012).

The modulation of memory function by CB1 receptors is often correlated with impairments in synaptic transmission and plasticity. Consistently, CB1 receptor activation by exogenous cannabinoids has been shown to modulate long-term forms of synaptic plasticity. It has been shown that acute application of the endocannabinoid 2-AG in brain slices impairs the induction of LTP *in vitro* in hippocampal circuits (Stella et al., 1997). Later on, it has also been shown that WIN 44212-2, a potent CB1 receptor agonist, can block the induction of LTP in a dose-dependent manner (Paton et al., 1998). Chronic THC administration (10 mg/kg) as well as of other synthetic cannabinoids have been shown to impair the induction of LTP in the hippocampus, a time-dependent effect relying on CB1 receptors (Hoffman et al., 2007, Fan et al., 2010). Chronic treatments for 3 and 7 days reduce or blunt LTP, respectively (Hoffman et al., 2007). It has been shown that THC withdrawal can rescue the impairment of LTP but not to vehicle levels, suggesting some kind of synaptic memory preventing the full recovery of the synapses (Hoffman et al., 2007). Furthermore, chronic treatments with cannabinoids down regulated NDMA receptor activity and content in hippocampal synapses (Fan et al., 2010). This observation supports the aforementioned lack of impaired capacity to induce LTP. In another recent study, repeated THC treatments impaired the induction of LTP in the same hippocampal circuits (Chen et al., 2013). The authors described a novel mechanism in which THC induced impairments in memory and synaptic plasticity by the induction of cyclooxygenase-2 (COX-2), doing so through a CB1 receptor-mediated mechanism. This modulation could in turn lead to the conversion of arachidonic acid to prostanoids (Chen et al., 2013). On the other hand, THC administration *in vivo* can induce an LTD via the activation of NMDA receptors and the internalization of AMPA receptors (Han et al., 2012). The modulation of neuronal networks by CB1 receptor agonists might not exclusively depend on their suppressive action on neurotransmission. The recently identified presence of CB1 receptors in the inner mitochondrial membranes (mtCB1) could play a role in the regulation of neuronal metabolism (Benard et al., 2012). Moreover, DSI which was previously attributed to presynaptic membrane CB1 receptors has been shown to be partially dependent on mtCB1 receptor activation (Benard et al., 2012). Interestingly, it has been recently shown that hippocampal mtCB1 is responsible for cannabinoid-induced long-term memory impairments, doing so by changing the mitochondrial capacity to modulate energy and metabolism (Hebert-Chatelain et al., 2016). MtCB1 receptors seem not to be required for normal long-term memory formation as the absence of mtCB1

receptors did not impair memory functions (Hebert-Chatelain et al., 2016). Still, it remains to be addressed what is the endogenous role of mtCB1 receptors in memory functions.

Although the contribution of specific CB1 receptor populations is yet to be properly understood, how the modulation of inhibitory and excitatory drive modulates memory remains an important line of research. Indeed, temporal coordination between excitatory and inhibitory neurons in the hippocampus is responsible for proper network activity that supports memory formation (Buzsaki, 2006). Natural and synthetic CB1 receptor agonists have been shown to decrease hippocampal local field potential oscillations (Robbe et al., 2006). They disrupt the temporal neuronal synchrony that is responsible for the modulation of memory functions suggesting that CB1 receptors in those cell types can modulate their function in the network (Robbe et al., 2006, Robbe and Buzsaki, 2009). Consistent with this idea, by using conditional genetic deletion of CB1 receptors in GABAergic and/or glutamatergic neurons, Monory and colleagues (2015) proposed that CB1 receptors can calibrate excitatory synaptic balance in hippocampal circuits (Monory et al., 2015). By studying LTP, the authors showed that the absence of CB1 receptors in GABAergic neurons decreased the amount of LTP induced whereas its lack in glutamatergic neurons induced a stronger potentiation (Monory et al., 2015). Furthermore, they showed that dendritic harbors of pyramidal neurons were consistently larger in mice lacking CB1 receptors in glutamatergic neurons as compared to mice lacking them in GABAergic neurons. However, it is important to note that this difference did not translate into better or worse memory performances in long-term (Puighermanal et al., 2009) or short-term memory (Han et al., 2012).

Overall, it is important to further underline that exogenous cannabinoid activation within a circuit might not be the same as the one endogenously activates. It is known that different agonists can impact differently CB1 receptor function and that CB1 receptors in specific populations can modulate several aspects of brain physiology. Thus, dissecting the endogenous function *per se* remains a very important aspect of CB1 receptor research which will provide in the future important insights on how CB1 receptor function or dysfunction is involved in the pathophysiology of brain function.

IV – Memory and astrocytes

The astrocytic modulation of neuronal circuits has been shown to play a role in learning and memory, sensory processing, locomotor activity and emotional processing (Adamsky and Goshen, 2017, Araque et al., 2014, Oliveira et al., 2015). There are an increasing number of studies that link the activity at the tripartite synapse with behavior (Oliveira et al., 2015). In the following paragraphs I will describe some key studies that address the significance of this relation and which establish possible links with the endocannabinoid system.

The modulation of learning and memory by astrocytes can be promoted at several levels, such as, by the availability of metabolic support or by direct impact through gliotransmission (Oliveira et al., 2015). For instance, astrocytes can modulate brain activity by metabolically supporting neuronal activity by increasing extracellular lactate levels during learning and memory (Steinman et al., 2016). Lactate is shuttled to neurons where it allows the formation of long-term memory and synaptic plasticity (Newman et al., 2011, Suzuki et al., 2011). A further analysis concluded that lactate synthetized from astrocytic glycogen and specifically transported to neurons, is able to modulate intracellular signaling cascades that lead to gene expression, LTP and memory formation (Suzuki et al., 2011, Yang et al., 2014). To confirm the role of astrocytes in the this gradient of lactate availability to neurons, Machler and colleagues (2016) have shown through 2-photon *in vivo* imaging, that astrocytes preferentially accumulate lactate, that is then shuttled to neurons during periods of necessity (Mächler et al., 2016). Although these effects point to a metabolic role in support of synaptic activity, it has been argued that L-Lactate can also act as an activity-dependent signaling molecule released via gliotransmission and acting directly on intracellular targets (Mosienko et al., 2015).

Sleep is believed to be important for learning and memory (Tononi and Cirelli, 2014). Thus, sleep deprivation can lead to cognitive impairments such as decreased attention and working memory, impaired long-term memory and decision-making (Alhola and Polo-Kantola, 2007). Interestingly, astrocytes have been shown to modulate sleep homeostasis by controlling adenosine availably at the synapse (Florian et al., 2011, Halassa et al., 2009). Furthermore, LTP and memory impairments induced by sleep deprivation could be reversed by decreasing adenosine receptor activity and vesicular gliotransmission (Florian et al., 2011).

Astrocyte-to-astrocyte interconnectivity is crucial for proper network activity (previously discussed in Part 3 – III). By impairing gap junction connectivity through genetically deleting Connexin 30 and 45 in astrocytes, the group of Huston has reported that it could modulate mouse exploratory activity, anxiety levels, efficiency of signaling molecules (*e.g.* dopamine) and memory functions (Dere et al., 2003, Frisch et al., 2003). Consistently, the decreased calcium

activity in astrocytic hippocampal networks impaired spatial memory and contextual fear memory (Tanaka et al., 2013). These results demonstrate that dynamic changes in astrocyte communication are crucial for normal functional of neuronal networks that underlie behavior.

Recognition memory has also been shown to be modulated by astrocytic activity. Lee and colleagues (2014) report that the inhibition of vesicular gliotransmission from astrocytes impaired the generation of gamma oscillations in the cortex and novel object recognition memory (Lee et al., 2014). Surprisingly other studies have reported that the ablation of putative vesicular release does not impair synaptic function such as LTP (Agulhon et al., 2010, Agulhon et al., 2012). However it has been shown that LTP is dependent on astrocytic calcium-dependent release of D-serine (Henneberger et al., 2010), a mechanism thought to be mediated by vesicular release (Martineau et al., 2013, Mothet et al., 2005). Could this suggest the existence of other main mechanisms to support calcium-dependent exocytosis of signaling molecules? As previously mentioned, D-serine is the main agonist of NMDA receptors in different brain regions and its function has been linked to several behavioral phenotypes (Henneberger et al., 2012, Oliveira et al., 2015). Consistently, it has been shown that modulation of D-serine levels in the brain can modulate behavior: *e.g.* increased brain levels of D-Serine can improve several mood disorders (Otte et al., 2013). In another recent study, it has been shown that D-serine levels can fluctuate during wakeful states in freely moving non-anesthetized mice (Papouin et al., 2017a). Furthermore, D-serine dynamics were shown to be dependent on astrocytic activity driven by the activation of acetylcholine and they can modify NMDA receptor activity (Papouin et al., 2017a). Their data suggest that D-Serine is an important regulator of brain function with a potential role in many pathophysiological conditions.

IV.A – Modulation of memory by astroglial CB1 Receptors

Mainly due to their low protein levels, the first evidence for the presence of CB1 receptors protein in astrocytes came from functional studies (Metna-Laurent and Marsicano, 2015). With the generation of conditional mutagenesis, it has become possible inquire about the role of CB1 receptors in specific neuronal and glial cell types underlying complex behaviors (Busquets-Garcia et al., 2015, Marsicano and Kuner, 2008). With the current knowledge that astrocytes can express CB1 receptors (Han et al., 2012), questions arise regarding their role in astrocyte-mediated brain functions (Metna-Laurent and Marsicano, 2015).

Exogenous cannabinoids and endocannabinoids can modulate memory by influencing the activity of several cell types in the brain (Busquets Garcia et al., 2016). Morris water maze is a robust behavioral paradigm that allows the study of hippocampal spatial memory in rodents (Morris et al., 1986). One of the best-known effects of cannabinoid intoxication is the CB1-dependent impairment of spatial working memory (Carlini, 2004). However, the location of CB1 receptors and the identity of the specific cell type(s) involved in this effect were not disclosed until recently. By using conditional deletion of CB1 receptors in GFAP-positive cells (*i.e.* mainly astrocytes, GFAP-CB1-KO mice), Han and colleagues (2012) demonstrated that astrocyte CB1 receptors are responsible for a cannabinoid-induced impairment of short-term working memory (Han et al., 2012). Furthermore, CB1 receptor deletion in both glutamatergic and GABAergic cells did not prevent the impairment of working memory caused by THC. Interestingly, Han and colleagues (2012) showed that GFAP-CB1-KO mice (although unresponsive to the effect of cannabinoids) in working memory did not have any endogenous phenotype regarding working memory, suggesting that CB1-dependent control of physiological astrocytic gliotransmission is not required for this type of memory. However, astrocytes have been reported to be involved in the modulation of long-term synaptic plasticity (Araque et al., 2014) and long-term memory formation (Oliveira et al., 2015).

Given that CB1 receptors modulate long-term memory and synaptic plasticity (Busquets Garcia et al., 2016), their presence in astrocytes suggests that they might participate in these functions. However, the physiological roles of astroglial CB1 receptors in synaptic plasticity and memory processes are mostly unknown, and thus they are one of the main subjects of my doctoral thesis.

PART 5 – DOPAMINERGIC SYSTEM AND THE ECS

The endogenous cannabinoid system is present in cell types that belong to important modulatory systems in the brain. One interesting case of this interaction regards the dopaminergic system, with evidence showing that CB1 receptors are present in neurons that express Dopaminergic type-1 (D1) receptors in several brain regions (Monory et al., 2007).

In the next section, I will introduce the dopaminergic system and how D1 receptors can functionally interact with CB1 receptors. Then I will mention how dopamine and endocannabinoid system can modulate memory formation and how plausible an interaction between both systems is.

I – The dopaminergic system

The dopaminergic system (DS) is a major modulatory system involved in many important brain functions such as the control of movement, emotion, reward, seeking behavior, motivation and learning and memory (Schetz and Sibley, 2007). The DS comprises neurons able to produce and release dopamine, enzymatic machinery responsible for the metabolism of dopamine, and cells expressing its target GPCRs of the D1-like (D1A-D and D5) and D2-like (D3, D4, and D5) family. The DS modulates neuronal function by stimulation or inhibition of adenylate cyclase, respectively (Schetz and Sibley, 2007). D1-receptor and D2-receptor family shows higher expression in the striatal structures (*i.e.* caudate nucleus, putamen and nucleus accumbens) and in the prefrontal cortex (Arias-Carrion and Poppel, 2007). The presence of several dopaminergic receptors in the brain, as well as their expression in different neuronal populations intermingled in packed space (*e.g.* striatal MSNs), makes the study of the dopaminergic system a complex challenge (Romanelli et al., 2010). Another level of complexity is the interaction of the dopaminergic system with other modulatory systems, including the ECS. Interestingly, DS-associated functions and distribution are often overlapped by those of the ECS, suggesting that in several domains, there might be a functional crosstalk that modulates synaptic plasticity and behavior.

From the several receptors that are currently known, I will focus on the D1 receptors because, together with the ECS, they have been shown to have an important role in the modulation of learning and memory, and part of the experiments present in this thesis were based on the deletion of the CB1 receptor in D1-positive cells.

II – Dopamine type-1 (D1) receptors

D1 receptors are heptahelical transmembrane spanning G protein-coupled receptors. They belong to the D1-like family that mainly bind to Gs proteins (Romanelli et al., 2010). They are the most abundant D1-like receptors and besides being mainly present in the striatum, D1 receptors can be also found in other brain regions such as the striatum, the cortex, the olfactory tubercle, the basolateral amygdala, the hypothalamus and the hippocampus (Bergson et al., 1995, Fremeau et al., 1991, Romanelli et al., 2010) .

Activation of D1 receptors by dopamine will initiates a Gs protein-dependent intracellular signaling cascade (Zhou et al., 1990). This leads to the production of cAMP via activation of adenylate cyclase, with consequent activation of PKA (Romanelli et al., 2010). PKA activation induces cAMP responsive element binding protein (CREB)-dependent gene transcription, activation of voltage- and ligand-gated ion channels that than promote neuronal excitability (Romanelli et al., 2010). D1 receptors can also modulate long-term effects at cellular level by the modulation of MAPK activity. Among the different pathways that can be activated by D1 receptors, the ERK pathway, which is known to be involved in neuronal plasticity and memory, is one of the most important (Gangarossa and Valjent, 2012).

At the cellular level, D1 receptors can be found in both glutamatergic and GABAergic neurons and astrocytes (Miyazaki et al., 2004, Nagatomo et al., 2017). D1 receptors have been characterized in neuronal cell bodies, in dendritic spines, in axon terminals (Bergson et al., 1995, Mansour et al., 1991) and also in fine astrocytic processes (Nagatomo et al., 2017). D1 receptors in the prefrontal cortex have been identified both at distal dendrites and spines of the glutamatergic pyramidal cells (Goldman-Rakic et al., 2000). D1 receptors are developmentally regulated and show higher densities during adolescence when compared with adulthood (Puig et al., 2014).

In the hippocampus, D1-positive cells have been described in the subiculum, in the granule cells of the dentate gyrus (Fremeau et al., 1991), in glutamatergic pyramidal cells (Bergson et al., 1995) and very recently in the GABAergic interneurons in the stratum radiatum area (Puighermanal et al., 2017). Until recently, there was no evidence regarding D1 presence in this sub region of the hippocampus (Gangarossa et al., 2012, Puighermanal et al., 2017). This illustrates how difficult it is to detect low amounts of D1-positive cells in certain brain regions. This is especially relevant for the fine astrocytic processes. Recently, it has been described that hippocampal astrocytes can increase intracellular calcium in response to dopamine depending on D1/D2 receptors (Jennings et al., 2017). This raises the question on whether there are functional D1/D2 receptors in the hippocampal astrocytes or if there is a secondary mechanism, dependent of the activation of these receptors elsewhere.

D1 receptors can also be present in intracellular compartments (*e.g.* endosomes) that are transported to the plasma membrane both constitutively and in an activity-dependent mechanism (Brismar et al., 1998). Interestingly, it has been reported that in striatal neurons, D1 and NMDA receptors can be assembled intracellularly in heteromers ready to be delivered to the synapses (Fiorentini et al., 2003). However the functional relevance of such interaction remains poorly explored.

III – The role of D1 receptors in LTP

Synaptic plasticity can also be modulated by the dopaminergic system. As synaptic plasticity is believed to be the cellular basis of memory formation, understanding how D1 receptors modulate synaptic plasticity might provide a working model to understand behavioral consequences of dopamine transmission in physiology and pathology. Dopaminergic transmission via D1 receptors have been associated with the modulation of LTP, LTD and with depotentiation (Hansen and Manahan-Vaughan, 2014). I will focus on the effect of dopaminergic transmission on LTP as it has been extensively characterized in the hippocampal CA3-CA1 synapses and it has been correlated with behavioral expression of learning and memory.

The involvement of dopamine in the modulation of LTP was primarily observed in the CA1 region of the hippocampus (Frey et al., 1990, Otmakhova and Lisman, 1996). By modulating dopamine receptors in slices, it was shown that although dopamine was not required for the induction of LTP, it was important for the maintenance (*i.e.* late phase) of the potentiation of synaptic transmission (Frey et al., 1990). Furthermore, it has been the established that the late phase of CA3-CA1 LTP, but not the induction, requires D1, but not D5, receptor function (Granado et al., 2008, Matthies et al., 1997). Interestingly, at the ultrastructural level, D1 receptors have been found to be mostly present in the excitatory dendritic spines whereas the D5 where abundant in the inhibitory GABAergic shafts. This could be an indication that these different classes could be specifically involved in the modulation of excitation or inhibition, respectively (Hansen and Manahan-Vaughan, 2014).

A direct protein-protein interaction between NMDA receptors and D1 receptors which can modulate NMDA receptor-dependent glutamatergic currents has been described (Lee et al., 2002). D1 receptor activity can modulate gene expression associated with normal LTP. Studies using constitutive deletion of D1 receptors in the brain have shown that in the absence of D1 receptors the expression of immediate early genes zif268 and arc that signal for protein synthesis in the hippocampus were impaired (Granado et al., 2008). The transition between early LTP and late LTP was shown to be dependent on newly synthetized proteins (Malenka and Bear, 2004). Large evidence indicates that D1-like receptors could modulate protein synthesis. For instance, the stimulation of D1-like receptors in the hippocampus can induce the synthesis and incorporation of the GluR1 of AMPA receptors at the synapse (Smith et al., 2005) and the D1-like dependent protein synthesis is involved the modulation of ERK ½ of the MAPK pathway, known to be necessary for the establishment of late LTP (Lisman et al., 2011). In an elegant study, Li and colleagues (2003) demonstrated that the exposure to spatial novelty (*i.e.* novel

environment) could induce a facilitation of LTP in the hippocampal CA3-CB1 synapses (Li et al., 2003). Interestingly, this facilitation could be blocked by the D1-like antagonist SCH23390, demonstrating that dopamine release and most likely D1 receptor activity might modulate *in vivo* LTP in the hippocampus (Li et al., 2003). Furthermore, the authors showed that by administrating a D1-like agonist, they could facilitate the induction of LTP, providing evidence that novelty-induced dopamine levels in the hippocampus trigger cellular mechanisms that lower the threshold necessary for LTP facilitation (Li et al., 2003). Consistent with this observation, Takeuchi and colleagues (2016) showed that optogenetic stimulation of locus coeruleus fibers that project to dorsal hippocampus induced a D1-like dependent facilitation of LTP (Takeuchi et al., 2016).

Dopaminergic transmission via D1-like family is involved in the modulation of memory (Hansen and Manahan-Vaughan, 2014). Dopamine levels in the prefrontal cortex and hippocampus can increase after exposure to novelty (Ihalainen et al., 1999) stressing the possible role of dopamine in supporting the consolidation of novel experiences (Lisman et al., 2011).

Working memory is the capacity to retain and elaborate information for short-term periods of time providing an interface between perception, long-term memory and action (Ma et al., 2014). In the prefrontal cortex, the importance of dopaminergic signaling via D1 receptors for working memory has been demonstrated in both in non-human (Vijayraghavan et al., 2007, Xing et al., 2012) and in human studies (McNab et al., 2009). For instance, in non-human primates, prefrontal D1 receptors are involved in the acquisition and not in the retention phase of the memory formation, suggesting that during aging or in pathological conditions, lower memory performance might be due to lower learning capacities caused by weak D1 receptor activity (Puig and Miller, 2012). In humans, the density of D1 receptors in prefrontal and parietal cortex (measured as binding potential) increased with training of working memory, a feature correlated with improved working memory performance (McNab et al., 2009).

Besides the known involvement of D1 receptors in the processing of short-term memory, it has also been shown that D1 receptors are involved in the storage and persistence of long-term memories in the hippocampus (da Silva et al., 2012, Xing et al., 2010). Although it is established that dopamine modulation is not required for maintenance and retrieval of previously established memories, dopaminergic transmission seems to be crucial for the establishment and stabilization of long-term memories (Lisman et al., 2011).

In a key study, Rossato and colleagues (2009) demonstrated that the pharmacological blockade of D1 receptors in the hippocampus by the specific antagonist SK38393 improved the stability of an aversive memory (Rossato et al., 2009). The mechanism proposed suggests that dopamine post-training is important for late post-acquisition stabilization of memories. The authors further demonstrated that dopamine released from VTA projections to the

hippocampus, possibly through the hippocampal-VTA loop (Lisman and Grace, 2005), were involved in this mechanism of long-term memory (Rossato et al., 2009).

Hippocampal D1 receptors can also modulate spatial memory (da Silva et al., 2012, Xing et al., 2010). Constitutive genetic deletion of D1 receptors impairs spatial memory in the Morris water maze task (Granado et al., 2008, Xing et al., 2010) and local administration of antagonist and agonist of D1/D5 receptors in the dorsal hippocampus can inhibit long-term memory formation or enhance long-term memory retention, respectively (da Silva et al., 2012). Recognition memory can also be modulated by D1 receptor activity. The administration of selective D1 receptor agonist SKF38393 has been shown to enhance recognition memory (de Lima et al., 2011).

Another interesting aspect of dopaminergic signaling in hippocampal memory is the origin of the fibers that project to the dorsal hippocampus. The dopaminergic system has been shown to be consistently involved in the modulation of spatial memory, which is greatly modulated by place cell activity in the dorsal hippocampus (Lisman et al., 2011). Although few fibers from the VTA innervate the dorsal hippocampus (in contrast with ventral hippocampus), recent evidence has shown that noradrenergic locus coeruleus fibers can also release dopamine in the dorsal hippocampus (Kempadoo et al., 2016, Takeuchi et al., 2016). Whereas it has been shown that dopamine release from neurons projecting from the VTA promoted spatial memory in the context of reward location (McNamara et al., 2014), dopamine from locus coeruleus improved spatial memory in the absence of a reward (Kempadoo et al., 2016). Furthermore, by using a different behavioral paradigm to assess novelty, Takeuchi and colleagues (2016) demonstrated that dopamine release in the dorsal hippocampus improved memory persistence in a D1-like family-specific manner (Takeuchi et al., 2016). Social learning can also be modulated by D1-receptors in the hippocampus. In a recent study, Matta and colleagues showed that local dorsal hippocampal injections of the D1 receptor antagonist SCH23390 impairs social learning in both males and females, without impacting on the capacity to sense food or disrupting meal patterns (Matta et al., 2017).

Altogether, this evidence shows that dopaminergic transmission via D1 receptors in the hippocampus can modulate short- and long-term memory functions with important consequences for proper brain function.

IV – D1 and CB1 receptors – A potential crosslink to modulate memory functions

The endocannabinoid system and the dopaminergic system are actively involved in the modulation of synaptic plasticity and memory functions. Whereas this functional relation in reward has been already established (Bloomfield et al., 2016) and is subject to scrutiny, its importance in cognition, more specifically in learning and memory, remains poorly explored.

CB1 receptor presence in D1-positive cells was anatomically characterized using *in situ* hybridization (ISH) analysis of CB1 receptor mRNA co-expression with D1 receptors in several brain regions (Hermann et al., 2002). CB1 receptors exhibit coexpression with D1 receptors in the caudate putamen, the nucleus accumbens, and the olfactory tubercle, the piriform cortex and the endopiriform nucleus (Hermann et al., 2002). More recently, by the use of a constitutive mouse line lacking CB1 receptors in D1-positive cells (D1-CB1-KO mice), Monory and colleagues (2007) showed that this deletion leads to reduction of CB1 receptor mRNA content in both principal glutamatergic neurons in the layer VI of the neocortex and in GABAergic MSNs in the striatum (Monory et al., 2007). The extent of deletion was much higher in the GABAergic medium spiny neurons in the striatum than compared with the lower deletion of glutamatergic neurons (Monory et al., 2007). Functional interaction between the CB1 and the D1 receptors have been widely studied in the striatum (Martin et al., 2008).

The modulation of memory function by the intercross between the dopaminergic system and the endocannabinoid system has been recently functionally assessed in the prefrontal cortex. In 2017, Scheggia and colleagues have shown that dysregulation of the catabolic enzyme involved in the dopamine degradation, catechol-O-methyltransferase (COMT), which decreases in dopamine availability in the prefrontal cortex, leads to the enhancement aversive remote memories by dysregulation of the endocannabinoid system in a CB1 receptor-dependent manner (Scheggia et al., 2017). It is known that the prefrontal cortex neurons express D1 receptors (Paspalas and Goldman-Rakic, 2005, Sawaguchi and Goldman-Rakic, 1991) and CB1 receptors (Marsicano and Kuner, 2008). However, it is not currently known if they are co-expressed in the same cell-types or if the results shown by Scheggia a colleagues are dependent on D1 receptors in that particular brain region. Further research will address these issues.

The hippocampus is another key brain region that, although it has been shown to express D1 and CB1 receptors, the functional relation between them is not currently known. In aversive learning, mice carrying a deletion of D1 receptors have prolonged retention and delayed extinction in conditioned fear responses (El-Ghundi et al., 2001). Novelty can induce

dopamine release and action in the hippocampus (Ihalainen et al., 1999, Menezes et al., 2015). Remarkably, it has been reported that novelty could facilitate the extinction of fear memories, an effect specifically mediated by D1 receptors in the hippocampus (Menezes et al., 2015). CB1 receptors have been shown to modulate fear memories (Marsicano et al., 2002) and mice lacking CB1 receptors in D1 positive cells display an impairment in extinction of aversive memories (Terzian et al., 2011). Furthermore, it has been shown that extinction of avoidance memories depends on CB1 receptors in D1-positive cells and local hippocampal inhibition of CB1 receptors in wild-type (WT) mice impairs this behavior (Micale et al., 2017). Although there is currently no anatomical evidence demonstrating the presence of CB1 receptors in hippocampal D1-positive cells, functional data support a potential crosslink between the two systems. Understanding and further demonstrating where and how this functional crosstalk occurs will provide novel information that will help to understand how dopamine via D1 receptors can modulate learning and memory via CB1 receptors.

SECTION II – RESEARCH OBJECTIVES

CB1 receptors in neuronal and glial populations are important modulators of learning and memory (Busquets-Garcia et al., 2017a). In the hippocampus, activation of neuronal CB1 receptors has been associated with decrease in neurotransmission, whereas activation of CB1 receptors in fine processes of astrocytes with the induction of gliotransmission (Araque et al., 2017). Past studies have addressed the exogenous and endogenous contribution of CB1 receptors in specific cell types (*i.e.* GABAergic, glutamatergic neurons) in the modulation of synaptic plasticity and transmission and in learning and memory. However, CB1 receptors have been recently identified in previously unknown locations (*e.g.* astrocytic processes and intracellular compartments). It is, therefore, necessary to investigate the role of these novel subpopulations of CB1 receptors in cellular and molecular mechanisms underlying behavior. For instance, whereas astroglial CB1 receptors can actively modulate synaptic transmission and plasticity and mediate the memory disruptive effects of cannabinoids on working memory (Han et al., 2012), their role in the endogenous modulation of memory functions is currently not known. Moreover, many studies show that neurons and astrocytes belong to different subpopulations, many of which are still to be identified (Chai et al., 2017, Haim and Rowitch, 2017). For instance, CB1 receptors have been shown to be present in cells expressing a major receptor from another important modulatory system (*i.e.* D1 receptors from the dopaminergic system). Because the number of these cells is reduced, it is very difficult to identify them anatomically. Nevertheless, functional evidence points to the existence of these cells within the hippocampal structure with a likely impact in hippocampal mediated functions. Thus, understanding the role of CB1 receptors in these cells is will provide novel insights regarding the modulation of memory functions by specific cells types expressing CB1 receptors. Globally, dissecting the role of CB1 receptors in different cellular populations in the physiological modulation of learning and memory is important to understand brain functions and will help to understand how dysregulations can lead to pathology.

The main objective of the thesis is to identify the cellular and molecular mechanisms by which specific CB1 receptors in discrete brain neuronal and glial populations contribute to the physiological modulation of learning and memory.

To address the main objective of this thesis we used a combination of genetics (constitutive and conditional mutagenesis of CB1 receptors in mice) coupled with behavioral, pharmacological and *in vitro* and *in vivo* electrophysiological approaches aiming at dissecting the contribution of CB1 receptors in the mechanisms memory formation.

First, we studied the cellular mechanisms involved in the physiological modulation of long-term memory by astroglial CB1 receptors. During this work we addressed several questions regarding: 1) the role of astroglial CB1 receptors in the modulation of long- and short-term memory formation, 2) the role of astroglial CB1 receptors in the modulation of LTP, 3) how CB1 receptors control astroglial functions to support gliotransmission and 4) we characterized the mechanism that underlie the astroglial CB1 control of memory functions.

To address these questions we used behavioral and *in vitro* and *in vivo* electrophysiological approaches in mice lacking CB1 receptors in brain astrocytes. Furthermore, we complemented our study with the use of general and local pharmacology, amino acid quantification and local genetic approaches to dissect the cellular and molecular mechanism involved in the astroglial CB1 modulation of memory functions. This first part of this thesis is resumed in the manuscript:

(Submitted)

Astroglial CB1 receptors determine synaptic D-serine availability to enable recognition memory

Laurie M. Robin*, Jose F. Oliveira da Cruz*, Valentin C. Langlais*, Mario Martin-Fernandez, Mathilde Metna-Laurent, Arnau Busquets-Garcia, Luigi Bellocchio, Edgar Soria-Gomez, Thomas Papouin, Ilaria Belluomo, Isabel Matias, Barbara Bosier, Filippo Drago, Ann Van Eeckhaut, Ilse Smolders, Francois Georges, Alfonso Araque, Aude Panatier, Stéphane H.R. Oliet# and Giovanni Marsicano#

*: 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. I participated in the writing of the manuscript.

Second, we studied the contribution of CB1 receptors in D1-positive cells in the physiological modulation of memory formation. During this work we aimed at exploring the role of 1) CB1 receptors in D1-positive cells in the modulating of short- and long-term object recognition memory functions and to 2) investigate the role of these receptors in the modulation of LTP.

To address these questions, we used a combination of behavioral, pharmacological and *in vivo* electrophysiological approaches in mice lacking CB1 receptors in D1-positive cells (D1-CB1-KO). The results obtained from this second aim are presented in a manuscript that is currently in preparation:

Deletion of CB1 receptors in hippocampal D1-positive cells impairs object recognition memory and associated synaptic plasticity

Jose F. Oliveira da Cruz[*], Arnau Busquets-Garcia[*], Luigi Bellocchio, Zhe Zhao, Filippo Drago, Marjorie Varilh, Giovanni Marsicano[#], Edgar Soria-Gomez[#]

*: equal contribution, #: equal supervision

My main contribution to this work was to perform and analyze data collected from *in vivo* recordings in anesthetized mice. 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 – ASTROGLIAL CB1 RECEPTORS DETERMINE SYNAPTIC D-SERINE AVAILABILITY TO ENABLE RECOGNITION MEMORY

Astroglial CB_1 receptors determine synaptic D-serine availability to enable recognition memory

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Abstract

Bidirectional communication between neurons and astrocytes shapes synaptic plasticity and behavior. D-serine is a necessary co-agonist of synaptic N-methyl-D-aspartate receptors (NMDAR), but the physiological factors regulating its impact on memory processes are not known. This study shows that astroglial CB_1 receptors are key determinants of object recognition by determining the synaptic availability of D-serine in the hippocampus. Mutant mice lacking CB_1 receptors from astroglial cells (GFAP-*CB_1*-KO) displayed impaired object recognition memory and decreased *in vivo* and *in vitro* long-term potentiation (LTP) at CA3-CA1 hippocampal synapses. Activation of CB_1 receptors increased intracellular astroglial Ca^{2+} levels and extracellular levels of D-serine in hippocampal slices. Accordingly, GFAP-*CB_1*-KO displayed lower occupancy of the co-agonist binding-site of synaptic hippocampal NMDARs. Finally, elevation of D-serine levels fully rescued LTP and memory impairments of GFAP-*CB_1*-KO mice. These data reveal a novel mechanism of *in vivo* astroglial control of memory and synaptic plasticity *via* the D-serine-dependent control of NMDARs.

Introduction

The endocannabinoid system is an important modulator of physiological functions. It is composed of cannabinoid receptors, their endogenous ligands (the endocannabinoids) and the enzymatic machinery for synthesis and degradation of endocannabinoids (Piomelli, 2003). The presence of type-1 cannabinoid receptors (CB_1) and the activity-dependent mobilization of endocannabinoids in many different brain regions, including the hippocampus, are particularly involved in the modulation of several types of memory and associated cellular processes (Kano et al., 2009; Marsicano and Lafenetre, 2009). Moreover, CB_1 receptors are expressed in different neuronal types in the brain, including inhibitory GABAergic and excitatory glutamatergic neurons, where their stimulation negatively regulates the release of neurotransmitters (Kano et al., 2009).

In addition to their localization on neurons, CB_1 receptors are also expressed in glial cells, particularly astrocytes (Andrade-Talavera et al., 2016; Han et al., 2012; Min and Nevian, 2012; Navarrete and Araque, 2008; Rasooli-Nejad et al., 2014). Astrocytes are the most abundant glial cells in the central nervous system (Verkhratsky and Butt, 2013) and, based on the fact that they are not able to generate action potentials, they were thought for more than a century to play an important supportive and nutritive role for neurons, without actively participating in brain information processing (Allaman et al., 2011; Araque et al., 2014). However, it is now known that astrocytes are much more complex and active cells and their functions overcome mere passive support and nutrition of neurons. For instance, peri-synaptic astroglial processes surrounding pre- and post-synaptic neuronal elements form the so-called “tripartite synapse”,

which is now considered an important unitary element underlying active brain plasticity where astrocytes actively contribute to information processing (Araque et al., 2014; Perea et al., 2009).

In vivo and *in vitro* studies showed that astroglial CB_1 receptor signaling indirectly stimulates glutamatergic transmission onto hippocampal pyramidal neurons (Han et al., 2012; Metna-Laurent and Marsicano, 2015; Navarrete and Araque, 2010; Oliveira da Cruz et al., 2016). For instance, the amnesic effect of exogenous cannabinoids on spatial working memory is mediated by astroglial CB_1 receptors through a NMDAR-dependent mechanism in the hippocampus (Han et al., 2012). Yet, the role of astroglial CB_1 receptors in long-term memory processes under physiological conditions and the precise mechanisms involved are still unknown (Metna-Laurent and Marsicano, 2015).

D-serine is the co-agonist of synaptic NMDARs and its action is required to induce different forms of synaptic plasticity (Henneberger et al., 2010; Panatier and Oliet, 2006; Papouin et al., 2012; Shigetomi et al., 2013; Sultan et al., 2015). Although the direct source of the aminoacid is still under debate (Araque et al., 2014; Wolosker et al., 2016), there is convergent consensus that its release within synapses requires Ca^{2+}-dependent astrocyte activity (Araque et al., 2014; Wolosker et al., 2016). However, the physiological mechanisms underlying the synaptic availability of D-serine are not known.

Using a combination of genetic, behavioral, electrophysiological and biochemical experimental approaches, in this study we asked whether the physiological activity of astroglial CB_1 receptors is involved in long-term object recognition memory and whether the mechanisms involved imply the regulation of glial-neuronal interactions. The results show that physiological activation of astroglial CB_1 receptors in the hippocampus is necessary for long-term object recognition memory consolidation *via* a mechanism involving the supply of D-serine to synaptic NMDARs and, consequently, the regulation of hippocampal synaptic plasticity. Thus, astroglial CB_1 receptors determine the time- and space-specific synaptic actions of astrocytes to promote memory formation.

Results

Hippocampal astroglial CB_1 receptors deletion impairs object recognition memory and in vivo NMDAR-dependent LTP

To study the physiological role of astroglial CB_1 receptors in memory, we tested conditional mutant mice lacking CB_1 receptors in glial fibrillary acidic protein (GFAP)-positive cells (GFAP-*CB_1*-KO mice) (Han et al., 2012) in a long-term novel object recognition memory task in an L-maze (NOR) (Busquets-Garcia et al., 2011; Puighermanal et al., 2013; Puighermanal et al., 2009). GFAP-*CB_1*-KO mice displayed a significant memory deficit as compared to their control littermates (**Figure 1A**, see also **Figure S1A**), with no alteration in total object exploration time (**Figure S1B**). Hippocampal NMDARs are involved in many forms of memory (Kandel, 2002; Puighermanal et al., 2009; Warburton et al., 2013). As the involvement of hippocampal NMDARs on NOR memory is still under debate and seems to depend on specific experimental conditions (Balderas et al., 2015; Warburton and Brown, 2015), we investigated the role of these receptors in our task. Intra-hippocampal injection (**Figure S1C**) of the NMDAR antagonist D-AP5 (15 µg/side) immediately after acquisition fully abolished memory performance in wild-type mice (**Figure 1B**, see also **Figure S1D**), with no alteration in total exploration time (**Figure S1E**). Thus, long-term object recognition memory in the NOR task specifically requires astroglial CB_1 receptors and hippocampal NMDARs signaling.

Activity-dependent plastic changes of synaptic strength, such as long-term potentiation (LTP), are considered cellular correlates of memory formation (Kandel, 2002; Whitlock et al., 2006). We recorded *in vivo* evoked field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA3-CA1 pathway of anesthetized wild-type and mutant mice. High-frequency stimulation (HFS) induced LTP in wild-type C57BL/6-N mice (**Figure 1C,D**) that was fully blocked by systemic treatment with the NMDAR antagonist MK801 (3 mg/kg, i.p., **Figure 1C,D**), confirming its NMDAR dependency. Notably, this form of LTP was abolished in GFAP-*CB_1*-KO mice (**Figure 1E,F)**, showing that CB_1 receptors expressed in GFAP-positive cells are necessary for hippocampal NMDAR-dependent LTP induction *in vivo*. Altogether, these data demonstrate that astroglial CB_1 receptors are essential for hippocampal NMDAR-dependent object recognition memory and LTP.

Activation of CB_1 receptors increases astroglial Ca^{2+} levels and D-serine release

Increase of astroglial intracellular Ca^{2+} modulates synaptic glutamatergic activity and plasticity *via* the release of gliotransmitters, whose identity likely depend on the brain region and the type of plasticity involved (Araque et al., 2014; Sherwood et al., 2017). Because activation of CB_1 receptors generate Ca^{2+} signals in astrocytes (Araque et al., 2014; Metna-

Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016), the impaired memory and synaptic plasticity in GFAP-CB_1-KO mice might result from alterations of astroglial Ca^{2+} regulation and signaling of specific hippocampal gliotransmitters.

First, we tested whether the CB_1 receptor-dependent modulation of intracellular Ca^{2+} levels (Gomez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010) depends on direct activation of astroglial CB_1 receptors. Local pressure application of the CB_1 receptor agonist WIN55,212-2 (WIN) induced a reliable increase of Ca^{2+} levels in somas and principal processes of hippocampal astrocytes in slices from GFAP-CB_1-WT mice (**Figure 2A-E**). As expected (Gomez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010), this effect was fully blocked by the CB_1 receptor antagonist AM251 (2 µM, **Figure 2B-E**). Notably, WIN had no effect in slices from GFAP-CB_1-KO littermates (**Figure 2B-E**), clearly indicating the direct impact of astroglial CB_1 receptor activation on intracellular Ca^{2+} levels.

Astrocytes can release several gliotransmitters *via* Ca^{2+}-dependent mechanisms, including glutamate and D-serine that would directly impact NMDAR activity (Araque et al., 2014). Therefore, we asked whether activation of CB_1 receptors might modulate the release of these amino acids. Application of WIN (5µM) to hippocampal slices did not alter intracellular tissue levels of several potential gliotransmitters (**Figure S2A-D**). However, the same treatment slightly, but specifically increased the extracellular levels of D-Serine (**Figure 2F-I**), indicating that activation of astroglial CB1 receptors can control the release of the gliotransmitter, which depends on intracellular Ca^{2+} signaling (Bohmbach et al., 2017; Henneberger et al., 2010).

Hippocampal LTP requires control of synaptic levels of D-serine by astroglial CB_1 receptors

D-serine is the co-agonist of hippocampal synaptic NMDARs and its presence is necessary for LTP induction (Bohmbach et al., 2017; Henneberger et al., 2010; Papouin et al., 2012). Thus, astroglial CB_1 receptors might control the activity of NMDARs and the hippocampal LTP by regulating the synaptic levels of D-serine. To test this hypothesis, we assessed the D-serine occupancy of the NMDAR co-agonist binding sites in CA1 hippocampal synapses, by measuring the impact of exogenous applications of the aminoacid on NMDAR-mediated fEPSPs in acute hippocampal slices (Papouin et al., 2012). Bath application of D-serine (50 µM) increased NMDAR-dependent fEPSPs in both GFAP-CB_1-WT and GFAP-CB_1-KO mice (**Figure 3A,B**). Strikingly, however, the effect of D-serine was twice more pronounced in the absence of astroglial CB_1 receptors (**Figure 3A,B**), indicating that astroglial CB_1 receptors are necessary to maintain appropriate concentrations of D-serine within the synaptic cleft and consequently ensuring a proper level of occupancy of NMDAR co-agonist binding site.

Next, we asked whether astroglial CB1 receptors control synaptic plasticity by regulating NMDAR signaling *via* D-serine. *In vitro* electrophysiological recordings revealed that GFAP-*CB_1*-WT and GFAP-*CB_1*-KO have comparable input-out relationships, indicating that the deletion of astroglial CB_1 receptors does not alter intrinsic dynamic excitability of hippocampal circuits (**Figure S3**). Similarly to *in vivo* electrophysiological results, however, HFS-induced LTP in hippocampal slices was significantly reduced in GFAP-*CB_1*-KO mice as compared to GFAP-*CB_1*-WT (**Figure 3C**). The exogenous application of D-serine (50 µM) had no effect in slices from GFAP-*CB_1*-WT mice, but it fully rescued *in vitro* LTP in GFAP-*CB_1*-KO littermates (**Figure 3D,E**). Similarly, the lack of *in vivo* LTP observed in GFAP-*CB_1*-KO was fully restored by the systemic administration of D-serine (50 mg/kg, i.p., **Figure 3F-H**). These results show that astroglial CB_1 receptors regulate the synaptic levels of the NMDAR co-agonist D-serine necessary for NMDAR-dependent *in vitro* and *in vivo* LTP.

Astroglial CB_1 receptors determine NOR memory* via *D-Serine

If, as shown above, astroglial CB_1 receptors determine the activity of NMDARs *via* the control of synaptic D-serine levels, this mechanism might underlie the processing of NOR memory. Strikingly, a sub-effective dose of D-serine (i.e. having no effect on memory performance *per se*, 50 mg/kg, i.p.; **Figure S4A**) reverted the memory impairment of GFAP-*CB_1*-KO mice (**Figure 4A** see also **Figure S4B,C**). This effect of D-serine in GFAP-*CB_1*-KO mice was not present when the injection occurred 1-hour after acquisition or immediately before test (**Figure S4D,E**), indicating that only the initial phase of NOR memory consolidation is altered in the mutant mice. Notably, administration of a sub-effective dose (**Figure S4F**) of the inhibitor of D-aminoacid-oxidase AS05278 (50 mg/kg, i.p.), which increases endogenous D-serine levels *in vivo* (Adage et al., 2008), also rescued the phenotype of GFAP-*CB_1*-KO mice (**Figure 4B**, see also **Figure S4G,H**). Moreover, post-acquisition intra-hippocampal injections of D-serine (sub-effective dose of 25 µg/side; **Figure S4I**) also restored NOR memory performance in GFAP-*CB_1*-KO mice (**Figure 4C**, see also **Figure S4J,K**). This suggests that the hippocampus is the brain region where astroglial CB_1 receptors control NMDAR-dependent memory formation *via* D-serine signaling. GFAP-*CB_1*-KO mice, however, carry a deletion of the *CB_1* gene in GFAP-positive cells in different brain regions (Bosier et al., 2013; Han et al., 2012), leaving the possibility that D-serine signaling in the hippocampus is remotely altered by deletion of astroglial CB_1 receptors elsewhere. To specifically delete the *CB_1* gene in hippocampal astrocytes, we injected an adeno-associated virus expressing the CRE recombinase under the control of the GFAP promoter (AAV-GFAP-CRE/mCherry) or a control AAV-GFAP-GFP into the hippocampi of mice carrying the "floxed" *CB_1* receptor gene (Marsicano et al., 2003) (**Figure 4D**). Mice injected with the CRE recombinase were impaired in NOR memory performance (**Figure 4E**, see also **Figure S4L,M**) and, notably, the systemic injection of D-serine (50 mg/kg, i.p.) fully reversed this phenotype

(**Figure 1E**, see also **Figure S4L,M**). Thus, hippocampal astroglial CB_1 receptors are required for NOR memory performance, *via* the control of D-serine signaling during the initial phases of memory consolidation.

Discussion

These results show that astroglial CB1 receptors are key determinants of physiological consolidation of object recognition memory in the hippocampus. *Via* Ca^{2+}-dependent mechanisms, they provide the synaptic D-serine levels required to functionally activate NMDARs and to induce LTP in the hippocampal CA1 region. In turn, this process is necessary upon learning to consolidate long-term object recognition memory (**Figure S5**). By causally linking the functions of a specific subpopulation of CB_1 receptors, astroglial control of NMDAR activity *via* the gliotransmitter D-serine and synaptic plasticity, these data provide an unforeseen physiological mechanism underlying memory formation.

These data might shed light onto the pathway underpinning D-serine availability at synapses. Indeed, whereas it has become increasingly clear that astrocytes control the activity of NMDARs at synapses through D-serine (Henneberger et al., 2010; Panatier and Oliet, 2006; Papouin et al., 2012; Shigetomi et al., 2013; Sultan et al., 2015), the physiological determinants that regulate D-serine availability at synapses have remained unknown. Our data clearly link the activity of astroglial CB_1 receptors to the activation of NMDARs *via* the modulation of D-serine occupancy of their co-agonist binding-site. Astrocytes occupy non-overlapping domains of the neuropil where they survey the activity of thousands of synapses (Bushong et al., 2002; Pannasch and Rouach, 2013; Papouin et al., 2017). On the other hand, endocannabinoids are locally mobilized at synapses in an activity-dependent manner and their actions are rather limited in space and time (Castillo et al., 2012; Kano et al., 2009; Piomelli, 2003). Therefore, astroglial CB_1 receptors may act as sensors integrating the overall intensity of local synaptic activity within the territory of specific astrocytes and this information may then be used to adjust the availability of D-serine and the activity of NMDARs. In this context, we propose that the astroglial CB_1-dependent regulation of D-serine supply is a major mechanism determining how much D-serine each astrocyte contributes to make available to NMDARs as a function of neuronal activity within its territory.

The direct release of D-serine by astrocytes has been recently questioned, suggesting that astrocytes release L-serine, which, in turn, shuttles to neurons to fuel the neuronal synthesis of D-serine (Wolosker et al., 2016). Our data do not directly address this issue, but they support the idea that astrocyte functions and synaptic D-serine actions are required for hippocampal LTP

(Henneberger et al., 2010; Sherwood et al., 2017; Wolosker et al., 2016). Thus, independently of their direct source, synaptic D-serine levels are under the control of CB_1 receptors specifically expressed in astrocytes, whose activation increases astroglial Ca^{2+} levels and promotes D-serine occupancy of synaptic NMDARs, eventually controlling specific forms of *in vivo* and *in vitro* LTP and object recognition memory.

Generalized activation or inhibition of CB_1 receptors does not reliably reflect the highly temporally- and spatially-specific physiological functions of the endocannabinoid system (Busquets Garcia et al., 2016; Busquets-Garcia et al., 2015). Indeed, previous data showed that deletion of astroglial CB1 receptors abolishes the impairment of hippocampal working memory by cannabinoid agonists, but it does not alter this form of short-term memory *per se* (Han et al., 2012), thereby leaving open the question of the physiological roles of astroglial CB_1 receptors in the hippocampus (Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). This question could not be addressed using global genetic or pharmacological inactivation of CB_1 receptors, because it is known that CB_1 receptors expressed in different cellular subpopulations have often very diverse and even opposite impact on brain functions (Busquets Garcia et al., 2016; Busquets-Garcia et al., 2015), and this is particularly true between neurons and astroglial cells (Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). Indeed, global pharmacological activation, blockade and genetic deletion of CB_1 receptors are not able to catch subtle but important effects of endocannabinoid signaling. For instance, recent data show that deletion of the *CB_1* gene in hippocampal GABAergic or glutamatergic neurons induces opposite alterations of *in vitro* LTP (Monory et al., 2015), suggesting that results obtained by global receptor manipulation might be confounded by contrary physiological functions of cell type specific subpopulations of CB_1 receptors. Thus, the present results determine an unforeseen link between endogenous activation of astroglial CB_1 receptor signaling and long-term memory consolidation. Moreover, by showing the involvement of D-serine and NMDAR in these processes, our data provide an unexpected synaptic mechanism for this physiological function.

The deletion of the *CB_1* gene in our study is induced by tamoxifen treatment of GFAP-*CB_1*-KO mice or local injection of AAV-Cre under the control of a GFAP promoter into the hippocampus of *CB_1*-flox mice. These procedures occur few weeks before testing, excluding potential compensatory confounding events during pre- and post-natal development. Moreover, the phenotypes of GFAP-*CB_1*-KO mice in NOR and LTP are rescued by increasing D-serine-dependent NMDAR signaling at the moment of memory consolidation or electrophysiological analysis. Therefore, we can conclude that the control of synaptic NMDAR plasticity and of NOR memory by astroglial CB_1 receptors is due to acute alterations of hippocampal circuitries during memory formation and LTP induction. An additional potential confounding factor is the role played by both D-serine (Sultan et al., 2015) and CB_1 receptors

(Galve-Roperh et al., 2007) on adult neurogenesis. Due to the expression of GFAP in precursor neurons, we cannot fully exclude that neurogenesis might play a role in the mechanisms described. However, CB_1 receptors expressed in GFAP-positive cells are necessary for LTP at CA3-CA1 hippocampal synapses that are likely not influenced by neurogenesis events, which are known to specifically impact dentate gyrus circuits (Massa et al., 2011).

The role of CB_1 receptors expressed in GFAP-positive cells in NOR appears to be limited to the early phases of memory consolidation. Indeed, whereas the injection of D-serine immediately after memory acquisition fully rescues the phenotype of GFAP-CB_1-KO mice in NOR, the same treatment as soon as 1 hour after or just before memory retrieval has no effect. This is notable, because it indicates a very early engagement of astrocyte signaling in memory processing, underlying the importance of glial-neuronal interactions at crucial phases of cognitive processes.

In conclusion, our data provide a novel neurobiological frame, where the tight interaction between astrocytes and neurons required for the formation of object recognition memory is under the control of astroglial CB_1 receptors. Thus, by determining the physiological availability of D-serine at NMDARs, astroglial CB_1 receptors are key causal elements of spatial and temporal regulation of glia-neuron interactions underlying synaptic plasticity and cognitive processes in the brain.

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Author Contributions

LMR, JFOdC and VCL performed behavioral, *in vivo* electrophysiology and *in vitro* electrophysiology experiments, respectively, and wrote the manuscript. MM-L, AB-G, LB and ES-G contributed to behavioral experiments. BB, IS, AVE, IM and IB contributed to the measurements of aminoacids. FD supervised part of the work. TP helped with *in vitro* electrophysiology. FG supervised *in vivo* electrophysiology. AP and SHRO supervised *in vitro* electrophysiology. GM conceived and supervised the whole project and wrote the manuscript. All authors edited and approved the manuscript.

Robin et al. Figure 1

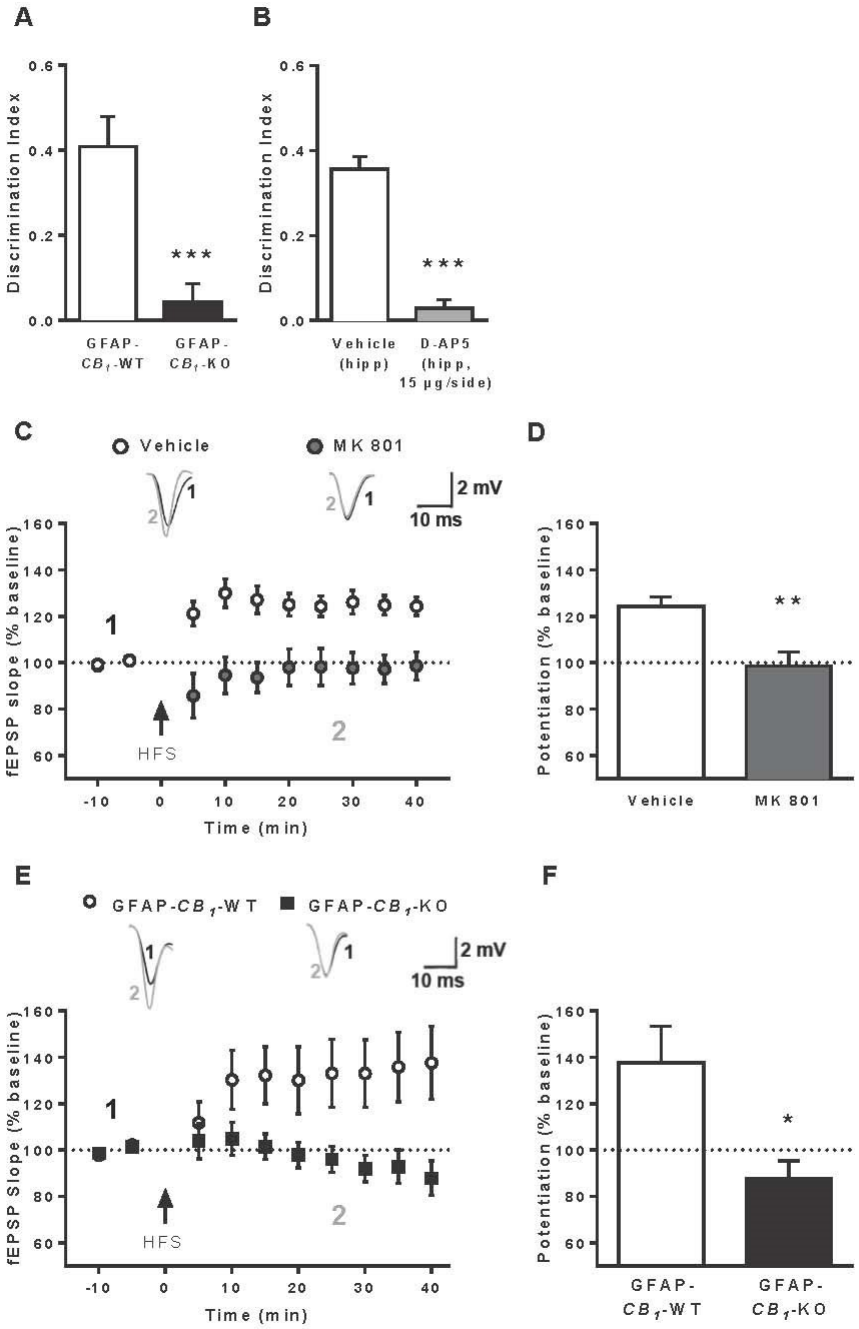

Figure 1. Hippocampal astroglial CB_1 receptors are necessary for NMDAR-dependent object recognition memory and *in vivo* LTP. (**A**) Memory performance of GFAP-*CB_1*-WT mice (n=10) and GFAP-*CB_1*-KO littermates (n=11) in the NOR task. (**B**) Effects of intra-hippocampal infusions of vehicle (n=10) or D-AP5 (15 µg/side; n=8) on NOR performance. (**C,D**) High frequency stimulation in the CA3 area of hippocampus induces NMDAR-dependent LTP in CA1 stratum radiatum. (**C**) Summary plots of normalized fEPSPs in anesthetized mice under vehicle (n=6) or MK 801 treatment (3 mg/kg; i.p.; n=5). (**D**) Bar histograms of normalized fEPSPs from experiment (**C**), 40 minutes after HFS. (**E,F**) *In vivo* LTP is absent in GFAP-*CB_1*-KO mice. (**E**) Summary plots of normalized fEPSPs in GFAP-*CB_1*-WT (n=9) and GFAP-*CB_1*-KO littermates (n=6). (**F**) Bar histograms of normalized fEPSPs from experiment (**E**), in 40 minutes after HFS. Data, mean ± SEM. *, P<0.05, **, P<0.01, ***, P<0.001. See **Tables S1 and S2** for detailed statistics.

Robin et al. Figure 2

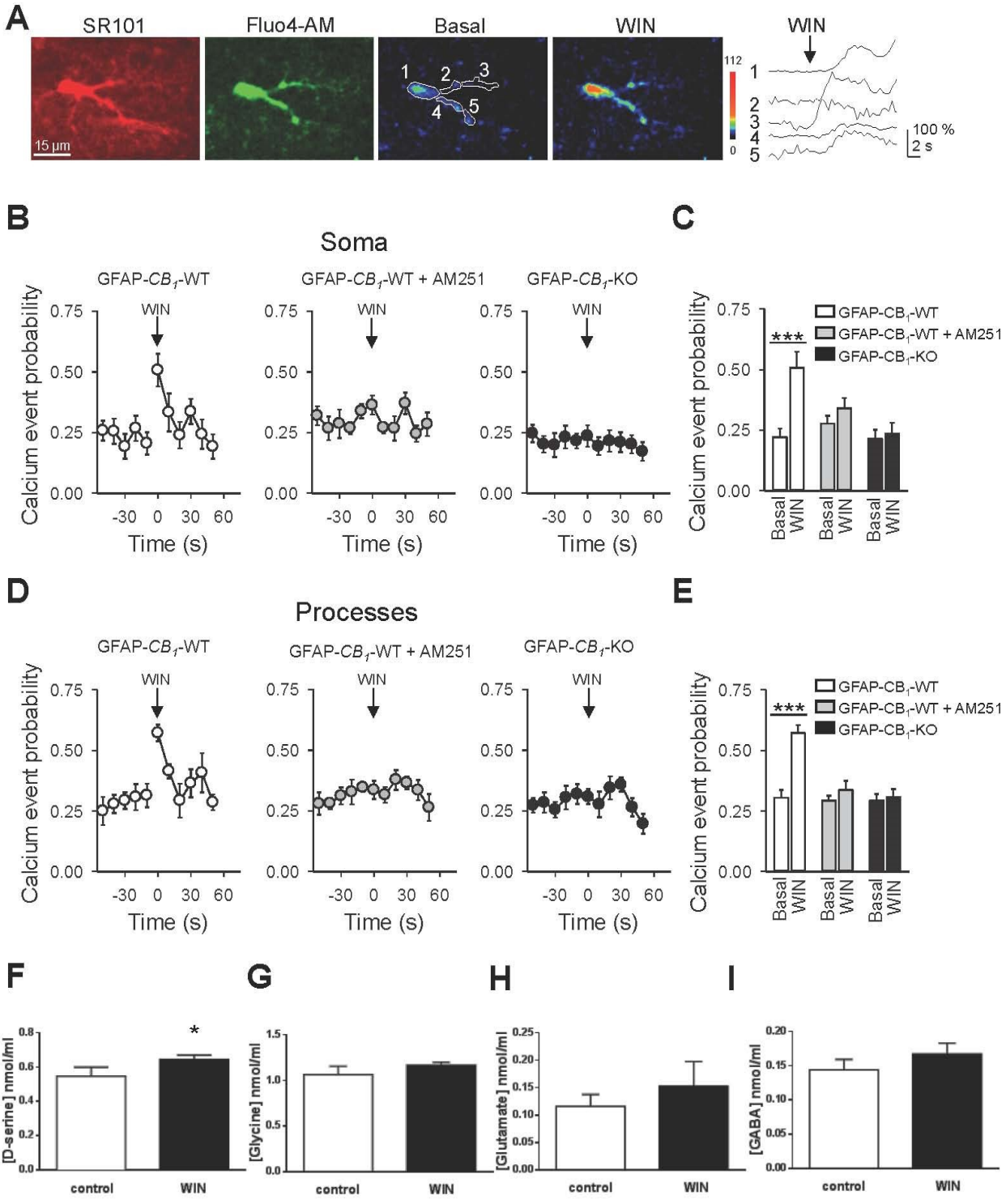

Figure 2. Activation of astroglial CB_1 receptors enhances intracellular Ca^{2+} levels in astrocytes and extracellular D-serine. (**A**) Representative image of a hippocampal astrocyte stained with SR101 and Fluo4, and pseudocolor images representing fluorescence intensities before and after WIN 515,212-2 (WIN) application, with the correspondent Ca^{2+} traces (numbers refer to different subcellular locations on the astrocyte). (**B**) Somatic calcium event probability before and after WIN (at time=0) in GFAP-CB_1-WT in control conditions (white) and in the presence of AM 251 (2 µM; grey), and in GFAP-CB_1-KO mice (black). (**C**) Somatic calcium event probability before and after WIN in GFAP-CB_1-WT in control conditions (white; n=9 slices and 79 somas) and in the presence of AM 251 (grey; n=12 slices and 159 somas), and in GFAP-CB_1-KO mice (black; n=16 slices and 145 somas). (**D**) Calcium event probability in the processes before and after WIN (at time=0) in GFAP-CB_1-WT in control conditions (white) and in the presence of AM 251 (2 µM; grey), and in GFAP-CB_1-KO mice (black). (**E**) Calcium event probability in the processes before and after WIN in GFAP-CB_1-WT in control conditions (white; n=8 slices and 171 processes) and in the presence of AM 251 (grey; n=8 slices and 140 processes), and in GFAP-CB_1-KO mice (black; n=10 slices and 189 processes). (**F-I)** Determination of D-serine, Glycine, L-glutamate and GABA as measured by capillary electrophoresis in extracellular solutions of acute hippocampal slices. Data, mean ± SEM. *, P<0.05, **, P<0.01, ***, P<0.001. See **Tables S1 and S2** for detailed statistics.

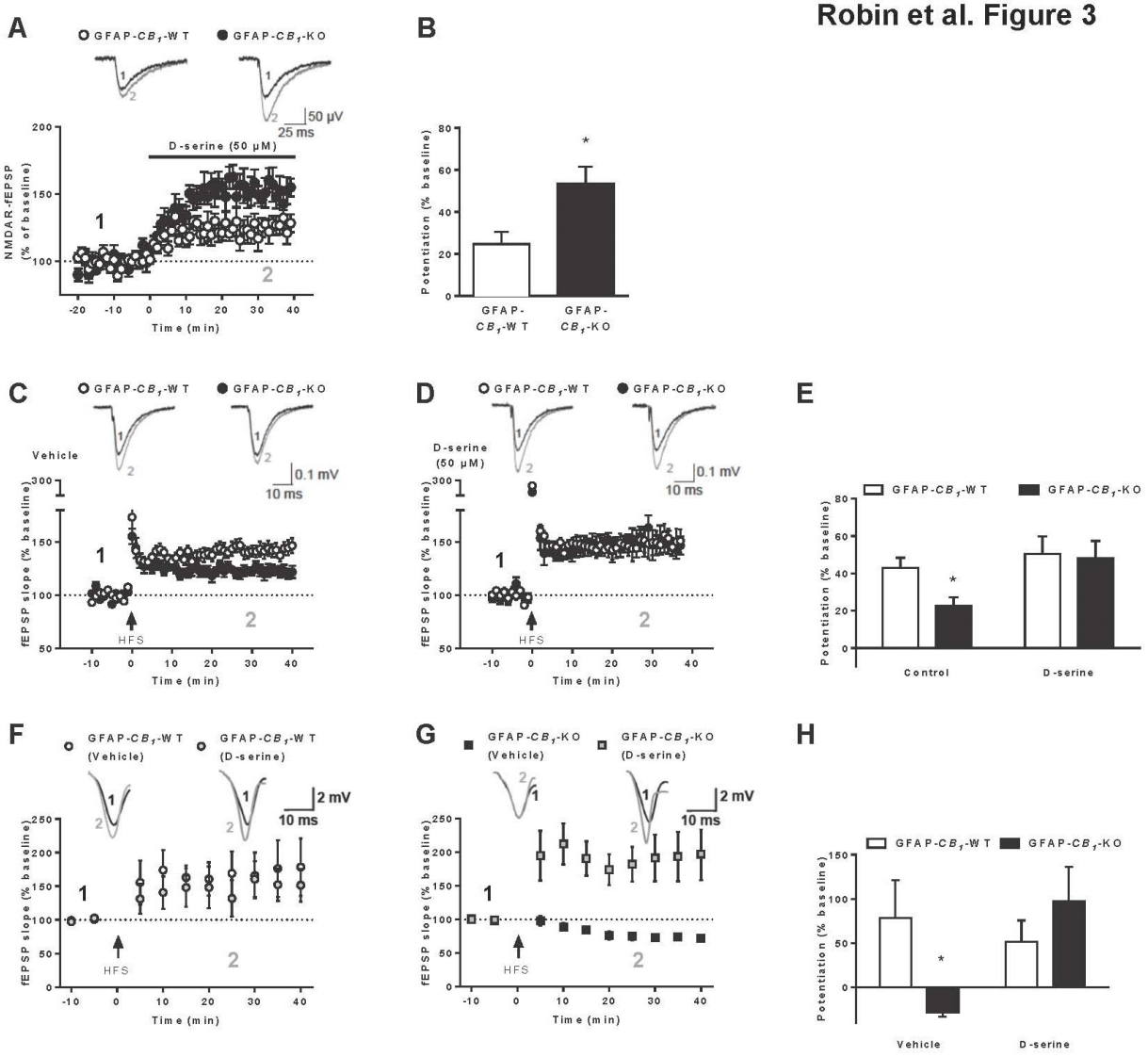
Robin et al. Figure 3
A
GFAP-CB1-WT
GFAP-CB1-KO
50 µV
25 ms
D-serine (50 µM)
NMDAR-fEPSP (% of baseline)
Time (min)
B
Potentiation (% baseline)
GFAP-CB1-WT
GFAP-CB1-KO
C
Vehicle
0.1 mV
10 ms
fEPSP slope (% baseline)
HFS
Time (min)
D
D-serine (50 µM)
0.1 mV
10 ms
fEPSP slope (% baseline)
HFS
Time (min)
E
Potentiation (% baseline)
Control
D-serine
F
GFAP-CB1-WT (Vehicle)
GFAP-CB1-WT (D-serine)
2 mV
10 ms
fEPSP slope (% baseline)
HFS
Time (min)
G
GFAP-CB1-KO (Vehicle)
GFAP-CB1-KO (D-serine)
2 mV
10 ms
fEPSP slope (% baseline)
HFS
Time (min)
H
Potentiation (% baseline)
Vehicle
D-serine

Figure 3. Astroglial CB_1 receptors control LTP induction through D-serine. (**A**) Summary plots showing the effect of D-serine application on NMDAR co-agonist binding site occupancy in slices from GFAP-*CB_1*-WT mice and GFAP-*CB_1*-KO littermates. Traces represent 60 superimposed NMDAR-fEPSPs before (1, black) and after (2, grey) D-serine application. (**B**) Bar histograms of normalized NMDAR-fEPSPs from experiment (**A**) measured 20-40 min after D-serine application. (**C**) *In vitro* LTP is impaired in GFAP-*CB_1*-KO mice. Summary plots of normalized fEPSPs in slices from GFAP-*CB_1*-WT (n=16) and GFAP-*CB_1*-KO mice (n=12) before (1) and after (2) high frequency stimulation (HFS). (**D**) D-serine application rescues LTP in slices from GFAP-*CB_1*-KO mice. Summary plots of fEPSPs showing the effect of D-serine (50 µM) on LTP in slices from GFAP-*CB_1*-WT (8) and GFAP-*CB_1*-KO mice (n=7). In (**C**) and (**D**), traces represent 30 superimposed successive fEPSPs before (1, black) and after (2, grey) the HFS stimulation (arrow). (**E**) Bar histograms of fEPSPs from experiments (**C,D**) measured 30-40 min after HFS. (**F,G**) Summary plots of normalized fEPSPs in GFAP-*CB_1*-WT (**F)** and GFAP-*CB_1*-KO littermates (**G**) treated with vehicle (GFAP-*CB_1*-WT, n=4; GFAP-*CB_1*-KO, n=7) or D-serine (GFAP-*CB_1*-WT, n=6; GFAP-*CB_1*-KO, n=5). (**H**) Bar histograms of normalized fEPSPs from experiment (**F** and **G**), 40 minutes after HFS. Top of each summary plot, representative traces of each recording before (1, black) or after (2, grey) HFS stimulation (arrow). . Data, mean ± SEM. *, $P<0.05$. See **Tables S1 and S2** for detailed statistics.

Robin et al. Figure 4

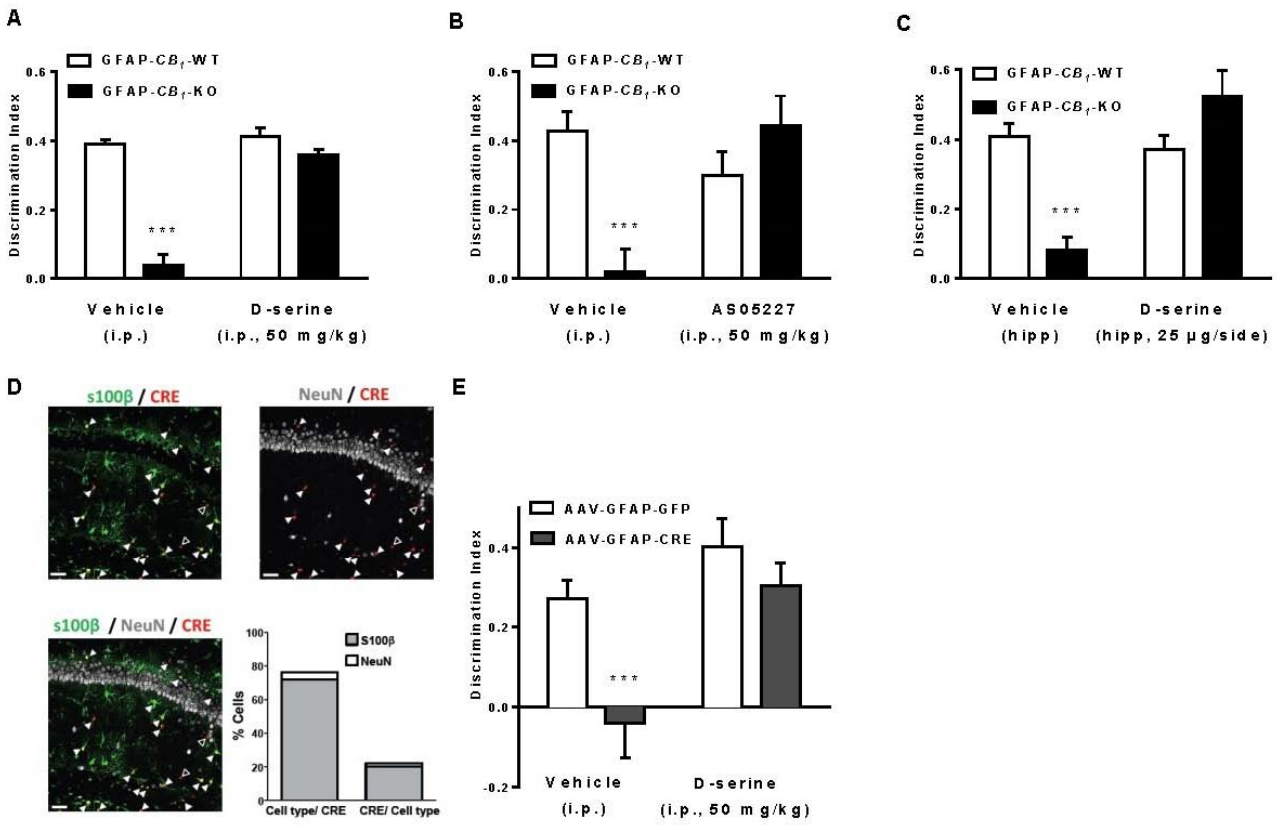

Figure 4. Hippocampal astroglial CB_1 receptors are necessary for object recognition memory through D-serine. (**A**) Memory performance of GFAP-*CB_1*-WT and GFAP-*CB_1*-KO mice injected with vehicle (n=5 both groups) or D-serine (50 mg/kg; i.p; GFAP-*CB_1*-WT, n=4; GFAP-*CB_1*-KO, n=5). (**B**) Memory performance of GFAP-*CB_1*-WT and GFAP-*CB_1*-KO mice injected with vehicle (GFAP-*CB_1*-WT, n=8; GFAP-*CB_1*-KO, n=9) or AS05278 (50 mg/kg; i.p; GFAP-*CB_1*-WT, n=9; GFAP-*CB_1*-KO, n=8). (**C**) Memory performance of GFAP-*CB_1*-WT and GFAP-*CB_1*-KO mice intra-hippocampally injected with vehicle (GFAP-*CB_1*-WT, n=5; GFAP-*CB_1*-KO, n=7) or D-serine (25 µg/side; GFAP-*CB_1*-WT, n=5; GFAP-*CB_1*-KO, n=7). (**D**) Immunofluorescence for s100β (green) and NeuN (white) in the hippocampus of mice injected with AAV-GFAP-CRE-mCherry (red). Filled arrows, cells co-expressing s100β and Cre. Empty arrows, cells expressing only Cre. Scale bar: 50µM. Bottom right, quantification of co-expression indicating the percentage of neurons (NeuN-positive) and astrocytes (S100 positive) containing Cre recombinase over the total Cre-positive cells (left superposed bars) and the percentage of Cre-positive cells over the whole population of neurons and astrocytes (right superposed bars). Data are from 2-3 sections per animal from 8 mice injected with AAV-GFAP-CRE. (**E**) Memory performance of *CB_1*-flox mice intrahippocampally injected with either an AAV-GFAP-GFP or a AAV-GFAP-CRE and treated with vehicle (AAV-GFAP-GFP, n=6; AAV-GFAP-CRE, n=8) or D-serine (50 mg/kg; i.p; AAV-GFAP-GFP, n=7; AAV-GFAP-CRE, n=8). (**F-H**) D-serine (50 mg/kg; i.p.) rescues *in vivo* LTP in GFAP-*CB_1*-KO mice. Data, mean ± SEM. ***, P<0.001. See **Tables S1 and S2** for detailed statistics.

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Supplemental Material for

Astroglial CB_1 receptors determine synaptic D-serine availability to enable recognition memory

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SUPPLEMENTARY ONLINE METHODS

Animals

All experiments were conducted in strict compliance with the European Union recommendations (2010/63/EU) and were approved by the French Ministry of Agriculture and Fisheries (authorization number 3306369) and the local ethical committee (authorization number A50120118). Two to three months-old naïve male C57BL/6N (JANVIER, France), CB_1 flox (mice carrying the "floxed" *CB_1* gene (*$CB1^{f/f}$*) and male GFAP-*CB_1*-KO mutant mice and GFAP-*CB_1*-WT littermates were used. Animals were housed in groups under standard conditions in a day/night cycle of 12/12 hours (light on at 7 am). Experiments were conducted between 2 and 5 pm. Mice undergoing surgery were housed individually after the operation.

GFAP-*CB_1*-KO mice were generated using the Cre/loxP system as previously described (Han et al., 2012). Mice carrying the "floxed" *CB_1* gene (*$CB_1^{f/f}$*) (Marsicano et al., 2003) were crossed with GFAP-CreERT2 mice (Hirrlinger et al., 2006), using a three-step backcrossing procedure to obtain *$CB_1^{f/f;GFAP\text{-}CreERT2}$* and *$CB_1^{f/f}$* littermates, called GFAP-*CB_1*-KO and GFAP-*CB_1*-WT, respectively. As CreERT2 protein is inactive in the absence of tamoxifen treatment (Hirrlinger et al., 2006), deletion of the *CB_1* gene was obtained in adult mice (7-9 weeks-old) by daily i.p. injections of tamoxifen (1 mg dissolved at 10 mg/ml in 90% sesame oil, 10% ethanol, Sigma-Aldrich, St Quentin, France) for 8 days. Mice were used 3-5 weeks after the last tamoxifen injection (Han et al., 2012).

Drug preparation and administration

For behavioral experiments, D-serine (Ascent Scientific, United Kingdom) was dissolved either in 0.9% saline for systemic injections in order to inject 10 ml/kg of body weight in each mouse. For intra-hippocampal infusions, D-serine was dissolved in artificial cerebro-spinal fluid (aCSF). AS05278 (Sigma-Aldrich, France) was dissolved in 0.9 % saline added with 2% DMSO, 10% ethanol. D-AP5 (Sigma-Aldrich, France) was dissolved in artificial cerebrospinal fluid (aCSF). All vehicles contained the same amounts of solvents. All drugs were prepared freshly before the experiments. All drugs were injected either intraperitoneally (i.p.) or intra-hippocampally immediately after the acquisition phase of the NOR task (see below), except for AS05278, which was injected 2 hours before, based on published data indicating the peak of endogenous D-serine at this time point (Adage et al., 2008) D-serine was also intraperitoneally injected 1 hour after the acquisition and right before the test session.

Intra-hippocampal drug infusions (see below) were performed with the aid of 30-gauge injectors protruding 1.0 mm from the end of the cannulae. The volume infused was: 0.3 µl at a rate of 0.3

µl/min. After infusion, injectors were kept in place for 60s to prevent outflow of injected solutions.

Intra-hippocampal drugs and virus delivery

Mice (8-12 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (100mg/kg, Imalgene 500®, Merial) and Xylazine (10mg/kg, Rompun, Bayer) and placed into a stereotaxic apparatus (David Kopf Instruments, CA, USA) with mouse adapter and lateral ear bars. For intra-hippocampal infusions of drugs, mice were bilaterally implanted with 23-gauge stainless steel guide cannulae (Bilaney, Germany) following stereotaxic coordinates (Paxinos and Franklin, 2001) aiming at the dorsal hippocampus (AP – 1.8, L ± 1, DV- 1.3 mm), Guide cannulae were secured with cement anchored to the skull by screws. Mice were allowed to recover for at least one week in individual cages before the beginning of the experiments. During the recovery period, mice were handled daily.

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). 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: dorsal HPC, AP -1.8; ML ±1; DV -2.0 and -1.5; ventral HPC: AP -3.5; ML ±2.7; DV -4 and -3. Following virus delivery, the syringe was left in place for 1 minute before being slowly withdrawn from the brain. CB1flox/flox mice were injected with AAV-GFAP-GFP (control) or AAV-GFAP-CRE (fused to mCherry, serotype 9, UNC Vector Core, USA) to induce deletion of the CB1 gene in hippocampal astroglial cells. Animals were used for experiments 4-5 weeks after injections. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments. To verify the correct pattern of CRE expression and localization, mice were transcardially perfused with paraformaldeyde and their brains were sliced with a vibratome. 40µm hippocampal sections incubated with primary antibody directed against S100 (Rabbit polyclonal, Sigma Aldrich, France) and NeuN (Mouse monoclonal, Millipore, France). Secondary antibodies incubation was performed in order to detect S100with Alexa488 (Thermo Scientific, France) and NeuN with Alexa647 (Thermo Scientific, France). Single plane confocal images were acquired with an SP8 confocal microscope (Leica, France) and minimally processed with ImageJ software. Automatic quantification of mCherry (CRE positive), s100β and /NeuN expressing cells was performed with ImageJ software as previously described (REF Bolte S et al., Journal of Microscopy 224 (3) December 2006). Briefly, after threshold subtraction and crosstalk correction, the number of cells co-expressing mCherry/S100 or mCherry/NeuN was automatically obtained by the “particle analysis” tool of the same software. mCherry/S100 co-expressing cells were expressed in percentage of CRE positive cells as well as percentage of total S100 cells. On the other hand,

mCherryNeuN co-expressing cells were reported as percentage of CRE positive cells as well as percentage of total NeuN cells.

Novel object-recognition memory task

We used the novel object recognition memory task in a L-maze (NOR) (Busquets-Garcia et al., 2013; Busquets-Garcia et al., 2011; Puighermanal et al., 2013; Puighermanal et al., 2009). As compared to other hippocampal-dependent memory tasks, this test presents several advantages for the aims of the present study: (i) the acquisition of NOR occurs in one step and previous studies revealed that the consolidation of this type of memory is deeply altered by acute immediate post-training administration of cannabinoids via hippocampal CB_1 receptors (Puighermanal et al., 2013; Puighermanal et al., 2009); (ii) the NOR test performed in a L-maze decrease variability and give strong and replicable results; (ii) this test allows repeated independent measurements of memory performance in individual animals (Puighermanal et al., 2013), thereby allowing within-subject comparisons, eventually excluding potential individual differences in viral infection.

The task took place in a L-shaped maze made of dark grey polyvinyl chloride shaped 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 scoring offline of animal's behavior.

The task consisted in 3 sequential daily trials of 9 minutes each. During the habituation session (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 acquisition session (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 memory test 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.

The apparatus as well as objects were cleaned with EtOH (70 %) before experimental use and between each animal testing.

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 a distance of less than 2 cm. Experienced investigators evaluating the exploration were blind of treatment and/or genotype of the animals.

***In vivo* electrophysiology**

GFAP-CB_1-KO and WT littermate mice were anesthetized in a box containing 5% Isoflurane (VIRBAC, France) before being placed in a stereotaxic frame (model SR-6M-HT, Narishige International, London, UK) in which 1.0% to 1,5% of Isoflurane was continuously supplied via an anesthetic mask during the complete duration of the experiment. The body temperature was maintained at 37°C using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA) and the complete state of anesthesia was assured through a mild tail pinch. Before surgery, 100 µl of the local anesthetic Lurocaine® (Vetoquinol, Lure, 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 correct alignment of the head, two holes were drilled in the skull to place: a glass recording electrode, inserted in the CA1 *stratum radiatum*, and one concentric bipolar electrode (Model NE-100, KOPF Instruments, Tujunga, CA, USA) in the CA3 region using the following coordinates: 1) CA1 *stratum radiatum*: A/P -1.5 mm, M/L -1.0 mm, DV 1.20 mm; CA3: A/P -2.5 mm, M/L -2.8, D/V -2.0 mm. The recording electrode (tip diameter = 1–2 µm, 4–6 MΩ) 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 an automatic micropositioner (MIM100-2, M2E, France). The stimulation electrode was placed in the correct area using a micromanipulator (UNI-Z, M2E, France). Both electrodes were adjusted to find the area with maximum response. *In vivo* recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 10 times by Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA, USA) before being further amplified 100 times and filtered (low pass at 1 Hz and high-pass at 5000Hz) *via* a differential AC amplifier (model 1700; A-M Systems, Sequim, WA, 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 300 sec. Test pulse intensities were typically between 50-250 µA with a duration of 500 µs. Basal stimulation intensity was adjusted to 30-40% of the current intensity that evoked a maximum field response. All responses were expressed as percent from the average responses recorded during the 10 min before high frequency stimulation (HFS). HFS was induced by applying 3 trains of 100

Hz (1 sec each), separated by 20 seconds interval. fEPSP were then recorded for a period of 40 minutes. In the specific group of mice the following treatments were applied: 1) MK 801 (Abcam, Cambridge, UK; 3 mg/kg, i.p., dissolved in saline, approx. 60 min before HFS) or vehicle (saline, i.p., approx. 60 min before HFS) 2) D-serine (Abcam, Cambridge, UK; 50 mg/kg, i.p., dissolved in saline) approx. 2 hours before HFS or vehicle (saline, i.p.). At the end of each experiment, the position of the electrodes was marked by iontophoretic infusion of the recording solution during 180s at -20 µA and continuous current discharge over 20 seconds at +20 µA for recording and stimulation areas, respectively. Histological verification was performed *ex vivo*.

In vitro Electrophysiology

Coronal hippocampal slices (350 µm) were prepared from adult GFAP-CB_1-WT or GFAP-CB_1-KO mice as described previously (Papouin et al., 2012). Briefly, mice were anesthetized with isoflurane and then decapitated. The brain was quickly extracted and placed in aCSF saturated with 95% O_2 and 5% CO_2. ACSF contained (in mM): 125 NaCl, 2.5 KCl, 1 Na_2HPO_4, 1.2 $MgCl_2$, 0.6 $CaCl_2$, 26 $NaHCO_3$ and 11 mM glucose (pH 7.4; 300 mosmol/kg). Coronal slices were cut from a block of tissue containing the hippocampus using a vibratome (Microm HM 650V). Slices were hemisected and maintained at 33°C during 30 min in ACSF containing this time, 2 mM $MgCl_2$ and 1 mM $CaCl_2$. Then, they were allowed to recover at room temperature for at least 1h.

Slices were transferred into a recording chamber perfused with ACSF (2.8 ml/min) containing 1.3 mM $MgCl_2$ and 2.5 mM $CaCl_2$, and maintained at 30°C. Field excitatory postsynaptic potentials (fEPSPs) slope were recorded with a Multiclamp 700A amplifier (Axon Instruments, Inc.) using pipettes (2-3 MΩ) filled with ACSF and placed in the *stratum radiatum* of CA1 area. Synaptic responses were evoked at 0.05 Hz by orthodromic stimulation (100 µs duration) of Schaffer collaterals using a concentric bipolar tungsten electrode placed >200 µm away from the recording electrodes. For LTP experiments, stimulation intensity was set to 35% of that triggering population spikes. After a stable baseline of at least 10 minutes, LTP was induced by applying a high-frequency stimulation (HFS) protocol consisting of a 100 Hz train of stimuli for 1 s repeated three times at 20 s intervals. NMDAR-fEPSPs were recorded in low Mg^{2+} ACSF (0.2 mM) with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10 µM) to block AMPA/kainate receptors. At the end of each experiment, D-AP5 (50 µM), was applied to isolate the remaining GABAergic component which was then subtracted from the responses to obtain pure NMDAR-fEPSPs. Average fEPSP and NMDAR-fEPSP traces correspond to 10 min and 20 min of stable recording, respectively. For clarity the stimulation artifact was deleted.

Signals were filtered at 2 kHz and digitized at 10 kHz. Data were collected and analyzed using pClamp9 software (Axon Instruments, Inc.).

Ca^{2+} Imaging

Ca^{2+} levels in astrocytes located in the stratum radiatum of the CA1 region of the hippocampus were monitored by fluorescence microscopy using the Ca^{2+} indicator fluo-4 AM (Molecular Probes, Eugene, OR). Slices were incubated with fluo-4 AM (2 µl of 2 mM dye were dropped over the hippocampus, attaining a final concentration of 2 µM and 0.01 % of pluronic for 20-30 min at room temperature. In these conditions, most of the cells loaded were astrocytes, as confirmed by their electrophysiological properties and SR101 staining. Astrocytes were imaged using a Leica SP5 multiphoton microscope and images were acquired at 1 to 2 Hz. Intracellular Ca^{2+} signals were monitored from astrocytic somas and processes and the signal was measured as fluorescence over baseline [(Fluorescence $_t$ - Background fluorescence $_t$) - (Fluorescence $_0$ - Background fluorescence $_0$)]/ (Fluorescence $_0$ - Background fluorescence $_0$) and cells were considered to display a Ca^{2+} event when the calcium signal increased three times the standard deviation of the baseline.

The astrocyte Ca^{2+} signal was quantified as the probability of occurrence of a Ca^{2+} event (calcium event probability). The Ca^{2+} event probability was calculated as the number of somas or processes starting a calcium event per time bin in a field of view, divided by the number of somas or processes in that field of view (8-12 somas and 15-20 processes in each field of view). Events were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca^{2+} event. The calcium event probability during 20 seconds before the WIN 515,212-2 (WIN) application (200 µM, 3 s , 10 psi) was compared with the calcium event probability in the time bin after the WIN application. WIN was dissolved in DMSO and then 36 µl of the DMSO-WIN solution was diluted in 1 ml of ACSF, obtaining a final concentration of 200 µM used in the pressure-pulse pipette. We estimate, based on quantifications of Alexa 594 fluorescence, that the WIN concentration becomes further diluted after being pressure ejected in the bath ACSF to approximately 1-10 µM around the recorded cells (Navarrete and Araque, 2008). In some cases, experiments were performed in the presence of the CB_1 antagonist AM251 (2 µM). Mean values were obtained from at least 5 slices and 2 mice in each condition.

Measurement of aminoacids in hippocampal slices.

For the simultaneous measurement of D-serine, glutamate, glycine and GABA, a capillary electrophoresis-laser induced fluorescence detection method was used.

Five hippocampi from adult C57Bl-6N mice (10-12 weeks old) were isolated from 350 µm slices and incubated in 350 µl oxygenated ACSF containing 0.5 µM TTX with either vehicle (1/4000 DMSO) or WIN 55,212-2 (5 µM in DMSO) during 30 min at 31°C. Extracellular medium was quickly extracted, frozen using liquid nitrogen and stored at -80°C. Extracellular levels of D-serine, glutamate, glycine and GABA were then determined. Briefly, pooled slices were

deproteinized by addition of cold trichloroacetic acid (TCA) to a 4% final concentration. The suspension was centrifuged at 16,800 g for 10 min, the TCA was extracted from the supernatant with water-saturated diethyl ether and stored at -80°C. Samples were analyzed with a commercial laser-induced fluorescence capillary electrophoresis (CE-LIF) (CE: Beckman Coulter (Brea, California, US), P/ACE MDQ; LIF: Picometrics (Labège, France), LIF-UV-02, 410 nm 20 mW) as following: samples were processed for micellar CE-LIF and were fluorescently derivatized at RT for 60 min with napthalene-2,3-dicarboxaldehyde (NDA) before being analyzed by CE using a hydroxypropyl-β-cyclodextrin (HP-b-CD) based chiral separation buffer. All electropherograms data were collected and analyzed using Karat 32 software v8.0 (Beckman Coulter, France). The extracellular amounts of D-serine, glutamate, glycine and GABA were normalized to the protein content determined from pooled hippocampal slices by the Lowry method using the BCA protein Pierce (ThermoScientific, CA) assay with bovine serum albumin (BSA) as standards. The quantity of D-serine, glutamate, glycine and GABA in the samples was determined from a standardized curve while peak identification was made by spiking the fraction with the amino acid.

Statistical analyses

Data were expressed as mean ± SEM or single data points and were analyzed with Prism 6.0 (Graphpad Software), using *t*-test (paired, unpaired or 1-sample), Mann Whitney test or ANOVA (One- or Two-Way), where appropriate. Dunnet's (One-Way ANOVA) or Bonferroni's (Two-Way ANOVA) *post-hoc* tests were used. Statistical details for each quantitative experiment are illustrated in **Table S1 (**for main figures) and **Table S2** (for supplemental figures).

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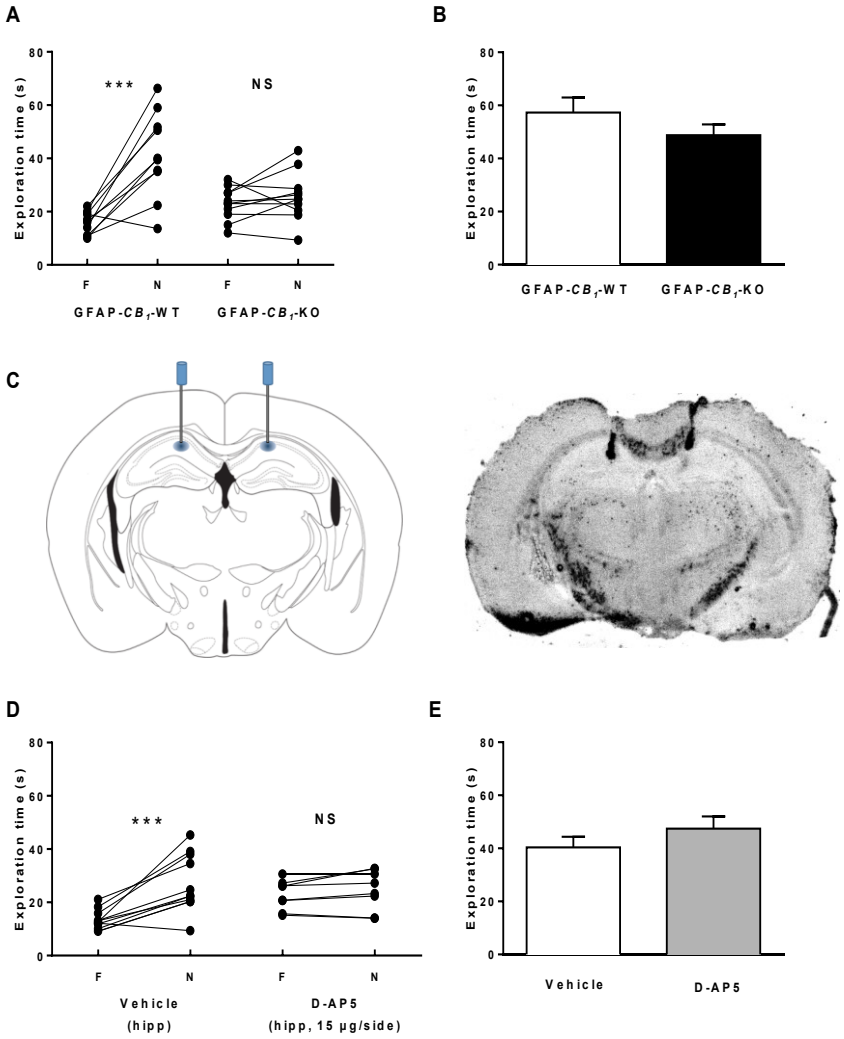

Robin et al. Figure S1

Figure S1. Related to Figure 1. (A) Exploration time of the familiar (F) and the novel object (N) of GFAP-*CB*$_1$-WT mice and GFAP-*CB*$_1$-KO littermates. (**B**) Total exploration time of GFAP-*CB*$_1$-WT mice and GFAP-*CB*$_1$-KO littermates. (**C**) Schematic drawing of local hippocampal injection (left) and representative image of injection sites (right). (**D**) Exploration time of the familiar (F) and the novel object (N) of C57BL/6-N and GFAP-*CB*$_1$-WT mice intra-hippocampally injected with vehicle or D-AP5 (15 µg/side) in the NOR task. (**E**) Total exploration time of C57BL/6-N and GFAP-*CB*$_1$-WT mice intra-hippocampally injected with vehicle or D-AP5 (15 µg/side). Data, mean ± SEM. ***, P<0.001, NS, not significant. See **Table S2** for detailed statistics.

Robin et al. Figure S2

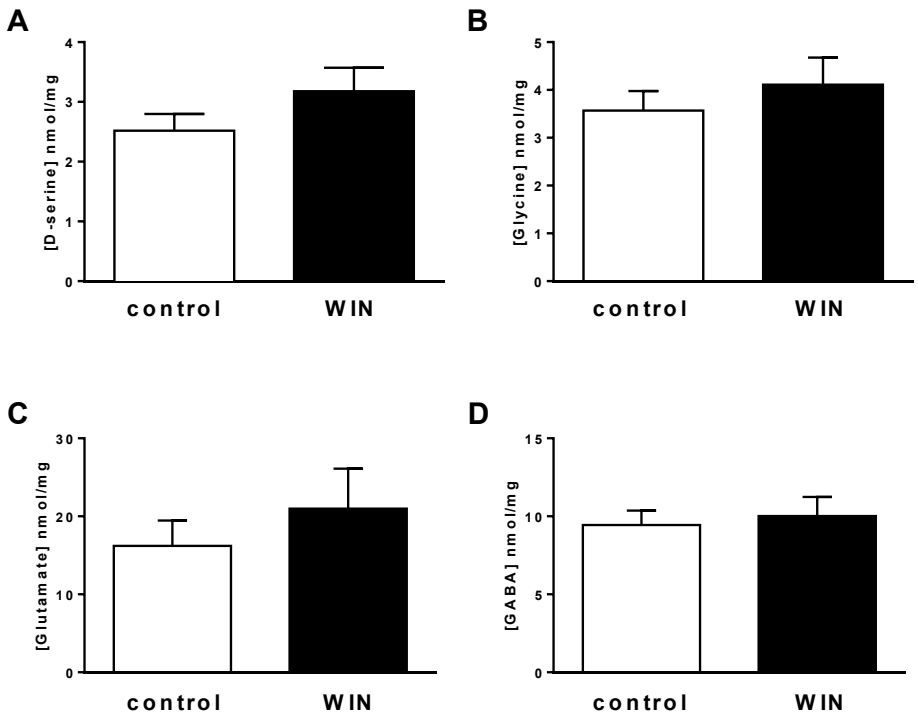

Figure S2. Related to Figure 2. Determination of D-serine (**A),** Glycine (**B),** L-glutamate (**C)** and GABA (**D)** as measured by capillary electrophoresis in mouse hippocampal slices treated with vehicle or WIN. Data, mean ± SEM. *, P<0.05 See **Table S2** for detailed statistics.

Robin et al. Figure S3

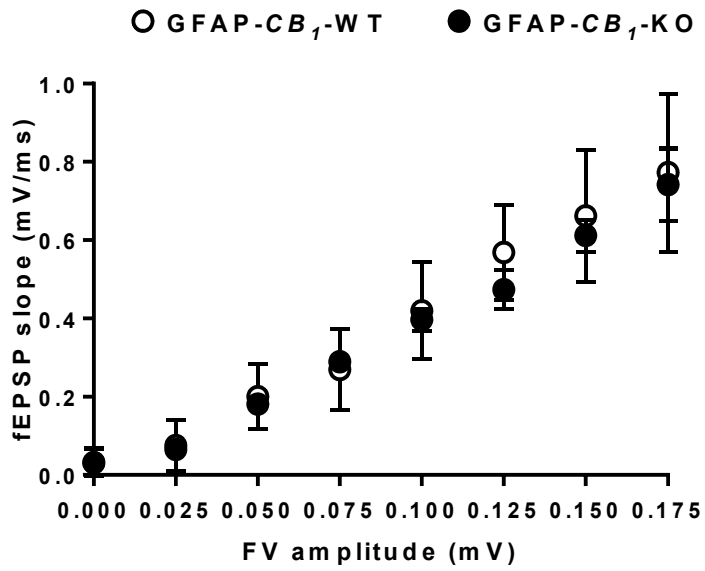

Figure S3. Related to Figure 3. Input-output curves of fEPSPs in GFAP-CB_1-WT (8) and GFAP-CB_1-KO mice (n=7). Data, mean ± SEM. See **Table S2** for detailed statistics.

Robin et al. Figure S4

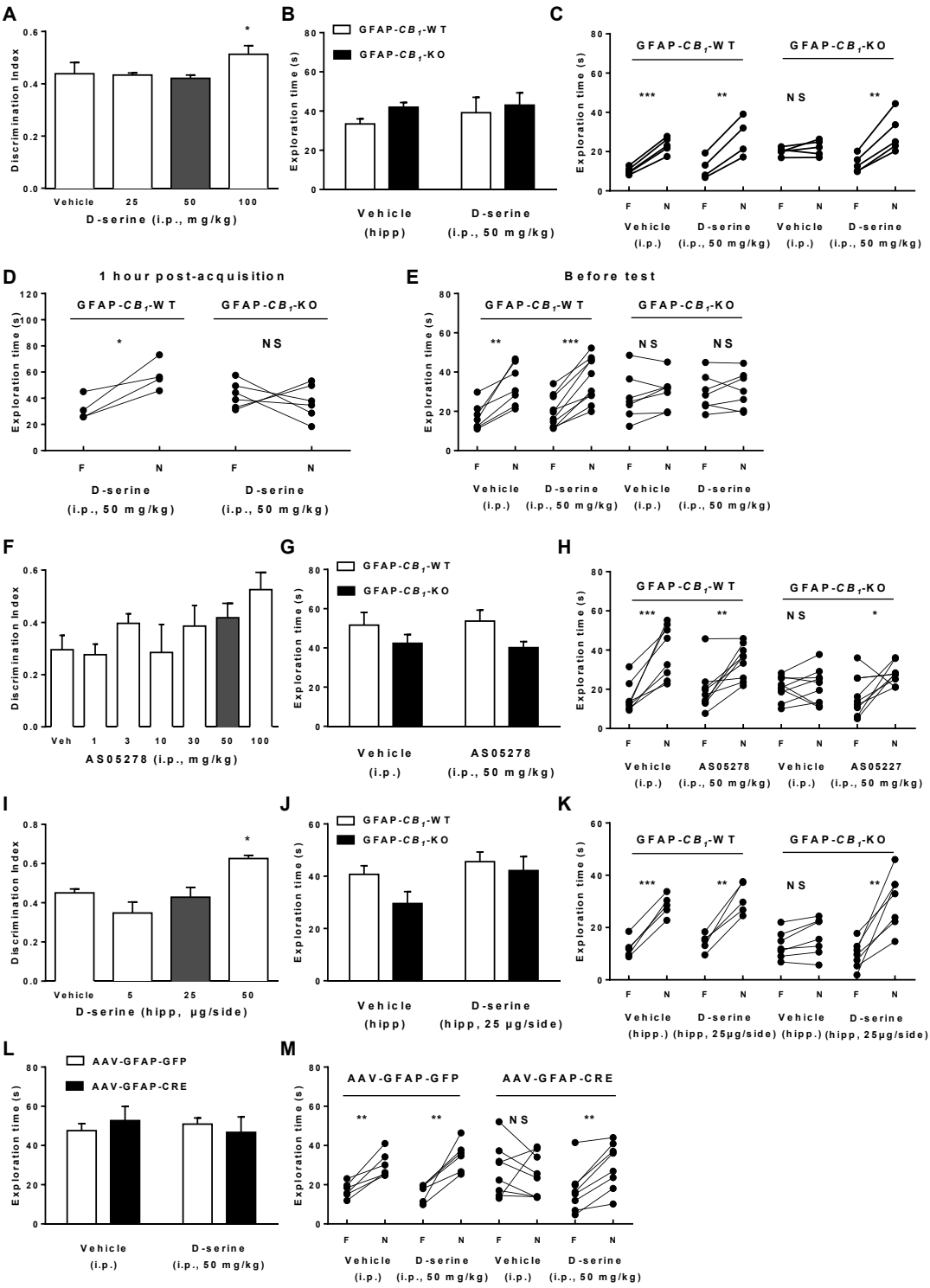

Figure S4. Related to Figure 4. Effects of D-serine and AS05278 on NOR task. (**A**) Effect of vehicle or different doses of D-serine (25, 50 or 100 mg/kg, i.p.) on memory performance in wild-type mice. Grey bar, sub-effective dose used in following experiments. (**B**) Total exploration time of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with vehicle or D-serine (50 mg/kg, i.p.). (**C**) Exploration time of the familiar and the novel objects of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with vehicle or D-serine (50 mg/kg, i.p.) immediately after acquisition. (**D,E**) Exploration time of the familiar and the novel objects of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with D-serine (50 mg/kg, i.p.) 1-hour after acquisition (**D**) and immediately before test (**E**). (**F**) Effect of vehicle or different doses of AS05278 (1, 3, 10, 30, 50 or 100 mg/kg i.p.) on memory performance of wild-type mice. Grey bar, sub-effective dose used in following experiments. (**G**) Total exploration time of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with vehicle or AS05278 (50 mg/kg, i.p.). (**H**) Exploration time of the familiar and the novel object of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with vehicle or AS05278 (50 mg/kg, i.p.). (**I**) Effect of intra-hippocampal vehicle or different doses of D-serine (5, 25 or 50 µg/side) on memory performances of wild-type mice. Grey bar, sub-effective dose used in following experiments. (**J**) Total exploration time of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with intra-hippocampal vehicle or D-serine (25 µg/side). (**K**) Exploration time of the familiar and the novel object of GFAP-CB_1-WT mice and GFAP-CB_1-KO littermates injected with intra-hippocampal vehicle or D-serine (25 µg/side). (**L**) Total exploration time of both objects of mice treated with vehicle or D-serine (50 mg/kg, i.p.;). (**M**) Object exploration time of the familiar and the novel object of CB_1-flox mice intra-hippocampally injected with either a AAV-GFAP-GFP or a AAV-GFAP-CRE, and treated with vehicle or D-serine (50 mg/kg, i.p.). Data, mean ± SEM. *, P<0.05, **, P<0.01, ***, P<0.001, NS, not significant. See **Table S2** for detailed statistics.

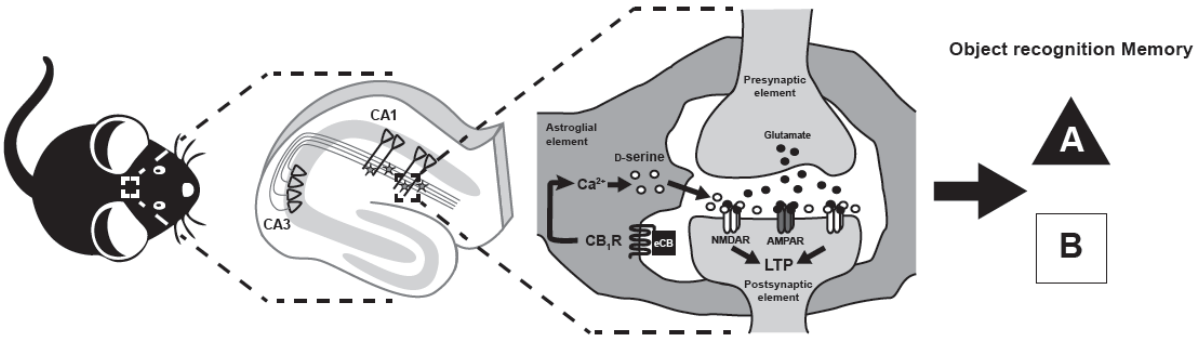

Figure S5. Schematic summary of the results. In adult mice, at the hippocampal CA3-CA1 synapse, astroglial CB1 receptors regulate cellular Ca^{2+} levels, synaptic D-serine availability and thus D-serine-dependent synaptic NMDAR gating. By this means, astroglial CB1 receptors control synaptic NMDAR-dependent long-term potentiation (LTP) and object recognition memory. eCB; endocannabinoid.

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Figure	Conditions	"n" (per group)	Analysis (post-hoc test reported in figures	Factors analyzed	F-ratios	P values
1A	GFAP-CB_1-WT vs GFAP-CB_1-KO	10-11	Unpaired *t-test*			P = 0.0002
1B	Vehicle vs D-AP5	8-10	Unpaired *t-test*			P < 0.0001
1C	Vehicle vs MK801 / time	5-6	2-WAY ANOVA	Treatment x Time	Treatment F(1,9) = 12.93	P = 0.0058
					Time F(9,81) = 5.60	P < 0.0001
					Interaction F(9,81) = 9.22	P < 0.0001
1D	Vehicle vs MK801	5-6	Unpaired *t-test*			P = 0.0054
1E	GFAP-CB_1-WT vs GFAP-CB_1-KO / time	6-9	2-WAY ANOVA	Genotype x Time	Genotype F(1,113) = 3.87	P = 0.071
					Time F(9,117) = 2.18	P = 0.0282
					Interaction F(9,117) = 4.28	P < 0.0001
1F	GFAP-CB_1-WT vs GFAP-CB_1-KO	6-9	Unpaired *t-test*			P = 0.0250
2C	GFAP-CB1-WT vs GFAP-CB1-KO basal vs treatment	9-16	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(2,34) = 3.149	P = 0.0556
					Treatment F(1,34) = 31.73	P < 0.0001
					Interaction F(2,34) = 12.72	P < 0.0001
2E	GFAP-CB1-WT vs GFAP-CB1-KO basal vs treatment	7-10	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(2,22) = 9.836	P = 0.0009
					Treatment F(1,22) = 20.01	P = 0.0002
					Interaction F(2,22) = 10.48	P = 0.0006
2F	Control vs WIN	7	Mann-Whitney test			P = 0.0379
2G	Control vs WIN	7	Mann-Whitney test			P = 0.0973
2H	Control vs WIN	7	Mann-Whitney test			P = 0.9015
2I	Control vs WIN	7	Mann-Whitney test			P = 0.3374
3A	GFAP-CB_1-WT vs GFAP-CB_1-KO/Time	7	2-WAY ANOVA (Bonferroni)	Genotype x Time	Genotype F(1,12) = 8.96	P = 0.0112
					Time F(59,708) = 18.26	P < 0.0001
					Interaction F(59,708) = 3.08	P < 0.0001
3B	GFAP-CB_1-WT vs GFAP-CB_1-KO	7	Unpaired *t-test*			P = 0.0121
3C	GFAP-CB_1-WT vs GFAP-CB_1-KO / Time	12-16	2-WAY ANOVA (Bonferroni)	Genotype x Time	Genotype F(1,26) = 7.965	P = 0.009
					Time F(50,1300) = 20.79	P < 0.0001
					Interaction F(50,1300) = 2.16	P < 0.0001
3D	GFAP-CB_1-WT vs GFAP-CB_1-KO / Time	7-8	2-WAY ANOVA (Bonferroni)	Genotype x Time	Genotype F(1,13) = 0.039	P = 0.8453
					Time F(47,611) = 30.92	P < 0.0001
					Interaction F(47,611) = 0.828	P = 0.7859
3E	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs D-serine	7-16	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,39) = 2.59	P = 0.1153
					Treatment F(1,39) = 5.61	P = 0.023
					Interaction F(1,39) = 1.68	P = 0.2019
3F	Vehicle vs D-serine / time	4-6	2-WAY ANOVA (Bonferroni)	Treatment x Time	Treatment F(1,8) = 0.31	P = 0.5920
					Time F(9,72) = 5.86	P < 0.0001
					Interaction F(9,72) = 0.43	P = 0.9163
3G	Vehicle vs D-serine / time	5-7	2-WAY ANOVA (Bonferroni)	Treatment x Time	Treatment F(1,10) = 18.31	P = 0.0016
					Time F(9,90) = 6.44	P < 0.0001
					Interaction F(9,90)=12.72	P < 0.0001
3H	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs D-serine	4-7	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,18) = 1.23	P = 0.2821
					Treatment F(1,18) = 3.21	P = 0.0901
					Interaction F(1,18) = 7.71	P = 0.0125

4A	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs D-serine	4-5	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,15) = 88.27	P < 0.0001
					Treatment F(1,15) = 63.23	P < 0.0001
					Interaction F(1,15) = 49.07	P < 0.0001
4B	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs AS05278	8-9	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,30) = 3.62	P = 0.0668
					Treatment F(1,30) = 4.39	P = 0.0447
					Interaction F(1,30) = 15.37	P = 0.0005
4C	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs D-serine	5-7	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,20) = 2.48	P = 0.1311
					Treatment F(1,20) = 14.12	P = 0.0012
					Interaction F(1,20) = 19.80	P = 0.0002
4D	Quantification of CRE/S110b and CRE/NeuN co-expression	16	2-WAY ANOVA (Bonferroni)	Cell type x CRE	Cell type F(1,60) = 444,4	P < 0.0001
					CRE F(1,60) = 175,3	P < 0.0001
					Interaction F(1,60) = 148,7	P < 0.0001
4E	AAV-GFAP-GFP vs AAV-GFAP-CRE Vehicle vs D-serine	6-8	2-WAY ANOVA (Bonferroni)	Virus x Treatment	Virus F(1,25) = 8.74	P = 0.0067
					Treatment F(1,25) = 11.70	P = 0.0022
					Interaction F(1,25) = 2.34	P = 0.1384

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Figure	Conditions	"n" (per group	Analysis (*post-hoc* test reported in figures)	Factor analyzed	F ratios	P values
S1A	Exploration Novel object vs familiar					
	GFAP-CB_1-WT	10	paired *t-test*			P = 0.0005
	GFAP-CB_1-KO	11	paired *t-test*			P = 0.2466
S1B	GFAP-CB_1-WT vs GFAP-CB_1-KO	10-11	Unpaired *t-test*			P= 0.2306
S1D	Exploration Novel object vs familiar					
	Vehicle	11	paired *t-test*			P = 0.0005
	AP5	8	paired *t-test*			P = 0.1284
S1E	Exploration Novel object vs familiar					
	Vehicle vs AP5	8-11	unpaired *t-test*			P = 0.2637
S2A	Control vs WIN	8	Mann-Whitney test			P = 0.1975
S2B	Control vs WIN	7	Mann-Whitney test			P = 0.5350
S2C	Control vs WIN	5	Mann-Whitney test			P = 0.4206
S2D	Control vs WIN	7	Mann-Whitney test			P = 1
S3D	GFAP-CB_1-WT vs GFAP-CB_1-KO fEPSP slope / FV amplitude	11-16	2-WAY ANOVA (Bonferroni)	Genotype x FV amplitude	Genotype F(1,24) = 2.63	P = 0.1063
					Fv amplitude F(7,237) = 119.5	P < 0.0001
					Interaction F(7,237) = 0.69	P = 0.6809
S4A	Dose of D-serine	5	1-way ANOVA (Dunnett's)		F(3,16) = 2.194	P = 0,0128
S4B	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs D-serine	4-5	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,15) = 1.50	P = 0.2394
					Treatment F(1,15) = 0.46	P = 0.5092
					Interaction F(1,15) = 0.22	P = 0.6455
S4C	Exploration Novel object vs familiar					
	GFAP-CB_1-WT / Vehicle	5	paired *t-test*			P = 0.0002
	GFAP-CB_1-WT / D-serine	4	paired *t-test*			P = 0.0062
	GFAP-CB_1-KO / Vehicle	5	paired *t-test*			P = 0.2666
	GFAP-CB_1-KO / D-serine	5	paired *t-test*			P = 0.0034
S4AD	Exploration Novel object vs familiar					
	GFAP-CB_1-WT	4	paired *t-test*			P = 0.0308
	GFAP-CB_1-KO	6	paired *t-test*			P = 0.5701
S4E	Exploration Novel object vs familiar					
	GFAP-CB_1-WT / Vehicle	7	paired *t-test*			P = 0.0032
	GFAP-CB_1-WT / D-serine	10	paired *t-test*			P < 0.0001
	GFAP-CB_1-KO / Vehicle	7	paired *t-test*			P = 0.2375
	GFAP-CB_1-KO / D-serine	7	paired *t-test*			P = 0.5468
S4F	Dose of AS05278	5-7	1-way ANOVA (Dunnett's)		F(6,37) = 2.117	P = 0.0744
S4G	GFAP-CB_1-WT vs GFAP-CB_1-KO Vehicle vs AS05278	8-9	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,32) = 5.10	P = 0.0309
					Treatment F(1,32) = 0.00	P = 0.9878
					Interaction F(1,32) = 0.18	P = 0.6771
S4H	Exploration Novel object vs familiar					
	GFAP-CB_1-WT / Vehicle	8	paired *t-test*			P = 0.001
	GFAP-CB_1-WT / AS05278	9	paired *t-test*			P = 0.0031
	GFAP-CB_1-KO / Vehicle	9	paired *t-test*			P = 0.5975
	GFAP-CB_1-KO / AS05278	9	paired *t-test*			P = 0.0392

S4I	Dose of D-serine (hipp.)	4-10	1-way ANOVA (Dunnett's)		F(3,23) = 5.043	P = 0.0079
S4J	GFAP-CB_1-WT vs GFAP-CB_1-KO/Vehicle vs D-serine (hipp.)	5-7	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,22) = 0.77	P = 0.0.3887
					Treatment F(1,22) = 2.68	P = 0.01159
					Interaction F(1,22) = 0.23	P = 0.6336
S4K	Exploration Novel object vs familiar					
	GFAP-CB_1-WT / Vehicle	5	paired *t-test*			P = 0.0002
	GFAP-CB_1-WT / D-serine	5	paired *t-test*			P = 0.0018
	GFAP-CB_1-KO / Vehicle	7	paired *t-test*			P = 0.3847
	GFAP-CB_1-KO / D-serine	7	paired *t-test*			P = 0.0017
S4L	AAV-GFAP-GFP vs AAV-GFAP-CRE: Vehicle vs D-serine	6-8	2-WAY ANOVA (Bonferroni)	Virus x Treatment	Virus F(1,25) = 0.01	P = 0.9419
					Treatment F(1,25) = 0.05	P = 0.8325
					Interaction F(1,25) = 0.55	P = 0.4649
S4M	Exploration Novel object vs familiar					
	AAV-GFAP-GFP / Vehicle	6	paired *t-test*			P = 0.0036
	AAV-GFAP-GFP / D-serine	7	paired *t-test*			P = 0.0019
	AAV-GFAP-CRE / Vehicle	8	paired *t-test*			P = 0.6639
	AAV-GFAP-CRE / D-serine	8	paired *t-test*			P = 0.0013

PART 2 – DELETION OF CB1 RECEPTORS IN HIPPOCAMPAL D1-POSITIVE CELLS IMPAIRS OBJECT RECOGNITION MEMORY AND ASSOCIATED SYNAPTIC PLASTICITY

Deletion of CB1 receptors in hippocampal D1-positive cells impairs object recognition memory and associated synaptic plasticity

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The endocannabinoid system is a major brain modulatory system controlling a plethora of different functions such as memory and learning mainly *via* the cannabinoid type-1 (CB1) receptor-dependent modulation of neuronal and glial activity (Metna-Laurent and Marsicano, 2015, Piomelli, 2003, Soria-Gomez et al., 2017). Hippocampal Dopamine type-1 (D1)-like receptor-mediated transmission has been shown to underlie the consolidation of learning and enhancement of long-term potentiation (LTP) (Lisman et al., 2011, Li et al., 2003). Although functional cross-talk between CB1 and D1-like receptors has been reported in other brain regions (Bloomfield et al., 2016, Hermann et al., 2002, Terzian et al., 2011), there is currently no evidence of the expression of CB1 receptors in D1-positive cells in the hippocampus and their physiological role in the modulation of hippocampal functions remains largely unknown.

To address this issue we tested mutant mice lacking CB1 receptors in D1-positive cells (D1-CB1-KO mice) (Monory et al., 2007) in a hippocampal long-term novel object recognition memory task (NORT) using the L-maze (Fig. 1A). D1-CB1-KO mice showed strong memory impairment in long-term memory performance as compared to their wild-type littermates (Fig. 1B), with no changes in the overall total exploration time (Fig. 1C). Furthermore, D1-CB1-KO mice displayed no impairment in the short-term memory version of NORT (Fig 1D, E, F), indicating a specific role of CB1 receptors expressed in D1-positive cells in long-term memory formation.

CB1 receptors are abundant in D1-positive medium spiny neurons of the striatum (Monory et al., 2007), a region that can indirectly modulate memory by the regulation of attentional and motivational states (Goldfarb et al., 2016, Tort et al., 2008). However, endogenous modulation of novel object recognition memory depends on CB1 receptors in the hippocampus (Robin et al., submitted). To address the specific contribution of each brain region to the consolation of long-term memory, we infused a viral vector leading to the Cre-dependent expression of the CB1 receptor (rAAV-CAG-DIO-CB1) or control (rAAV-CAG-DIO) in either the hippocampus or the striatum of D1-CB1-KO mice. Thanks to the Cre-dependency of the viral expression vector, this manipulation should lead to the selective re-expression of CB1 in cells where its gene is deleted in the mutant mice. Anatomical studies are currently being performed to confirm this directly. The infusion of rAAV-CAG-DIO-CB1 in the striatum of D1-CB1-KO mice was not able to rescue the memory performance of the mutant mice (Fig. 2A, B). Strikingly, however, the same manipulation in the hippocampus fully rescued the NORT memory phenotype of D1-CB1-KO mice (Fig. 2A, B), indicating that hippocampal CB1 receptors in D1-positive neurons are necessary for the consolidation of long-term memory.

Activity-dependent plasticity of synaptic transmission is thought to be one of the cellular mechanisms of hippocampal long-term memory formation (Nicoll, 2017, Whitlock et al., 2006). CB1 receptors in the hippocampus have been shown to be necessary for the induction of LTP (Robin et al., submitted). To address the role of CB1 receptors in D1-positive cells in the modulation of synaptic plasticity, we recorded *in vivo* evoked field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA3-CA1 pathway of anesthetized mutant mice (Fig. 3 A, E). High Frequency Stimulation (HFS) was able to induce a LTP of synaptic fEPSPs in both D1-CB1-KO and wild-type littermates (Fig. 3 B, C, D), suggesting that CB1 receptors expressed in D1-positive cells are not necessary for LTP induction or maintenance in basal conditions. Exposure to novelty has been shown to enhance long-term memory and hippocampal LTP by modulating dopamine activity through hippocampal D1-like receptors (Lemon and Manahan-Vaughan, 2006, Li et al., 2003), suggesting that learning itself induce molecular changes that influence plasticity. To test the impact of learning on LTP, we trained animals in NORT and immediately after we checked for the induction and maintenance of LTP (Fig. 3F). Interestingly, exposition to training before HFS induced an LTP in wild-type control animals (Fig. 3 G, H) that is impaired in D1-CB1-KO littermates (Fig. 3 G, H). Thus, physiological activation of CB1 receptors in D1-positive cells is not involved in basal conditions (*i.e.* homecage), but it is required for learning-induced modulation of LTP.

This study provides novel functional evidence that CB1 receptors expressed in D1-positive cells control recognition memory and learning-induced modulation of LTP. The modulation of *in vivo* LTP and memory functions through D1 receptors in hippocampus has been consistently shown (Frey et al., 1990, Granado et al., 2008, Li et al., 2003, Lemon and Manahan-

Vaughan, 2006) however the mechanism is not currently known. The presence of CB1 receptors in cells expressing D1 receptors in the hippocampus has been suggested (Monory et al., 2007) and functional behavioral evidence connects its function with the modulation of aversive memories (Micale et al., 2017). Although there is currently no definitive anatomical evidence showing CB1 receptor in D1-positive cells in the hippocampus, it has been recently reported that they co-localize with D1-positive CCK- and VGluT3-positive cells GABAergic interneurons in the hippocampus (Puighermanal et al., 2017). Previous results show that CB1 receptors in astrocytes are necessary for object recognition memory formation and LTP (Robin et al., submitted). D1-positive astrocytes have been recently described in the brain (Nagatomo et al., 2017) and although it has never been reported the presence of D1 receptors in hippocampal astrocytes, it has been recently shown that this cell type respond to D1-agonists (Jennings et al., 2017). Thus, our data suggests that D1-expressing cells in the hippocampus might also be a subclass of astrocytes.

Overall, these results provide further functional evidence of the role of CB1 receptors in the complex cell type-dependent regulation of long-term recognition memory and synaptic plasticity.

Author Contributions

JFOdC performed and analyzed *in vivo* electrophysiology an experiments and wrote the manuscript. AB-G and ESG performed and analyzed behavioral. LB contributed to behavioral experiments. ZZ and MV helped with the analysis of the data. GM and ES-G conceived and supervised the whole project and wrote the manuscript. All authors edited and approved the manuscript.

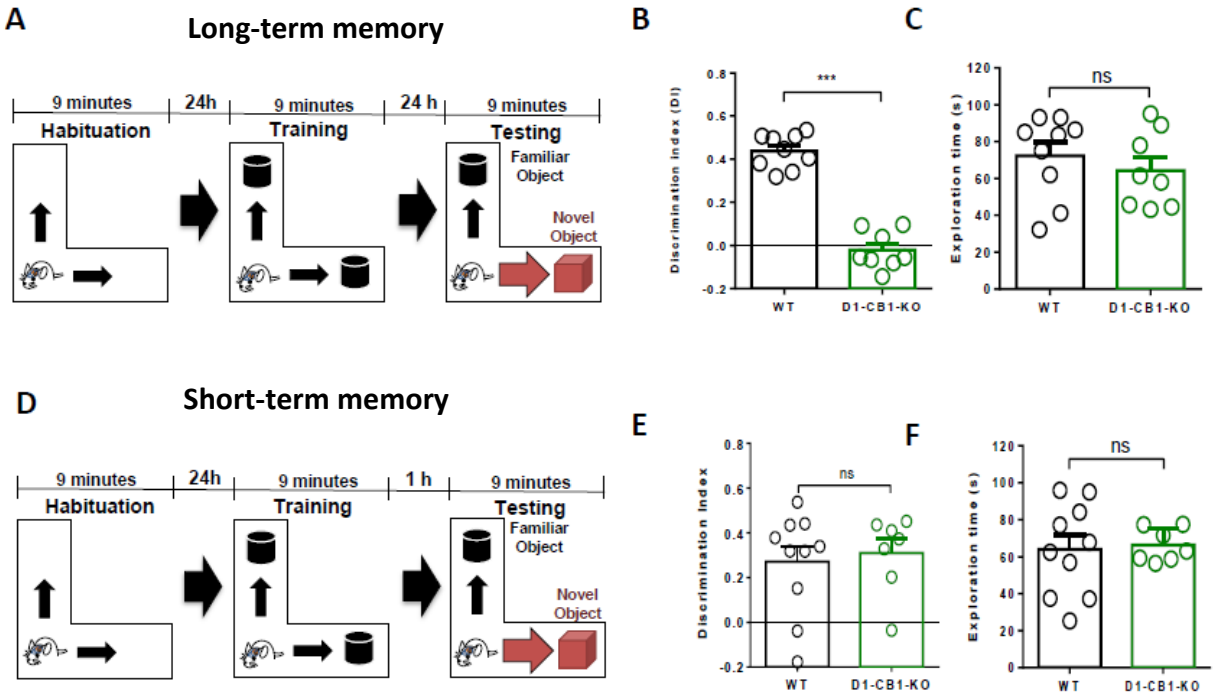

Figure 1 – CB1 receptors in D1-positive cells modulate the consolidation of long-term memory formation.

(**A, D**) Schematic representation of the Novel Object Recognition Task (NORT) in an L-Maze to evaluate long- and short-term memory, respectively. (**B**) Memory performance of D1-CB1-WT mice (n=9) and D1-CB1-KO (n=8) littermates in the NOR task for long-term memory (**E**) Memory performance of D1-CB1-WT mice (n=9) and D1-CB1-KO (n=7) littermates in the NOR task for short-term memory. (**C, F**) Total object exploration during the testing phase for D1-CB1-WT and D1-CB1-KO in long- and short-term memory, respectively. Data mean ± SEM. ***, P<0.001. ns, not significant (D1-CB1-WT vs D1-CB1-KO)

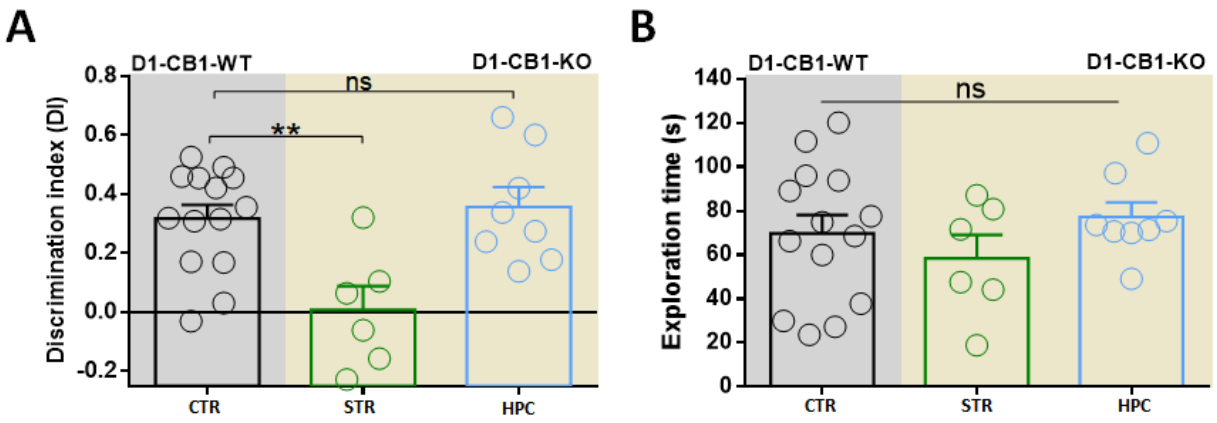

Figure 2 – Hippocampal D1-positive cells are responsible for the consolidation of long-term memory formation.

(**A**) Effects of viral re-expression of CB1 receptors (rAAV-CAG-DIO-CB1) in the hippocampal (n = 8) and striatal (n = 6) neurons of D1-CB1.KO mice and control virus (rAAV-CAG-DIO) (CTR) in the D1-CB1-WT mice (n = 14). (**B**) Total exploration during testing. ** P<0.01; ns, not significant; One-way ANOVA (Dunnett's), F (2, 25) = 7,449; P=0.0029.

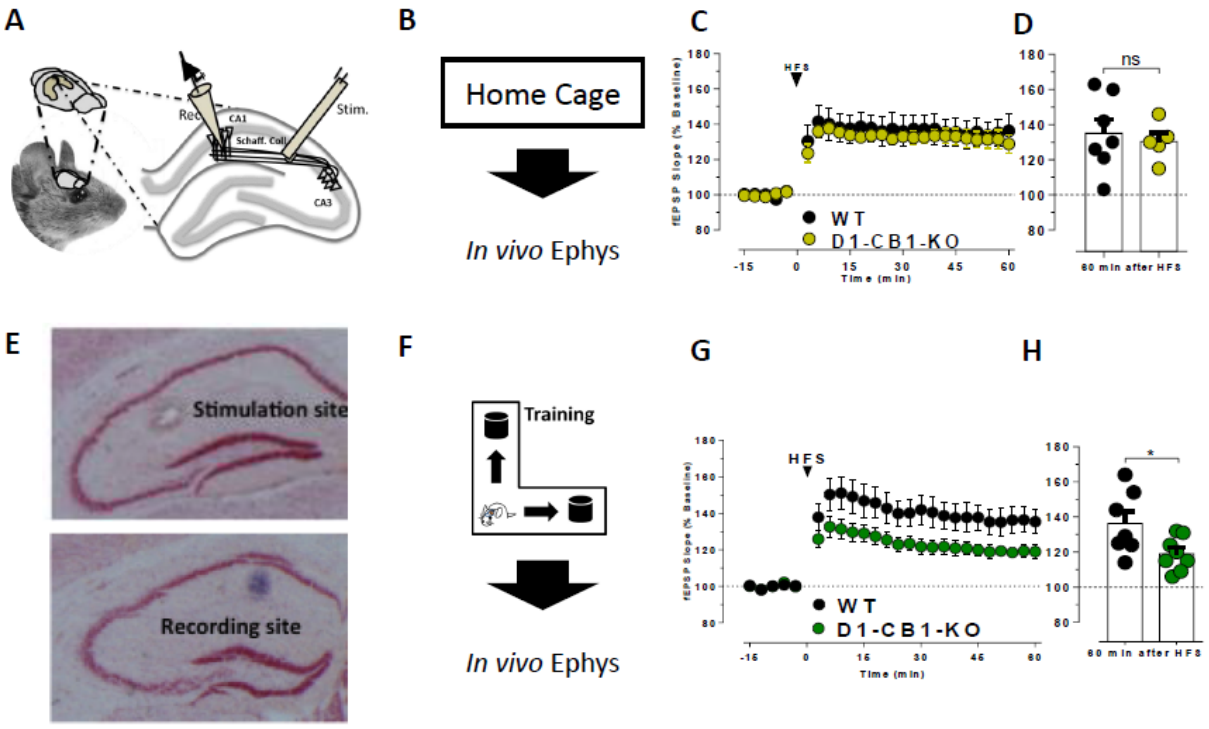

Figure 3 - CB1 receptors in D1-positive cells modulate *in vivo* LTP after learning

(**A, E**) Schematic representation of the experimental design of *in vivo* electrophysiology. Mouse hippocampus was implanted with a stimulation electrode (Stim.) at the CA3 Shaffer collateral (Schaff.) fibers and a recording electrode (Rec.) at the CA1 *stratum radiatum.* (**B, C**) High frequency stimulation (HFS) in the CA3 area of hippocampus from D1-CB1-KO (n=5) and wild-type littermates (n=7) after homecage induces LTP in the CA1 *stratum radiatum*. (**F, G**) HFS in the CA3 area of hippocampus from D1-CB1-KO (n=8) and wild-type littermates (n=7) after training induces LTP in the CA1 *stratum radiatum*. (**D, H**) Summary plots of normalized fEPSPs 60 min after HFS in anesthetized mice after homecage and after training. Traces represent 90 superimposed evoked fEPSP. Data mean ± SEM. *, P<0.05. ns, not significant (D1-CB1-WT vs D1-CB1-KO, 60 min after HFS).

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SUPPLEMENTARY ONLINE METHODS

Animals

Two to three months-old naïve male D1-CB1-KO mutant mice and D1-CB1-WT littermates were used (Monory et al., 2007). Animals were housed in groups under standard conditions in a day/night cycle of 12/12 hours (light on at 7 am).

Novel object-recognition memory task

We used the novel object recognition memory task in an L-maze (NOR) (Busquets-Garcia et al., 2013, Busquets-Garcia et al., 2011, Puighermanal et al., 2009, Puighermanal et al., 2013). As compared to other hippocampal-dependent memory tasks, this test presents several advantages for the aims of the present study: (i) the acquisition of NOR occurs in one step and previous studies revealed that the consolidation of this type of memory is deeply altered by acute immediate post-training administration of cannabinoids via hippocampal CB_1 receptors (Puighermanal et al., 2009, Puighermanal et al., 2013); (ii) the NOR test performed in a L-maze decrease variability and give strong and replicable results; (ii) this test allows repeated independent measurements of memory performance in individual animals (Puighermanal et al., 2013), thereby allowing within-subject comparisons, eventually excluding potential individual differences in viral infection.

The task took place in a L-shaped maze made of dark grey polyvinyl chloride shaped 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 scoring offline of animal's behavior.

The task consisted in 3 sequential daily trials of 9 minutes each. During the habituation session (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 acquisition session (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 memory test 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.

The apparatus as well as objects were cleaned with EtOH (70 %) before experimental use and between each animal testing.

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 a distance of less than 2 cm. Experienced investigators evaluating the exploration were blind of treatment and/or genotype of the animals.

In vivo electrophysiology

GFAP-CB_1-KO and WT littermate mice were anesthetized in a box containing 5% Isoflurane (VIRBAC, France) before being placed in a stereotaxic frame (model SR-6M-HT, Narishige International, London, UK) in which 1.0% to 1,5% of Isoflurane was continuously supplied via an anesthetic mask during the complete duration of the experiment. The body temperature was maintained at 37°C using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA) and the complete state of anesthesia was assured through a mild tail pinch. Before surgery, 100 µl of the local anesthetic Lurocaine® (Vetoquinol, Lure, 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 correct alignment of the head, two holes were drilled in the skull to place: a glass recording electrode, inserted in the CA1 *stratum radiatum*, and one concentric bipolar electrode (Model NE-100, KOPF Instruments, Tujunga, CA, USA) in the CA3 region using the following coordinates: 1) CA1 *stratum radiatum*: A/P -1.5 mm, M/L -1.0 mm, DV 1.20 mm; CA3: A/P -2.5 mm, M/L -2.8, D/V -2.0 mm. The recording electrode (tip diameter = 1–2 µm, 4–6 MΩ) 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 an automatic micropositioner (MIM100-2, M2E, France). The stimulation electrode was placed in the correct area using a micromanipulator (UNI-Z, M2E, France). Both electrodes were adjusted to find the area with maximum response. *In vivo* recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 10 times by Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA, USA) before being further amplified 100 times and filtered (low pass at 1 Hz and high-pass at 5000Hz) *via* a differential AC amplifier (model 1700; A-M Systems, Sequim, WA, 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 300 sec. Test pulse intensities were typically between 50-250 µA with a duration of 500 µs. Basal stimulation intensity was adjusted to 30-40% of the current intensity that evoked a maximum field response. All responses were expressed as percent from the average responses recorded during the 10 min before high frequency stimulation (HFS). HFS was induced by applying 3 trains of 100 Hz (1 sec each), separated by 20 seconds interval. fEPSP were then recorded for a period of 40 minutes. At the end of each experiment, the position of the electrodes was marked by iontophoretic infusion of the recording solution during 180s at -20 µA and continuous current discharge over 20 seconds at +20 µA for recording and stimulation areas, respectively. Histological verification was performed *ex vivo*.

Intra-hippocampal virus delivery

Mice (8-12 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (100mg/kg, Imalgene 500®, Merial) and Xylazine (10mg/kg, Rompun, Bayer) and placed into a stereotaxic apparatus (David Kopf Instruments, CA, USA) with mouse adapter and lateral ear bars. 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). Mice were injected directly into the hippocampus (HPC) or striatum (STR) (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; 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. CB1flox/flox mice were injected with rAAV-CAG-DIO (empty vector as control) or AAV-CAG-DIO-CB1 to induce repression of the CB1 gene in hippocampal or striatal D1-positive cells. CB1 coding sequence was cloning in rAAV-CAG-DIO vector using standard molecular cloning technology. The coding sequence was cloned inverted in orientation to allow CRE-dependent expression of CB1 receptors (Atasoy et al., 2008). Animals were used for experiments 4-5 weeks after injections. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments.

Statistical analyses

Data were expressed as mean ± SEM or single data points and were analyzed with Prism 6.0 (Graphpad Software), using *t*-test (unpaired) or one-way ANOVA (post-hoc dunnett's test).

SECTION IV – GENERAL DISCUSSION

PART 1 – ASTROGLIAL CB1 RECEPTORS DETERMINE SYNAPTIC D-SERINE AVAILABILITY TO ENABLE RECOGNITION MEMORY

The first objective of this thesis was to investigate the physiological role of astroglial CB1 receptors in the modulation of memory functions. Here we show that astroglial CB1 receptors in the hippocampus are necessary for the formation of object recognition memory via astrocyte-mediated gliotransmission. The results presented in this thesis suggest that the activity-dependent calcium increase via astroglial CB1 receptors releases D-serine into the synapse, leads to the activation of NMDA receptors and allows the induction of LTP in CA1 region of the hippocampus. Thus, this work provides a novel physiological mechanism involved in the control of memory processes by coupling astroglial CB1 receptors and memory formation.

It has been reported that astrocytes modulate memory and synaptic plasticity (reviewed in (Araque et al., 2014, Oliveira et al., 2015)). However, the question on how physiological activation of the ECS via astroglial CB1 receptors could control gliotransmission to modulate synaptic plasticity and behavior has not been addressed until now. The first results obtained during this work are that conditional deletion of astroglial CB1 receptors can impair the formation of object recognition memory thus demonstrating for the first time that endogenous astroglial CB1 receptors participate in important brain functions. Previous results have shown that constitutive deletion of CB1 receptors does not alter novel object recognition memory (Hebert-Chatelain et al., 2016, Puighermanal et al., 2009). However, global deletion of receptors can yield different results compared with precise manipulations of CB1 receptors in cell-type specific populations. For instance, whereas CB1 receptor deletion in both glutamatergic and GABAergic neurons does not impair object recognition memory, specific deletion of CB1 receptors in GABAergic cells but not glutamatergic protected mice against the memory disruptive effects of cannabinoids (Puighermanal et al., 2009). In this line, it is important to address several issues in order to understand how CB1 receptors can be involved in the modulation of memory by exogenous or endogenous cannabinoids and also how the experimental model (*e.g.* the use of genetic modified mice or different behavioral paradigms) might impact on the function of CB1 receptors. Indeed, previous data showed that constitutive genetic deletion and re-expression of proteins in genetic KO models to access necessity and sufficiency, respectively, of any specific function might not provide straightforward answers. It is possible that in constitutive KO models, due to the lack of the gene of interest in all cells and at all developmental stages, the organism develops compensatory mechanisms involved in a given function, thus biasing the conclusions. One way to bypass the developmental problems of constitutive deletion of CB1 receptors in brain cells is the use of conditional mutagenesis by the

CRE/LoxP system. In the present work, we adopted this strategy to target astroglial CB1 receptors. This approach offers two main advantages: the first is that it allows temporal recombination and deletion of CB1 receptors in only adult population of GFAP-expressing cells (*i.e.* mainly astrocytes) and, second, it avoids compensatory mechanisms that might occur during development (Hirrlinger et al., 2006). It is known that astrocytes and CB1 receptors are key in development (Clarke and Barres, 2013, Fernández-Ruiz et al., 2000). However, whether specific astroglial CB1 receptors play a role in such processes is not known.

Functional evidence regarding the study of CB1 receptors in astrocytes in synaptic transmission and plasticity in the brain rely mostly in the study of the developmental brain [reviewed in (Oliveira da Cruz et al., 2016)]. Recently, it has been shown that another important receptor for astroglial functions, the mGlu5 receptor, can be differentially expressed from early stages of development to adulthood (Sun et al., 2013). The levels of mGlu5 receptor in adolescent mice are substantially higher as compared to adults. Because mGlu5 receptor activity have been shown to be important for adult astrocytic networks *in vivo* (Araque et al., 2014, Wang et al., 2006), this differential expression throughout development levels raised key questions regarding their functional role (Grosche and Reichenbach, 2013). However, a golden rule of brain biology is that "quantity is not quality", with proteins expressed at very low levels exerting important functions (Busquets-Garcia et al., 2015). Thus, whereas high amounts of mGlu5 receptor protein could be important to proper circuit function during development, adult low levels of expression could be involved in the fine tuning of synaptic physiology with important behavioral consequences (Grosche and Reichenbach, 2013). Current evidence suggests that astroglial CB1 receptors are expressed at very low quantity in the fine astrocytic processes that unsheathe the synapses (Han et al., 2012, Metna-Laurent and Marsicano, 2015). Consistently, CB1 receptor expression in the brain is considerably higher during early developmental periods than in adulthood (Laprairie et al., 2012). Because astrocytes are key to proper circuit maintenance and wiring during the critical developmental period (Clarke and Barres, 2013), it is tempting to imagine a potential functional role of astroglial CB1 in the modulation of these circuits during development.

To reduce the component of developmental compensation in our study, we achieved deletion of astrocytic CB1 receptors in adulthood, by using the inducible version of the Cre recombinase (CreERT2) and also by local injection of AAV-CRE into the hippocampus of CB1-flox mice. These procedures were able to clearly show that CB1 receptors in hippocampal astrocytes mediate novel object recognition memory and LTP. Also, acute treatment of D-serine aiming at restoring D-serine-dependent NMDA receptor signaling during behavioral or electrophysiological procedures reinforces the idea that the phenotypes observed In GFAP-CB1-KO mice are due to acute alterations of hippocampal circuits.

NMDA receptor-dependent LTP in the hippocampus is a key process for learning and memory which is controlled by astrocytic activity (Henneberger et al., 2010, Whitlock et al., 2006). The results presented in this thesis further demonstrate that astrocytes *in vivo* modulate synaptic plasticity *via* astroglial CB1 receptors. Furthermore, these results indicate that astrocytic regulation of D-serine availability in the synapse is a key determinant in the modulation of NMDA receptor-dependent LTP. Although it is currently accepted that D-serine is a major player in modulating synaptic plasticity and memory, the origin of D-serine at the synapse remains controversial. While some groups report that impacting astrocytic gliotransmission can impair D-Serine synthesis and release (Papouin et al., 2017b), other groups reported that D-serine is not synthetized or released by astrocytes, but by neurons (Wolosker et al., 2016). Part of the controversy regards the localization of SR in the brain (Wolosker et al., 2017). While it was first proposed to be present in astrocytes, it has latter been shown that it is mostly expressed in neurons (Wolosker et al., 2017). One hypothesis that is currently uphold suggests that astrocytes are not responsible for the direct release of D-Serine but by the production of L-serine, which is then shuttle to neurons in order to be converted by SR into D-serine and released in the synapse to modulate NMDA receptor activity (Wolosker et al., 2016). Although our evidence supports the model in which D-serine is release from astrocytes, our results can also be explained by the other astrocyte-to-neuron L-serine hypothesis as astroglial CB1 receptors could potentially control this shuttle activity. Nevertheless, our data demonstrate that D-serine is key for NMDA receptor activity and that astrocytes *via* astroglial CB1 receptors are important for D-Serine availability at the synapse.

Besides their role in the modulation of LTP, astrocytes have been implied in the modulation of several other forms of plasticity such as Spike-time Dependent plasticity or LTD (De Pitta et al., 2016). The latter is a form of plasticity that has been related with the modulation of object recognition memory formation (Kemp and Manahan-Vaughan, 2004). Interestingly, exogenous cannabinoid acting at astroglial CB1 receptors can induce an LTD in the hippocampus (Han et al., 2012). Therefore, this kind of plasticity might be also actively modulated by endogenous astroglial CB1 receptors. Although both LTP and LTD have been suggested to be involved in the modulated of recognition memory, their physiological role is yet to be determined as well as the role of astrocytes (and astroglial CB1 receptors) in these forms of plasticity. It is known that following LTP or LTD induction the synapses are not uniformly potentiated or depressed: some synapses get potentiated, some depressed while others unaltered. Several groups have long questioned the nature of such modulation. For instance, according to the synaptic tagging hypothesis proposed by Morris and Frey (1997), persistence of long-term forms of plasticity (*i.e.* LTP or LTD) will depend on the molecular underpinnings that prepare a synapse (mRNA trafficking, local protein synthesis, cytoskeleton dynamics, etc.) prior to the stimulus, so that it is primed for the persistence forms of plasticity to achieve (Frey and

Morris, 1997). Astrocytes have been shown to support and modulate neurons during times of high demand (*e.g.* high network activity). It is tempting to speculate that astrocytes might by responsible for the tagging of certain synapses for potentiation or depression, depending on the requirement of their activity. We know that individual astrocytes have the capacity to modulate synaptic activity at different domains (*i.e.* local synaptic, regional branches and global whole-astrocyte changes) (Araque et al., 2014) and that astroglial CB1 receptors regulate astrocytic activity (Martin et al., 2015, Min and Nevian, 2012, Navarrete and Araque, 2008). As CB1 receptors are known to be an effective feedback mechanism during times of synaptic activity, it is tempting to suggest that astroglial CB1 receptors might control which synapses are potentiated or depressed during synaptic plasticity. Though our results suggest that at least LTP is controlled by astrocytes *via* astroglial CB1 receptor activity, further studies will address whether also LTD is modulated by similar mechanism.

Although our evidence supports D-serine transmission from the astrocyte to the synaptic cleft as responsible for the impairment in LTP and memory shown in the mice lacking astroglial CB1 receptors, global CB1 deletion in the hippocampus does not impair object recognition memory. Above, I gave some possible explanations (*e.g.* compensatory developmental mechanisms) for the apparent contradiction with our current results. However, one possible reason might be a deregulation of neuronal CB1 receptor signaling. Potentially, deletion of astroglial CB1 receptors might impact endocannabinoid signaling to the point that it could act on other CB1 receptors (for instance, GABAergic and/or glutamatergic cells) and this would be the responsible for the phenotype. For instance, in the hippocampus, CB1 receptors are mostly expressed in the GABAergic basket cells in both the pyramidal layer and stratum radiatum and also in glutamatergic pyramidal cells (Marsicano and Lutz, 1999). Although we did not account for this possibility, one possible way to address this issue is by acute inhibition of CB1 receptors by Rimonabant. However, based on evidence that D-Serine transmission is the main mechanism of impairment of LTP and memory, we predict that this manipulation will not restore the phenotype as most likely, it is not an issue of neuronal supply of neurotransmitters but rather a lack of the D-serine gliotransmission from astrocytes.

Besides the role of astroglial CB1 receptors in the modulation of recognition memory it would be important to investigate whether other forms of long-term memory are also impacted. For instance, it has been shown that learning in the hippocampus produces a LTP in the CA3-CA1 synapses (Whitlock et al., 2006). The authors used the inhibitory avoidance behavioral paradigm in which mice have to use hippocampal-related information (*i.e.* contextual cues) to avoid receiving an electrical shock. They observed that this learning procedure could produce an LTP in a subset of synapses (Whitlock et al., 2006). In this context, as CB1 receptors modulate aversive long-term memory formation (Marsicano et al., 2002) and astrocytes are important to memory and LTP (Oliveira et al., 2015), it would be important to address whether

astroglial CB1 receptors might play a role in this type of memory. Nevertheless, it is important to keep in mind that aversive memories, besides the involvement of hippocampus, have a strong influential drive of the limbic regions and that could recruit different circuits than the ones used in non-aversive long-term memories.

Another speculative aspect regarding astroglial modulation of synaptic transmission and plasticity is the mechanism of gliotransmission *per se*. Our results indicate that astroglial CB1 receptor activation is responsible for the intracellular calcium increase in astrocytes. However it is not well understood how gliotransmission and calcium activity might affect D-serine availability at the synapse. There is currently a huge debate around the calcium-dependent gliotransmission with some groups reporting calcium increases dependent on specific PLC-dependent mechanisms and others reporting that calcium activity in astrocytes does not have any physiological role in synaptic function (Agulhon et al., 2012, Bazargani and Attwell, 2016, Henneberger et al., 2010). One way that CB1 receptor activity might help untangling this issue comes from the observation that CB1 receptors are present in neuronal mitochondria (Hebert-Chatelain et al., 2016). As many receptor expressed in neurons have been reported in glial cells, it is tempting to speculate about a possible expression of mtCB1 in astrocytic mitochondria. One of the mechanism by which astroglial CB1 receptors modulate synaptic function is by a Gq-dependent mechanism (Navarrete and Araque, 2008), which is known to induce PLC activity with generation of IP3 and DAG (De Pittà et al., 2009). Whereas it has been described that IP3 receptors activation in the endoplasmic reticulum induce an increase in intracellular calcium in the astrocyte, subsequent studies have demonstrated that knocking out the main type of these receptors (*i.e.* IP3R2) do not impair synaptic plasticity (Agulhon et al., 2010) (but see (Sherwood et al., 2017)). However, subsequent studies showed that there are IP3-independent calcium rises in the astrocytes, especially at the fine processes that are closely associated with the synapses (Srinivasan et al., 2015). Interestingly, mitochondria were found in the astrocytic processes (Agarwal et al., 2007) and besides their main role in the production of ATP, they can also act as calcium buffers (Nicholls, 2009). Notably, it has been also demonstrated that mitochondrial calcium is involved in the release of gliotransmitters both *in vitro* and *in vivo* (Agarwal et al., 2017). The functional consequences of these findings are great and can be the missing link between astroglial CB1 receptor activation and the release of gliotransmitters. Indeed, astrocytes are competent producers of endocannabinoids (reviewed in (Metna-Laurent and Marsicano, 2015)) and one of the two molecules generated by Gq activation of PLC is DAG: a precursor of the endocannabinoid 2-AG (Hu and Mackie, 2015). Thus it is likely that membrane astroglial CB1 receptors, via the Gq mechanism, trigger a molecular cascade that control large global calcium waves via IP3 to IP3R activity. This pathway could on one hand increase local calcium-induced gliotransmission, but on the other hand, it could induce production of 2-AG which might act at mitochondrial CB1 receptors. Indeed, unpublished results

from our laboratory show that astroglial mtCB1 induces calcium rise in mitochondria, which might then contribute to gliotransmission.

Interestingly, also L-Serine to D-serine conversion has been recently linked with mitochondrial activity in astrocytes (Suzuki et al., 2015). In this particular study the authors show that changes in the way astrocytes process the glycolysis (anaerobic production of energy at the cytoplasm) or oxidative phosphorylation (aerobic production of energy by mitochondria) can modulate D-serine availability at the synapse. Altogether, astroglial CB1 receptors (both at mitochondria and plasma membrane) might be potential contributors to the modulation of gliotransmission by modulating intracellular astrocytic calcium signaling.

Astrocytes possess very mobile tripartite synapse-associated fine processes that are dynamically modulated by synaptic transmission (Panatier et al., 2006). Interestingly, CB1 receptor agonists have been shown to change neuronal morphology and growth by interacting with cytoskeleton proteins (Roland et al., 2014). As CB1 receptors are also present in astrocytes, one might suggest that D-Serine availability is not only controlled by gliotransmission, but also by the decreased coverage of the synapse by the astrocytic processes. Functional morphological analysis of astrocytes *in vitro* and *in vivo* remain mostly unknown and further studies aiming at imaging the astroglial processes under the control of CB1 receptors will provide more evidence on such a possible scenario.

Endocannabinoids are thought to be produced and delivered “on demand”. However, it is not known how different endocannabinoids might act on closely located CB1 receptors (*e.g.* neuronal *vs* astrocytic). One possibility is that specific endocannabinoids are involved in the modulation of either neuronal CB1 or astroglial CB1 receptors. Current evidence indicates that CB1 receptors, probably between different neuronal populations, under certain conditions might have different affinities for specific endocannabinoids (Turu and Hunyady, 2010). Also, the G proteins that are recruited by a certain ligand might differ depending on the ligands (Turu and Hunyady, 2010). Furthermore, other potential intracellular pools of CB1 receptors (*i.e.* endosomes or mitochondria) and other complementary signaling proteins might also have a role in this process. Thus, mobility of the astrocytic processes during synaptic activity might impact on the binding of endocannabinoids to CB1 receptors. For instance, if the astrocytic process retracts during synaptic activity (which produces endocannabinoids), binding of endocannabinoids would be reduced and this could impact on the activation of the receptors in astrocytes. Further studies will clarify this problematic.

Astrocytes have been shown to modulate independent non-overlapping territory domains (Bushong et al., 2002, Chai et al., 2017). Thus, understanding how single astrocytes can modulate thousands of glutamatergic synapses and how different astrocytes coordinate the

activity among themselves is a challenging question both technically and theoretically. Recently, it has been shown in the striatum that different astrocytes were closely associated to specific synapses from different classes of neurons and that "domain-specific" astroglial network activity was dependent on astroglial CB1 receptors (Martin et al., 2015). This suggests that similar domain-specific astrocytes might exist in the hippocampus and astroglial CB1 receptors might be involved in the modulation of their functions. The formation of hippocampal memory trances (*i.e.* engrams) relies on the establishment of specific cell assemblies during the acquisition of a memory (Liu et al., 2014). Because astrocytes can participate in circuit specific modulation of neuronal networks in the striatum, it is tempting to suggest that a similar mechanism in the hippocampus plays a role in the formation of hippocampal engrams, ultimately controlling memory formation.

One of the disadvantages of *in vivo* electrophysiological investigations of synaptic plasticity is its "artificial" nature, for which strong exogenous stimulations are applied to neuronal circuits. This creates a strong limitation in searching causal relationships between what we observe and what is indeed relevant in normal brain physiology. Another way to investigate the endogenous role of astroglial CB1 receptor functions would be to investigate their putative role in the study of hippocampal oscillations. As previously discussed in the introduction, brain oscillations are thought to be drivers of cognition by the coordination of neuronal networks. It has been shown that impairing astrocytic interconnectivity can impact the generation of certain rhythms of the local field potentials (Lee et al., 2014). As astrocytes, *via* astroglial CB1 can modulate homo- and heterosynapses (Navarrete and Araque, 2010) it would be important to investigate their role in the modulation of the brain rhythms.

Sharp wave ripples (SPW-Rs) in the hippocampus represent a highly synchronous population pattern in the brain that is present during consummatory behaviors and is known to be affected by several neuromodulators, endocannabinoids included (Buzsaki, 2015). Among several other functions, SPW-Rs have been suggested to support memory consolidation. Since astroglial CB1 receptors mediate memory consolidation in the hippocampus and acute cannabinoids treatment impair the generation of SPW-Rs in the hippocampus (Robbe et al., 2006, Robbe and Buzsaki, 2009), possible functional crosstalk between these two phenomena might exist. Besides SPW-R, the hippocampus possess other rhythmic activity such as theta and gamma oscillations that are likely involved in several behavioral functions (Buzsaki, 2006). Recently, vesicle-mediated gliotransmission has been shown to modulate gamma oscillations in the hippocampus, which the authors correlated with memory impairment in an object-recognition task (Lee et al., 2014). As we report that astroglial CB1 receptors control the formation of recognition memory, it would be important to investigate whether gamma oscillations could be affected. Direct evidence showing causal relations between both activities

would provide a novel framework demonstrating that astroglial CB1 receptors modulate behavior by the modulation of important circuit functions that underlie memory formation.

One of the biggest technical challenges in endocannabinoid research in glial cells is that the experimental procedures used can bias the conclusions obtained. For instance, astrocytes in cell cultures are less complex than their *in vitro* slices or *in vivo* counterparts with differences in the content, shape (*i.e.* less complexity with fewer ramifications and fine processes) and receptor expression (Verkhratsky et al., 2012b). *In vitro* electrophysiology (both cultured and acute slices) is a powerful technique to investigate neuronal circuits. However, it is an artificial condition in which the preparations are subject to unavoidable insults such as slicing of the brain, extensive washing and temperature changes that can have important impact on the interpretation of results. Overall, the current technical diversity of conditions used in the *in vitro* electrophysiology studies pose problems in the replication of results and must be taken in consideration during direct comparisons between *in vivo* and *in vitro* results. Astrocytes are interconnected in a far-reaching syncytium that allows the global and local modulation of astrocytic networks (Araque et al., 2014). Slicing the brain to produce *in vitro* preparations or dissociated cultures profoundly disrupt this network and impair global communication amongst astrocytes and their ability to modulate fine-tuned synaptic processes. Furthermore, it is known that astrocytic processes are very mobile, a feature shown to be important in the modulation of synaptic plasticity (Panatier et al., 2006). Because mobility of biological membranes depends on the temperature of the brain, it is important to keep in mind that this important feature might be also severely compromised in *in vitro* models where temperature is well below the physiological range. Astrocytes are important modulators of metabolism in the brain by providing metabolites locally and globally by the astrocytic syncytium (Bazargani and Attwell, 2016, Harris et al., 2012). If the network during *in vitro* preparation is compromised, there is an important supportive function performed by astrocytes that is impaired. Also, the chemical concentration and content in the aCSF will also affect the astrocytic network and the neuronal capacity to sustain similar patterns of action. In the study of hippocampal glutamatergic transmission, GABAergic transmission is often blocked by specific inhibitors, a condition that is not doable in vivo due to practical reasons and that can alter the physiological properties of cells, including astrocytes. Another issue that might arise from ECS study in the *in vitro* model regards the circulating levels of endocannabinoids that might have a role in the tonic, rather than phasic, modulation of CB1 receptors (Alger and Kim, 2011). In *in vitro* electrophysiology, extensive washing of the slices during slice recovering and non-physiological recording temperatures (*i.e.* below 37 degrees) due to experimental constraints in keeping slices viable, can have a profound impact on the endocannabinoid tone. Endocannabinoids are lipid signaling molecules that are regulated by temperature and cellular activity (Hajos et al., 2004, Piomelli, 2003). Another problem of *in vitro* electrophysiology is that studies often use juvenile animals

because of the much better longevity of brain cells and easier possibility to perform long-term experiments, which dramatically decreases in older slices (Ting et al., 2014). As the ECS is developmentally regulated (Laprairie et al., 2012), direct comparisons between juvenile *in vitro* recordings and adult behaviors should be done carefully (Oliveira da Cruz et al., 2016). The use of *in vivo* models allows the study of the brain during long-term recordings (days or weeks depending on the approach), and avoids most of the *in vitro* technical limitations described above. Overall, these are aspects that are avoided by in *vivo* preparations and might explain why LTP *in vitro* in GFAP-CB1-KO mice that we report is only mildly impaired whereas *in vivo* it is completely abolished. It further reinforces the idea that highly dynamic systems must be studied in preparations that preserve the best their original conditions.

Some variants of *In vivo* electrophysiological methodologies have also important drawbacks, such as the use of general anesthesia. Isoflurane is a general volatile anesthetic which can be administered in combination with air and oxygen to induce and maintain deep anesthesia and induce both muscular relaxation and decreased pain sensitivity (Campagna et al., 2003). Although the mechanism of action is yet to be identified, it has been reported that isoflurane can induce anesthesia by decreasing tissue excitability by diminishing gap junction mediated cell-to-cell coupling and by modulating channels that control action potential propagation (Campagna et al., 2003). It is currently known that inhaled anesthetics can impair learning and memory by unknown mechanisms (Saab et al., 2010). For instance, in *in vitro* and *in vivo* models it has been shown that anesthesia can acutely modulate the activity of $GABA_A$ receptors (Saab et al., 2010), impair long-term synaptic plasticity (Simon et al., 2001, Uchimoto et al., 2014), and induce intracellular apoptotic cascades (Zhang et al., 2008). However, it has been also described that isoflurane could improve learning and memory and synaptic plasticity (Rammes et al., 2009). Isoflurane can also impact astrocytic physiology. Whereas some studies reported that some features of astrocytic morphology are changed thought without impact on viability, proliferation, motility, and ability to support synapses (Culley et al., 2013), others have reported that astrocytes exposed to isoflurane have decreased capacity to support neuronal development (Ryu et al., 2014). Because the experimental conditions are different (*e.g.* time of exposition and type of animal model used), it is difficult to make a full comparison between different studies.

In humans it has been shown that general anesthesia induced by isoflurane during cardiac surgery reduces plasma Anandamide concentrations (Weis et al., 2010). Although such effects were not yet reported in rodent models, it is not possible to exclude that isoflurane might affect the endocannabinoid system. Overall, it is important to acknowledge that isoflurane is not a neutral drug and has important side effects in brain physiology that must be properly experimentally controlled. In our case, isoflurane did not impair the induction of LTP in WT mice but we cannot exclude that it might have an effect in the overall brain physiology.

In conclusion, our data provide a novel neurobiological frame, where the tight interaction between astrocytes and neurons determine physiological cognitive processes. The control of D-serine gliotransmission by astroglial CB1 receptors and the modulation of NMDA receptor-dependent LTP constitute the cellular mechanisms by which astroglial CB1 receptors modulates neuron-glia interactions to control learning and memory.

PART 2 – DELETION OF CB1 RECEPTORS IN HIPPOCAMPAL D1-POSITIVE CELLS IMPAIRS OBJECT RECOGNITION MEMORY AND ASSOCIATED SYNAPTIC PLASTICITY

The results obtained in the second part of this thesis show that CB1 receptors in hippocampal D1-positive cells are necessary and sufficient for the modulation of *in vivo* LTP and object recognition memory consolidation. These results provide novel evidence for a potential functional crosstalk between the endocannabinoid and the dopaminergic systems in the modulation of memory functions.

We show that specific deletion of CB1 receptors from D1-positive cells impairs long-term, but not short-term, object recognition memory formation. The formation of long-term memories depends on several factors that act during different time scales to stabilize a previously acquired experience. Our results indicate that CB1-D1-KO mice have impaired consolidation of memory but normal acquisition. Dopamine is thought to mediate the modulation of memory stabilization and consolidation, but not the acquisition, of new memories by a mechanism dependent on D1-like receptors (Lisman et al., 2011). Although it is quite well established the role of dopamine in these important functions, the mechanism of this modulation remains poorly explored. Thus, my thesis data show that short-term memory formation is not impaired while the mechanism for stabilization of long-term memories depends on CB1 receptors in D1-positive cells.

Memory formation does not rely solely on hippocampal function for its consolidation but it requires also other components in order to successfully preserve the memories. Attention to the task and/or motivation to execute the task are two key factors for memory formation that are modulated by dopaminoceptive cells in the striatum (Palmiter, 2008). Although there are not currently known direct connections between striatal structures and the hippocampus, it is known that striatum can indirectly modulate several parameters of hippocampal activity (Goldfarb et al., 2016, Sales-Carbonell et al., 2013, Tort et al., 2008) and that striatal D1-positive MSNs express functional CB1 receptors (Monory et al., 2007). In line with this evidence, it is possible to speculate that CB1 receptors in D1-positive cells in the striatum can modulate memory formation by indirectly modulating attentional and motivational states required for learning. However, local re-expression of CB1 receptors in the hippocampus but not the striatum of D1-CB1-KO mice is able to reverse the memory impairment displayed by these animals. Thus, we provide evidence that hippocampal CB1 receptors in D1-positive cells are necessary and sufficient for the consolidation of object recognition memory.

Dopaminergic transmission via D1 receptors has been implicated in the modulation of other types of memory (*i.e.* spatial and aversive) (Lemon and Manahan-Vaughan, 2006). Thus, it would be interesting to assess if CB1 in D1 positive cells also participates in these mechanisms. It has already been reported that mice lacking CB1 receptors in D1-positive cells have impaired extinction of freezing in fear conditioning tasks (Micale et al., 2017), suggesting that this crosstalk is important also in aversive memory. On the other hand, D1-dependent transmission in the hippocampus has been shown to modulate spatial learning (Xing et al., 2010). As CB1 receptors in the hippocampus are important for spatial memory formation (Riedel and Davies, 2005), one can speculate that CB1 receptors in D1-positive cells might be also involved in this function.

Dopamine transmission in the hippocampus facilitates the induction of LTP *in vitro* and *in vivo* by the modulation of D1-like receptors (Lisman et al., 2011). Our results shows that deletion of CB1 from D1-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 D1-CB1-KO mice as compared with their WT littermates. These results demonstrate that the presentation of a novel stimulus enhances the LTP, which cannot be done in the absence of CB1 receptors in the D1-positive cells. Literature suggests that learning induces hippocampal dopamine release that modulates consolidation of memory and LTP by acting on D1 receptors in the hippocampus (Kempadoo et al., 2016, Takeuchi et al., 2016). Our results could indicate that a CB1-dependent mechanism downstream to D1 receptor activation could be responsible for the consolidation of memory. However, it remains to be tested in our protocol whether pharmacological blockage of D1 receptors in the hippocampus can block LTP after object exposition of if indeed dopamine levels are increase in the hippocampus following NORT. Nevertheless, this indicates that CB1 receptors in D1-positive cells modulate LTP enhancement, which ultimately will allow the stabilization of new experiences allowing long-term memory formation.

It has been shown that D1-positive cells can be glutamatergic neurons, GABAergic neurons and astrocytes. Although it has been extensively described that dopamine in the hippocampus can modulate memory and synaptic plasticity, the nature cells responding to dopamine and the mechanism supporting such functions remains mostly unknown. One of the challenges in assessing the presence or absence of these cells in the hippocampus resides on the low level of expression of these receptors and the specificity of the tools available to proceed with the identification. In the hippocampus, CB1 receptors have been identified in the vicinities of cell bodies to D1 positive cells labeled as CCK- and Vglu3-positive cells (Puighermanal et al., 2017). Because CB1 receptors are mostly present in the terminals, the authors could not quantify with precision the presence of CB1 in these cells. However, it seems clear that D1-positive interneurons can express CB1 protein (Puighermanal et al., 2017). One of the

observations from this work is that, although CB1 receptors are mainly expressed in GABAergic cells of the hippocampus (Marsicano and Lutz, 1999, Marsicano and Kuner, 2008), these CB1 receptors in D1-positive cells represent most likely a subclass within GABAergic-expressing CB1 interneurons. This raises questions regarding the functional role of such small population and their impact during the modulation of memory and synaptic plasticity. On the other hand, it has been previously described that deletion of CB1 receptors from GABAergic cells (achieved using the Dlx5/6 promoter) does not impair object recognition memory (Puighermanal et al., 2009). However, one might argue that complete obliteration of CB1 from all inhibitory cells expressing CB1 is not the same as fine tune specific changes in the modulation a sub population of GABAergic interneurons. Further investigations will address the identification of the nature of these cells.

Another possibility is that CB1 in D1-positive cells might be present in astrocytes. Interestingly, it has been reported that astrocytes in hippocampal slices can induce intracellular calcium responses that are dependent on D1 receptors (Jennings et al., 2017). Furthermore, astrocytes expressing D1 receptors have been recently identified in the substantia nigra (Nagatomo et al., 2017), suggesting that possibility they could also be expressed in the hippocampus. Furthermore, evidence from the first part of this thesis showed that CB1 receptors in astrocytes are necessary for *in vivo* LTP and object recognition memory formation. However, we do not currently know 1) if sole CB1 receptor expression in astrocytes is sufficient to modulate object recognition memory, 2) if there is the participation of other cell-type in such process (for instance, CB1 receptors in D1-positive cells) or 3) the existence and involvement of a specific subclass of astrocytes in the hippocampus expressing D1 and CB1 receptors.

Nevertheless, current evidence does not support the identity of D1-CB1 positive cells as astrocytes, at least, in the hippocampus. First, D1 receptors mRNA is not detectable in hippocampal astrocytes, contrasting with astrocytes from the striatum and the cortex (Chai et al., 2017). Second, our results show that deletion of astroglial CB1 impairs *in vivo* LTP while deletion of CB1 in D1-positive cells does not impair LTP per se but rather prevent the enhancement induced by learning. Third, deletion of astroglial CB1 receptors also impairs the formation of short-term memory whereas it is conserved in D1-CB1-KO mice.

In conclusion, the results presented in the second part of this thesis, provide a novel link between CB1 receptor-mediated activity in the D1-positive cells in the facilitation of hippocampal LTP and in the consolidation of object recognition memory. Thus, CB1 receptors in D1-positive cells act as gateway to the consolidation of memory function.

SECTION V – REFERENCES

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SECTION VI – ANNEX

ASTROGLIAL TYPE-1 CANNABINOID RECEPTOR (CB1): A NEW PLAYER IN THE TRIPARTITE SYNAPSE

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REVIEW

ASTROGLIAL TYPE-1 CANNABINOID RECEPTOR (CB_1): A NEW PLAYER IN THE TRIPARTITE SYNAPSE

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Abstract—The endocannabinoid system is an important regulator of physiological functions. In the brain, this control is mainly exerted through the type-1-cannabinoid (CB_1) receptors. CB_1 receptors are abundant at neuron terminals where their stimulation inhibits neurotransmitter release. However, CB_1 receptors are also expressed in astrocytes and recent studies showed that astroglial cannabinoid signaling is a key element of the tripartite synapse. In this review we discuss the different mechanisms by which astroglial CB_1 receptors control synaptic transmission and plasticity. The recent involvement of astroglial CB_1 receptors in the effects of cannabinoids on memory highlights their key roles in cognitive processes and further indicates that astrocytes are central active elements of high-order brain functions.

This article is part of a Special Issue entitled: Astrocyte-Neuron Interact.

Key words: astroglial cells, tripartite synapse, cannabinoid receptors, synaptic plasticity, working memory.

Contents

INTRODUCTION

The discovery of the endocannabinoid system (ECS) as the endogenous target of the main active compound of the plant *cannabis sativa* (Marijuana), Δ^9-tetrahydrocannabinol (THC), revealed a complex and multimodal system involved in the regulation of many physiological processes (Piomelli, 2003; Kano et al., 2009).

The ECS is broadly present in the body (Piomelli, 2003; Pacher et al., 2006) and is composed of cannabinoid receptors (mainly CB_1 and CB_2), their endogenous lipophilic ligands called endocannabinoids (eCBs), including 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the enzymatic machinery responsible for eCBs production and degradation (Piomelli, 2003). Both CB_1 and CB_2 receptors are present in the central nervous system (CNS) and their activity directly affects glial functions (Stella, 2010). However, CB_1 receptors are the most important responsible elements of (endo)cannabinoid effects and functions in the CNS. Thus, for the sake of brevity, this review will focus on the astroglial roles of CB_1 receptors and we refer the reader to recent reviews describing the role of CB_2 receptors and other elements of the ECS in glial cells (Walter and Stella, 2004; Stella, 2009, 2010).

CB_1 receptors have been extensively described at the membrane of neuronal presynaptic terminals, where they are responsible for intracellular mechanisms leading to retrograde inhibition of neurotransmitter release (Piomelli, 2003; Kano et al., 2009; Castillo et al., 2012). Yet, during the last decade, an increasing number of studies reported the presence of CB_1 receptors at other locations such as postsynaptic terminals (Bacci et al., 2004; Marinelli et al., 2009), intracellular organelles such as mitochondria (Benard et al., 2012) and also on astrocytes

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazloe propionic acid receptors; CB-LTD, cannabinoid-induced LTD; DSE, depolarization-induced suppression of excitation; eCBs, endocannabinoids; ECS, endocannabinoid system; GLT-1, glutamate transporter-1; GPCRs, G protein-coupled receptors; LTD, long-term depression; LTP, long-term potentiation; mGluR1, metabotropic glutamate receptors type-1; mGluRs, metabotropic glutamate receptors; NMDAR, N-methyl-D-aspartate receptor; THC, Δ^9-tetrahydrocannabinol; t-LTD, spike-timing-dependent depression; VGCC, voltage-activated Ca^{2+} channels; WM, working memory.

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(Navarrete and Araque, 2008, 2010; Han et al., 2012) although their functional roles at these locations are far from being completely understood.

Astrocytes constitute the large majority of glial cells in the CNS, and they are mainly thought to metabolically support neurons and to keep a stable homeostatic environment for correct neuronal functions (Magistretti, 2006; Belanger and Magistretti, 2009). Despite their lack of electrical properties, astrocytes are highly organized and can communicate among themselves through extensive networks (Giaume et al., 2010). Moreover, in the past 15 years, it has been proposed that astrocytes were not mere supporters of neuronal survival and functions, but they could also be part of bidirectional communication with neurons (Araque et al., 1999). This growing view of astrocytes as powerful integrators of synaptic information together with recent findings linking ECS and astroglial functions lead to an outlook where the ECS might be a key modulator of astrocytic activity (Navarrete et al., 2014; Metna-Laurent and Marsicano, 2015).

In this review, we describe the presence of the ECS in astrocytes and its known roles in some astrocytic functions. Particularly, we discuss the role played by CB_1 receptors in the modulation of the electrophysiological activity of the tripartite synapse in the brain and its potential impact on behavioral processes.

INTRODUCTION TO THE TRIPARTITE SYNAPSE

Because astrocytes are unable to generate action potentials, it was thought for a long time that these cells did not take part in the exchange or integration of information in the CNS but rather had a passive and structural role (Koob, 2009). However, it is now clear that astrocytes form, together with pre- and post-synaptic neurons, an important functional entity that has been called the tripartite synapse (Araque et al., 1999). The concept of tripartite synapse has been recently reviewed (Araque et al., 2014). Therefore, we will here just summarize few ideas that are necessary to further understand the emerging roles that CB_1 receptors play in neuro–astroglial interactions at the synaptic level.

In different areas of the CNS, astrocytes have a close anatomical relationship with synapses and they are a key element of synaptic transmission (Araque et al., 2014). Indeed, astrocytes are able to detect synaptic signals coming from neurons. For instance, they play a key role in the clearance of K^+ and glutamate from the synaptic cleft. A prerequisite for this function is the abundant coupling of astrocytes through gap junctions, allowing them to redistribute elevated K^+ levels from sites of excessive neuronal activity to sites of lower extracellular K^+ concentration (Kofuji and Newman, 2004). Although both astrocytes and neurons possess glutamate transporters, the astrocytic glutamate transporter-1 and glutamate aspartate transporter (GLT-1 and GLAST, respectively) are responsible for up to 90% of extracellular glutamate clearance (Belanger and Magistretti, 2009). Furthermore, the highly dynamic membrane diffusion of GLT-1 modulates synaptic transmission (Murphy-Royal et al., 2015).

Synaptic neurotransmitters and neuromodulators activate astroglial G protein-coupled receptors (GPCRs) that, in turn, trigger the production of inositol 1,4,5-trophosphate (IP3), eventually leading to Ca^{2+} release from the endoplasmic reticulum (Verkhratsky et al., 2012). For instance, glutamate can increase astroglial intracellular Ca^{2+} via metabotropic glutamate receptors (mGluRs) (Araque et al., 2014). At a finer resolution, recent studies using high-resolution Ca^{2+} imaging of hippocampal slices demonstrated that even astrocytic thin processes are able to respond to activity of single synapses with local Ca^{2+} elevations (Di Castro et al., 2011; Panatier et al., 2011).

The functional meaning of Ca^{2+} elevations in astrocytes is far from being fully understood. However, several pieces of evidence indicate that one of the most important responses of astrocytes to increases in intracellular Ca^{2+} is the release of so-called gliotransmitters (Araque et al., 2014). Despite the fact that the detailed mechanisms of this release are under debate [discussed in (Hamilton and Attwell, 2010; Parpura and Zorec, 2010; Gucek et al., 2012)], molecules such as ATP, glutamate, D-serine and others are known to be released by astrocytes and to act at neighboring neuronal synaptic elements, actively modulating synaptic transmission and plasticity (Araque et al., 2014).

Astrocytes are in close proximity with neuronal synaptic elements, can "listen" to neurons by responding to neurotransmitters and can "talk" back to neuronal elements via the release of gliotransmitters. They thereby are key active players in synaptic transmission and plasticity and justify the concept of "tripartite synapse" as an important functional unit of the CNS activity (Araque et al., 2014).

THE ECS IN ASTROCYTES

CB_1 receptors are likely the most abundant GPCRs in the brain (Herkenham et al., 1990) and they are widely expressed in several brain regions such as the hippocampus, the neocortex, the amygdala, the striatum, the substantia nigra, the hypothalamus, the cerebellum and the brainstem (Marsicano and Kuner, 2008; Kano et al., 2009). Classically, CB_1 receptors are described as mainly present in the presynaptic terminals, mostly of GABAergic interneurons, but also, although at lower levels, on many other neuronal types, such as glutamatergic, serotonergic, cholinergic and others (Marsicano and Kuner, 2008). Although CB_1 receptor expression levels can vary between different populations, it is important to state that differential expression of CB_1 receptors is not directly linked with equivalent functional relevance (Marsicano and Kuner, 2008; Bellocchio et al., 2010). This is particularly true when considering astroglial CB_1 receptors. Indeed, the presence of CB_1 receptors on astrocytes has been for a long time challenged by apparently contradictory results,

mostly because of the low levels of their detectable expression in astrocytes [reviewed in (Kano et al., 2009; Stella, 2010; Metna-Laurent and Marsicano, 2015)]. One important issue regarding astrocytic functions is that cultured astrocytes are different and behave differently than astrocytes *in vivo*, and these differences may produce results that might not correspond to natural mechanisms and thus must be carefully evaluated (Verkhratsky et al., 2012). Nevertheless, during the past 15 years, many studies were able to confirm both *in vitro* and *in vivo* that astrocytes functionally express CB_1 receptors, which are involved in important mechanisms that underlie brain functions (Navarrete and Araque, 2008, 2010; Han et al., 2012; Bosier et al., 2013).

The production and release of eCBs are also important aspects of ECS functioning. Believed to be produced and released "on demand", the two mainly studied eCBs, AEA and 2-AG, are lipid-derived signaling molecules capable of diffusing freely through cell membranes and activate CB_1 receptors mostly in a paracrine way (Piomelli, 2003), although autocrine mechanisms have been described (Bacci et al., 2004). The production of eCBs by astrocytes has been addressed by several studies, showing that astrocytes are able to efficiently synthesize these signaling molecules, mainly through Ca^{2+}- and ATP-dependent pathways (Stella, 2010). Interestingly, recent data indicate that astroglial CB_1 receptors participate in the turnover of eCBs in the brain (Belluomo et al., 2015). This eCB turnover by astrocytes might control the retrograde neuronal signaling of CB_1 receptors.

Another interesting aspect of astroglial CB_1 receptors refers to the mechanism of intracellular signaling. Classically, neuronal CB_1 receptors are believed to exert an inhibitory effect through the activation of $G_{i/o}$ proteins. Thus, the activation of neuronal CB_1 receptors leads to (1) an inhibition of adenylyl cyclase with subsequent decrease of cyclic adenosine monophosphate (cAMP) production and inhibition of protein kinase A, (2) an inhibition of voltage-activated Ca^{2+} channels (VGCC) and (3) the stimulation of inwardly rectifying K^+ channels (Howlett et al., 2010). Altogether, these effects induce an overall hyperpolarization of the presynaptic terminals and a consequent reduction of neurotransmitter release (Kano et al., 2009; Castillo et al., 2012). In astrocytes, evidence supports a mechanism dependent on G_q signaling with consequent mobilization of internal Ca^{2+}, thus leading to increases of intracellular Ca^{2+} levels (Navarrete and Araque, 2008; Perea et al., 2014). Thus, the intracellular consequences of CB_1 receptor signaling appears to emerge from its cellular localization, rather than being an intrinsic property of the protein. Moreover, CB_1 receptor activation leads also to the modulation of several intracellular pathways, such as the extracellular regulated kinases (ERKs) and others (Howlett, 2002). We will not discuss these issues in detail here, but some of these signaling pathways appear to be specific of certain cell types, including glial cells (Stella, 2009, 2010).

ASTROGLIAL CB_1 RECEPTOR SIGNALING IN THE TRIPARTITE SYNAPSE – ELECTROPHYSIOLOGICAL EVIDENCE

The broad range of overlapping physiological functions modulated by the ECS and astrocytes suggests their close functional association in vast domains such as energy and metabolism, neuroprotection and synaptic plasticity (Metna-Laurent and Marsicano, 2015). Regarding the purposes of this review, we will limit our discussion to the role played by the ECS in the modulation of synaptic transmission through astrocytes.

As previously described, the neuron–astroglial interactions represented in the tripartite synapse have begun to challenge important concepts regarding the individual contribution of both neurons and astrocytes to the synaptic outcome (Araque et al., 1999, 2014). The early identification of neuronal CB_1 receptors at both excitatory and inhibitory synapses led to the characterization of several types of ECS-dependent short- and long-term forms of synaptic plasticity (Chevaleyre et al., 2006; Kano et al., 2009; Castillo et al., 2012). Still, as several studies in the past decade reported the existence of the functional expression of CB_1 receptors in astrocytes, important questions were raised regarding the contribution of these receptors to synaptic functions.

The first functional evidence of this contribution appeared in 2008, when Navarrete and Araque (2008) reported that activity-dependent postsynaptic release of eCBs by CA1 pyramidal neurons is able to induce an intracellular Ca^{2+} increase in neighboring astrocytes through the activation of astroglial CB_1 receptors (Navarrete and Araque, 2008). The authors also showed that this Ca^{2+} increase depends on phospholipase C (PLC) activation and it is not abolished in the presence of pertussis toxin, thus suggesting that astroglial CB_1 receptor signaling is not dependent on classical $G_{i/o}$ proteins but likely on $G_{q/11}$ protein signaling (Navarrete and Araque, 2008). Interestingly, the same authors showed that these specific intracellular Ca^{2+} increases are responsible for the induction of N-methyl-D-aspartate receptor (NMDAR)-dependent glutamate-mediated slow inward currents (SIC) in proximal neurons, an effect dependent on postsynaptic NMDAR (Navarrete and Araque, 2008) (Fig. 1A).

It is known that hippocampal astrocytes occupy specific areas that are not overlapped by other astrocytes (Bushong et al., 2002), and that Ca^{2+} increases act as an integrator allowing astrocytes to dynamically modulate neuronal excitability and synaptic plasticity (Perea et al., 2014). Taking this into consideration, another interesting study evaluated the role of this eCB-mediated neuron-astroglial signaling. In paired electrophysiological recordings of hippocampal pyramidal neurons, Navarrete and Araque (2010) observed that while eCBs acting at homosynaptic neuronal CB_1 receptors produced a classical depolarization-induced suppression of excitation (DSE), they could also lead to a heterosynaptic short-term facilitation of synaptic transmission through astroglial CB_1 receptors (Navarrete and Araque, 2010). Mechanistically, the authors reported that

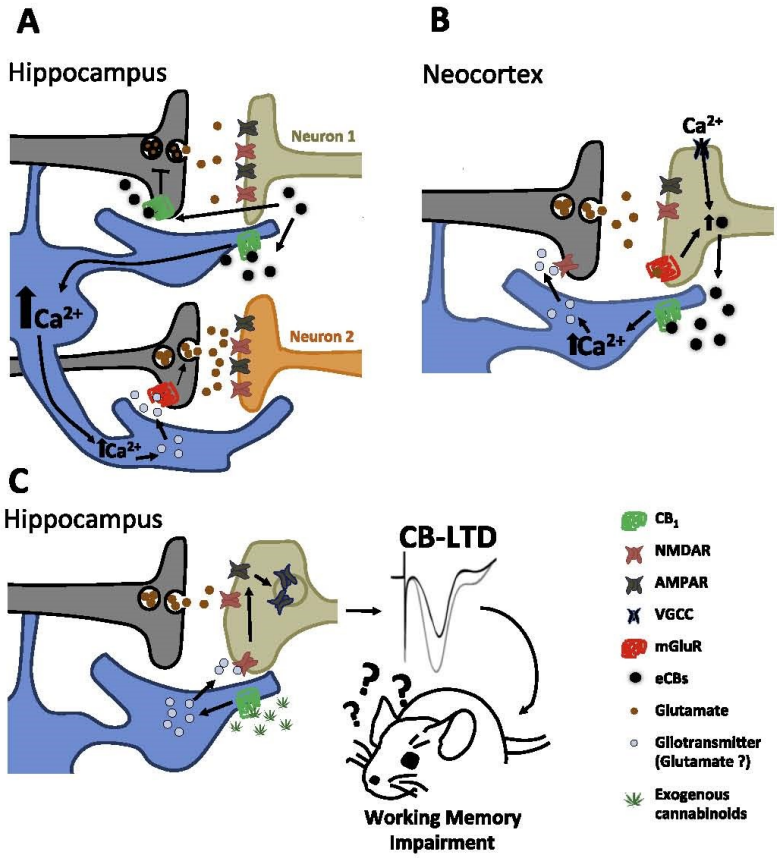

Fig. 1. ECS modulates synaptic function through astroglial CB_1 receptor-dependent signaling. (A) Activity-dependent production of eCB elicits a decrease of neurotransmitter release through the activation of presynaptic CB_1 receptor. In parallel, astroglial CB_1 receptor activation generates an intracellular Ca^{2+} increase in astrocytes responsible for a mGluR1-dependent lateral potentiation of synaptic transmission in distant single synapses (Navarrete and Araque, 2010; Gomez-Gonzalo et al., 2014). (B) eCBs produced by a t-LTD-inducing protocol produces astroglial CB_1 receptor-dependent transient Ca^{2+} increase in astrocytes. Both postsynaptic mGluR activation and VGCC-dependent Ca^{2+} influx induced by a back propagating action potential are involved in the synthesis of eCBs. In astrocytes, the Ca^{2+} increase is responsible for the putative release of glutamate that, by acting at presynaptic NMDAR, induces a synaptic long-term depression (t-LTD) (Min and Nevian, 2012). (C) Exogenous cannabinoid administration elicits an astroglial CB_1 receptor-dependent *in vivo* long-term depression of synaptic transmission (CB-LTD). Astroglial CB_1 receptor activation induces the putative release of glutamate which, through the activation of postsynaptic NMDAR, leads to internalization of AMPAR and to depression of synaptic transmission, likely responsible for the working memory impairment (Han et al., 2012).

Ca^{2+} increases elicited by astroglial CB_1 receptor stimulation lead to the activation of presynaptic metabotropic glutamate receptors type-1 (mGluR1), likely through glutamate (Navarrete and Araque, 2010) (Fig. 1A).

Several studies showed the existence of CB_1-mediated forms of plasticity, reported initially in juvenile animals, which are attenuated or even not present in adult animals, hence suggesting an important developmental effect over the ECS (Castillo et al., 2012). As *in vitro* electrophysiological data supporting the astroglial CB_1 receptor-dependent modulation of synaptic plasticity are based on the use of juvenile mouse brains (Navarrete and Araque, 2008, 2010), it would be important to address whether these forms of plasticity are developmentally regulated. Another issue that remains elusive is the amount of astroglial CB_1 receptors in astrocytes. Recently, another astroglial receptor, mGluR5, has been described to have differential expression throughout brain development starting with higher protein levels in juvenile animals and decreasing with age (Sun et al., 2013). Since astroglial CB_1 is found in extremely low quantities, though functionally very important, in adult mice (Han et al., 2012), it would be interesting to investigate whether a similar developmental pattern of regulation is associated with this receptor. Altogether, these results (Navarrete and Araque, 2008, 2010) are

particularly interesting not only for the demonstration of a broader CB_1 receptor modulatory role in bridging neuron–astrocyte interactions but also by proposing a new, different, non-canonical astroglial CB_1 receptor-mediated intracellular mechanism of action (Fig. 1A).

The astroglial CB_1 receptor-mediated modulation of long-term synaptic plasticity has been also reported in the somatosensory neocortex and in the hippocampus (Han et al., 2012; Min and Nevian, 2012; Gomez-Gonzalo et al., 2014). Spike-timing-dependent plasticity (STDP) can induce long-term potentiation (LTP) or long-term depression (LTD) depending on the order and the temporal interval between activation of the presynaptic and postsynaptic elements (Feldman, 2012). In the neocortex, the ECS is known to mediate a spike-timing-dependent depression (t-LTD) in the excitatory synapses (Sjostrom et al., 2003). This t-LTD is dependent on both CB_1 receptors and presynaptic NMDA receptor activations (Sjostrom et al., 2003). Recently, Min and Nevian (2012) provided functional evidence that astroglial CB_1 receptor activation mediates the t-LTD in the somatosensorial neocortical L4 to L2/3 synapses (Min and Nevian, 2012). The authors reported that eCBs released through post-before-presynaptic activity stimulate astroglial CB_1 receptors and induce an intracellular Ca^{2+} increase. Furthermore, this internal signal cascade is proposed to be responsible for a mechanism of gliotransmission that activates presynaptic NMDA receptors through the release of glutamate, thus inducing t-LTD (Min and Nevian, 2012) (Fig. 1B).

Also LTP can be modulated by astroglial CB_1 receptors. Gomez-Gonzalo and colleagues (2014) reported that eCB release is capable of inducing both a homosynaptic DSE and a lateral heterosynaptic LTP at single hippocampal synapses through the activation of astroglial CB_1 receptors. The mechanism, similarly to what have been described previously (Navarrete and Araque, 2010), relies on an astroglial CB_1 receptor-dependent Ca^{2+} increase. This event presumably causes the release of glutamate that induces an LTP through the activation of mGluR1 at presynaptic terminals (Gomez-Gonzalo et al., 2014).

The CB_1-dependent modulation of synaptic transmission and plasticity is thought to be one of the main mechanisms underlying many THC-induced psychotropic effects (Di Marzo et al., 2004; Castillo et al., 2012). In 2012, Han and colleagues reported that the administration of THC induces an *in vivo* LTD of excitatory transmission in the CA3–CA1 hippocampal synapses (Han et al., 2012). Furthermore, the genetic deletion of CB_1 receptors from astrocytes abolishes this cannabinoid-induced LTD (CB-LTD), whereas the genetic deletion of neuronal CB_1 receptors does not (Han et al., 2012). In addition, the authors also reported a signaling pathway involving the activation of NMDA receptors, possibly through astroglial glutamate release, which induces the CB-LTD via alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) internalization (Han et al., 2012). These results not only constitute the first *in vivo* direct evidence regarding the astroglial CB_1 receptor modulation of synaptic transmission but also support the validity of previous functional studies (Fig. 1C).

Although astroglial CB_1 receptors are proposed as the mediators of both CB-LTD (Han et al., 2012) and the indirect lateral eCB-mediated LTP (Gomez-Gonzalo et al., 2014), these studies, which are apparently contrasting, are not incompatible. In the first study (Han et al., 2012), the proposed mechanism is based on a massive administration of exogenous cannabinoids that might lead to a temporally prolonged and spatially widespread activation of astroglial CB_1 receptors. On the other hand, the endogenous eCBs production elicited by neuronal depolarization (Gomez-Gonzalo et al., 2014) might produce a substantially shorter and more localized activation of astroglial CB_1 receptors, thereby promoting a potential "fine-tuned" mechanism responsible for the observed LTP. Nevertheless, further investigations are needed to elucidate whether astroglial CB_1 receptor activation produces different effects depending on the nature of the agonists (e.g. endogenous versus exogenous cannabinoids), if astroglial CB_1 receptors behave *in vivo* differently from the main currently used models *in vitro*, or even the functional role of the astroglial CB_1 receptor-dependent signaling on the coordination of neuro-glial networks.

ROLE OF ASTROGLIAL CB_1 RECEPTORS IN BEHAVIORAL FUNCTIONS

The characterization of exogenous cannabinoids, and later of the ECS, brought an impressive number of experimental studies characterizing on the one hand the behavioral consequences of exogenous cannabinoids administration, and on the other hand the endogenous functions of the ECS in behavioral processes. Indeed, CB_1 receptors have been implicated in many behaviors including the control of food intake, of emotion- motivation- and stress-related responses, as well as the expression of cognitive processes (Carlini, 2004; Marsicano and Lafenetre, 2009; Puighermanal et al., 2009; Bellocchio et al., 2010; Riebe and Wotjak, 2011).

Genetic mouse models allowed determining which cell types are involved in the control of CB_1 receptors on some of these functions (e.g. Bellocchio et al., 2010; Dubreucq et al., 2012; Metna-Laurent et al., 2012). In particular, the GFAP-CB_1-KO mouse line, bearing a specific deletion of the CB_1 receptor gene in astrocytes (Han et al., 2012), is a unique model for studying the role of astroglial CB_1 receptors *in vivo*, and, more generally, an interesting tool to describe astrocyte-mediated functions in behavior.

The accumulating evidence of a direct control of astrocytic signaling on synaptic plasticity led several groups to examine the behavioral correlates of such a control, especially regarding memory functions (Suzuki et al., 2011; Stehberg et al., 2012). A reproducible effect of exogenous cannabinoids in both animals and humans is the impairment of short-term working memory (WM) (Lichtman et al., 1995; Hampson and Deadwyler, 1999; Ranganathan and D'Souza, 2006). GFAP-$CB_1^{-/-}$ mice

do not display any spontaneous phenotype in the acquisition of the WM task but they are insensitive to THC-induced WM impairment, suggesting that the endogenous activation of astroglial CB_1 receptors are dispensable for WM, whereas it is required for WM-disrupting effects of cannabinoids (Han et al., 2012). However, the WM impairment induced by acute injections of THC in mice was present in neuron-specific conditional CB_1 receptors mutant mice (i.e. lacking the *CB_1* gene in cortical glutamatergic or forebrain GABAergic neurons), strengthening that astroglial, but not neuronal, CB_1 receptors mediate the THC-induced disruption of WM performance (Han et al., 2012) (Fig. 1C).

Further experiments revealed that similar mechanisms underlie both the cannabinoid-induced impairment of WM and CB-LTD (see above), including the activation of NMDAR containing the GluN2B subunit and the endocytosis of AMPAR, suggesting a causal relationship between the cannabinoid effects on synaptic plasticity and on behavioral performance (Han et al., 2012)(Fig. 1C).

Although these results uncovered the necessity of astroglial CB_1 receptors for the THC-induced impairment of WM, the endogenous roles of these receptors on astrocytes need to be further investigated. A thorough evaluation of GFAP-*CB_1*-KO mice in other behavioral paradigms will likely provide information regarding the endogenous role of astroglial CB_1 receptor signaling in learning and memory and other behavioral processes.

Learning and memory processes are also regulated by adult neurogenesis (Abrous et al., 2005; Koehl and Abrous, 2011). In adult animals, particular brain regions such as the subventricular zone or the dentate gyrus contain GFAP-expressing cells that can give rise to newborn neurons and astrocytes (Bordey, 2006; Galve-Roperh et al., 2008). CB_1 receptors are likely expressed on progenitor GFAP-expressing cells of the subventricular zone and the dentate gyrus (Moldrich and Wenger, 2000; Galve-Roperh et al., 2008) and are necessary for normal adult neurogenesis through unknown mechanisms (Jin et al., 2004; Galve-Roperh et al., 2008). CB_1 receptors expressed in GFAP-positive cells might thus also contribute to the modulation of memory processes via the regulation of adult neuronal or astroglial proliferation and differentiation.

CONCLUSION

In this short review, we addressed the growing importance of CB_1 receptors expressed in astroglial cells in the regulation of synaptic transmission and plasticity and its likely consequences at behavioral level. The interactions between the ECS and astrocytes are mostly unexplored with many interesting questions to be addressed (Box 1). Their role in the regulation of high-order brain functions is a very exciting and new field of research, which will provide interesting surprises in the next future, shading additional light onto general mechanisms of brain functioning.

Box 1 Questions regarding the ECS in astrocytes.

(1) The level of expression of CB_1 receptors in astrocytes is low, yet the functional consequences of their activation are striking. Could this increased functional efficacy be explained by specific downstream signaling pathways? Can astroglial CB_1 receptors form homodimers or heterodimers? Is CB_1 present in astrocytic organelles such as mitochondria?
(2) Are CB_1 receptors in neurons and astrocytes differentially targeted and activated? If so, what could mediate the different regulation of the same receptor in a so close associated space? Are different ligands or different affinities to eCB involved in this differential modulation?
(3) How can astrocytes modulate the production and degradation of eCBs? Are astrocytes involved in the production of specific eCB? Do astrocytes produce eCB for autocrine or paracrine action?
(4) Astrocytes have an important role in brain energy by the supply of metabolites to neurons and many of the effects of CB_1 activation involve the regulation of brain metabolism with consequent changes in brain energy states (Metna-Laurent and Marsicano, 2015). What is the role of astroglial CB_1 on the main metabolic pathways and metabolites availability *in vivo*? Are these changes cell type specific?
(5) Astrocytes are highly dynamic cells (Heller and Rusakov, 2015). Can astroglial CB_1 activation modulate astrocytic mobility? Does it impact synaptic plasticity?
(6) Calcium is an internal integration signal of astrocytic information (Araque et al., 2014). Astroglial CB_1 activation induces synaptic plasticity through the increase of astrocytic calcium (Navarrete and Araque, 2008, 2010). Can astroglial CB_1 receptor-dependent calcium modulation impact at network level with consequent modulation of neuronal circuits and behavioral functions? Is the calcium rise induced by CB_1 receptors different in nature and consequences as compared to the one induced by other mechanisms?
(7) Does astroglial CB_1 receptor control other cognitive or behavioral functions than cannabinoid-induced working memory impairment?

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