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Laurie Robin

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POUR OBTENIR LE GRADE DE
DOCTEUR DE L'UNIVERSITÉ DE BORDEAUX

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Spécialité - Neurosciences

Laurie ROBIN

**ROLES OF ASTROGLIAL CANNABINOID TYPE 1
RECEPTORS (CB₁) IN MEMORY AND SYNAPTIC
PLASTICITY.**

Sous la direction de : Giovanni MARSICANO

Soutenue le 30 novembre 2018

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ROLES DU RECEPTEUR AUX CANNABINOÏDES DE TYPE 1 DES ASTROCYTES DANS LA MEMOIRE ET LA PLASTICITE SYNAPTIQUE.

Le système endocannabinoïde est un important modulateur des fonctions physiologiques. Il est composé des récepteurs aux cannabinoïdes, de ses ligands lipides endogènes (les endocannabinoïdes) et de la machinerie enzymatique pour leur synthèse et leur dégradation. Les récepteurs aux cannabinoïdes de type 1 (CB₁) sont exprimés dans différents types cellulaires dans le cerveau et sont connus pour être impliqués dans les processus mnésiques. Les endocannabinoïdes sont mobilisés dépendamment de l'activité notamment dans les régions cérébrales impliquées dans la mémoire telle que l'hippocampe. Dans cette région, les récepteurs CB₁ sont exprimés au niveau des terminaisons neuronales pré-synaptiques où leur stimulation inhibe la libération de neurotransmetteurs, modulant ainsi différentes formes d'activité synaptique.

Outre leur expression sur les neurones, les récepteurs CB₁ sont également exprimés par les astrocytes. Avec l'élément pré- et post-synaptique, les astrocytes font partis de la « synapse tripartite » où ils participent à la plasticité synaptique et les processus mnésiques associés. De manière intéressante, la stimulation des récepteurs CB₁ astrocytaires facilite la transmission glutamatergique dans l'hippocampe. Dans cette région, les astrocytes régulent l'activité des N-méthyl-D-aspartate receptors (NMDARs) à travers le contrôle des niveaux synaptiques de leur co-agoniste, la D-serine, modulant ainsi la plasticité synaptique à long-terme. Cependant, le mécanisme entraînant la libération de D-serine par les astrocytes n'est pas identifié. De manière intéressante, notre laboratoire a montré que les effets délétères des cannabinoïdes exogènes sur la mémoire de travail spatial sont médiés par les récepteurs CB₁ astrocytaires à travers un mécanisme dépendant des NMDARs dans l'hippocampe. Cependant, le rôle physiologique des récepteurs CB₁ astrocytaires restent méconnus.

Une des formes de mémoire impliquant le récepteurs CB₁ est la mémoire de reconnaissance d'objet (NOR). La stimulation exogène des récepteurs CB₁ hippocampique inhibe la consolidation de la NOR mais la délétion constitutive des récepteurs CB₁ n'affecte pas la NOR, suggérant que la signalisation des récepteurs CB₁ endogènes n'est pas nécessaire. Cependant, de récentes études soulignent que la délétion globale du gène CB₁ pourrait masquer le rôle des récepteurs CB₁ des différents types cellulaires. Ceci indique la nécessité de nouveaux outils plus sophistiqués afin de totalement comprendre le rôle physiologique du système endocannabinoïde dans des comportements complexes.

Dans cette étude, nous avons étudié le rôle physiologique des récepteurs CB₁ astrocytaires dans la formation de la NOR et la plasticité synaptique. En utilisant une combinaison d'approches génétiques, comportementales, électro-physiologiques, d'imagerie et de biochimie, nous avons montré que l'activation endogène des récepteurs CB₁ astrocytaires est nécessaire pour la consolidation de la NOR à long-terme, et ceci à travers un mécanisme impliquant l'apport en D-sérine, afin de stimuler l'activité des NMDARs synaptiques de l'hippocampe dorsal.

Cette étude révèle un mécanisme inattendu à la base de la libération de D-sérine, entraînant l'activité des NMDARs et la formation de la mémoire à long-terme.

Mot-clés: Récepteurs CB₁ astrogliaux, D-serine, Récepteur NMDA , Mémoire, Synapse LTP *In vitro*, LTP *In vivo*, Hippocampe, Astrocytes.

ROLES OF ASTROGLIAL CANNABINOID TYPE 1 RECEPTORS IN MEMORY AND SYNAPTIC PLASTICITY.

The endocannabinoid system is an important modulator of physiological functions. It is composed of cannabinoid receptors, their endogenous lipid ligands (the endocannabinoids) and the enzymatic machinery for endocannabinoid synthesis and degradation. The type-1 cannabinoid receptors (CB1) are expressed in different cell types of the brain and are known to be involved in memory processes. Endocannabinoids are mobilized in an activity-dependent manner in brain areas involved in the modulation of memory such as the hippocampus. In this brain region, CB1 receptors are mainly expressed at neuronal pre-synaptic terminals where their stimulation inhibits the release of neurotransmitters, thereby modulating several forms of synaptic activity.

Besides their expression in neurons, CB1 receptors are also expressed in astrocytes. Along with the pre- and post-synaptic neurons, astrocytes are part of the “tripartite synapse”, where they participate in synaptic plasticity and associated memory processes. Interestingly, modulation of astroglial CB1 receptors has been proposed to facilitate glutamatergic transmission in the hippocampus. In this brain area, astrocytes regulate the activity of N-methyl-D-aspartate receptors (NMDARs) through the control of the synaptic levels of their co-agonist D-serine, thereby mediating long-term synaptic plasticity. However, the mechanisms inducing D-serine release by astrocytes are still not identified. Interestingly, our laboratory showed that the negative effect of exogenous cannabinoids on spatial working memory is mediated by astroglial CB1 receptors through a NMDAR-dependent mechanism in the hippocampus, but the physiological role of astroglial CB1 remains unknown.

One of the forms of memory involving CB1 receptors is novel object recognition (NOR) memory. The exogenous stimulation of hippocampal CB1 receptors inhibits the consolidation of long-term NOR formation. Constitutive global deletion of CB1 receptors in mice leaves NOR memory intact, suggesting that endogenous CB1 receptor signaling is not necessary for long-term NOR. However, recent studies pointed-out that, likely due to compensatory mechanisms, the global deletion of the *CB1* gene might mask cell type-specific roles of CB1 receptors, indicating that more sophisticated tools are required to fully understand the physiological roles of the endocannabinoid system in complex behavioral functions.

In this work, we investigated the physiological role of the astroglial CB1 receptors on NOR memory formation and synaptic plasticity. By using a combination of genetic, behavioral, electrophysiological, imaging and biochemical techniques, we showed that endogenous activation of astroglial CB1 receptors is necessary for the consolidation of long-term NOR memory, through a mechanism involving the supply of D-serine to enhance synaptic NMDARs-dependent plasticity in the dorsal hippocampus.

This study uncovers an unforeseen mechanism underlying D-serine release, triggering NMDARs activity and long-term memory formation.

Key words: Astroglial CB₁ receptors, D-serine, NMDA receptors, Memory, Synapse, *In vitro* LTP, *In vivo* LTP, Hippocampus, Astrocytes, Synapse.

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Key words: Astroglial CB₁ receptors, D-serine, NMDA receptors, Memory, Synapse, *In vitro* LTP, *In vivo* LTP, Hippocampus, Astrocytes, Synapse.

*A ma sœur Emilie
une des femmes les plus fortes que je connaisse.*

*« Le principal fléau de l'humanité n'est pas l'ignorance, mais le refus de savoir »
(Simone de Beauvoir)*

LIST OF PUBLICATIONS

Robin, L.M.*, Oliveira da Cruz, J.F.*, Langlais, V.C.*, Martin-Fernandez, M., Metna-Laurent, M., Busquets-Garcia, A., Bellocchio, L., Soria-Gomez, E., Papouin, T., Varilh, M., Sherwood MW., Belluomo I., Balcells G., Matias I., Bosier B., Drago F., Van Eeckhaut A., Smolders I., Georges F., Araque A., Panatier A., Olier SHR.#, Marsicano G.# (2018). **Astroglial CB₁ Receptors Determine Synaptic D-Serine Availability to Enable Recognition Memory**. *Neuron* 98, 935-944.e5.

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LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
AAV	Adeno-Associated Virus
ABD	Agonist Binding Site
AC	Adenylate Cyclase
ACEA	Arachidonyl-2'-chloroethylamine
AEA	Arachidonylethanolamide; Anandamide
AMPA	α -Amino-3-hydroxy-5-Methyl-4-isoxazole propionic acid receptors
ANLS	Astrocyte-neuron lactate shuttle
AP5	2-amino-5-phosphonovalérique acid
ASCT	Alanine-Serine-Cysteine-Transporter
ATP	Adenosine Tri Phosphate
b.C.	before Christ
Best-1	Bestrophin 1
CaMKII	Calmodulin-dependent protein kinase II
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
cAMP	Cyclic adenosine monophosphate
Ca²⁺	Calcium
CB-LTD	Cannabinoid-induced Long-Term Depression
CB1R	Cannabinoid type 1 receptor
CB2R	Cannabinoid type 2 receptor
CeM	Central amygdala
CNS	Central Nervous System
CSF	Cerebro-Spinal Fluid
Cx	Connexin
DAAO	D-Amino Acid Oxidase
DAG	Diacylglycerol
DAGL	Diacylglycerol Lipase
DREADDs	Designer Receptor Exclusively Activated by Designer Drugs
DSE	Depolarization-induced Suppression of Excitation
DSI	Depolarization-induced Suppression of Inhibition
EAAT	Excitatory Amino Acid Transporter
eCB	Endocannabinoid
eCB-LTD	Endocannabinoid-mediated LongTerm Depression
eCB-STD	Endocannabinoid-mediated Short-Term Depression
ECS	Endocannabinoid System
EM	Electron Microscopy
EPSC	Excitatory Post-Synaptic Currents
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase

LIST OF ABBREVIATIONS

ERT	Estrogen Receptor
FAD	Flavine Adenine Dinucleotide
FAAH	Fatty Acid Amid Hydrolase
GABA	Gamma-Aminobutyric Acid
GAT	GABA Transporters
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter
GLT-1	Glutamate Transporter
Glu	Glutamate
Gln	Glutamine
GlyRs	Glycine Receptors
GPCR	G-Protein Coupled Receptors
I1	Phosphate Protein 1 Inhibitor 1
iGluR	Ionotropic Glutamate Receptor
IP₃	Inositol 1,4,5-trisphosphate
IP₃R	IP3 Receptor
IPSCs	Inhibitory Post-Synaptic Currents
JNK	c-Jun N-terminal Kinase
K⁺	Potassium
kDA	Kilo Dalton
Kir	Inward-rectifying potassium channels
KO	Knock-Out
LTD	Long Term Depression
LTDi	Long-Term Depression of inhibition
LTM	Long Term Memory
LTP	Long-term Potentiation
MAGL	Monoacylglycerol Lipase
MAGUK	Membrane-Associated Guanylate Kinases
MAPK	Mitogen Activated Protein Kinase
MCTs	MonoCarboxylate Transporters
mGluR	Metabotropic Glutamate receptor
Mg²⁺	Magnesium
Mhb	Medial Habenula
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
mtCB1	Mitochondrial CB1 receptors
Na⁺	Sodium
NAT	N-acetyltransferase
NAPE	N-Arachidonoyl Phosphatidyl Ethanol
nm	nanometer
NMDAR	N-Methyl-D-Aspartate Receptor
NO	Nitric Oxide
NOR	Novel Object Recognition
NORT	Novel Object Recognition Memory Task

LIST OF ABBREVIATIONS

NT	Neurotransmitter
NTD	N-terminal Domain
PAP	Perisynaptic Astrocytic Processes
PCP	Phencyclidine
PKA	Protein Kinase A
PLA	Phospholipase A
PLC	Phospholipase C
PNS	Peripheral Nervous System
PP1	Phosphate Protein 1
PTPN22	Protein Tyrosine Phosphatase non-receptor type-22
PSD	Post-synaptic Density
SIC	Slow Inward Current
SLMV	Synaptic-Like Microvesicles
SNARE	Soluble -ethylmaleimide-sensitive fusion protein attachment protein receptor
SR	Serine Racemase
STED	Stimulation-Emission-Depletion
STM	Short-Term Memory
TCA cycle	TriCarboxylic Acid cycle
TDM	Transmembrane Domain
THC	Δ^9 -tetrahydrocannabinol
t-LTD	time-dependent Long Term Depression
TM	TransMembrane
TRPA1	Transient Receptor Potential Ankyrin 1
TRPV1	Transient Receptor Potential Vanilloid type 1 ion channel
VAMP2	Synaptobrevin
VAMP3	Cellubrevin
VRAC	Volume-Regulated Anion Channel
VGCC	Voltage Gated Calcium Channel
WT	Wild-Type
μm	Micrometer

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CHAPTER I

Glial Cells and the tripartite synapse

Glial cells are non-neuronal cells in the brain, in the spinal cord and also in the peripheral nervous system (PNS). Rudolf Virchow described them as a connective substance and a “kind of glue” in which nervous elements are embedded (Die Cellularpathologie, 1858). Glial cells are diverse in shape and functions, and they have been historically classified in oligodendrocytes, microglia and astrocytes. Classically, they are considered to play supportive roles to neurons, by several mechanisms. Thus, oligodendrocytes provide myelin isolation to neuronal axons, microglia represent “immune cells” in the brain, and astrocytes deliver nutritional structural support to neurons (Del Río-Hortega, 1922; Ramon y Cajal, 1903; Ramon y Cajal, 1904). As they are not electrically excitable, the potential roles of glial cells in brain information processing have been long underestimated. Nowadays, however, we know that glial cells are directly and actively involved in synaptic transmission in the central and peripheral nervous system. In particular, new theories propose that astrocytes can encode information through calcium (Ca^{2+}) signaling and cooperate with neurons to exert all brain functions. In this frame, modern hypotheses postulate that, as members of the “tripartite synapse”, astrocytes directly participate in synaptic transmission and information exchange (Araque et al., 1999).

I. History of the discovery of glial cells

Glial cells were discovered in the mid-1800s by a group of scientists including Robert Remak, Theodor Schwann and Rudolf Virchow. We consider that Robert Remak was the first to mention a type of glial cells in his thesis published in 1836 where he described “nerve fibers and their surrounding sheaths”, that will be later called Schwann cells. However, discovery of glial cells is more often assigned to Rudolf Virchow, who introduced the concept of neuroglia in 1856 as a “kind of connective tissue in which the nervous elements are planted”. We owe the first drawings of a star-shaped glial cell to Otto Deiters whose work was published posthumously in 1865. These cells were called later astrocytes by Michael von Lenhossek (Parpura and Verkhratsky, 2012) Other drawings of glial networks were produced some years later by Jacob Henle and Friedrich Merkel (Henle & Merkel, 1864).

In contrast to extensive studies on various neuronal functions, glial cells remained poorly studied. This is likely due to the fact that glial cells are electrically non-excitabile (Orkand et al., 1966; Sontheimer, 1994), as they do not fire action potentials, with the exception of a recently discovered particular subpopulation, NG2 cells (Lin and Bergles, 2002). Thus, glial cells would not be able to be part of the genesis, exchange and integration of fast electrical information and neurophysiologists kept the idea of glia as passive cells supporting neurons (Orkand et al., 1966; Seifert and Steinhäuser, 2001; Sontheimer, 1994; Verkhratsky and Steinhäuser, 2000).

Thanks to histological studies, Camillo Golgi (1889) and Santiago Ramon y Cajal proposed that glial cells could play a role in metabolic support to neurons (Ramon y Cajal, 1903; Ramon y Cajal, 1911). In cortical slices, they observed cells with thin and long processes in contact with both blood vessels and neurons (**Figure 1, page 2**), and proposed a “physiological importance of these cells in the regulation of brain

CHAPTER I: Glial cells and the tripartite synapse

microcirculation”, establishing the so-called “*Neuron Nutrition Theory*” for their function. Finally, although Ramon y Cajal proposed for a long time that axon secreted myelin, he published with Del Río-Hortega in 1922 a bold hypothesis: “*We are inclined to believe, however, that both kinds of cells (oligodendroglia and Schwann cell) carry out identical functions of support, isolation, and nutrition connected with nerve conduction*”(Del Rio Hortega, 1922).

Although research on glial cells started 200 years ago, it is only recently that brain scientists showed a real craze for those cells. Through the development of tools and imaging techniques, it was shown that these cells could actively participate in synaptic communication (Allen and Barres, 2005; Haydon, 2001). These observations started a new wave of interest for glial cells which seems essential since they represent 50% of the volume of the brain (Laming, 2000). Nowadays, it is generally accepted that glia-neurons ratio is 1:1 in adult human brain (Azevedo et al., 2009) However, this ratio can vary in different brain regions and species. For example, it is more accurate to say that glia-neuron ratio in the mouse brain is about 3:1(Herculano-Houzel et al., 2006).

Firstly described as a "cement" for neurons, a wide number of studies showed that glial cells are not only supportive cells for neurons, but they also display a large repertoire of properties and active functions.



Figure 1: Cajal's drawing showing “neuroglia”.

Glial cells of the pyramidal layer and *stratum radiatum* of the Ammon horn (from adult man autopsied 3h after death). Original labels: A, large astrocyte embracing a pyramidal neuron; B, twin astrocytes forming a nest around a cell, C, while one of them sends two branches forming another nest, D; E, cell with signs of “autolysis”; F, capillary vessel.

Image from Navarette & Araque, 2014 and adapted.

II. Glial cells and their functions

Based on their morphology and origin, glial cells are currently classified into 2 groups: microglial and macroglial cells. Microglia contains microglial cells only, whereas macroglia contains oligodendrocytes, NG2 cells, Schwann cells, ependymal cells and astrocytes (**Figure 2, page 5**). The present PhD Thesis deals with synaptic active roles of astrocytes. In the following, I will briefly describe the general functions of most glial cells subtypes, for which active roles in information processing have been proposed. Besides certain macroglial subtypes such as ependymal and Schwann cells (Jiménez et al., 2014; Kidd et al., 2013), active roles in synaptic information processes have been suggested for all glial cells in the central nervous system (CNS). In the following, I will briefly introduce the functions of each glial subtype, indicating their "classical" and "novel" proposed roles.

A. MICROGLIAL CELLS

Microglial cells are considered to form the immune system of the CNS. Indeed, they have common properties with macrophages and are essential to protect neurons. At the basal state, they sense the environment thanks to their highly mobile processes. In case of damages in the CNS, they are "activated" (Ransohoff and Cardona, 2010). They multiply and migrate to the lesion (Davalos et al., 2005), where they can act as phagocytes. They are also able to secrete cytokines that can modulate cell survival and fate (Ransohoff and Cardona, 2010). Apart from their role as immune cells, microglia seems to participate in active information processing (Bessis et al., 2007). Indeed, they are able to communicate with neurons and astrocytes to modulate synaptic transmission and could be the 4th partner of a putative "quadripartite" synapse (Hristovska and Pascual, 2015; Panatier and Robitaille, 2012).

B. MACROGLIAL CELLS

i. Oligodendrocytes

The main function of oligodendrocytes is to provide support and insulation to axons in the CNS by creating the myelin sheath (Gill and Binder, 2007; Nave, 2010). Along neuronal axons, myelin sheaths are separated by 1-2 μm from each other, forming the so-called Ranvier nodes (also known as myelin-sheath gaps), where the neuronal membrane is exposed to the extracellular space. Because Nodes of Ranvier are uninsulated and enriched in ion channels such as sodium (Na^+) channels, they are key elements of ions exchanges to generate action potentials and to allow their so-called "saltatory" propagation (Kaplan et al., 1997, 2001; Salzer, 1997). Recent evidence pointed out the existence of a sort of "axomyelinic neurotransmission" involving several oligodendrocyte-dependent novel mechanisms (Micu et al., 2018).

ii. NG2 cells

NG2 cells are the most recently identified glial cell subtypes. They are characterized by the expression of the proteoglycan NG2 and are considered as

oligodendrocyte precursor cells in the CNS (Nishiyama et al., 2009). These cells are still poorly studied, but accumulating evidence indicates that they have many specific properties and functions in the CNS. For instance, they can interact with synapses, representing a likely additional functional component (Bergles et al., 2000; Nishiyama et al., 2009).

iii. Schwann cells

Schwann cells are the principal glia of the PNS, analogue to oligodendrocytes in the CNS. They produce the myelin sheaths around neuronal axons to ensure protection to neurons and the propagation of action potentials (Voyvodic, 1989). Schwann cells also assist in neuronal survival and signal the formation of various structures within the PNS. The Schwann cell offers trophic support to developing neurons whose axons have not yet reached their targets (Riethmacher et al., 1997). Finally, recent studies suggest that Schwann cells could be active synaptic partners, particularly at the neuromuscular junction, where these cells regulate synaptic efficacy (Rousse and Robitaille, 2006; Sugiura and Lin, 2011).

iv. Ependymal Cells

Ependymal cells are part of CNS glia. Ependymal cells form the epithelial lining of the ventricles in the brain and the central canal of the spinal cord. They are involved in the production of cerebrospinal fluid (CSF) (de Reuck and Vanderdonck, 1986) and they have been shown to serve as a reservoir for neuroregeneration (Carlén et al., 2009). One type of ependymal cells, known as tanycytes are found only on the floor of the third ventricle and play an important role in facilitating the transport of hormones and other substances in the brain (Langlet et al., 2013).

v. Astrocytes

Firstly described as star-shaped glial cells, astrocytes represent following recent evaluations approximately 20-40% of total glia in the brain, depending on brain regions and species (Herculano-Houzel, 2014). We know now that they are not all star-shaped but they have thin and ramified processes that interact with both neurons and blood vessels, allowing them to put these elements in communication (Pellerin and Magistretti, 2012). After neurons, they are the most studied CNS cells. Indeed, simple PubMed searches using cell names as key words reveal the following numbers of published papers: > 600.000 for neuron, > 53.000 papers for astrocyte, > 29.000 for oligodendrocyte, > 28.000 for microglia, > 16.000 for Schwann cells, > 4.000 for ependymal cells and in the order of some hundreds for NG2 cells. In particular, astrocytes have been recently shown to participate in many physiological functions such as sleep, reproduction and memory (Ben Achour and Pascual, 2012; Halassa et al., 2009). Ongoing research focuses on determining novel specific cellular and functional properties of astrocytes, and new subpopulations of these cell types are continuously proposed (Oberheim et al., 2012). In the following, I will briefly describe established general properties of astrocytes providing useful information for the experimental part of this Thesis.

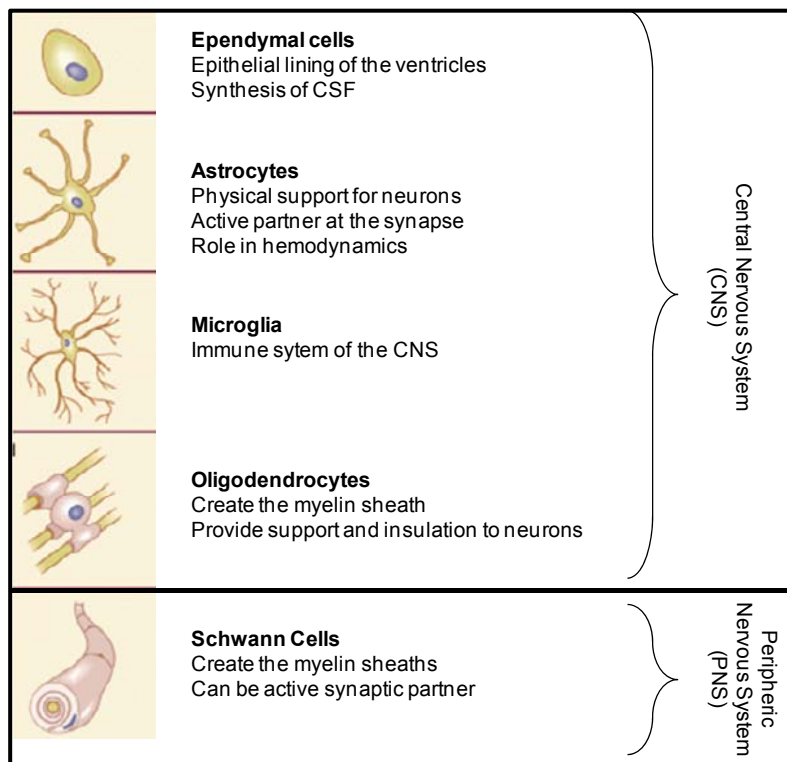


Figure 2: Classification, main functions and structures of glial cells.
Adapted from the book Neuroscience, Dale Purves & George J. Augustine. 2015

III. Cell biology of astrocytes

A. MORPHOLOGY

Following classical morphological classifications, there are 3 types of astrocytes (**Figure 3, page 6**): protoplasmic, fibrous and radial astrocytes (Miller and Raff, 1984). Protoplasmic astrocytes are located in the grey matter and have numerous and short ramifications. On the contrary, fibrous astrocytes are in the white matter, they have less but longer and thinner ramifications (Zhang and Barres, 2010). Radial glia, with the exception of Müller cells of the retina and cerebellar Bergmann glia, are generally present only during development. They are placed perpendicular to ventricles and their main function is to help the migration of developing neurons (Rakic, 2003). Within these types, astrocyte morphology is heterogeneous since it depends on the species and the brain regions (Matyash and Kettenmann, 2010; Oberheim et al., 2012). In general, there are no clear parameters to define astrocytes. However, some features, such as the star-like shape, the presence of thin and ramified processes in contact with blood vessels and neurons and the expression of the intermediate filament of the cytoskeleton Glial Fibrillary Acidic Protein (GFAP) and/or of specific transporter proteins, are used to identify these cell types. Because they represent the main subject of the present Thesis work, we will mainly focus on protoplasmic astrocytes of the hippocampus in the following sections.

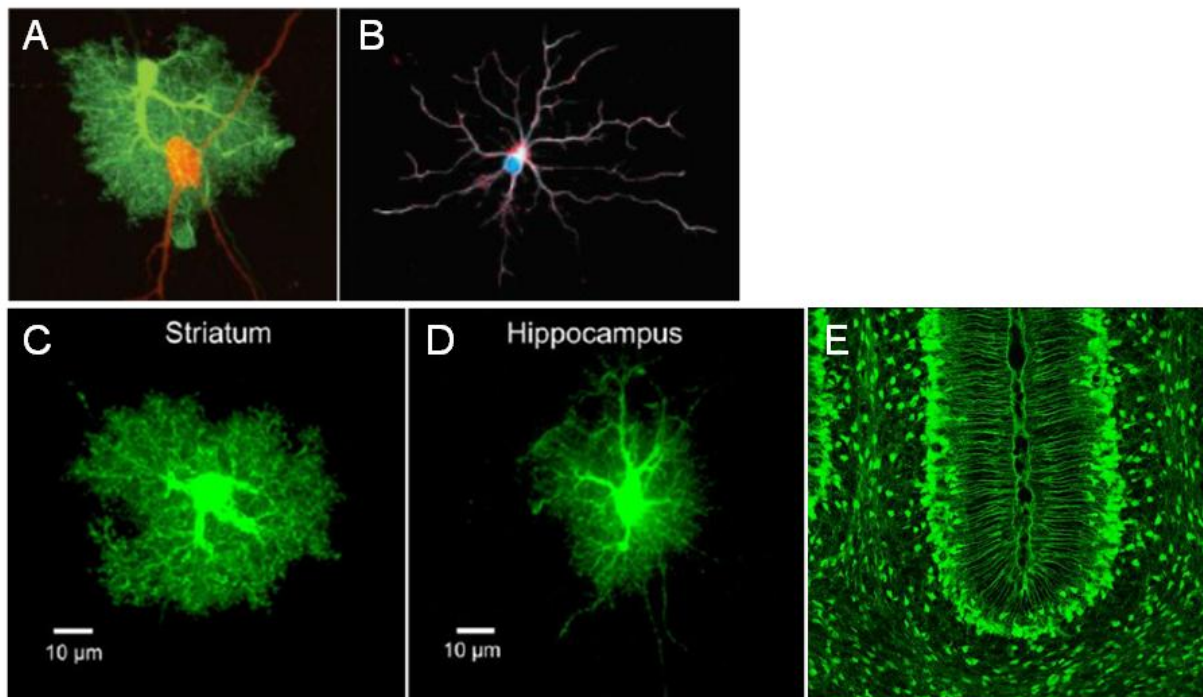


Figure 3: Example of different types of astrocytes.

(A) Example of protoplasmic astrocyte (green) in close association with neuronal cell body and processes (red). (B) Example of Fibrous astrocyte. (C-D) Representative pictures of protoplasmic astrocytes from the mouse striatum and hippocampus, respectively. (E): Example of Bergmann glia.

(A) Adapted from Allen and Barres, 2009.

(B) Adapted from https://fhs.mcmaster.ca/fxar/astrocytes_gallery.html

(C-D) Adapted from Chai et al., 2017.

(E) Adapted from <http://www.gensat.org/imagenavigator.jsp?imageID=10578>

i. Processes and astrocyte territories

Primary processes of hippocampal protoplasmic astrocytes are quite thick and rich in GFAP while the distal processes are very small, containing a limited amount of cytoplasm, lacking most of the organelles as well as GFAP (Theodosis et al., 2008). It has been calculated that cell body and primary processes represent only 15% of the total volume of astrocytes (Bushong et al., 2002), implicating that 85% of the cellular volume is formed by very tiny elements that are extremely difficult to observe and study (Bushong et al., 2002; Freeman, 2010).

Therefore, one aim of recent studies is to develop precise methods to observe astrocyte processes. For example, filling astrocytes with appropriate markers such as Lucifer Yellow or Alexa Fluor 568 revealed that astrocytes processes extend from 50 to 100 µm from the cell body and that different astrocytes display a very limited spatial overlap (~5%; **Figure 4, page 7**), suggesting that each cell occupies a distinct territory in the hippocampus (Wilhelmsson et al., 2006) and, importantly, that 2 astrocytes are unlikely to contact the same synapse (Bushong et al., 2004; Oberheim et al., 2006).

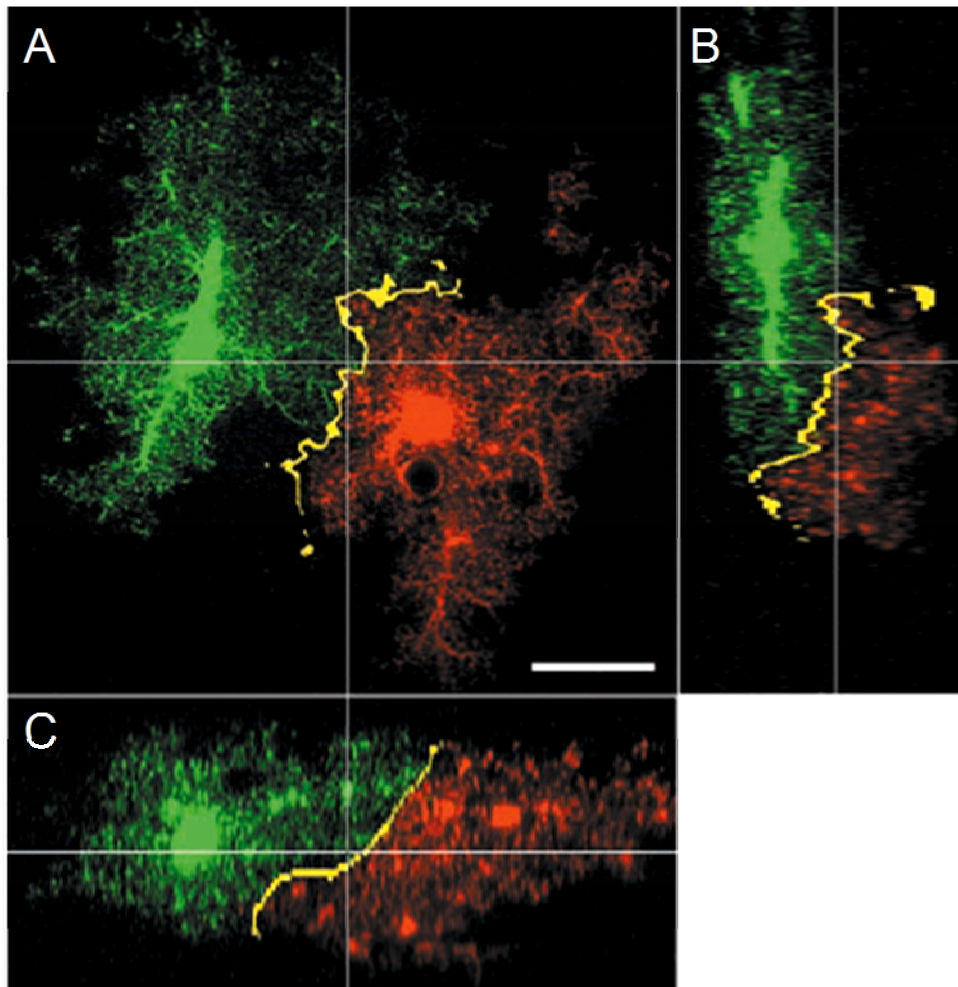


Figure 4: The discrete region of interaction across the fine processes of protoplasmic astrocytes.

(A-C) Respective x–y, y–z and x–z views of hippocampal astroglial processes occupying specific territories called “domains”. Domains of different astrocytes (in green and in red) display a very limited spatial overlap (~5 %; in yellow). Scale bar, 20 μ m.

Adapted from Bushong et al., 2002.

B. ASTROCYTIC NETWORK

Astrocytes were thought for a long time that astrocytes are organized as a global syncytium, which is a mass of cytoplasm that has many nuclei and an enclosing membrane but no individual cells (Brightman and Reese, 1969). Indeed, astrocytes are connected through gap-junctions and some molecules can freely move from one cell to another (Pappas et al., 1996). However, it is now clearly admitted that astrocytes are organized as delimited networks. Using some markers that can diffuse through those gap-junctions, it was shown that the number of connected astrocytes is different depending on the brain regions and physiological states of the animals (Blomstrand et al., 2004; D’Ambrosio et al., 1998; Giaume and Theis, 2010; Konietzko and Müller, 1994; Rouach et al., 2008). For instance, in the CA1 region of

the hippocampus, recent data proposed that astrocytes' networks are likely composed of hundreds of cells (Rouach et al., 2008). Gap-junction coupling is an efficient way to maintain intercellular communication and coordinate information exchanges between, because these junctions allow the exchange of ions and small signaling molecules (under 1,5 kDA) (Giaume and Liu, 2012; Giaume and Theis, 2010).

Gap junctions are composed of 2 juxtaposed hemichannels called connexons. Each of these connexons is composed of 6 transmembrane proteins called connexins (Cx). In the astrocytes the main 2 types of Cx are Cx30 and Cx43. Moreover, when alone, connexons act as hemichannels allowing the entry or exit of molecules. For instance, it was proposed that hemichannels can participate in the release of some gliotransmitters such as glutamate (Ye et al., 2003).

C. MAIN PHYSIOLOGICAL FUNCTIONS OF ASTROCYTES

Astrocytes play major roles in the CNS. Some of these functions are described below.

i. Glutamate uptake

Astrocytic processes surrounding synaptic elements express transporters for a variety of neurotransmitters and neuromodulators including glutamate (Gadea and López-Colomé, 2001). These transporters participate in the rapid removal of neurotransmitters released into the synaptic cleft (**Figure 5, page 9**), which is essential for the termination of synaptic transmission and maintenance of neuronal excitability, with important implications in protecting neurons against glutamate-induced excitotoxicity (Yi et al., 2005).

At rest, extracellular glutamate levels are maintained in the low micromolar range (2 μM) but they increase dramatically during glutamatergic neurotransmission, reaching up to 1 mM for a few milliseconds in the synaptic cleft (Clements et al., 1992). This concentration of glutamate would cause extensive neuronal injury in the absence of highly efficient mechanisms for its removal at the synapse. This is primarily achieved by the astrocyte-specific sodium-dependent high affinity Glutamate Transporters GLT-1 and the astroglial Glutamate Aspartate Transporter GLAST (corresponding to human EAAT2 and EAAT1, respectively) and to a lesser extent by the neuronal glutamate transporters EAAC1 (human EAAT3) and EAAT4 (Danbolt, 2001).

ii. Potassium buffering

Propagation of action potentials causes substantial local increases of extracellular potassium ions (K^+) in the extracellular space, going from 3 μM at rest to around 10 μM . Such an increase can alter the neuronal membrane potential and lead to neuronal hyperexcitability (Walz, 2000). This is prevented by the buffering of K^+ by astrocytes (Holthoff and Witte, 2000), which occurs mainly through by 2 types of mechanisms.

The first one is passive absorption of K^+ through the numerous K^+ channels such as the inward-rectifying potassium channels (Kir) (Chen and Nicholson, 2000; Sibille et

al., 2015). K^+ ions are then distributed in the astrocytic network (Bellot-Saez et al., 2017).

A second, active and energy-costing mechanism involves the Na^+/K^+ /ATPase pump. For every Adenosine-tri-Phosphate (ATP) molecule that the pump uses, three sodium (Na^+) ions are exported and two K^+ ions are imported into astrocytes (Figure 5, page 9). Thus the pump enables astrocytes to accumulate the excessive extracellular K^+ , which can then travel in the astrocytes through gap junctions, following its concentration gradient (D'Ambrosio et al., 2002). This allows the spatial dispersion of K^+ from areas of high concentration to areas of lower concentration where it can be extruded either into the extracellular space or the circulation, thus maintaining the overall extracellular K^+ concentration within the physiological range.

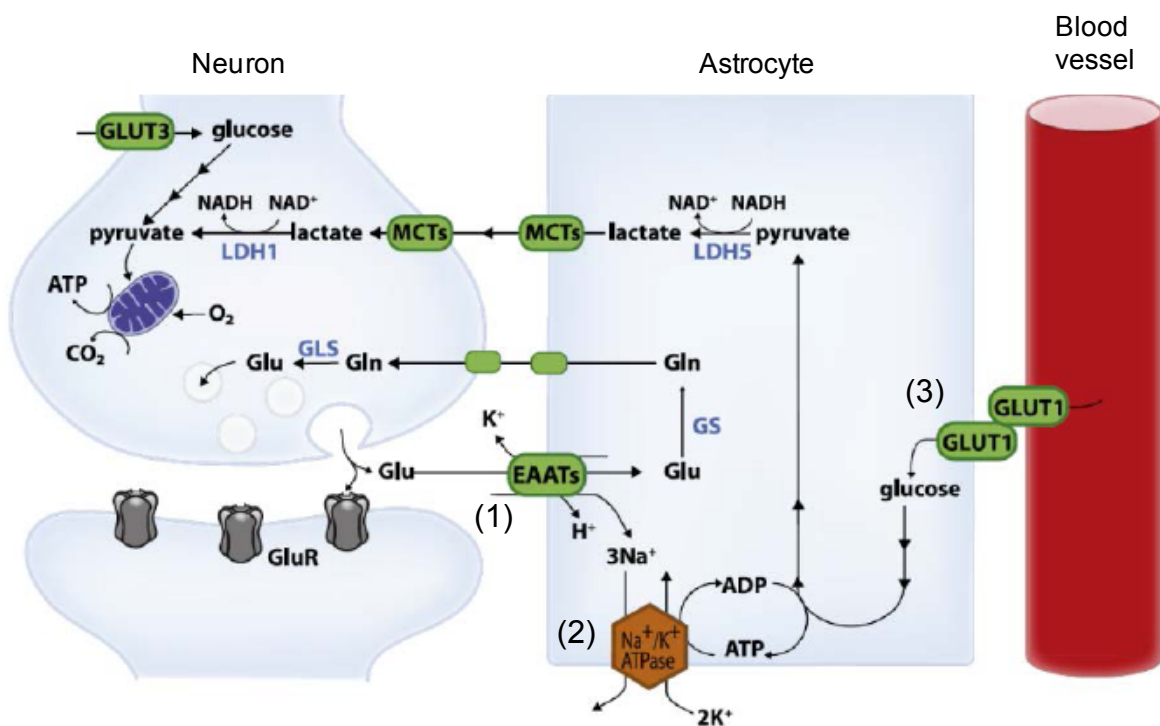


Figure 5: Astrocytes maintain synaptic homeostasis.

(1) During excitatory synaptic transmission, glutamate (Glu) is released by neurons. Then, Glu is uptaken by astrocytes through Excitatory Amino Acid Transporters (EAATs), such as GLT-1. Glu is converted to glutamine (Gln), which is uptaken by neuron to support Glu production. (2) Astrocytes uptake the extracellular K^+ , mainly through the Na^+/K^+ ATPase exchanger (3) Finally, astrocytes take glucose from blood and transform it into lactate, which is then released and taken up by neurons via monocarboxylate transporters (MCTs) to fuel neuronal metabolism.

Adapted from Bélanger et al., 2011.

iii. Neuro-Vascular coupling

Astrocytes are positioned between neurons and blood vessels. They have a particularly intimate relationship with blood vessels in the brain through their endfeet processes that completely envelop all cerebral blood vessels and can uptake glucose (Abbott et al., 2006). Although neurons can import glucose directly from the extracellular space, astrocytes have been proposed to play a key role in coupling neuronal activity and brain glucose uptake, through a mechanism called the astrocyte-neuron lactate shuttle (ANLS; **Figure 5, page 9**) (Pellerin et al., 2007; Simpson et al., 2007). This hypothesis originally proposed by Pellerin and Magistretti (1994) is based on the observation that glutamate uptake by astrocytes following synaptic release by neurons stimulate glycolysis in nearby astrocytes, and the lactate so produced is then released by astrocytes into the extracellular space and taken up by neurons via monocarboxylate transporters (MCTs) to fuel neuronal metabolism (Pellerin and Magistretti, 1994; Simpson et al., 2007). Once in neurons, lactate can be used as an energy substrate *via* its conversion to pyruvate by the action of lactate dehydrogenase and subsequent oxidation in the mitochondrial tricarboxylic acid cycle (TCA cycle). Over the years, experiments from several laboratories have supported the ANLS hypothesis (for reviews see (Magistretti and Allaman, 2015; Weber and Barros, 2015), and more recent *in vivo* experiments showed a lactate gradient between astrocytes and neurons (Mächler et al., 2016). Electrophysiological evidence also indicates that lactate released by astrocytes and taken up by neurons is necessary to sustain neuronal activity (Morgenthaler et al., 2006; Rouach et al., 2008).

The astrocytic functions described here are essential to maintain physiological neuronal activity (Bélanger et al., 2011). However, we know now that astrocytes are not only essential to maintain but they can also actively participate in synaptic transmission (Araque et al., 1999; Bains and Oliet, 2007).

IV. The tripartite synapse

Since the 1990's, accumulating evidence supports the presence of dynamic and bidirectional interactions between astrocytes and synaptic neuronal elements. This evidence indicated that astrocytes can detect synaptic neuronal activity and respond by modulating synaptic transmission. These findings suggested that astrocytes are an active protagonist of synaptic transmission and plasticity, and they established the concept of the "tripartite synapse" formed by pre- and post-synaptic elements and the surrounding astroglial processes (Araque et al., 1999). However, before describing the tripartite synapse in more details, it is important to introduce the notion of synapse and more particularly the excitatory synapse.

A. CANONICAL VIEW OF THE GLUTAMATERGIC SYNAPSE

The human brain contains approximately 10^{11} - 10^{12} of neurons (Kandel et al., 2000). Brain functions rely mainly on a functional unit: the synapse, a structure that permits

a neuron to pass an electrical or chemical signal to another cell and enables rapid signal transmission.

Chemical synapses are composed of the pre- and the post-synaptic element that transmits and receives the information, respectively. They are separated by the synaptic cleft, and connected through trans-synaptic protein-based nanocolumns (Tang et al., 2016).

Glutamate is the main excitatory neurotransmitter in the CNS and the glutamatergic synapse represents the major type of synapses in the brain. Glutamate released from pre-synaptic terminals binds to the post-synaptic glutamate receptors. Glutamate receptors are transmembrane proteins that specifically bind to glutamate on the extracellular side of the membrane. Upon binding of glutamate, glutamate receptors transduce the signal into intracellular responses. Glutamate receptors are divided in two groups: metabotropic receptors (mGluR), which are G protein-coupled receptors (GPCR), and ionotropic receptors (iGluR), which are glutamate-gated ion channels. iGluRs are the main responsible of fast synaptic transmission and can be divided into three main groups: N-Methyl-D-Aspartate receptors (NMDARs), α -Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid receptors (AMPA) and Kainate receptors (Dingledine et al., 1999a; Hollmann and Heinemann, 1994).

Excitatory glutamatergic synapses are defined as “asymmetric synapses” (**Figure 6, page 12**) because of their characteristic post-synaptic density (PSD), which faces the pre-synaptic active zone (Gray, 1959). PSD is identified by electron microscopy (EM) as an electron-dense region formed by protein complexes binding the post-synaptic membrane.

PSD is a disc-like structure measuring in average 30-60 nm thick and a few hundred nanometers wide (Carlin et al., 1980). The size of the PSD correlates with the size of the dendritic spine and with the number of postsynaptic glutamate receptors (Kasai et al., 2003).

With electron and super-resolution microscopy imaging, recent studies dissected the anatomy of the PSD (Maglione and Sigrist, 2013). Proteins within the PSD are located in different sites along the axo-dendritic axis of synapses, in this order :

- 1) Membrane receptors and cell adhesion molecules
- 2) membrane-associated guanylate kinases (MAGUK)
- 3) SAPAP, SH3 and SHANK scaffolds
- 4) The actin cytoskeleton contacting the interior face of the PSD

This dense protein complex does not only provide structural support, but also facilitates rapid and efficient synaptic transmission by gathering various signaling components and pathways (Boeckers, 2006; Kennedy, 1997; Kim and Sheng, 2004). The modulation of synapse activity constitutes a major strategy to control brain physiology and functions. However, a third partner is necessary to the proper function of brain physiology and synaptic activity, the astrocyte.

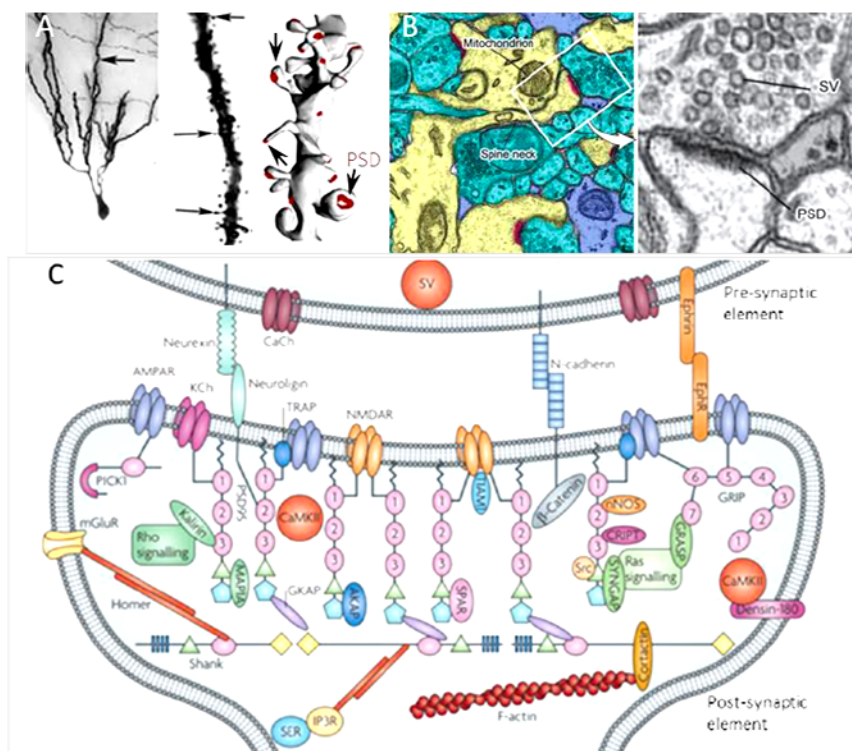


Figure 6: The glutamatergic synapse.

(A) Golgi impregnated hippocampal neurons and a representative dendrite covered by dendritic spines (arrows), 3D reconstruction materializes the presence of post-synaptic densities (PSD) on dendritic protrusions and spines (arrows). (B) EM morphology of a glutamatergic synapse. Dendritic elements appear in yellow, axons in turquoise, astrocytes in blue and PSD in pink. The pre-synaptic element containing synaptic vesicles loaded with glutamate (SV) faces the post-synaptic element with the typical electron-dense PSD. (C) Schematic protein organization of the glutamatergic synapse. Cell adhesions proteins, transmembrane proteins, scaffold proteins and component of the cytoskeleton help maintaining the synaptic architecture. Receptors and ion channels at the cell surface as well as scaffold and signaling protein mediate synaptic transmission.

Figure by Julie Jezequel, 2016 and adapted from Stewart et al. 2014; Sheng & Hoogenraad, 2007; Feng & Zhang, 2009.

B. ANATOMY OF THE TRIPARTITE SYNAPSE

The tripartite synapse is composed of the pre- and the post-synaptic elements along with the astroglial processes ensheathing them (Araque et al., 1999). In the CNS, astrocytes are closely associated with synapses thanks to their intricate cellular prolongations, known as perisynaptic astrocytic processes (PAPs; **Figure 7, page 13**), which facilitate transmitter exchange (Dallérac et al., 2013; Ghézali et al., 2016). PAPs are found in all brain regions, but the proportion of synapses bearing them and the level of synaptic coverage may vary within the brain regions, synapses and conditions (For review, see Bernardinelli et al., 2014). In the neocortex, astrocytes processes unsheath partially pre- or post-synaptic glutamatergic synapses (Ventura and Harris, 1999), while in the Bergmann glia, processes enwrap them completely (Grosche et al., 1999).

In vivo studies in the barrel cortex demonstrated that 24 hours of whisker stimulation and associated synaptic activity trigger the movement of astrocyte processes to increase PAP synaptic coverage (Genoud et al., 2006). In the hippocampus, around 60% of synapses are covered by PAPs (Araque et al. 1999), but 20% would not have any coverage (Witcher et al., 2007). Independently of the brain region, the coverage by PAPs also depends on the size of dendritic spines and synapses (Witcher et al., 2007). It is important to notice that these results were often obtained by electron microscopy, which reflects a steady-state and does not always snapshot dynamic physiological events. Indeed, we know that astrocyte processes display particular dynamic morphological properties (Haber et al., 2006). The first studies on PAPs revealed the high mobility of PAPs in astrocytic culture (Cornell-Bell et al., 1990). More recent studies in brain slices support those results, demonstrating spontaneous remodeling within the time range of a minute (Haber et al., 2006; Hirrlinger et al., 2004). Moreover, their motility can depend on the physiological state of the subject (Theodosis et al. 2008).

The induction of long-term potentiation (LTP) also induces changes of the coverage of synapses by PAPs within minutes (Bernardinelli et al., 2014). Using live-imaging of astrocytes-synapses interactions during the induction of LTP in hippocampal brain slices and in the barrel cortex *in vivo*, Bernardinelli and colleagues (2014) showed that PAPs are highly mobile. During the induction of LTP, PAPs' motility occurs mainly towards the active synapses, leading to their enhanced astrocytic coverage (Bernardinelli et al., 2014).

In the close future, thanks to the development of high resolution live microscopy such as STED (Stimulation-emission-depletion), studies of the tripartite synapse at the nanoscale level will bring a better understanding of neuron-glia interactions (Heller et al., 2017; Panatier et al., 2014).

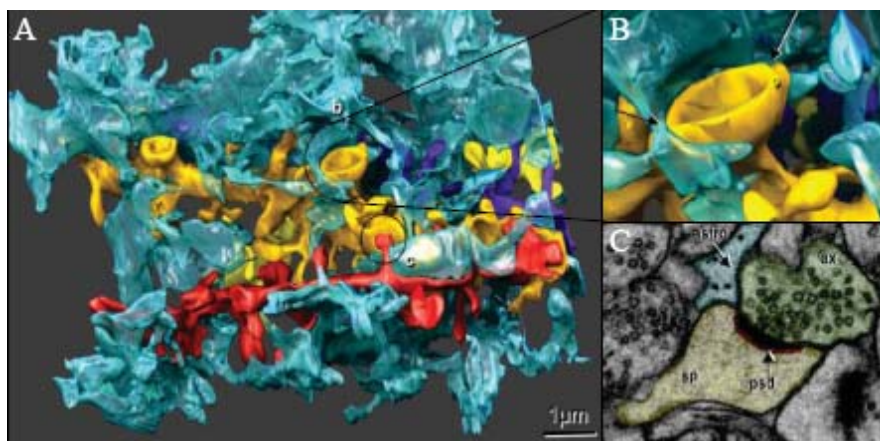


Figure 7: Anatomy of the tripartite synapse.

(A) Fine processes (blue) enwrap four dendrites (orange, yellow, purple and red). (B) Enlargement of (A), fine processes enwrap the synapse. (C) Electron microscopy image showing tight interactions between neurons and astrocytes at the synapse. *sp*, dendritic spine; *astro*, astrocyte ; *ax*, axon ; *psd*, post-synaptic density.

Adapted from Witcher et al., 2007.

C. SYNAPTIC SIGNAL DETECTION

Synaptic transmission is based on the classical scheme of release of transmitter by the pre-synaptic neuron, binding on post-synaptic receptors and clearance from the synaptic cleft by diffusion, uptake and/or degradation in the extracellular space.

Astrocytes play an important role in the transmission since they express transporters, ionotropic and metabotropic receptors that can detect signals and in turn modulate synaptic activity.

i. Membrane transporters

Astroglial transporters regulate the extracellular concentration of neurotransmitters, which is essential for the integrity of synaptic transmission.

As we previously saw, glutamate released by neurons in the synaptic cleft is mainly uptaken by glutamate transporters in astrocytes (Bergles and Jahr, 1998; Rothstein et al., 1994). In the brain, 95 % of glutamate uptake is performed by GLT-1 which is the most prominently expressed glutamate transporter in the mammalian forebrain (Danbolt, 2001) and found almost exclusively on astrocytes. GLAST is also contributing to synaptic glutamate clearance (Rothstein et al., 1994). GLT-1-mediated glutamate removal from the tripartite synapse is conducted in an activity-regulated manner, through the membrane diffusion of GLT-1 (Murphy-Royal et al., 2015). By quickly removing glutamate from the synaptic cleft, astrocytes control the inappropriate diffusion of the neurotransmitter and limit the communication between neurons. Thus, they preserve the specificity and quality of the synaptic communication (Piet et al., 2004; Theodosis et al., 2008). Moreover, glutamate uptake is concomitant with a Na⁺ co-transport (Meeks and Mennerick, 2007). This large entrance of Na⁺ is important because it plays a signaling role. Indeed, it triggers the entry of glucose into astrocytes from blood vessels to provide energy to neurons in an activity-dependent manner (Pellerin and Magistretti, 2004): a bigger neuronal activity triggers high amount of glutamate released, higher Na⁺ entrance into astrocytes and higher recruitment of glucose.

Besides glutamate transporters, astrocytes also express γ -aminobutyric acid (GABA) transporters (GAT-1 to 3), which can also influence synaptic activity. It was recently shown that activation of GAT-3 in the hippocampus leads to the release of ATP/adenosine by astrocytes, which then diffusely inhibits neuronal glutamate release *via* activation of pre-synaptic adenosine receptors (Boddum et al., 2016).

The discovery of the control of neurotransmitter uptake by astrocytes suggested the new hypothesis that astrocytes play active roles in synaptic transmission (Kofuji and Newman, 2004).

ii. Receptors

The role of astrocytes is not limited to uptake of neurotransmitters. Indeed astrocytes can also detect synaptic activity through the expression of a wide variety of iono- and metabotropic neurotransmitter receptors (Hamilton and Attwell, 2010; Perea and Araque, 2005a; Volterra and Meldolesi, 2005), which do not seem much different than the ones expressed by neurons. Receptors for neuropeptides, for purines, for

lipids, as well as iono- and metabo-tropic GABA and glutamate receptors are all present in both neurons and astrocytes. The differences between astroglial and neuronal receptors seem to rely on the levels of expression as well as their subunit composition. Interestingly, the same receptors expressed either in neurons or in astrocytes could activate different intracellular cascades, as, for instance, is the case for cannabinoid type 1 receptors (CB₁) (Navarrete and Araque, 2008) (see also Chapter III & Chapter IV).

Through the expression of transporters and receptors, astrocytes can detect neuronal activity at the level of the network. However it was recently shown that astrocytes can also detect activity at the level of a unique synapse. In the CA1 region of the hippocampus, astroglial mGluRs can be activated by glutamate release by the pre-synaptic neuron (Panatier and Robitaille, 2016; Panatier et al., 2011) and can detect the intensity and the frequency of the message (Panatier et al., 2011; Pasti et al., 1997; Perea and Araque, 2005b).

D. SIGNAL INTEGRATION: Ca²⁺ SIGNALING IN ASTROCYTES

Astrocyte membranes are not electrically excitable and, therefore, these cells cannot use action potentials to propagate fast information like neurons. Indeed, they do not express voltage-dependent Na⁺ channels, they express numerous leakage channels and have low membrane potential (Barres et al., 1990). Thus, they are electrically silent and they were considered as non excitable cells.

Nevertheless, astrocytes are able to detect exogenous or endogenous signals, such as neuronal activity within a range of milliseconds. Astrocytes express a variety of ion channels and membrane receptors to react to environmental changes by slight variation of membrane potentials and mostly by increase of intracellular Ca²⁺ concentrations (Barres et al., 1990; MacVicar and Tse, 1988; Marrero et al., 1989; McCarthy and Salm, 1991; Usovich et al., 1989). These discoveries were essential to reconsider astrocytes, and glial cells in general as active partners of information processing in the CNS.

i. Mechanisms of Ca²⁺ excitability

Ca²⁺ increase mechanisms in astrocytes are different from the ones in neurons. While Ca²⁺ increase in neuronal cell bodies happen in a range of milliseconds, it is a hundred to thousand times slower in astrocyte somas (Parpura and Haydon, 2000). This can be explained by the fact that Ca²⁺ increase in neurons is largely due to fast opening of plasma membrane voltage-dependent ion channels, whereas it requires slower and molecularly more complex intracellular signaling cascades in astrocytes (Golovina and Blaustein, 2000; Haydon, 2001; Petravic et al., 2008; Scemes, 2000; Scemes and Giaume, 2006; Sheppard et al., 1997).

The most widely accepted mechanism for astrocytic Ca²⁺ increase is the canonical phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP₃) pathway (**Figure 8, page 16**). Upon activation of GPCRs (seven-transmembrane membrane proteins coupled to G protein signaling), PLC hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and IP₃, leading to the activation of

IP₃ receptor (IP₃R) and Ca²⁺ release from intracellular organelles, such as the endoplasmic reticulum (ER).

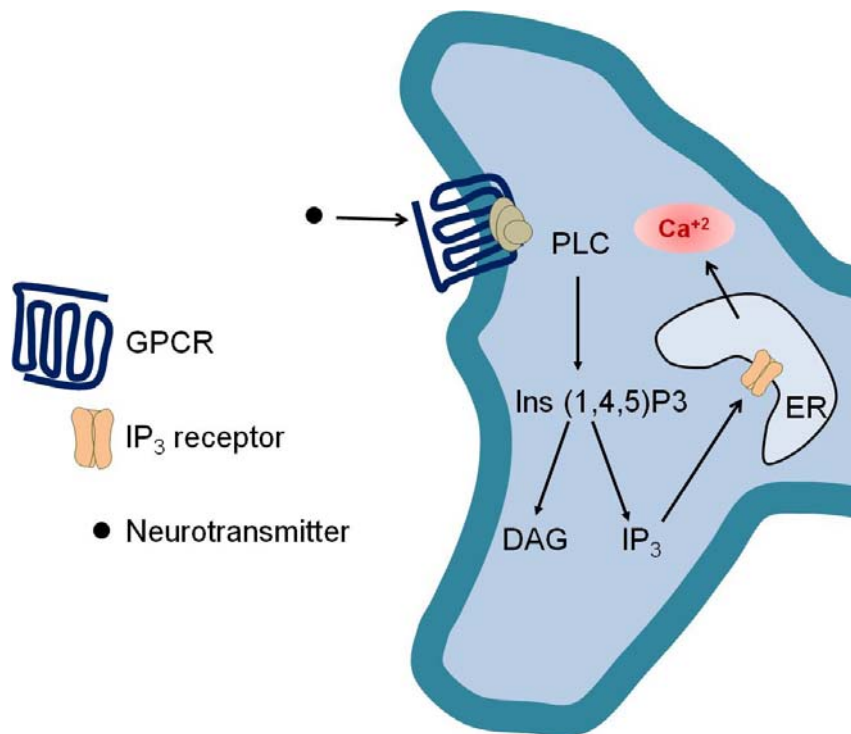


Figure 8: Schematic representation of the main mechanism of Ca²⁺ excitability in astrocytes. NT: Neurotransmitter; GPCR: G-Protein Coupled Receptor; PLC: Phospholipase C; Ins(1,4,5)P₃: phosphatidylinositol 4,5-bisphosphate; DAG: diacylglycerol; IP₃: Inositol tri-phosphate; ER: Endoplasmic reticulum; IP₃R₂: IP₃ receptor type 2; Ca²⁺: Calcium.

IP₃Rs are a family of 3 different receptor isoforms that are permeable to Ca²⁺ and are expressed at the ER membrane. We thought for a long time that astrocytes only expressed the IP₃R2 isoform (Petraovic et al., 2008; Sheppard et al., 1997; Weerth et al., 2007). However, whilst functional IP₃R1 and IP₃R3 have not been reported in hippocampal astroglia, mRNA for IP₃R1 and IP₃R3 were detected in acutely isolated mouse astrocytes (Cahoy et al., 2008), and IP₃R3 immunoreactivity was detected in rodent brain sections (Sharp et al., 1999; Yamamoto-Hino et al., 1995). The expression of multiple IP₃R isoforms in astrocytes is particularly interesting, because recent studies revealed that Ca²⁺ activity in large and finer processes of astrocytes from null mutant mice lacking the most abundant receptor (IP₃R2-KO mice) is still present (Di Castro et al., 2011; Hausteiner et al., 2014; Kanemaru et al., 2014; Rungta et al., 2016; Srinivasan et al., 2015). These observations indicate the existence of unidentified Ca²⁺ sources in astrocytic processes, raising the possibility that IP₃R1 and IP₃R3 subtypes could be functional in astrocytes. This hypothesis was recently confirmed by Sherwood et al., who, by using genetic deletion of the different IP₃R isoforms in organotypic slices, demonstrated that multiple IP₃R subtypes contribute to Ca²⁺ signaling in CA1 hippocampal astrocytes (Sherwood et al., 2017).

Astrocyte Ca^{2+} increases do not only occur at the level of the cell body (Panatier et al., 2011). Indeed, more and more studies currently focus on Ca^{2+} events in fine processes, where activity is smaller, but faster and more frequent than in cell bodies (Bindocci et al., 2017; Di Castro et al., 2011; Panatier et al., 2011; Shigetomi et al., 2013, 2016).

Finally, Ca^{2+} efflux could derive from other sources than ER. For instance, mitochondria can release Ca^{2+} in the cytoplasm of astrocytes *via* the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Parnis et al., 2013). Indeed, Agarwal et al. (2017) recently proposed that at least some of the Ca^{2+} signals of microdomains in fine processes are independent of IP_3Rs and they are rather due to efflux from mitochondria (Agarwal et al., 2017).

E. GLIOTRANSMISSION

Following the detection and integration of neuronal activity, astrocytes are able in turn to modulate neuronal activity and/or synaptic transmission by releasing various molecules, which are often but not always the same ones released by neurons. For example, glutamate, GABA, ATP, taurine and D-serine are likely released by astrocytes and by analogy with neurotransmission, these molecules have been called gliotransmitters and the phenomenon, gliotransmission (**Figure 11, page 21**). The detailed cellular and molecular mechanisms of gliotransmission are not fully elucidated and are under intense scrutiny and debate (Araque et al., 2014a; Hamilton and Attwell, 2010; Wolosker et al., 2017). Nevertheless, several possibilities have been proposed with different levels of experimental demonstration. In the following, I will shortly describe the most important.

i. Vesicular release

Astrocytes release of gliotransmitters has been mainly studied using approaches and points of view typical of the better known field of neurotransmitter release. Thus, in analogy to Ca^{2+} -dependent release of neurotransmitters in neurons, intracellular Ca^{2+} increase in astrocytes has been proposed to trigger exocytosis of vesicles containing the gliotransmitters.

In neurons, vesicular proteins VAMP2 (synaptobrevin) interact with membrane proteins SNAP-25 and syntaxin to form so-called a SNARE complexes (Soluble N-ethylmaleimide-sensitive fusion protein Attachment Protein REceptor). The fast exocytosis is catalyzed when Ca^{2+} interact with synaptogamin-1 (**Figure 9, page 18**). Astrocytes express VAMP2 and/or VAMP3 (Cellubrevin), and the SNAP-25 analog SNAP-23. They also contain a synaptogamin Ca^{2+} sensor (forms 4, 7 and/or 11), making it possible that intracellular Ca^{2+} release from internal stores trigger the fusion of gliotransmitter-containing vesicles (**Figure 9, page 18**) and (For review, see (Hamilton and Attwell, 2010). In accordance with these data, both pharmacological (e.g. by tetanic or botulinum toxins) and genetic inhibition of SNARE functions block the release of many gliotransmitters (Araque et al., 2000; Bezzi et al., 2004; Coco et al., 2003; Halassa et al., 2009; Jourdain et al., 2007; Pascual et al., 2005; Pasti et al., 1997).

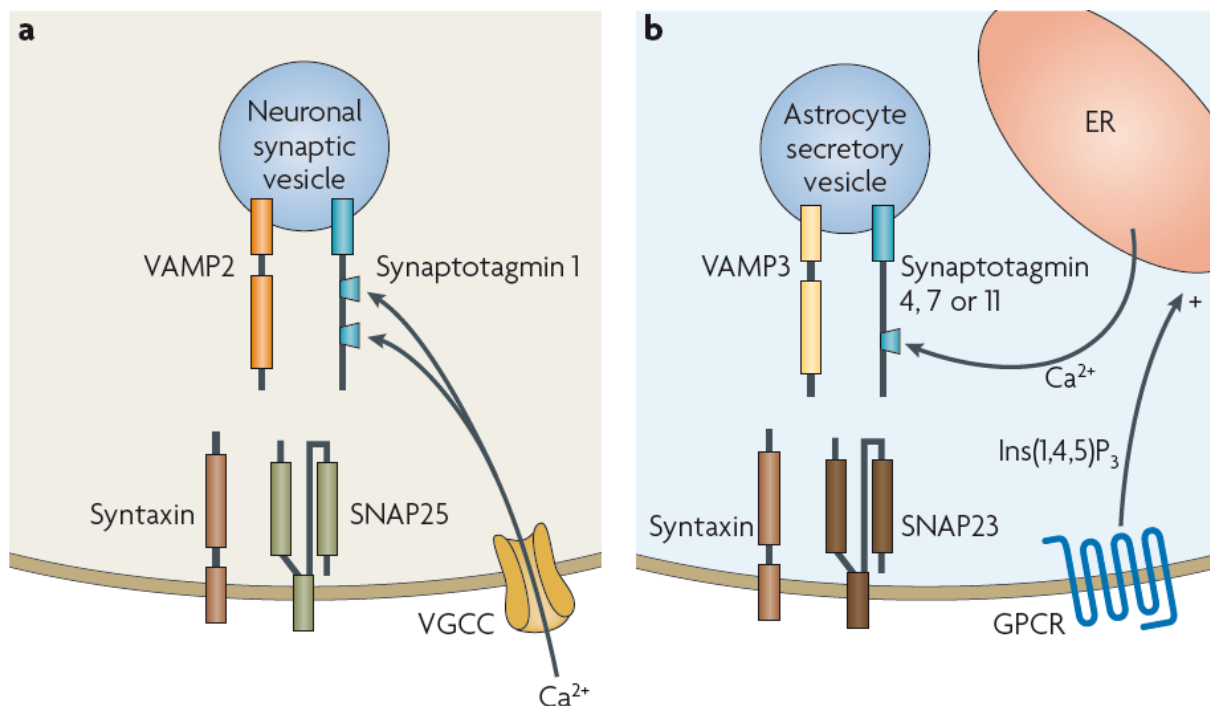


Figure 9: Proteins proposed to mediate exocytosis from neurons and astrocytes.

(a) For the formation of a functional SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex that mediates vesicle fusion, syntaxin and synaptosomal-associated protein 25 (SNAP25) at the neuronal plasma membrane bind to vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin 2). This is regulated by Ca^{2+} , normally entering from outside the cell through voltage-gated Ca^{2+} channels (VGCCs), binding to two sites of the Ca^{2+} sensor synaptotagmin 1. (b) In astrocytes, SNAP23 has an analogous role to neuronal SNAP25, and VAMP3 (also known as cellubrevin) has an analogous role to VAMP2. The Ca^{2+} sensor may be synaptotagmin 4 or synaptotagmin 11 (each of which has one Ca^{2+} -binding site, as shown) or synaptotagmin 7 (which has two Ca^{2+} -binding sites). Activation of G protein-coupled receptors (GPCRs) at the plasma membrane generates inositol-1,4,5-trisphosphate [Ins(1,4,5)P₃], which binds to its receptor on the endoplasmic reticulum (ER) and triggers the release of Ca^{2+} from the ER, resulting in vesicle fusion.

Adapted from Hamilton & Attwell, 2010

ii. Non-vesicular release

As mentioned above, vesicular release of gliotransmitters is under intense debate and some authors argue against its functional value (Hamilton and Attwell, 2010, 2010). Despite this debate, it is now clear that astrocytes can release synaptically active molecules, even in the absence of vesicular mechanisms. Indeed, there are several other possible non-exocytotic release mechanisms (Hamilton and Attwell, 2010). For instance, membrane transporters (Ribeiro et al., 2002; Ye et al., 2003), hemi-channels (Stehberg et al., 2012; Stout et al., 2002; Ye et al., 2003), Volume-Regulated Anion Channels (VRACs) (Takano et al., 2005) or channel-receptors to ATP, P2X7 (Duan et al., 2003; Fellin and Carmignoto, 2004; Pan et al., 2015) have been all proposed to participate in the mechanisms releasing gliotransmitters from astrocytes (Figure 10, page 19).

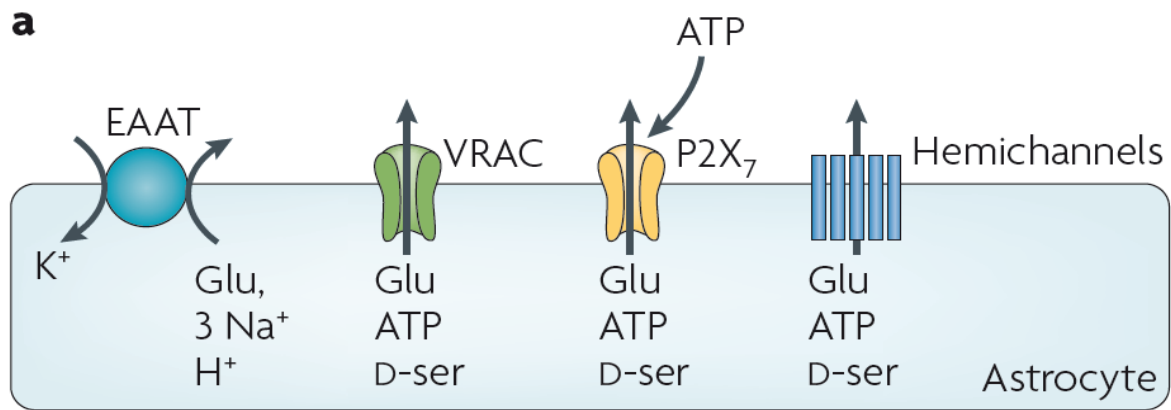


Figure 10: Potential non-exocytotic mechanisms for gliotransmitters release.

(a). Non-exocytotic transmitter release can occur by reversal of plasma membrane glutamate (Glu) transporters (excitatory amino-acid transporters (EAATs), or by efflux through volume-regulated anion channels (VRACs), ATP-gated P2X purinoceptor 7 (P2X₇) receptor channels or gap junctional hemichannels formed by connexins or pannexins for Glu, ATP and D-serine (D-ser).

Adapted from Hamilton & Attwell, 2010.

The exact mechanisms of gliotransmitter release remains unclear. Nevertheless, there is general substantial consensus that astrocytes are indeed able to release various gliotransmitters such as glutamate, GABA, ATP, taurine or D-serine (Araque et al., 1999; Henneberger et al., 2010; Kozlov et al., 2006; Mori et al., 2002; Newman, 2003).

F. GLIOTRANSMITTERS

Here, I will briefly summarize the current knowledge about major gliotransmitters, underlying the mechanisms for their release and the downstream synaptic consequences.

i. Glutamate

Astroglial Ca²⁺ increase is necessary to induce glutamate release by astrocytes (Angulo et al., 2004; Araque et al., 1999; Fellin and Carmignoto, 2004; Nedergaard, 1994; Parpura et al., 1994; Parri et al., 2001; Perea and Araque, 2005b). Glutamate from astrocytes activates in turn neuronal mGluR or iGluR (Nedergaard, 1994; Parpura et al., 1994). For example, astroglial glutamate release has been proposed to stimulate glutamatergic synaptic transmission by activating hippocampal pre-synaptic mGluR1 (Navarrete and Araque, 2008, 2010). Post-synaptic iGluRs such as NMDARs are also activated by astroglial glutamate (Araque et al., 1998). Activation of pre-synaptic NMDARs by astroglial glutamate stimulates synaptic transmission in the dentate gyrus (Jourdain et al., 2007) and is necessary for time-dependent long-term depression of excitatory transmission in the neocortex (t-LTD; (Min and Nevian, 2012). Extra-synaptic NMDARs are also stimulated by astroglial glutamate, enabling

the synchronization of neuronal activity through slow inward current (SIC) in the pyramidal neurons of the CA1 region of the hippocampus (Angulo et al., 2004; Fellin et al., 2004).

ii. GABA

GABA is released by astrocytes in different brain regions. In the olfactory bulb, mechanical stimulation of astrocytes triggers GABAA receptors-dependent slow currents in granular and mitral cells (Kozlov et al., 2006). A decrease of the extracellular osmolarity favors the release of transmitter from astrocytes. By this technique, in the hippocampus, astroglial GABA release triggers transient inhibitory currents (Le Meur et al., 2012). Finally, astroglial GABA participates in the tonic inhibition of GABAA receptors in the cerebellum *via* the release of GABA through the astroglial Ca²⁺-activated anion channel, Bestrophin 1 (Best-1) (Lee et al., 2010).

iii. ATP

Ca²⁺-dependent release of ATP from astrocytes affects neuronal activity (Newman, 2003) and plays a major role in the regulation of synaptic transmission (Pascual and Haydon, 2003). After release, ATP is rapidly converted into adenosin by extracellular ectonucleotidases (Newman, 2003; Zimmermann and Braun, 1996), thereby activating A1 or A2a purinergic receptors. At the CA3-CA1 synapse, pre-synaptic A1 receptors inhibit excitatory synaptic transmission (Pascual et al., 2005). On the contrary, A2a stimulation increases basal synaptic transmission (Panatier et al., 2011). Finally, ATP released by astrocytes could directly activate post-synaptic P2 receptors of CA1 pyramidal neurons and stimulate synaptic transmission (Chever et al., 2014a).

iv. Taurine

Taurine is an agonist of glycine receptors (GlyRs). In the hypothalamus, astroglial taurine is release after an osmotic challenge, inducing an inhibition of vasopressin release in the blood (Hussy et al., 2000). Taurine is also an endogenous ligand of GlyRs in cortical areas (Mori et al., 2002), but its origin has been ascribed mainly to neurons during development (Flint et al., 1998).

v. D-serine

Despite recent debate (Papouin et al., 2017a; Wolosker et al., 2017) D-serine is an amino-acid that is likely released by astrocytes (Henneberger et al., 2010; Papouin et al., 2017b). D-serine can work as a co-agonist at the co-agonist site of NMDAR (Mothet et al., 2000; Panatier et al., 2006). As D-serine is a central aspect of the present Thesis, I will dedicate the full next CHAPTER II to its biological and functional aspects.

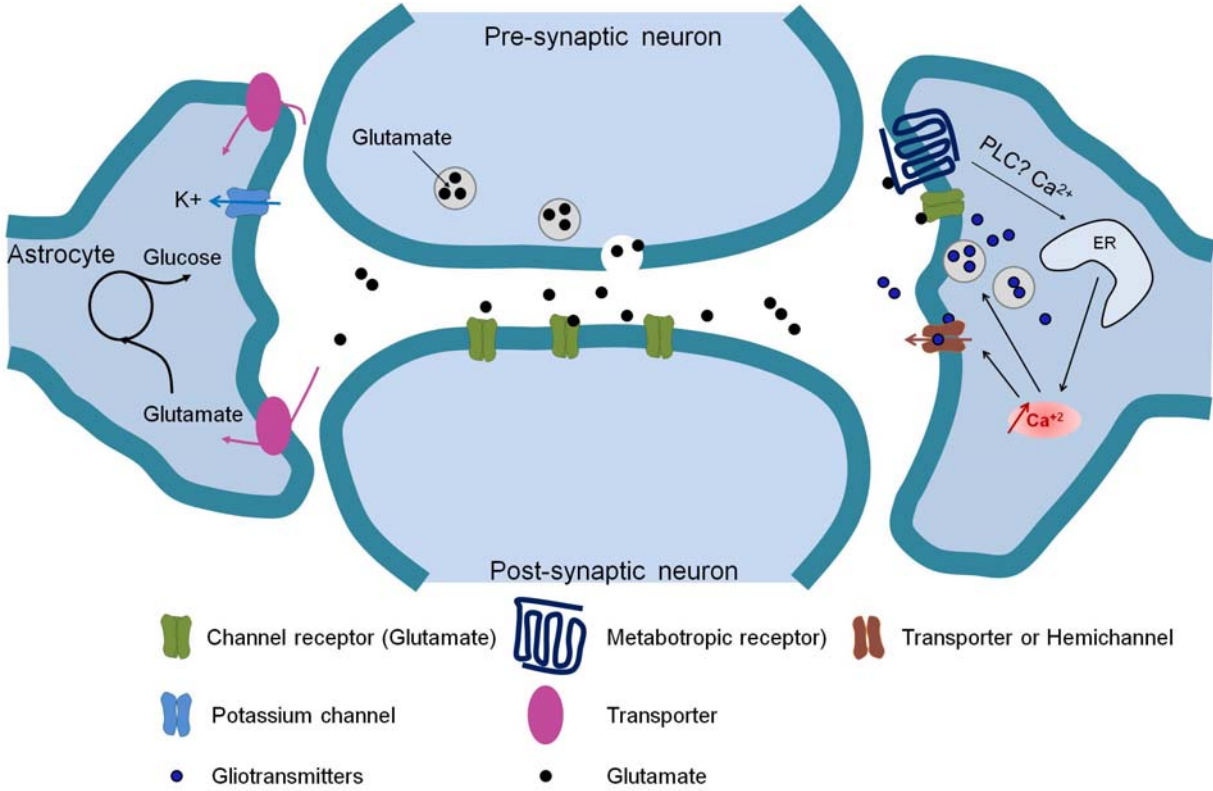


Figure 11 : Summary scheme of gliotransmission processes. Neurotransmitter released (glutamate) by pre-synaptic neuron activates astroglial receptors, triggers intracellular calcium (Ca²⁺) increase and gliotransmitters release. Adapted from Papouin et al., 2011

CHAPTER II

**D-serine, a gliotransmitter and a co-agonist
of NMDARs**

As previously seen, astrocytes can release various gliotransmitters such as glutamate, GABA, taurine, ATP and D-serine. D-serine is a particularly interesting gliotransmitter because it is known to be an endogenous ligand of NMDARs, thereby playing key roles in the modulation of synaptic plasticity and memory formation. Despite the fact that its nature as gliotransmitter has been recently questioned (Wolosker et al., 2017), large evidence still indicate that astrocytes can produce and release the amino-acid to regulate synaptic transmission and plasticity. In this Chapter, evidence for both ideas will be presented

I. D-serine metabolism

A. DE NOVO SYNTHESIS: SERINE RACEMASE

It was first proposed that D-serine could derive from exogenous sources such as food intake (Friedman, 1999), but whether this source is involved in the maintenance of endogenous levels of D-serine is still unclear. However, *de novo* synthesis of D-serine in the CNS was discovered after intraperitoneally injections of radiolabeled L-serine which led to accumulation of radiolabeled D-serine, highlighting a direct metabolic link between exogenous support of L-serine and endogenous production of D-serine. The enzyme that generates D-serine directly from L-serine was discovered in 1999 and named serine-racemase (SR; 339 amino-acids, 37 kDa) (Wolosker et al., 1999a). Consensus exists that this enzymatic racemisation reaction of L-serine is the only source of D-serine in the brain (De Miranda et al., 2000; Wolosker et al., 1999a).

Whereas SR is highly expressed in the hippocampus, the corpus callosum and the cortex, this enzyme is present at low levels in the amygdala, the adult cerebellum, the thalamus and subthalamic nucleus (**Figure 14, page 24**) (De Miranda et al., 2000; Panatier et al., 2006; Wolosker et al., 1999a). Although recently under debate, SR seems to be mainly expressed in astrocytes in the hippocampus (Wolosker et al., 1999a) but see (Papouin et al., 2017a; Wolosker et al., 2017) and in the hypothalamus (Panatier et al., 2006), amongst other brain regions (Miraucourt et al., 2011; Sullivan and Miller, 2010).

SR is a bidirectional enzyme (**Figure 12, page 23**), it can both convert L-serine into D-serine or D-serine into L-serine (Foltyn et al., 2005). SR is also a bifunctional enzyme because it converts part of the substrate L- or D-serine into pyruvate (De Miranda et al., 2000; Foltyn et al., 2005; Wolosker, 2011). Thus, D-serine is produced through racemisation of L-serine (Wolosker et al., 1999b) while pyruvate is obtained after α - β -elimination (Foltyn et al., 2005). SR is able to use indifferently L-serine or D-serine as substrates of the α - β -elimination, which occurs at higher rate than the racemisation (Wolosker, 2011). Thus, from 4 L-serine molecules, 1 D-serine and 6 pyruvate molecules are synthesized (De Miranda et al., 2002).

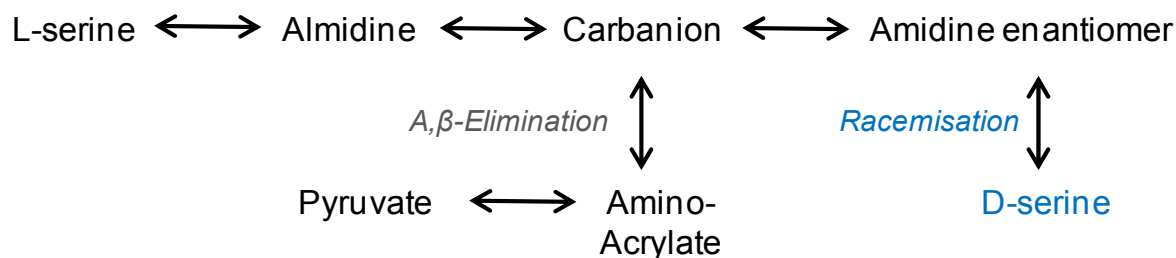


Figure 12 : Simplified reaction of racemisation and A-β-Elimination by serine racemase.

D-serine and pyruvate are the products of reactions. The other components are intermediate compounds due to the catalytic site of the enzyme.

B. DEGRADATION OF D-SERINE: DAAO (D-AMINO OXIDASE)

A bacterial degradation enzyme was early identified as the flavoprotein D-amino acid oxydase (DAAO; Kreb, 1935, (Pollegioni et al., 2007) and various studies suggest that DAAO is expressed in the vertebrate CNS and catalyzes D-serine (Konno and Yasumura, 1983; Konno et al., 1988; Park et al., 2006).

DAAO catalyzes the oxydative deamination of D-serine and potentially other D-amino acids (Sacchi et al., 2013). This enzymatic reaction requires a cofactor named flavine adenine dinucleotide (FAD) and produces imino-serine. Imino-serine is then hydrolyzed to produce ammonium (NH_4^+) and hydroxyppyruvate (**Figure 13, page 24**; (Pollegioni et al., 2007).

DAAO is present throughout the all CNS, with cerebellum, basal ganglia, hippocampus, cortex and thalamus displaying the highest levels (**Figure 14, page 24**, (Horiike et al., 1994; Moreno et al., 1999; Park et al., 2006; Schell et al., 1995). However, the observation that there is no direct correlation between the levels of expression of DAAO and the degradation of D-serine suggested that other so-far uncharacterized proteins might be involved in this enzymatic activity (Horiike et al., 1994; Schell et al., 1995).

At the cellular level, DAAO is mainly expressed in astroglial peroxysomes (Arnold et al., 1979) but a substantial expression is also found in neurons (Horiike et al., 1994; Moreno et al., 1999; Schell et al., 1995).

It is still under debate whether DAAO is the only enzyme involved in the D-serine degradation, but *in vitro* pharmacological studies showed that inhibition of DAAO activity increases D-serine levels (Park et al. 2006). Genetic studies using mutant mice lacking DAAO also showed an increase of D-serine levels in the brain region where D-serine levels are usually low. However, they described no change in the cortex or the hippocampus where D-serine levels are high (Hamase et al., 1997; Nagata et al., 1992). These results suggest that DAAO is not the only enzyme involved in the degradation of D-serine. As mentioned above, D-serine could potentially be degraded by SR itself (Foltyn et al., 2005; Wolosker, 2011).

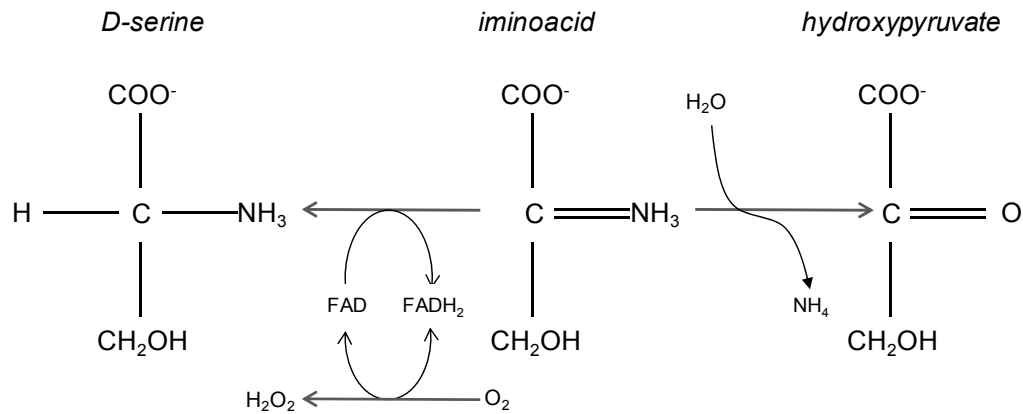


Figure 13 : Enzymatic degradation of D-serine by the enzyme DAAO.

The degradation of D-serine is possible if the co-enzyme FAD is present. The degradation of D-serine produces NH_4^+ and H_2O_2 .

II. D-serine distribution in the brain

A. BRAIN DISTRIBUTION OF D-SERINE IN THE CNS

Immunohistochemical and HPLC studies revealed the localization of D-serine in the rodent CNS (Schell et al., 1995, 1997; Wolosker et al., 1999b). Interestingly, D-serine presence correlates with the distribution of SR and DAAO enzymes. Thus, D-serine is abundant in regions where SR is high and DAAO is low and *vice versa* (Schell et al., 1995, 1997; Wolosker et al., 1999b).

After birth, D-serine is found in different brain regions such as cerebellum, midbrain or forebrain (Hashimoto et al., 1995). During adulthood, D-serine is high in the retina, thalamus, striatum and the hippocampus but not in the brainstem or cerebellum (Schell et al., 1995; Schell et al., 1997), suggesting possible developmental roles of the amino-acid (**Figure 14, page 24**).

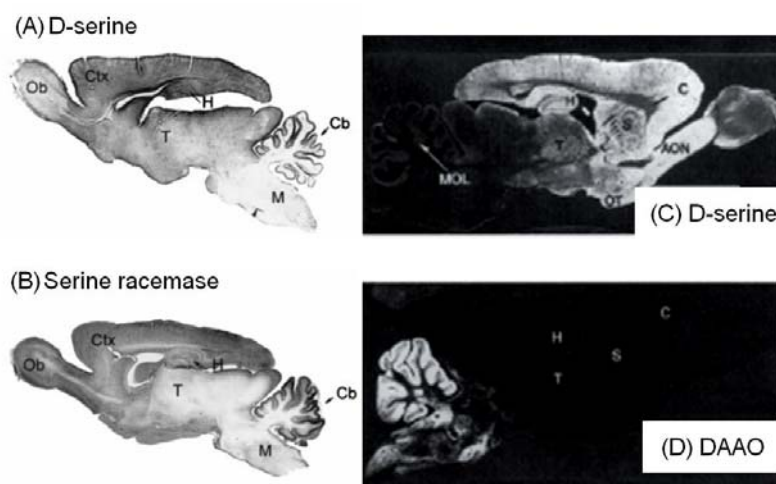


Figure 14 : D-serine, serine-racemase and DAAO distribution in the rodent brain.

(A-B) Colocalization of serine racemase and endogenous D-serine in the brain, Immunohistochemical staining for D-serine (A) and serine racemase (B).

(C-D) Immunoreactivity for DAAO and D-serine.

Adapted from Schell et al., 1995 and Wolosker et al., 1999.

B. CELLULAR DISTRIBUTION OF D-SERINE IN THE CNS

Initially, D-serine was associated to a population of astrocytes ensheathing glutamatergic synapses (Schell, 2004). Electronic microscopy in the cortex showed that D-serine was exclusively present in the podocytes and astroglial processes contacting dendritic spines (Schell et al., 1997). The analysis of SR and DAAO expression reinforced its localization in astrocytes. Indeed, in the hippocampus, SR is almost exclusively expressed in astrocytes (Wolosker et al., 1999b). However, more recent studies also report neuronal localization (Balu et al., 2014). SR expression could even be up to 65% in hippocampal neurons compared to 15% in astrocytes (Benneyworth et al., 2012) and a direct release of D-serine by astrocytes has been recently questioned (Wolosker et al., 2016). These data are contradictory with other studies directly assessing the role of astroglial Ca^{2+} in the activation of the NMDAR co-agonist site by D-serine (Henneberger et al., 2010; Papouin et al., 2017b; Sherwood et al., 2017). One recent publication also showed the contribution of astrocytes to daily oscillations of D-serine availability using dnSNARE mice (Papouin et al., 2017b), in which the formation of a SNARE complex is impaired in astrocytes reducing the exocytotic release of gliotransmitters, such as D-serine (Papouin et al., 2017b; Pascual et al., 2005; Sultan et al., 2015). Still, we cannot exclude that D-serine is released by neurons and it is possible that both astroglial and neuronal cells might contribute to the presence of the amino acid in the brain.

III. D-serine release

As for other gliotransmitters, the mechanisms underlying D-serine release are still debated. Although, most of the studies mention a vesicular release by astrocytes, D-serine could be also released via hemichannels, P2X7 receptors, volume-regulated anion channels (VRACs) or Alanine-Serine-Cysteine-Transporter (ASCT) (Kaplan et al., 2018; Utsunomiya-Tate et al., 1996) and see review (Hamilton and Attwell, 2010). However, here I will specifically focus on the evidence pointing to vesicular release of D-serine.

A. VESICULAR RELEASE OF D-SERINE BY ASTROCYTES

A seminal study by Mothet et al. in 2005 strongly argued for the exocytotic release of D-serine by astrocytes (Mothet et al., 2005). These authors showed that this release depends on both Ca^{2+} signaling and SNARE proteins. Moreover, in the adult hippocampus, it was convincingly suggested that D-serine is stored in “synaptic-like microvesicles” (SLMV), which possess all the molecular tools involved in exocytosis processes (Bergersen et al., 2012; Martineau et al., 2008, 2013). These vesicles assemble in clusters close to the plasma membrane (< 100 nm) where this is juxtaposed to neuronal NMDARs (Bergersen et al., 2012; Bezzi et al., 2004). These observations are in agreement with data showing that glutamate can also be stored in SLMV and released by Ca^{2+} -dependent exocytosis (Bezzi et al., 2004; Crippa et al., 2006). Thus, D-serine and glutamate are present in the same compartments of astrocytes, and could be stored in the same vesicle populations (Mothet et al., 2005). Moreover, the transport of D-serine inside astroglial vesicles was recently

characterized (Martineau et al., 2013). The vesicular D-serine transporter is proposed to be a D-serine/chloride co-transporter (Martineau et al., 2013), strongly suggesting a vesicular release of D-serine by astrocytes.

B. Ca^{2+} -DEPENDENT RELEASE OF D-SERINE

The release of D-serine by astrocytes is mainly dependent on an increase in cytosolic Ca^{2+} and on SNARE proteins (**Figure 9, page 18**), indicating Ca^{2+} -regulated exocytosis as a release mechanism (Martineau et al., 2014). Indeed, disrupting Ca^{2+} signaling inside astrocytes reduces D-serine release not only from astrocytes in culture and in hippocampal slices (Henneberger et al., 2010; Mothet et al., 2005; Shigetomi et al., 2013) but also from astrocytes *in vivo* (Takata et al., 2011).

Ca^{2+} is certainly a critical signaling aspect of D-serine release from astrocytes, but intracellular mechanisms involved are still poorly understood. As we described above, as a general rule for gliotransmitters release, we could speculate that D-serine release could depend on the activation of GPCRs, associated with the recruitment of Ca^{2+} from IP₃R (Zorec et al., 2012). Indeed, studies have shown that D-serine release is triggered by agonists of the ionotropic and metabotropic glutamate and of nicotinic acetylcholine receptors (Martineau et al., 2008; Mothet et al., 2005; Papouin et al., 2017b; Schell et al., 1995). The astroglial receptors able to trigger D-serine release are likely not all belonging to the GPCR family. For instance, TRPA1 (Transient Receptor Potential Ankyrin 1) ion channels (Shigetomi et al., 2013) Ephrin B3 receptor (Zhuang et al., 2010) have been shown to regulate extracellular D-serine levels.

An additional speculative possibility is that the source of Ca^{2+} involved in D-serine release might be different from ER. For instance, mitochondria are involved in the Ca^{2+} signaling necessary for glial glutamate release (Reyes and Parpura, 2008) and neuronal activity enhances microdomain Ca^{2+} signals by mitochondria in astrocytes processes (Agarwal et al., 2017), suggesting that these organelles might participate in the control of D-serine release.

C. ACTIONS OF D-SERINE

Interestingly, the only known molecular target of the amino-acid D-serine is the NMDARs, which are glutamate-gated, Ca^{2+} -permeable ion channels that mediate synaptic transmission. At these receptors D-serine acts as a necessary co-agonist binding the so-called glycine-site (Mothet et al., 2000; Schell et al., 1995). In other words, independently of the levels of glutamate, synaptic NMDARs are not able to work in the absence of D-serine, making this amino-acid a key element of synaptic transmission and plasticity (Henneberger et al., 2010; Mothet et al., 2000; Panatier et al., 2006; Papouin et al., 2012, 2017b). Due to the importance of NMDARs in the context of the present Thesis, I will dedicate the next sub-chapter to a specific description of their structure, roles and mode of functioning.

IV. NMDARs, composition, expression, functions and regulation

A. NMDARS COMPOSITION

i. Subunit and genes

NMDARs are tetrameric complexes, meaning they are formed with 4 monomers, which are called subunits. Cloning studies revealed the existence of 7 different subunit classified in 3 families (Moriyoshi et al., 1991; Paoletti et al., 2013) that can form di- or tri-heteromeric complexes.

The GluN1 subunit is encoded by a single gene GRIN1. Post translational modifications happen at the level of the 3 alternatively spliced exon sites (exon 5, 21, 22), generating 8 different functional isoforms, GluN1-1a–GluN1-4a and GluN1-1b–GluN1-4b (Dingledine et al., 1999a; Moriyoshi et al., 1991). There are 4 GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) coded by 4 different genes (Ishii et al., 1993; Monyer et al., 1992) and two GluN3 subunits (GluN3A and GluN3B) arising from two distinct genes.

ii. Structure of NMDARs subunits

Like other ionotropic glutamate receptors, NMDARs subunits have a typical structure whether they are from the GluN1, GluN2 or the GluN3 family. Indeed, each subunit consists of 4 domains (**Figure 15, page 27**; (Mayer, 2006; Paoletti and Neyton, 2007) as listed below:

- 1) the N-terminal domain (NTD) which is involved in subunit assembly and allosteric regulation.
- 2) the ABD domain (Agonist Binding Domain) involved in the binds of two co-agonists: glycine (or D-serine) in GluN1 and GluN3 subunits and glutamate in GluN2 subunit.
- 3) The TMD domain (Transmembrane Domain) form the ion channel and define the receptor conductance, ion selectivity and affinity for the Mg^{2+} block (see below). It is composed of M1, M3 and M4 segments and a re-entrant pore loop (M2).
- 4) A cytoplasmic C-terminus domain involved in interaction with other receptors and intracellular proteins for anchoring, trafficking and signaling.

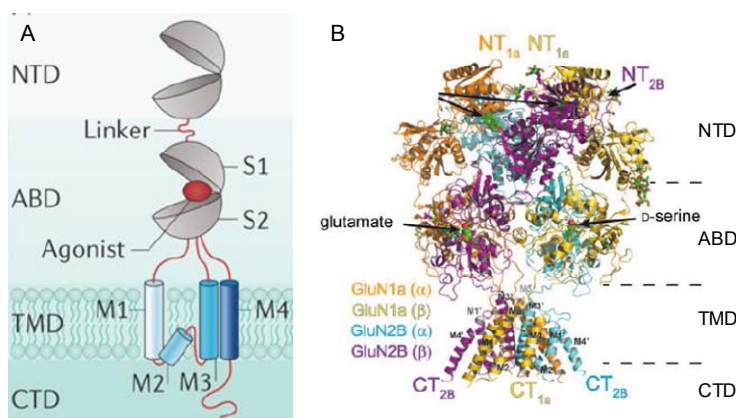


Figure 15: Molecular architecture of the NMDAR.

(A): Topology of the NMDAR. Each GluN subunit is organized in 4 layers (see text). **(B):** Crytallographic studies revealed that the structure of the GluN1-GluN2B NMDAR resembles a mushroom.

Adapted from Paoletti et al., 2013 and Karakas & Furukawas, 2014.

B. ACTIVATION OF NMDARS AND PERMEABILITY

A specificity of NMDARs is that binding of two co-agonists is required for receptor activation. Thus, NMDAR composed of GluN1 and GluN2 subunits require the binding of the agonist glutamate, concomitant with the binding of the co-agonist glycine or D-Serine (Johnson and Ascher, 1987; Mothet et al., 2000; Schell et al., 1995). At resting membrane potential the NMDAR is blocked by extracellular Mg^{2+} ions (Mayer et al., 1984; Nowak et al., 1984), which impedes its activation by ligands (glutamate, glycine, NMDA, D-serine). Ion influx only occurs when agonists are present and the cell membrane hosting the receptor is depolarized allowing the removal of the Mg^{2+} block, such as upon simultaneous stimulation of both pre-synaptic and post-synaptic neurons (Seeburg et al., 1995). Importantly, however, the necessary presence of agonists (glutamate) and co-agonists (glycine or D-Serine) expands the regulation of NMDAR activity.

i. Agonist binding at the GluN2 subunit

Glutamate, as an agonist of NMDARs binds on ABD of the GluN2 subunits (**see Figure 15, page 27**), (Furukawa et al., 2005), affinity of glutamate for NMDARs depends on the subunit composition but it is in the order of few μM and it is higher than its affinity to AMPARs or Kainate receptors (Attwell and Gibb, 2005). For example, NMDARs composed of GluN1 and GluN2B have the best affinity to glutamate compared to heterodimers GluN1/GluN2A, GluN1/GluN2C, GluN1/GluN2D. This observation is interesting because the couple GluN1/GluN2B is described as being extrasynaptic, where the concentration of glutamate is lower than within the synapse.

Other endogenous ligands of the GluN2 exists in the CNS. For instance, D-aspartate binds the GluN2 subunit of the NMDAR (Errico et al., 2011; Hashimoto and Oka, 1997) but the endogenous role of D-aspartate remains poorly known (Errico et al., 2014).

ii. (Co)-agonist binding at the GluN1 subunit

In addition to the glutamate binding, activation of NMDARs requires the simultaneous binding of a second agonist (or co-agonist). D-Serine and glycine are both co-agonists of the NMDARs and the work of Kleckner and Dingledine in 1988 (Kleckner and Dingledine, 1988) showed that they bind to another subunit (See **Figure 15, page 29**). The co-agonist binding site (historically called glycine-site) is localized on the ABD in the GluN1 subunit (Paoletti et al., 2013) and is not sensitive to strychnine, a competitive antagonist of inhibitory glycine receptors (GlyRs), thereby showing that glycine binds a different site (Bristow et al., 1986; Johnson and Ascher, 1987). Interestingly, the affinity of D-Serine and glycine for the GluN1 subunit depends on the composition of the GluN2 subunit. Thus, it seems that D-Serine and glycine has a better affinity for GluN2A and GluN2B dimers, respectively (Papouin et al., 2012).

iii. Activation of the NMDARs.

The activation of NMDARs depends solely on ABD and transmembrane domains. Mechanistically, NMDAR channel opening is triggered by the following sequence of events :

- 1) Agonists and co-agonists bind the clamshell-like ABD, each ABD containing a single agonist site (NMDAR activation requires occupation of the four agonist binding sites).
- 2) Agonist binding promotes cleft-closure of the “Venus Fly Trap” motif of the ABD, increasing the space between two adjacent ABDs.
- 3) This separation in turn exerts tension on the linkers connecting the ABD to the transmembrane segments, eventually leading to reorientation of the transmembrane domains and channel gate opening (Paoletti 2011).

This general activation sequence seems to be conserved across the different subunit-compositions of NMDARs, even though subtle differences exist between receptor subclasses (Furukawa et al., 2005; Mayer, 2006; Paoletti and Neyton, 2007; Traynelis et al., 2010).

iv. Permeability of NMDARs

The NMDAR channel is permeable to monovalent cations (Na^+ and K^+) and Ca^{2+} . The NMDAR permeability to Ca^{2+} is one of the highest across all ionotropic receptors (Rogers and Dani, 1995). The Ca^{2+} permeability depends on the subunit composition. For example, GluN2A-containing NMDARs is less permeable to Ca^{2+} than NMDARs encompassing GluN2B subunits (Dingledine et al., 1999b; Paoletti, 2011). Moreover, due to the Mg^{2+} block mentioned above, NMDARs permeability depends on the membrane potential. The equilibrium potential is close to 0 mV. Then, at resting potential (-70 mV), Mg^{2+} ions occupy the ionic channel of the NMDARs. Consequently, Ca^{2+} cannot circulate through the channel. Thus, a depolarization of the membrane is necessary to release the Mg^{2+} ions allowing the flow of Ca^{2+} through the channel.

C. LOCALISATION OF NMDARS WITHIN THE BRAIN

NMDARs are definitely the most abundant excitatory receptors in the CNS. However, different brain regions and developmental stages present diverse predominance of subunit composition and the sub-cellular distribution plays important functional roles

i. Regional brain distribution

GluN1 is highly expressed within the CNS, although temporal and regional expression of the different isoforms can vary. In the CA1 region of the hippocampus, the GluN1a splice variant is the main isoforms at all developmental stages (Dingledine et al., 1999b; Laurie and Seeburg, 1994). GluN2 expression is highly regulated (Szczurowska and Mareš, 2013) in the hippocampus, as well as the whole

CNS. GluN2A is virtually undetectable during early development, and it gradually appears in the neocortex, the hippocampus, the olfactory bulb and the cerebellum starting from the second post-natal week to eventually spread to the whole CNS at adulthood. During development, GluN2B is highly expressed in the whole embryonic CNS, peaking at 5-10 days post-natal to be then restricted to anterior structures, such as the telencephalon at 1 month of age. GluN2C expression starts during the first 2 post-natal weeks and it is limited to the olfactory bulb and cerebellum. Similarly to GluN2B, GluN2D is highly expressed during embryonic development, but it is specifically enriched in the diencephalon and the brainstem. Finally, whereas GluN3A expression is low before birth, maximal at 1 week post natal and decreases until adulthood when it is only detectable in the olfactory bulb, GluN3B is present in the whole CNS at all developmental stages (Henson et al., 2010; Low and Wee, 2010; Wee et al., 2008).

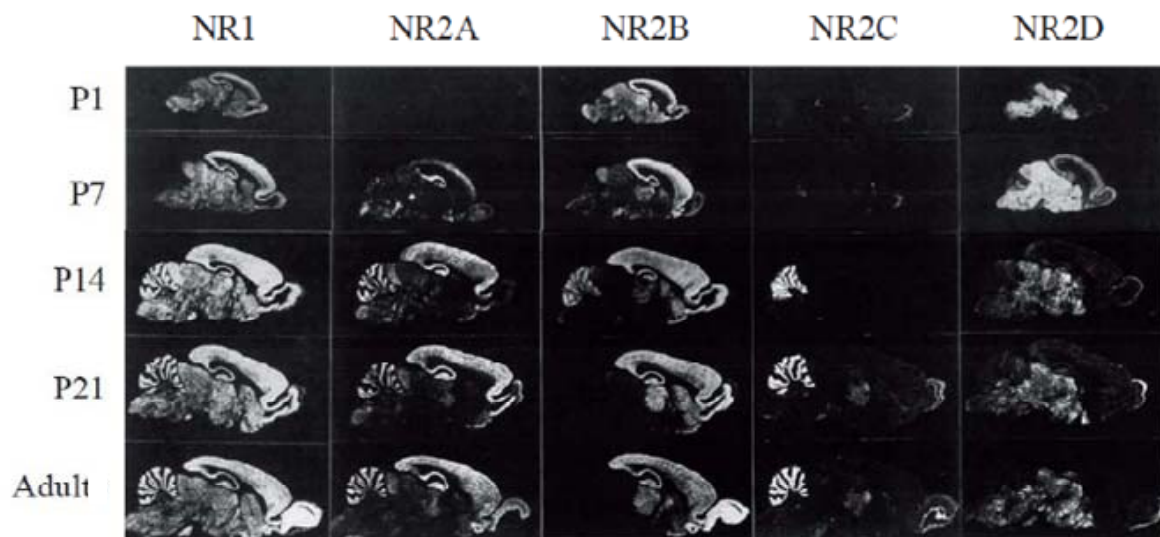


Figure 16: Distribution of NMDAR subunits in the rat brain during development.

In situ hybridization micrographs illustrating the mRNA expression of the GluN1 (NR1), GluN2A (NR2A), GluN2B (NR2B), GluN2C (NR2C) and GluN2D (NR2D) subunit during development from post-natal day 1 (P1) to adulthood.

Adapted from Akazawa, 1994.

ii. *Subcellular distribution of NMDARs*

Pre-synaptic NMDARs

The expression and the role of pre-synaptic NMDARs remain controversial, but physiological and anatomical evidence strongly suggest this localization (Aoki et al., 1994; Charton et al., 1999; McGuinness et al., 2010). Their role seems to be the increase of glutamate release probability at the synapse after LTP induction (Banerjee et al., 2016) or time-dependent LTD (t-LTD), an associative form of plasticity based on the precise temporal relationship between pre- and post-synaptic activity (Andrade-Talavera et al., 2016; Castillo, 2012).

Post-synaptic NMDARs

Excitatory neurotransmitter receptors have long been known to be concentrated in the post-synaptic density (Boeckers, 2006), but it is well established now that NMDARs occupy both synaptic and extrasynaptic locations. Indeed, subcellular immunogold labeling of NMDAR subunits provided high-resolution EM visualization of receptors at synaptic and extrasynaptic sites on spines, dendrites, somata, and within intracellular compartments (**Figure 17, page 31**; (Petralia et al., 2010).

- Extrasynaptic NMDARs

NMDARs are defined as extrasynaptic (**Figure 17, page 31**) when they are located at more than 100 nm from the PSD, which characterize excitatory synapses in the CNS (Petralia et al., 2010). These extrasynaptic NMDARs are activated by glutamate only when the synaptic activity happens at high frequencies (Harris and Pettit, 2007). During adulthood, they represent 1/3 of total NMDARs in the CA1 region of the hippocampus. They are mainly composed of GluN2B subunits (Papouin et al., 2012) and are involved in many neuronal functions (Papouin and Oliet, 2014), including the modulation of neuronal excitability (Sah et al., 1989), the control of neuronal synchronicity (Angulo et al., 2004; Fellin et al., 2004), and the induction or LTD (Papouin et al., 2012).

- Synaptic NMDARs

Synaptic NMDARs (**Figure 17, page 31**) represent 2/3 of total NMDARs in the CA1 region of the hippocampus. By definition, they are confined within 100 nm from the synapse, where they interact with the PSD. They are mainly composed of GluN2B subunits at early stages of development and GluN2A during adulthood (Le Bail et al., 2015; Papouin et al., 2012). Synaptic NMDARs are directly involved in long term synaptic plasticity (Citri and Malenka, 2008; Malenka and Bear, 2004) and in most memory processes.

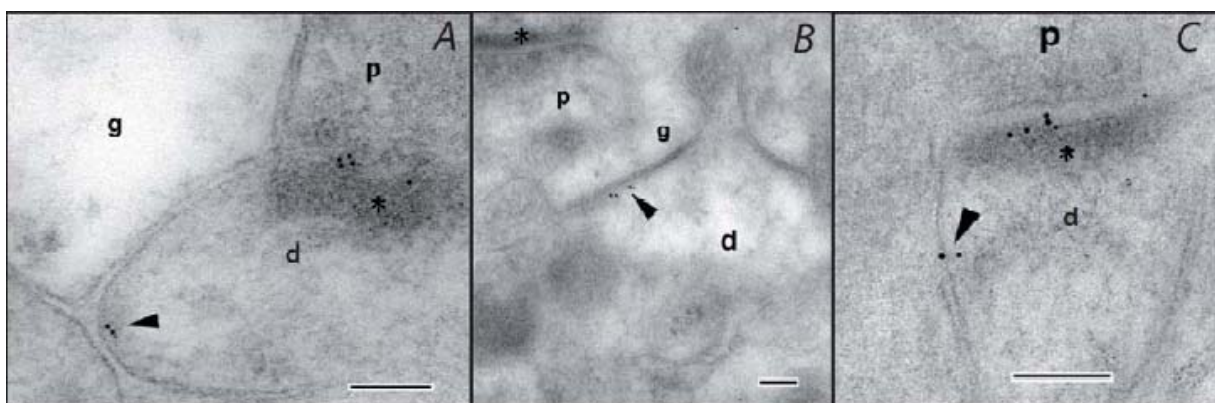


Figure 17 : Subcellular distribution of NMDARs.

Electron microscopy images from hippocampal slices in rodents showing the cellular distribution of NMDARs.

g : glia ; p : pre-synaptique ; d : dendrite. The black arrows show the extra-synaptic NMDARs. Stars represent the post-synaptic density (PSD). **(A-B)**: low density of extra-synaptic NMDARs and a close astroglial process. **(C)**: example of a extra-synaptic NMDARs (arrow).

Scale bar : 100 nm.

Adapted from Petralia et al., 2010.

In the 1940's, Donald Hebb proposed that associative memory would be a consequence of the increase of the strength of synaptic connections when the pre-synaptic activity is correlated to a post-synaptic discharge (Hebb, 1949), thereby forming a mnesic trace. During the 1970s, experimental evidence supported this theory. Bliss & colleagues discovered that repetitive activation of excitatory synapses induced a persistent increase of synaptic transmission in the hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). This phenomenon was called long term potentiation (LTP) and 10 years later Collingridge & colleagues demonstrated that LTP induction at the CA3-CA1 synapse depends on NMDARs (Collingridge et al., 1983). Indeed, when NMDARs are blocked by the selective antagonist 2-amino-5-phosphonovalérique acid (AP5), LTP is blocked.

i. Induction

At the CA3-CA1 synapse, LTP is obtained after 1 or several high frequency electric stimulations (HFS, e.g. 50-100 Hz during 1 sec) at the Schaffer collateral, triggering a long-term increase of synaptic transmission between CA3 and CA1 neurons (Bliss and Collingridge, 1993; Malenka, 1994). Since then, many other stimulation protocols have been shown to induce NMDAR-dependent LTP at many CNS synapses (Malenka, 2003; Nicoll, 2017).

The synaptic activity induced by HFS triggers the release of glutamate, which in turn activates AMPARs and create a post-synaptic depolarization. Upon depolarization the Mg^{2+} block of the NMDARs is expelled from the pore, allowing Na^+ , K^+ , and, importantly, Ca^{2+} ions to flow through the channel. As a consequence of coincident depolarization and glutamate-binding, Ca^{2+} influx through NMDARs is maximal, activating intracellular signaling cascades that ultimately are responsible for the increased synaptic efficacy.

The Ca^{2+} entry is a key element in LTP induction. Indeed, Ca^{2+} chelators inhibiting Ca^{2+} variations block LTP induction (Lynch et al., 1983). Consistently, increased post-synaptic Ca^{2+} levels can trigger *per se* a potentiation of synaptic transmission (Malenka et al., 1988). Indeed, a brief and strong elevation of Ca^{2+} levels is sufficient to activate the Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2002) that, upon autophosphorylation, is essential to the intracellular machinery triggering LTP (Malinow et al., 1989). In turn, Ca^{2+} /calmodulin complex formation activates adenylate cyclase (AC) to synthesize cAMP, stimulating protein Kinase A (PKA). PKA then phosphorylate the Phosphate Protein 1 (PP1) inhibitor 1 (I1), thereby allowing LTP induction (**Figure 18, page 33**); (Brown et al., 2000).

ii. Expression

LTP expression is characterized by a long-lasting increase of synaptic transmission, which can be explained by two alternative or complementary mechanisms. Thus, either an increased release of neurotransmitter by the pre-synaptic neurons and/or an enhanced in the post-synaptic sensitivity in response to the same concentration of neurotransmitter can explain LTP expression. Different studies on NMDAR-dependent LTP indicate a major role of post-synaptic mechanisms, but other evidence suggest a role of a modification of pre-synaptic glutamate release in LTP [For Review see (Lüscher and Malenka, 2012)]. However, it was shown than during a NMDAR-dependent LTP protocol, retrograde messengers could be released by the

post-synaptic neurons, like Nitric Oxide (NO) that could act pre-synaptically to modify neurotransmitter release (Arancio et al., 1996).

The early phase of NMDAR-dependent LTP is characterized by the redistribution of AMPARs *via* exocytotic and/or lateral trafficking processes leading to their post-synaptic accumulation allowing quick and efficient changes in the strength of synaptic connections (Collingridge et al., 2004; Kessels and Malinow, 2009; Lüscher and Frerking, 2001; Lüscher et al., 1999; Malinow and Malenka, 2002; Nicoll, 2003).

iii. Maintenance

Once induced, LTP can last for hours or even days (Malenka and Bear, 2004). LTP maintenance requires the synthesis of new proteins through the involvement of nuclear processes (Abraham and Williams, 2003; Lynch, 2004; Pittenger and Kandel, 1998). However, interesting data show that the translation of these proteins can also occur locally, at the level of dendrites (Sutton and Schuman, 2006). For instance, mRNAs coding for CaMKII or AMPAR subunits are present at dendrites and are locally translated. It is also important to notice that LTP is associated with morphological changes, such as the enlargement of the dendritic spine head, the decrease of the size of its neck and the expansion of the PSD, all contributing to increased synaptic currents (Dalva et al., 2007; Yuste and Bonhoeffer, 2001).

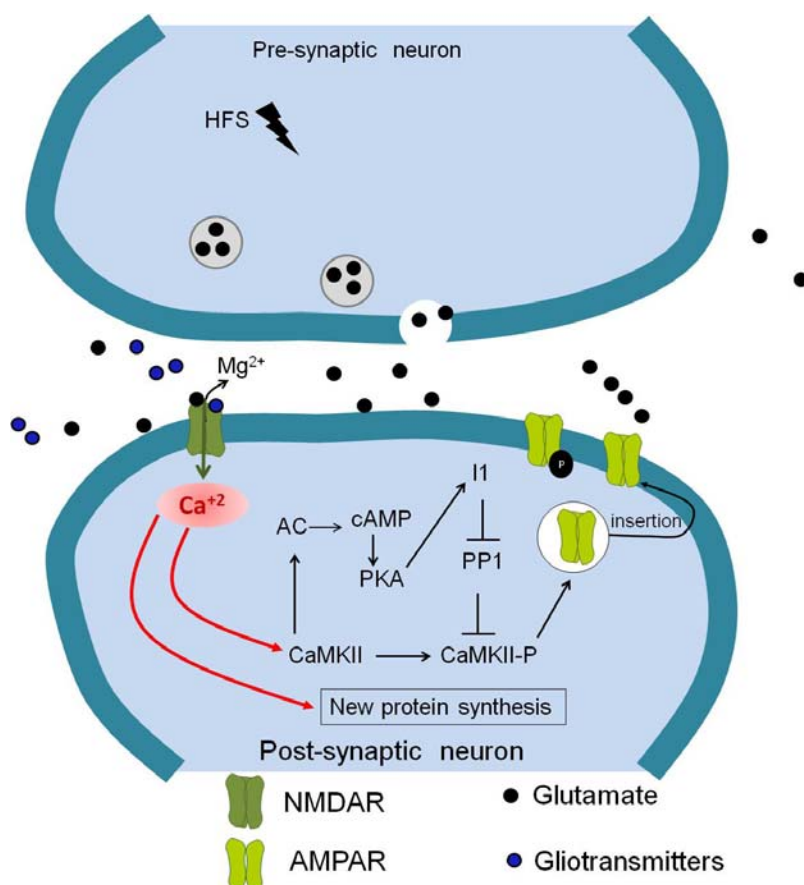


Figure 18 : Schematic representation of the NMDAR-dependent long-term potentiation (LTP).

Glutamate is released by the pre-synaptic terminal after high frequency stimulation (HFS). Glutamate binds post-synaptic NMDARs, allowing the entry of Ca^{2+} through the channel. This phenomenon induces the phosphorylation of CamKII (CaMKII-P). In turn, CamKII-P phosphorylates AMPARs increasing their conductance and favoring their insertion at the postsynaptic membrane. These 2 mechanisms increase synaptic transmission.

See also text.

CHAPTER III

The endocannabinoid system

I. A bit of history: from China to pharmaceutical industries

A. ASIA

Although the geographical origins of Marijuana remain not clear, the first pieces of evidence of the use of *cannabis* were found in China approximately 6000 years ago, when it was grown for textile and paper extracts from fibers rather than for its psychoactive properties (Li, 1974). However, the oldest evidence for pharmacological use of *cannabis* was found in China, where a pharmacopeia mentions the use of *cannabis* against pain, intestinal and reproductive troubles about 2000 years b.C. At the same time, *cannabis* was mentioned as a sacred plant in the Atharva Veda (one of the holy books of hindu religion, 2000 b.C in India (Touw, 1981). Indeed, the hemp plant has been historically used in many cultures for its therapeutic properties such as analgesic/anesthetic, anticonvulsant, sedative, antibiotic, antiparasite, antispasmodic, pro-digestive, appetite stimulant, diuretic and aphrodisiac (Zuardi, 2006).

B. EUROPE

The greek historian Herodotus (450 b.C) mentioned collective fumigation sessions that brought great hilarity and it has been suggested that *cannabis* was at the base of these effects.

In antiquity, Germanic populations grew hemp plant for fabric purpose and boat ropes but there is no proof that *cannabis* was used for medicine or its psychotropic properties (Godwin, 1966).

It is only at the beginning of the 19th century that medicinal *cannabis* was introduced into Western Europe. The British physician O'Shaughnessy introduced *cannabis* into British medicine, after observing its use in Calcutta as anti-rheumatism, -convulsions and -muscular spasms, and evaluated the plant's toxicity in animals (O'Shaughnessy, 1839a,b).

C. THE BEGINNING OF SCIENTIFIC STUDIES

In the 1840s', Jacques-Joseph Moreau published 'Du Hashish et de l'Aliénation Mentale' where he compared the psychomimetic effects of *cannabis* resin (Hashish) from his self or students' consumption reports to the symptoms observed in his mental patients, arguing that *cannabis* intoxication represents a powerful mean to investigate mental illness (Moreau, 1845). This publication led to the multiplication of studies on *cannabis* medical value both in Europe and North America. Indeed, the selling of plant extracts by private laboratories (e.g. Merck in Germany or Eli Lilly in USA) from the second half of the 19th century allowed the experimental identification of several effects such as sedation, analgesia, appetite-promotion and others (Mikuriya, 1969).

Despite the long history, research on the medical asset of *cannabis* declined at the beginning of the 20th century because the mechanism of action remained unknown as well as the active component of the plant. Moreover, the banning of the plant from pharmacopeia and the "Marijuana Tax Act" by the USA government in 1937, which prohibited the production of *cannabis* and hemp, contributed decreasing wide scientific interest in the properties and mechanisms of action of plant extracts.

D. IDENTIFICATION OF THE PSYCHOACTIVE COMPONENT OF *CANNABIS SATIVA*

Starting in the 1940s', however, scientific studies led to the purification and the identification of the chemical identities of specific *cannabis* molecules, called the cannabinoids such as cannabiniol, cannabidiol or tetrahydrocannabinol (Adams et al., 1940; Jacob and Todd, 1940). This early chemical research was completed with the precise identification of the chemical structure of the main component of *cannabis*, the Δ^9 -tetrahydrocannabinol (THC, (Mechoulam and Gaoni, 1965). These findings led to numerous pharmacological studies both in humans and animals showing that THC mediates the main psychoactive properties of the plant *cannabis sativa* (Carlini et al., 1974; Kiplinger et al., 1971). At the same time, Mechoulam et al. completed the synthesis of the pure molecules, established their molecular structures and studied their structure-activity relationships (Mechoulam et al., 1972; Razdan, 1986). The already known effects of *cannabis* consumption could then be precisely studied by systemic injection of various doses of pure THC in animals (Kiplinger et al., 1971).. Interestingly, THC was found to mediate *cannabis*-induced memory impairments (Carlim and Kramer, 1965).

E. SHORT OVERVIEW OF CANNABINOID RESEARCH

Although in 1937 *cannabis* use was prohibited in the United States followed in 1941 by its removal from the *American pharmacopeia*, it is interesting to notice that in parallel, hedonic and recreational *cannabis* consumption drastically increased until the 1970's. Nowadays, after coffee alcohol and tobacco, *cannabis* is amongst the most widely diffused drugs of abuse and its consumption keeps increasing across the different groups of age (Mikuriya, 1969; Sznitman, 2008) and (World Drug Report 2017).

However, due to the progress in the understanding of *cannabis*' effect on physiological processes, the therapeutic use of *cannabis*-based compounds has been authorized in several countries (e.g: USA, Spain, Canada) for the symptomatic treatment of several conditions, such as spasticity (as in Multiple Sclerosis), nausea and vomiting, loss of appetite and chronic pain (Borgelt et al., 2013; Grotenhermen and Müller-Vahl, 2012). Pharmaceutical preparations such as Marinol® (dronabinol) or Sativex® (THC and cannabidiol) are indeed currently authorized and commercialized in several countries (US, Canada, Germany, Australia and New Zealand; (Russo, 2007). In parallel, the recreational use of *cannabis* is nowadays being authorized under different legal frames in many Western countries.

The clear identification of the chemical structures of cannabinoids and the possibility to study their effects in different biological models raised a large amount of interest, which is reflected by the exponential growth of papers published on the subject from 1965 to middle of the Seventies (**Figure 19, page 36**). However, due to the infructuous attempts to find a specific mechanism of action of these drugs, this enthusiasm decreased until the late Eighties (**Figure 19, page 36**). With the identification and the cloning of cannabinoid receptors (1990), and the purification of their endogenous ligands (Anandamide and 2-Arachidonoylglycerol (2-AG), in 1992 and 1995 respectively), the scientific attention to this field grew again to very high numbers of publications per year, maintaining nowadays a stable plateau of approximately 1000.

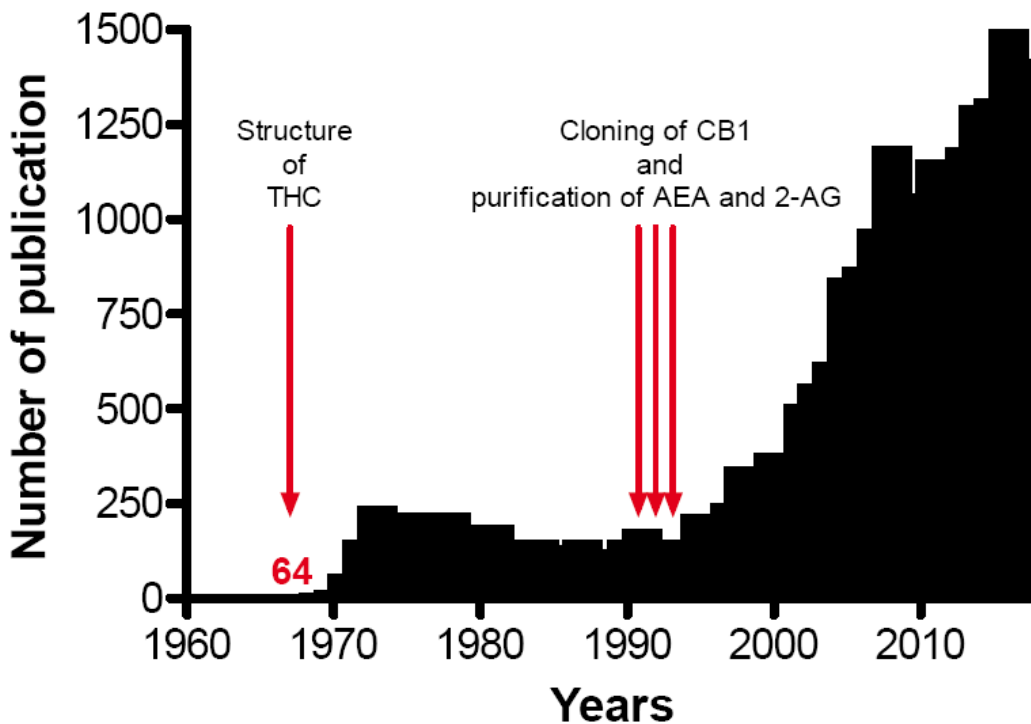


Figure 19 : Number of publications per year produced in recent decades, in relation to cannabinoid research.

The values were obtained with a Medline search (<http://www.ncbi.nlm.nih.gov/entrez/query>), using as keywords "cannabinoid OR marijuana" Key discoveries are indicated with the red arrows.

II. The endocannabinoid system

The endocannabinoid system is composed by the cannabinoid receptors, their endogenous ligands (endocannabinoids) and the enzymes involved in their synthesis and degradation.

A. CANNABINOID RECEPTORS

i. Structure and general distribution

As mentioned above, cannabinoid research regained the interest of the scientific community when the first unequivocal evidence for the presence of a specific cannabinoid receptor was found (Devane et al., 1988). This discovery was also the first evidence of the existence of the endocannabinoid system.

Cannabinoids exert their pharmacological effects through the activation of at least 2 receptors. The first identified receptor, named type-1 cannabinoid receptor (CB₁), was cloned in rat (Matsuda et al., 1990), in human (Gérard et al., 1991), in mouse

(Chakrabarti et al., 1995) and in other species, including mammals, fishes and birds. The characterization and the cloning of the other well known cannabinoid receptor, designated CB₂, were realized subsequently in human (Munro et al., 1993), mouse and rat (Shire et al., 1996).

CB₁ and CB₂ receptors are members of a large family of GPCRs. They both contain seven transmembrane (TM) domains, connected by three intracellular (I1, I2, I3) and three extracellular loops (E1, E2, E3), an intracellular C-terminus region which begins with a small helical domain (oriented parallel to the membrane surface). Both CB₁ and CB₂ receptors contain several potential sites for post-translational modifications, such as phosphorylation, palmitoylation and N-glycosylation, whose impact on the protein functions are not fully clarified yet (Shim, 2010).

CB₁ and CB₂ receptors share 48% of amino-acid sequence and their sensitivity to exogenous or endogenous ligands is similar, although not fully overlapping (Mechoulam and Parker, 2013). Dimerization of cannabinoid receptors with other GPCRs has been reported (Mackie, 2005). For instance, CB₁ receptors can be found in the monomeric, homodimeric (Wager-Miller et al., 2002) or heteromeric forms with CB₂ receptors (Callén 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).

CB₁ receptors are found ubiquitously but are highly enriched in the brain and the spinal cord (Howlett, 2002). At the peripheral level, CB₁ receptors are expressed in several metabolic and endocrine organs, such as the adrenal and thyroid glands, adipose tissue, muscle and heart, liver, lungs, kidneys and the gastrointestinal tract (Pagotto et al., 2006). In contrast, CB₂ receptors are mainly expressed in cells of the immune system such as leukocytes. At a lower extent, CB₂ receptors are found on peripheral nerve terminals and current research suggests that these receptors play a role in nociception (Calignano et al., 1998; Klein et al., 2003). In the brain, CB₂ receptors are mainly expressed by microglial cells (Walter and Stella, 2004), although some evidence exists that neurons might express them. Consequently, the original concept that CB₁ receptors play an exclusive role in the brain, and CB₂ receptors in the immune system, has evolved into the idea that both cannabinoid receptors can control both central and peripheral functions. Moreover, functional studies have suggested that the activation of CB₂ receptors by the administration of exogenous ligands may open novel therapeutic avenues for a number of brain pathological conditions such as neurodegenerative disorders, stroke, and neuropathic pain (Mackie, 2006).

Numerous pharmacological studies suggest the existence of additional cannabinoid receptors. The transient receptor potential vanilloid type 1 (TRPV1) ion channel, which interacts with endocannabinoid ligands such as anandamide, was shown to be involved in some effects of cannabinoids (Marsch et al., 2007; Starowicz et al., 2007). Two other GPCRs, the G protein-coupled receptor 55 (GPR55) and the G protein-coupled receptor 119 (GPR119) were affiliated as novel potential cannabinoid receptors (Brown, 2007).

However, CB₁ receptors are known to mediate most of the cannabinoid-induced behavioral effects. Thus, it is essential to study the role of CB₁ receptors in brain physiology and pathology. In agreement with the aim of this thesis and in sake of

space and clarity, I will limit the focus of the following paragraphs to CB₁ receptors and their role in physiology, behavior and synaptic plasticity.

ii. Brain distribution

CB₁ receptors are considered as the most abundant GPCR in the brain and their distribution has been well characterized both in rodents (**Figure 20, page 39**; (Herkenham et al., 1991; Tsou et al., 1998) and humans (Westlake et al., 1994). These receptors are particularly rich in certain brain areas of the CNS such as basal ganglia, cerebellum and the hippocampus. They are also found in other brain areas including the amygdala, hypothalamus and the spinal cord, among other structures (Marsicano and Kuner, 2008; Pertwee, 1997).

In neurons, CB₁ receptor protein is mainly located at pre-synaptic terminals where they modulate the release of a variety of neurotransmitters such as glutamate, GABA, acetylcholine, noradrenaline, dopamine, serotonin and cholecystokinin, among others (Howlett, 2002; Pertwee and Ross, 2002; Szabo and Schlicker, 2005). Indeed, ultrastructural analysis has detected CB₁ receptors on the terminals of GABA and glutamatergic neurons (Eggan and Lewis, 2007; Katona et al., 2006; Mátyás et al., 2008). In the neocortex, the striatum and the hippocampus, CB₁ receptor expression is higher on GABA- than glutamatergic terminals (Kawamura et al., 2006). The reasons of this differential distribution remain unknown, but this fact might explain some biphasic behavioral effects of cannabinoid agonists, such as in food intake, anxiety or locomotion (Bellocchio et al., 2010; Genn et al., 2004; Häring et al., 2011; Rey et al., 2012; Wiley et al., 2005).

Thus, CB₁ receptors are abundant in the brain, mainly localized at neuronal terminal membranes, and expressed on several types of neurons. This wide distribution explains the complexity of the mechanisms by which CB₁ receptors modulate brain functions. However, such a picture is still too simplistic. For instance, due to their high liposolubility, most cannabinoids can easily cross the plasma membrane and thereby act intracellularly. Mitochondria are intracellular organelles regulating the energy metabolism of living cells. In presence of oxygen, mitochondria metabolize glucose to produce ATP, an energetic source directly used by cells. Using electron microscopy approaches, Bénard and colleagues in our laboratory recently demonstrated the presence of CB₁ receptors on the mitochondrial membrane of hippocampal neurons (Bénard et al., 2012). That is, THC affects mitochondrial functions, such as energy production, by acting at mitochondrial CB₁.

Besides the neuronal expression of CB₁ receptors, several *in vitro* and *ex vivo* studies have indicated the existence of CB₁ receptors on brain glial cells (Gutiérrez-Rodríguez et al., 2018; Han et al., 2012; Stella, 2010). Indeed, the presence of CB₁ receptors in astrocytes is nowadays well documented, where it is located at different sub-cellular compartments ((Gutiérrez-Rodríguez et al., 2018). As this localization of CB₁ receptors is a central issue of the present Thesis, I will provide a thorough discussion of the known roles of the endocannabinoid system in astroglial functions in Chapter III, part V.

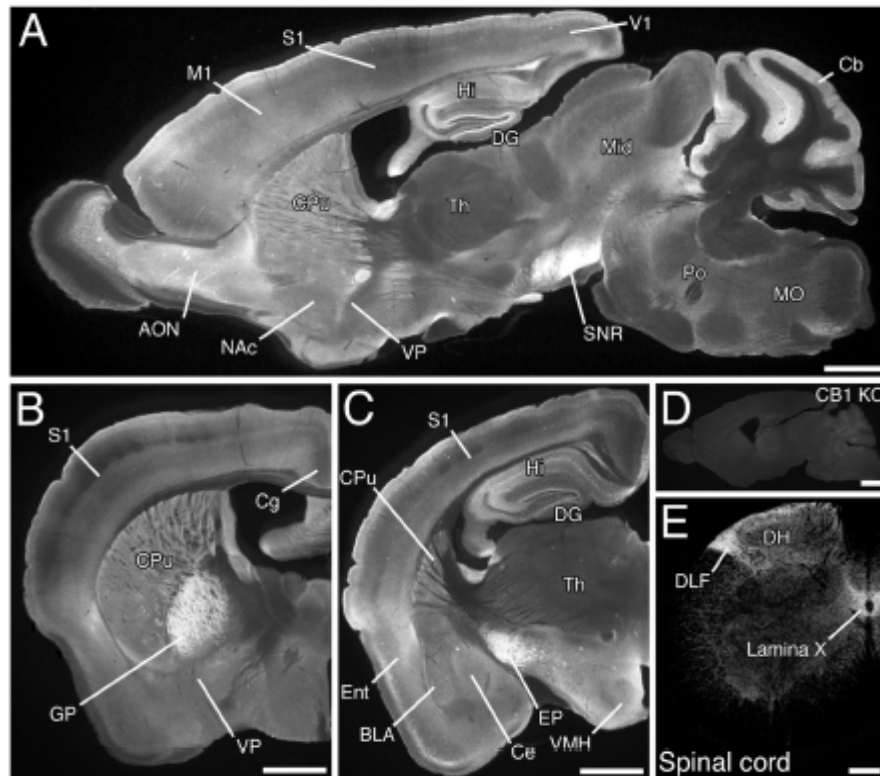


Figure 20 : Brain distribution of the mouse CB₁ receptor.

Immunolabelling of the CB₁ receptor protein in brain slices of wild-type (A) and CB₁-KO mice (D), and in coronal sections of wild type mice (B, C and E). High levels of CB₁ expression are found in the anterior olfactory nucleus (AON, A), neocortex (A-C), caudate putamen (CPu, A-C), hippocampus (Hi, A,C), thalamus (Th, A,C), basolateral (BLA) and central (Ce) amygdaloid nuclei (C), cerebellum (CB, A) and spinal cord (E). CB₁ protein is absent in the CB₁-KO mouse brain (D).

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, entopeduncular nucleus; VMH, ventromedial hypothalamus; DH, dorsal horn; DLF, dorsolateral funiculus.

Scale bars: 1 mm (A-C, E), 200 μm (D)

From Kano et al., 2009.

B. THE ENDOCANNABINOIDS

The discovery of cannabinoid receptors in the late 1980s' immediately prompted the research for their endogenous ligands (called endocannabinoids). The first endocannabinoid to be discovered in 1992 was arachidonylethanolamide (AEA). It was termed anandamide from the Sanskrit work *ananda* signifying bliss (Devane et al., 1992). A second endocannabinoid, 2-arachidonoylglycerol (2-AG) was discovered in 1995 (Mechoulam et al., 1995; Sugiura et al., 1995) and others were soon described, such as 2 arachidonoylglycerol ether (Hanus et al., 2001), N-arachidonoyldopamine (Huang and Walker, 2006) and O-arachidonylethalamine (virodhamine) (Porter et al., 2002). In addition, recent evidence points to the existence of a class of peptide ligands derived from hemoglobin (so-called pepcans)

that have been described to bind and modulate in different ways the activity of cannabinoid receptors (Bauer et al., 2012). However, anandamide and 2-AG are the best characterized endocannabinoid so far and the physiological relevance of other compounds has not been fully identified yet. Anandamide and 2-AG are lipid derivatives of arachidonic acid (Piomelli, 2003). Therefore, unlike conventional neurotransmitters, these endocannabinoids are not stored in vesicles and are thought to be synthesized “on demand” from membrane phospholipids in response to several cellular stimuli, such elevations of intracellular Ca^{2+} (Di Marzo et al., 2005).

Similarly to THC, anandamide has been proposed to act as a partial agonist at both CB_1 and CB_2 receptors, and also as an endogenous ligand for TRPV1. 2-AG is the most prevalent endocannabinoid in the brain and it acts as a full agonist for both CB_1 and CB_2 receptors. endocannabinoids are lipids and the balance between synthesis and inactivation finely regulates their levels.

i. Synthesis and degradation of 2-AG and anandamide

Synthesis

Different and redundant enzymatic pathways are implicated in the synthesis and degradation of anandamide and 2-AG (**Figure 21, page 41**), and novel mechanisms are continuously proposed. In the classic view, anandamide synthesis starts with the conversion of the phosphatidylethanolamines by N-acetyltransferase (NAT) into the precursor N-arachidonoyl phosphatidyl ethanol (NAPE) (Piomelli, 2003). NAPE is synthesized by the Ca^{2+} -independent N-acyl-transferase (Jin et al., 2009) and is then hydrolyzed to anandamide by a specific phospholipase D (NAPE-PLD) (Di Marzo et al., 1994; Wang and Ueda, 2009), which is the first pathway that has been discovered. The second is the NAPE-phospholipase C (NAPE-PLC) (Lu and Mackie, 2016). In this 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 (Liu et al., 2006).

The endocannabinoid 2-AG is synthesized mainly by two principal mechanisms. First, 2-AG precursor diacylglycerol (DAG) is formed from enzymatic cleavage of membrane phospholipid precursors by the enzyme PLC (Basavarajappa, 2007). DAG is then hydrolyzed by the diacylglycerol lipase (DAGL) to generate 2-AG (Bisogno et al., 1999). The second main pathway to regulate 2-AG production is *via* the generation, by the action of phospholipase A (PLA), of the intermediate molecule phosphatidyl inositol, which is then converted into 2-AG by the enzyme lyso-PLC (Lu and Mackie, 2016).

Degradation

The enzymes that degrade endocannabinoids have been quite thoroughly characterized. Once produced, anandamide is quickly degraded (**Figure 21, page 41**) by the enzyme fatty acid amid hydrolase (FAAH; (Cravatt et al., 1996). In the hippocampus, FAAH has been shown to be an integral membrane bound protein found in the soma and dendrites of pyramidal cells that are innervated by CB_1 receptor-positive axon terminals ((Gulyas et al., 2004; Hu and Mackie, 2015).

The degradation of 2-AG (**Figure 21, page 41**) is mainly processed by the enzyme monoacylglycerol lipase (MAGL; (Dinh et al., 2002a). This enzyme belongs to the family of the serine hydrolase, highly expressed in the CNS (Dinh et al., 2002b), and it converts 2-AG into arachidonic acid and glycerol (Ahn et al., 2008). In hippocampal neurons, MAGL is expressed mainly pre-synaptically in glutamatergic and GABAergic terminals, in contrast to FAAH, which is mainly post-synaptic (Dinh et al., 2002b) 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).

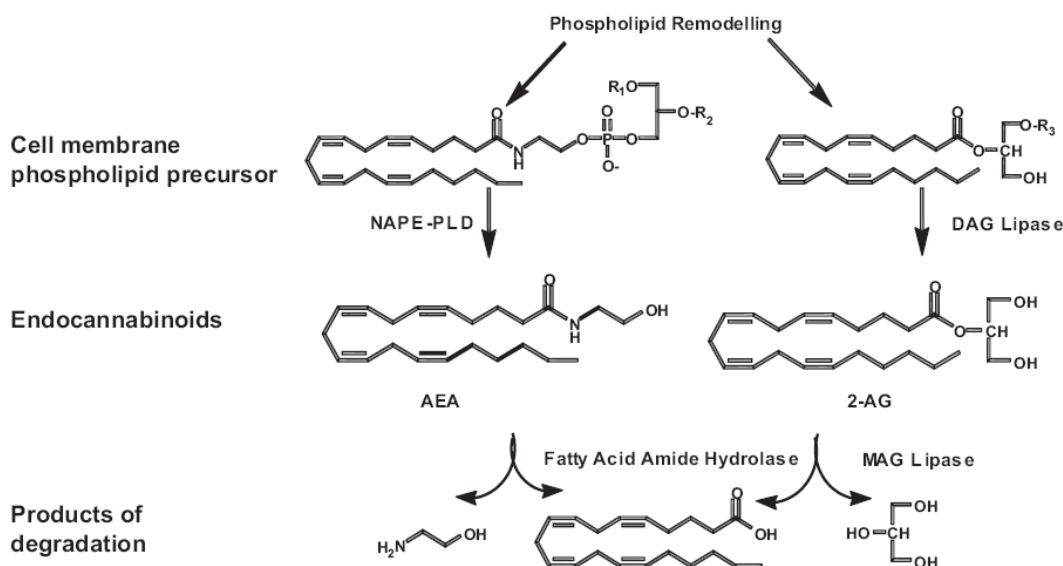


Figure 21: Schematic representation of the synthesis and degradation pathways of the two main endocannabinoids AEA and 2-AG.

DAG, diacylglycerol; MAG, monoacylglycerol; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D.

Adapted from Gonthier et al., 2010

III. Methods to dissect CB₁ receptor functions

CB₁ receptors are widely expressed in different brain regions, types of cells and subcellular compartments, making the endocannabinoid system difficult to study in details. 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.

A. Pharmacological tools

The use of pharmacological tools is very important to identify and dissect specific CB₁ receptor functions from the molecular to the behavioral level.

Several synthetic molecules (**Figure 22, page 42**) can act as full agonists with high activity and partial agonists with mild affinity to the receptor. Also, antagonists can block the action of CB₁ and inverse agonists decrease the activation of the receptor

below a threshold of basal activity. There are also allosteric modulators that through binding at allosteric rather than orthosteric sites can modify the function of the receptor (Mackie, 2008).

Besides THC, which is a natural agonist, there are several synthetic ligands currently used in the field of cannabinoid research to study the specific role of CB₁ receptors (**Figure 22, page 42**).

The agonist HU-210 has a high affinity and potency; CP55940, a potent agonist has high affinity (but inferior to HU-210), WIN 55212-2 and arachidonyl-2'-chloroethylamine (ACEA), both are highly selective and potent agonists (Pertwee et al., 2010). The best characterized CB₁ receptors antagonist are SR141716A (= Rimonabant) and AM-251 (Pertwee et al., 2010).

As mentioned, pharmacological compounds are powerful tools to activate or inhibit CB₁ receptors. However, they intrinsically lack cell type specificity, thereby limiting the information they can provide on specific circuits where actions of CB₁ receptor occur during brain functioning.

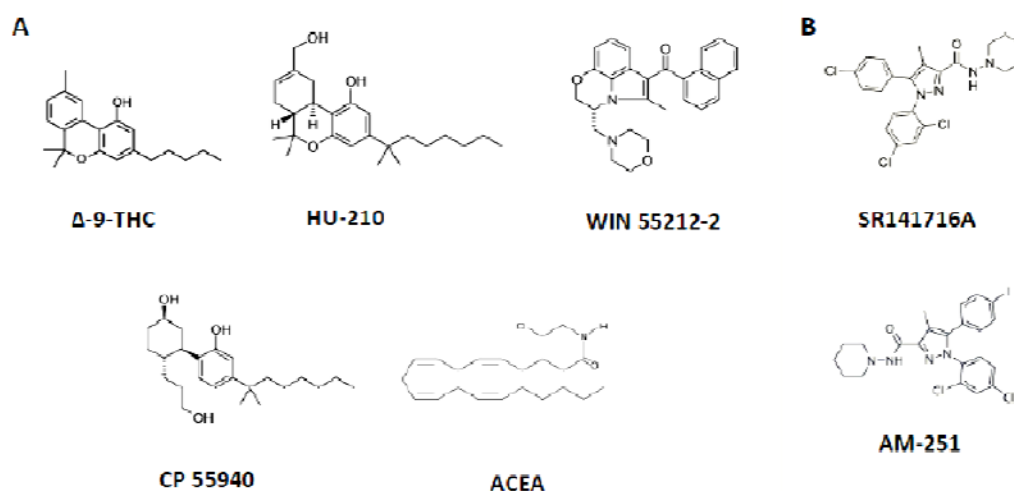


Figure 22: Chemical structures of exogenous natural and synthetic ligands of cannabinoid receptors.

(A) Agonists (B). Antagonists

Adapted from Piomelli, 2003

B. GENETIC TOOLS

Together with drugs, null CB₁ mouse mutant lines characterized by an ubiquitous deletion of CB₁ receptors (Ledent et al., 1999; Marsicano et al., 2002; Zimmer et al., 1999) are very powerful tools to study the general functions of the endocannabinoid system. However, since CB₁ receptors are expressed on many types of cells, the consequences of systemic or even local manipulations of CB₁ receptor activity reflect a “net” effect of CB₁ signaling in various target cells and a gene alteration may exert its effects in multiple different cells and tissue types, creating a complex phenotype in which it is difficult to distinguish its direct functions in particular cells. Therefore, methods have been developed to control conditions such as the timing and/or the cell type specificity of gene activation or repression.

The Cre/LoxP system (**Figure 23, page 43**) is an approach for generating tissue-specific knockout mice (Sauer and Henderson, 1989). Briefly, two different genetically engineered mouse lines are needed to achieve a tissue-specific gene deletion. In most cases, Cre- and loxP-containing strains of mice are developed independently and crossed to generate offspring bearing the tissue-specific gene knock-out. The first mouse strain contains the targeted gene sequence flanked by two loxP (“locus of crossover P1”) sites (“floxed” gene). The loxP sites are 34-base pair DNA sequences placed on each side of a target gene sequence by homologous recombination in mice (Sauer and Henderson, 1989). The small dimensions of the loxP sites guarantee that the mutation does not alter the expression of the floxed gene. Thus, floxed mouse lines can be considered as phenotypically wild-type. The Cre recombinase is a member of the integrase family of recombinases, which catalyzes recombination between two loxP sites properly oriented. Indeed, the Cre excises the DNA segment between two loxP sites, resulting in a single remaining loxP site (Sauer and Henderson, 1989). Once the normal expression of the gene is verified, the floxed mouse line can thus be crossed to any transgenic mouse line expressing the Cre recombinase under the control of a promoter that is specific for a particular cell type (referred to as Cre line). Offspring of this crossing will express both the floxed gene and the Cre-expressing transgene. In the cells where the Cre recombinase is expressed, the DNA segment flanked by the loxP sites will be excised, and consequently inactivated. The floxed gene remains active in the cells that do not express the Cre (Sauer and Henderson, 1989). By this genetic system, several cell-type specific CB_1 knock-out (CB_1 -KO) mouse lines were generated. It is important to note that constitutive as well as the available conditional CB_1 -KO mice are viable, fertile, develop normally and do not have severe deficits that can distinguish them from the control wild-type littermates.

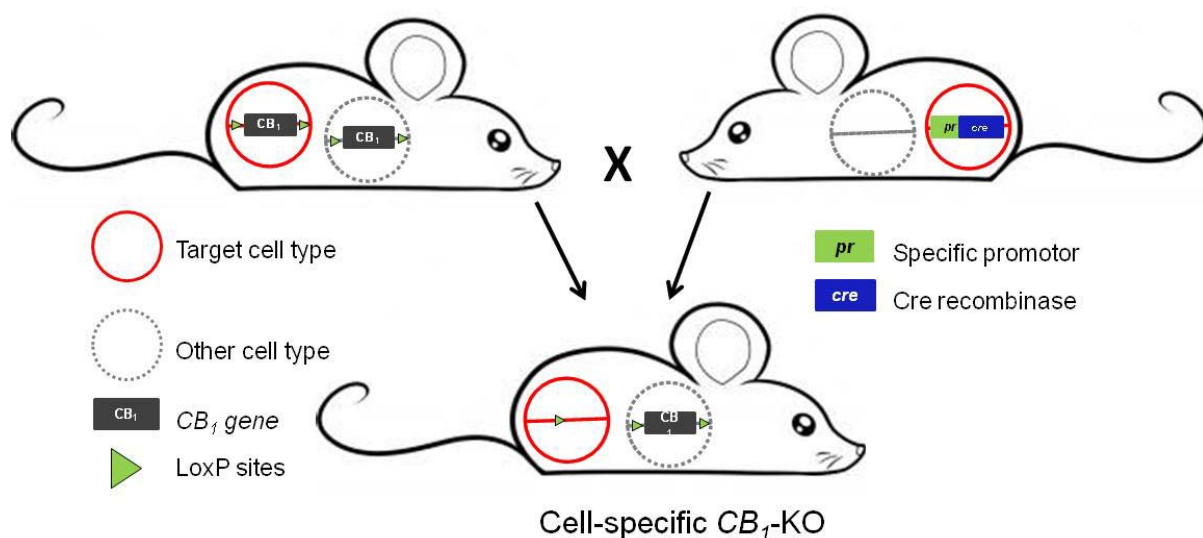


Figure 23: Cell type-specific deletion of the CB_1 gene by the Cre/loxP system strategy in mice.

The generation of cell-type specific CB_1 -KO mouse lines reposes on the crossing of two different lines. The CB_1 -floxed line (upper left) is characterized by the flanking of the CB_1 sequence by two loxP sites in all the cells. The Cre line (upper right) is characterized by the expression of the Cre recombinase under the control of a promoter specific to the targeted cell type. In the offspring of this crossing (lower), Cre will excise the CB_1 gene in the targeted cells, leaving the normal expression of the receptor in the remaining cells of the body. Alternatively, the Cre protein can be locally delivered using viral vectors

Conditional deletion of CB₁ receptors is a powerful tool to examine the cell type-specific roles of the endocannabinoid system 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, GFAP is a cytoskeleton protein that is commonly used as a marker for astrocytic identification (Brenner et al., 1994). 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 has been provided by a system allowing time-dependent inducible gene deletion. In the case of astrocytes, to achieve cell-type specific KO, Hirrlinger and colleagues (Hirrlinger et al., 2006) used the tamoxifen-inducible Cre-ERT2/loxP system (Sauer & Henderson, 1989). In this model the Cre is fused to a mutated ligand binding domain of the estrogen receptor (ERT). Through the binding of heat-shock proteins to the ERT domain, this modification impedes the nuclear translocation of the Cre recombinase. Thus, the Cre-ERT2 is expressed in the cells that have GFAP but it is only active after treatment with the selective estrogen ligand tamoxifen, which removes the binding to heat-shock proteins (**See Material and Methods**). 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 functions 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 functions in different brain processes.

C. VIRAL APPROACH

Mice carrying conventional null mutations have been very useful to study the roles of various genes in the functions of the nervous system. However, the possibility of compensatory changes during development is often raised as well as the lack of region specificity. During the last decades, viral approaches have been developed to obviate these issues. In particular, the recombinant adeno-associated virus (AAV) has been shown to have the potential to mediate the delivery and stable transduction of genes in the CNS. As we mentioned in the previous part, the development of the Cre-loxP system has provided powerful tools for the manipulation of genes. There are several strains of mice that express Cre under the control of promoters with various tissue specificities. These can be bred with animals carrying loxP-flanked genes to produce gene inactivation in different tissues. However, there are clear advantages to being able to deliver Cre to specific groups of cells in adult animals.

This technology is based on the construction of adeno-associated virus expressing the Cre recombinase (AAV-Cre). The AAV delivers Cre recombinase to the target region of the adult mice. Then, the Cre recombinase excises DNA sequences located between 2 *LoxP* sites that surround the targeted gene. Many studies have shown the utility of AAV (Anton and Graham, 1995; Rohlmann et al., 1996) to deliver functional Cre recombinase to the mouse *in vivo* and reports on AAV vectors have shown long-term gene expression in the CNS (Clark et al., 1999; Kaplitt et al., 1994).

Stereotaxic injections of AAV expressing the Cre recombinase (AAV-cre) into the target brain region of a floxed mice can allow the deletion of the gene of interest in specific location. Moreover, to avoid the recombination in all cell-types, it is possible to place the viral Cre recombinase under the control of a cell-selective promoter.

In the specific case of CB₁ receptor, studies showed the efficacy of this viral approach (Monory et al., 2006). Monory and colleagues were the first to use this approach to specifically delete CB₁ receptors in the dentate gyrus of the hippocampus by injecting a AAV-Cre in CB₁-floxed mice and notice a complete deletion of CB₁ gene. Later, other groups used this methods. For example, in 2015, Soria-Gomez et al., addressed the specific role of CB₁ receptor injecting a AAV-Cre in the medial habenula (Mhb) of CB₁-floxed mice (Marsicano et al., 2003) to obtain MHb-CB₁-KO mice, lacking CB₁R expression in MHb neurons and found that the expression of aversive memories is under the control of CB₁ receptor in MHb neurons (Soria-Gómez et al., 2015).

Overall, these genetic approaches have provided the possibility of exploring the role of genes in selected brain regions and cell types, and at specific time points.

IV. Signaling of CB₁ receptors and modulation of synaptic plasticity

The CB₁ receptor, which controls endocannabinoid-mediated retrograde signaling, is an important feedback mechanism that modulates synaptic transmission (Figure 24) ((Freund et al., 2003; Piomelli, 2003). Several features of CB₁ contribute to this function.

A. INTRACELLULAR PATHWAYS

Neuronal CB₁ receptors are generally coupled with the Gi/o subunits of G proteins (Howlett and Fleming, 1984; Howlett et al., 1986). Thus, the activation of CB₁ receptors by endocannabinoids inhibits adenylyl cyclase and decreases cAMP levels, thereby decreasing the activity of the cAMP-dependent 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 voltage-gate Ca²⁺ channels (VGCCs; (Fisyunov et al., 2006; Mackie et al., 1995) and the activation of inwardly-rectifying K⁺ channels (**Figure 24, page 46**; (Guo and Ikeda, 2004; Mackie et al., 1995). These effects contribute to a hyperpolarization of the pre-synaptic neuron and a decrease of neurotransmitter release into the synaptic cleft (Castillo et al. 2012). Furthermore,

CB₁ receptor activation by its different ligands modulates several intracellular cascades, such as the mitogen-activated protein kinases (MAPK) pathway, including extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), p38 MAPK or ERK5 proteins (Turu and Hunyady, 2010). 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).

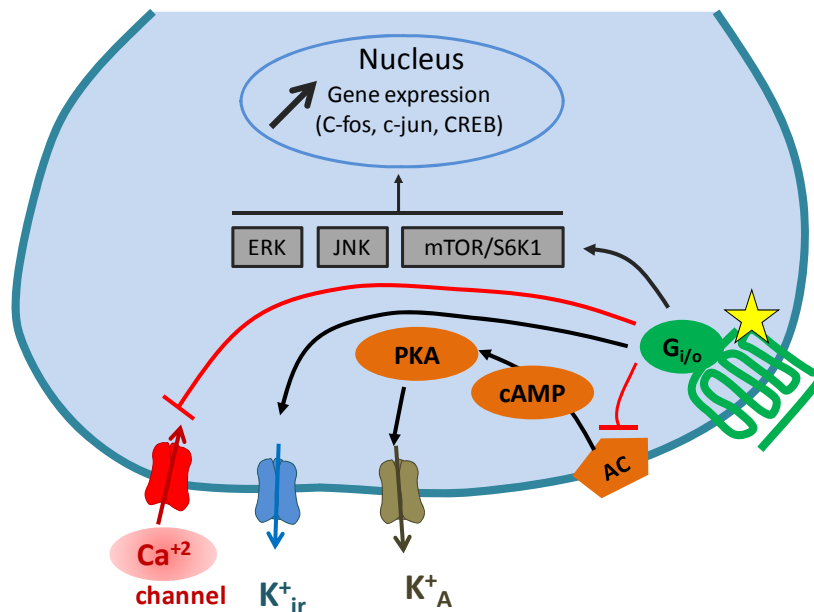


Figure 24 : Main intracellular CB₁ receptor signaling pathways.

Classically, stimulation of CB₁ receptors leads to 3 main cascades of events. A direct modulation of ion channels conductance including an inhibition of Ca²⁺ channel and activation of K^{ir} channels. Activation of A-type K⁺ channels (K⁺_A) can be induced through the inhibition of adenylyl cyclase (AC). Activation of several protein kinases including ERK, JNK and mTOR leads to *de novo* gene expression.

Adapted from Pagotto et al., 2006.

Besides the canonical pathways described above, different intracellular cascades can be activated by CB₁ receptors, depending on specific conditions and also on the chemical structure of the agonist (Turu and Hunyady, 2010). For instance, it has been shown that, in certain conditions, CB₁ receptor can recruit G_s (Glass and Felder, 1997) or G_q (Lauckner et al., 2005; Navarrete and Araque, 2008) rather than Gi/o proteins (Turu and Hunyady, 2010). Moreover, the CB₁ receptor agonist WIN 55212-2 can induce G_q-dependent Ca²⁺ increase in hippocampal neurons whereas THC or HU-210, other CB₁ receptor agonists, do not (Lauckner et al., 2005). These findings might explain why different agonists sometimes do not induce the same effect, though all of them are specific to CB₁ receptors. In the next future and for the development of efficient therapy, it will be interesting to better understand which intracellular pathways are recruited by different agonists *in vivo*, as well as which CB₁ receptor configurations and cellular/subcellular localization are involved in these differential effects.

B. SUPPRESSION OF NEUROTRANSMITTER RELEASE

In general, pharmacological activation of neuronal CB₁ receptors inhibits the release of neurotransmitters (Schlicker and Kathmann, 2001), including glutamate (Lévénés et al., 1998) and GABA (Szabo et al., 1998). For instance, the cannabinoid agonist WIN55,212-2 suppresses excitatory post-synaptic currents (EPSCs) in the hippocampus. This effect is blocked by the specific antagonist SR141716A, confirming the involvement of CB₁ receptors in the reduction of glutamate release (Shen et al., 1996). The reduction of glutamate release was later confirmed in many different brain regions such as the cerebellum, striatum, and cortex (Schlicker and Kathmann, 2001).

The inhibitory effects of cannabinoids on GABA release were first reported in the striatum and substantia nigra pars reticulata (Chan and Yung, 1998; Szabo et al., 1998). WIN55,212-2 suppressed GABAergic inhibitory post-synaptic currents (IPSCs), but not the post-synaptic response to exogenous GABA, indicating a pre-synaptic site of action. This effect was also blocked by SR141716A confirming the involvement of CB₁ receptors. As for glutamate release, the effect of cannabinoids on GABA release was confirmed in different brain regions including the hippocampus, cerebellum, and nucleus accumbens (Schlicker et al., 2001).

C. CB₁ RECEPTOR-MEDIATED MODULATION OF SYNAPTIC TRANSMISSION AND PLASTICITY

Endocannabinoids mediate retrograde signaling at synapses (Castillo, 2012; Kano et al., 2009; Piomelli, 2014). Already in the 1990s, it was observed that depolarization of post-synaptic neurons causes transient suppression of transmitter release (Alger et al., 1996; Pitler and Alger, 1992). However, the nature of the retrograde signal mediating this phenomenon remained elusive for many years. In 2001, three almost simultaneous independent papers appeared showing that endocannabinoids and CB₁ receptors are the main responsible of such retrograde inhibition of synaptic transmission (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Since then, many groups reported similar retrograde mechanisms many brain regions and under different stimulation protocols (Iremonger et al., 2013; Kano et al., 2009; Ohno-Shosaku and Kano, 2014; Piomelli, 2014). Briefly, upon post-synaptic depolarization and/or receptor activation, endocannabinoids are mobilized from post-synaptic neurons and activate pre-synaptic CB₁ receptors, inducing transient suppression of transmitter release called endocannabinoid-mediated short-term depression (eCB-STD) (Castillo et al., 2012). Moreover, endocannabinoid can mediate LTD called endocannabinoid-induced long-term depression (eCB-LTD) in the hippocampus and other brain regions (Castillo et al., 2012). Apart from these forms of synaptic plasticity, many others have been described over the last 20 years (Bacci et al., 2004; Marinelli et al., 2009; Maroso et al., 2016). In the next section, I will briefly summarize the best characterized form of endocannabinoid-induced plasticity.

i. DSI and DSE

Endocannabinoid mobilization, pre-synaptic activation of CB₁ receptors and transient decrease of neurotransmitter release can be induced by a large post-synaptic Ca²⁺ elevations alone. This form of eCB-STD (**Figure 25, page 48**) includes DSI

(depolarization-induced suppression of inhibition (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and DSE (depolarization-induced suppression of excitation) (Kreitzer et al., 2001).

In 1991, Llano et al. discovered that depolarization of cerebellar Purkinje cells induces transient suppression of inhibitory inputs to the depolarized cells (Llano et al., 1991) and the same phenomenon was reported in hippocampal CA1 neurons and termed “DSI” (Pitler and Alger, 1992). DSI lasts from tens of seconds up to 1 min (Gerdeman and Fernández-Ruiz, 2008). Endocannabinoids mediate retrograde signaling for DSI in the hippocampus (Ohno-Shosaku et al., 2001; Wilson et al., 2001), and the same mechanisms have been then reported in various brain regions including the striatum, globus pallidus, substantia nigra, cerebral cortex, amygdala, ventral tegmental area and hypothalamus (for review, see Kano et al., 2009).

At the same time, Kreitzer and Regehr discovered that depolarization of cerebellar Purkinje cells induces transient suppression of excitatory transmission (Kreitzer & Regehr; 2001), named DSE. Similar to DSI, DSE is induced by post-synaptic Ca^{2+} increase and is mediated by retrograde endocannabinoid signaling DSE has been reported in many brain regions including the hippocampus, cerebral cortex, hypothalamus, ventral tegmental area, and dorsal cochlear nucleus (for review, see (Kano et al., 2009).

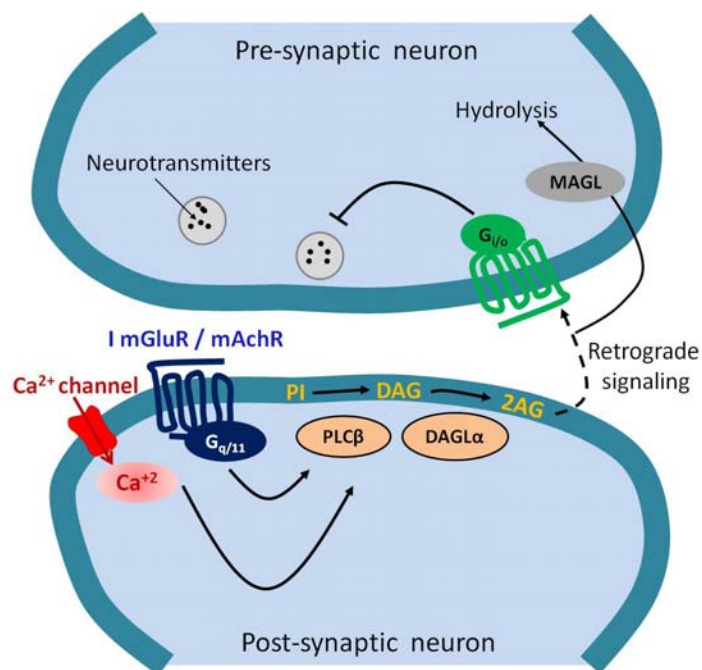


Figure 25: Molecular mechanisms of eCB-STD.

When a large Ca^{2+} elevation is caused by activation of voltage-gated Ca^{2+} channels or $G_{q/11}$ -coupled receptors (I-GluR and mAChR), 2-AG is generated in a DAGL-dependent manner. Synthesis of 2-AG from postsynaptic neurons, activates presynaptic CB $_1$ receptors, and induces transient suppression of transmitter release. 2-AG is degraded by presynaptic MAGL

Reproduced with modification from Kano 2014.

ii. Long-term forms of endocannabinoid-mediated plasticity

Besides their role in the modulation of transient changes in synaptic transmission, CB $_1$ receptors can also mediate long-term forms of synaptic plasticity (**Figure 26,**

page 49; Castillo et al., 2012). In 2002, long term effects of retrograde endocannabinoid signaling were described in the amygdala and called LTDi for long-term depression of inhibition (Marsicano et al. 2002). A similar phenomenon was later identified in the hippocampus, where it was named I-LTD with the same meaning (Chevaleyre and Castillo, 2003). Similarly, Robbe and colleagues showed that endocannabinoids mediate LTD of excitatory neurotransmission in the nucleus accumbens (Robbe et al., 2002) and Gerdeman and colleagues identified a similar phenomenon in the dorsal striatum (Gerdeman et al., 2002). Since then, long-term depression (LTD) of synaptic transmission dependent on retrograde endocannabinoid signaling (eCB-LTD) has been described in several areas of the brain CB₁ (Hashimoto et al., 2007). Below, I will summarize the main features of this phenomenon, whose details slightly vary depending on the brain region and experimental conditions.

In general eCB-LTD depends on glutamate release from neuronal afferents induced by different protocols of synaptic stimulation. This glutamate activates in turn mGluRs at the post-synaptic neurons, inducing post-synaptic Ca²⁺ increase. Finally endocannabinoids are mobilized post-synaptically and activate presynaptic CB₁ receptors triggering intracellular modifications resulting in long-term decrease of synaptic strength. The nature of these intracellular modifications is not fully elucidated, but recent evidence points, for instance, to the involvement of presynaptic phosphatase (calcineurin) signaling. Interestingly, the endocannabinoids involved in these phenomena seem to depend on the brain region and on the type of synaptic transmission investigated. For instance, whereas anandamide is suggested to mediate LTDi in the amygdala (Azad et al., 2004; Marsicano et al., 2002) and LTD of excitatory transmission in the striatum (Gerdeman et al. 2002), on demand 2-AG mobilization seems to be required for I-LTD in the hippocampus (Chevaleyre & Castillo, 2003).

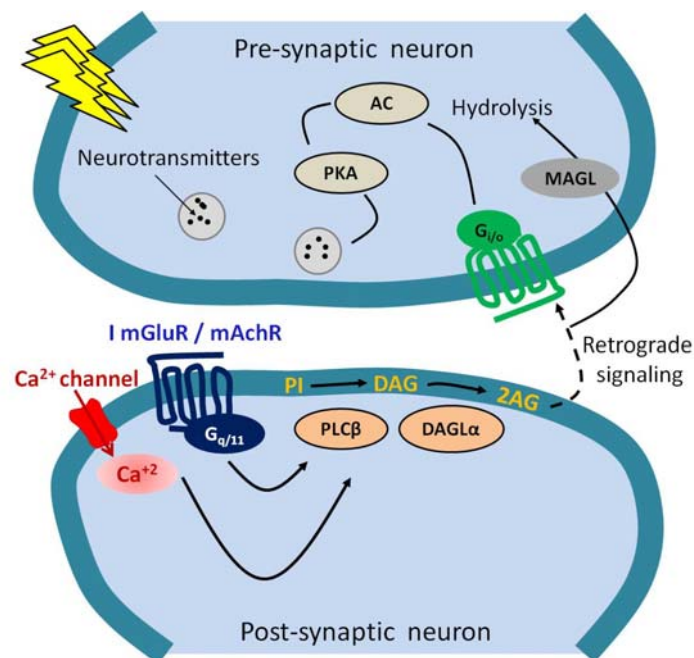


Figure 26 : Established mechanisms of endocannabinoid-mediated long-term synaptic plasticity.

Sustained patterns of activity can induce a long-term depression (LTD) of excitatory or inhibitory terminals via presynaptic CB₁ receptors by postsynaptically produced endocannabinoids.

Adapted from Castillo et al., 2012

V. Astroglial CB₁ at the tripartite synapse

A. LOCALISATION AND METABOLISM

The expression of astroglial CB₁ receptors was debated for a long time. It was first observed in primary cultures from rats or humans (Bouaboula et al., 1995; Molina-Holgado et al., 2002; Sheng et al., 2005), but the same techniques used in mice revealed contradictory results (Sagan et al., 1999; Walter and Stella, 2003). The debate was reinforced because of the difficulty to identify astroglial CB₁ mRNA or protein expression in brain sections (Metna-Laurent and Marsicano, 2015). These issues were somehow settled by the work of Navarrete and Araque (Navarrete and Araque, 2008, 2010) clearly showing functional evidence of astroglial expression of CB₁ in hippocampal slices. These studies showed that endocannabinoids regulate synaptic activity not only acting at pre-synaptic neurons, but also *via* activation of astroglial CB₁ receptors and consequent release of specific gliotransmitters (Navarrete and Araque, 2008, 2010). This functional evidence was later confirmed by anatomical experiments using electromicroscopy coupled to immunostaining, directly revealing the presence of low, but significant amounts of CB₁ receptors in GFAP-positive astrocytes (**Figure 27, page 50**; (Han et al., 2012).

Interestingly, astrocytes can also produce endocannabinoids. Studies conducted in primary cultures showed that astrocytes can produce even more endocannabinoid than neurons, through a ATP- and Ca²⁺- dependent mechanisms (Stella, 2010; Walter and Stella, 2003). Moreover, astrocytes express the endocannabinoids degradation machinery (Metna-Laurent and Marsicano, 2015), suggesting that astrocytes might play a role in the endocannabinoid system functions by both paracrine or autocrine modes of action.

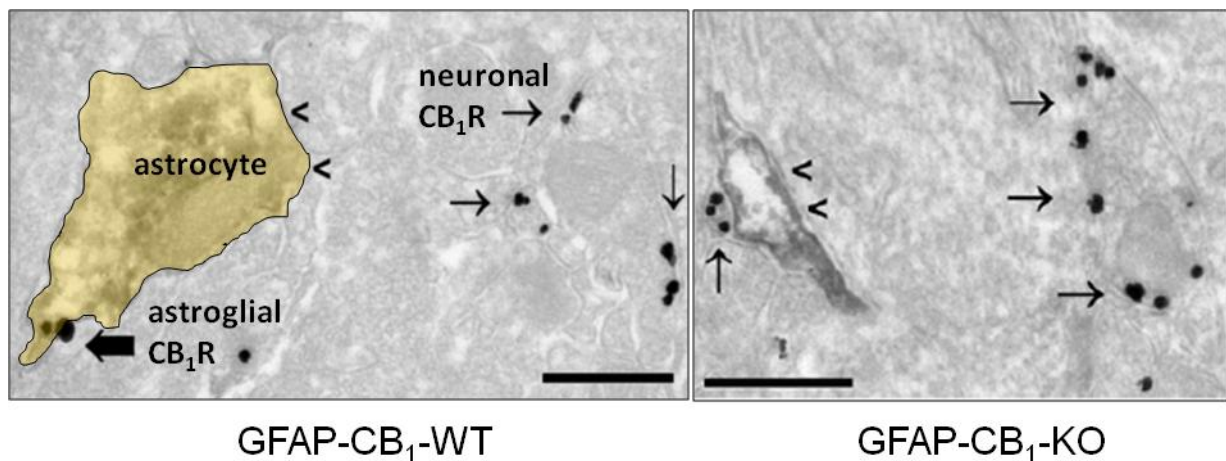


Figure 27: CB₁ receptor is present in astrocytes.

Electron microscopic images show a high density of CB₁ receptor immunopositive silver grains (small arrows) in axons/terminals of GFAP-CB₁-WT and GFAP-CB₁-KO mice, and a low density of silver grains (large arrow) in DAB-stained astrocytes (arrowheads) of GFAP-CB₁-WT mice but not of GFAP-CB₁-KO littermates. The scale bar represents 500 nm.

Adapted from Han et al., 2012

B. PHYSIOLOGICAL IMPACT OF ASTROGLIAL CB₁ RECEPTORS

In the CA1 region, stimulation of astroglial CB₁ receptors triggers an increase of intracellular Ca²⁺ concentration (**Figure 28, page 51**) (Navarrete and Araque, 2008, 2010). This increase is suggested to be due to the coupling of the astroglial CB₁ receptors to G_q proteins leading to PLC activation (Lauckner et al., 2005; Navarrete and Araque, 2008). Indeed, while neuronal signaling (DSE, DSI) is inhibited by pertussis toxin (G_{i/o} protein inhibitor), the astroglial Ca²⁺ increase is not (Navarrete and Araque, 2010). CB₁-dependent astroglial signaling can modulate heterosynaptic neuronal transmission in the hippocampus (Navarrete and Araque, 2008) but also in other structures such as the striatum or the amygdala (Martín et al., 2015; Martín-Fernandez et al., 2017). At the CA3-CA1 synapse, it was shown that endocannabinoid release induced by the depolarization of neurons triggers a potentiation of synaptic transmission at neighboring synapses *via* astroglial Ca²⁺ increase and consequent release of gliotransmitters, such as glutamate (Navarrete and Araque, 2008, 2010). In turn, astroglial-derived glutamate is proposed to activate mGluR1 receptors on neurons to eventually potentiate their synaptic functions. Thus, astroglial CB₁ receptors have been functionally associated with the release of glutamate (Navarrete and Araque, 2008, 2010).

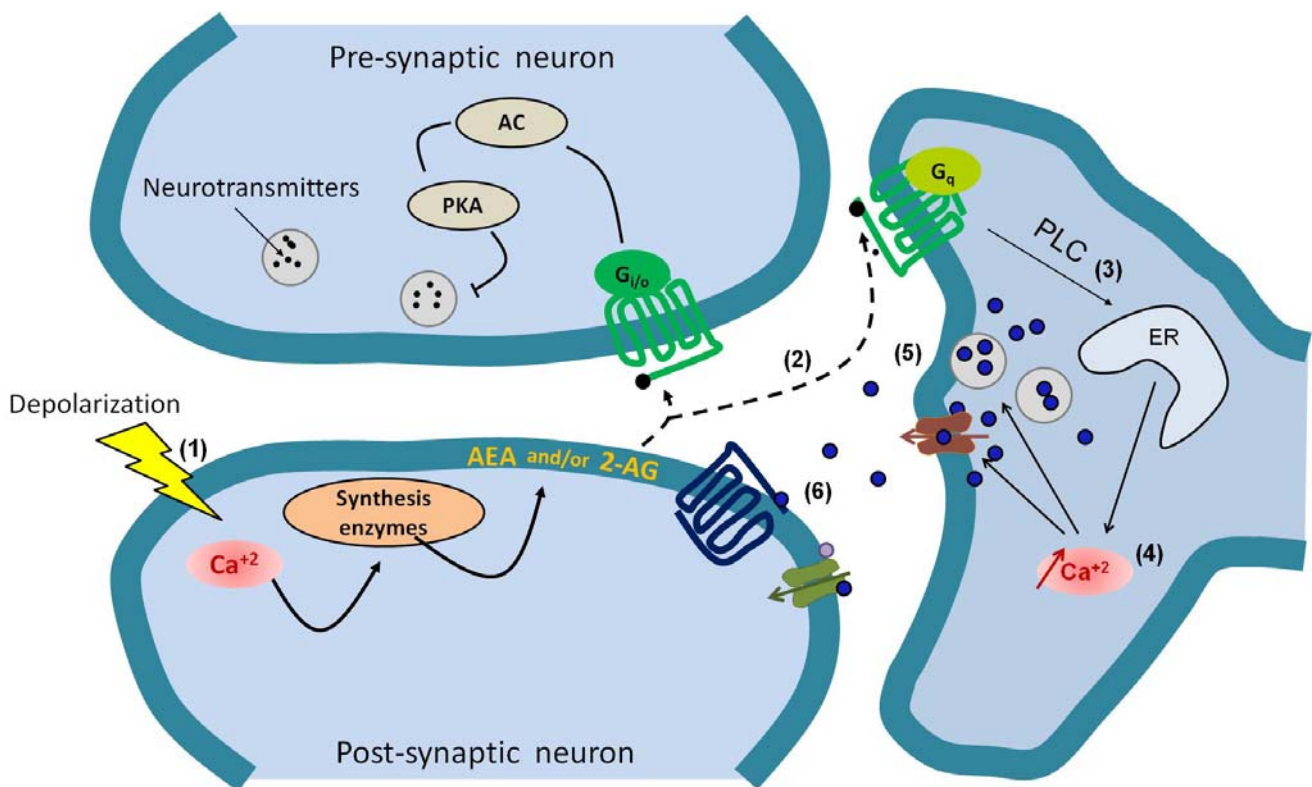


Figure 28: Summary and putative scheme of endocannabinoid-induced gliotransmission.

(1). Stimulating endocannabinoid release by neuronal depolarization. The intracellular pathway and the endocannabinoid involved are unknown. (2). Mobilization of endocannabinoid in the synaptic cleft. (3) Endocannabinoid-stimulation of astroglial CB₁R_s coupled to G_q activates phospholipase C (PLC). (4). Activation of PLC increases astroglial Ca²⁺ levels through the mobilization of Ca²⁺ from internal stores that could be the endoplasmic reticulum (ER). (5). Ca²⁺-dependent vesicular release, or non-vesicular release of glutamate (and/or other gliotransmitters). (6). Glutamate binds the post-synaptic NMDAR or mGluR1 receptors on neurons to eventually potentiate their synaptic functions.

Adapted from Navarrete & Araque, 2008; 2010.

Finally, exogenous applications of cannabinoids *in vivo* also induce a form of cannabinoid-dependent long-term depression (CB-LTD; Han et al., 2012). The use of mutant mouse models where astroglial CB₁ receptors have been deleted (GFAP-CB₁-KO mice) demonstrated that this *in vivo* synaptic effect of cannabinoids depends on this specific subpopulation of CB₁ receptors.

Importantly, recent evidence associated these effects of endogenous or exogenous activation of astroglial CB₁ receptors to the modulation of behaviors such as the control of fear responses in the amygdala (Martin-Fernandez et al., 2017) or the impairing effect of cannabinoids on working memory in the hippocampus (Han et al., 2012).

There are very few publications onto the role of astroglial CB₁ receptors on behavior because of the difficulty to manipulate the receptor without affecting neuronal activity. Genetic models and new technologies will allow us to better understand its role in physiology and more particularly in learning and memory.

CHAPTER IV

Memory

I. Neuroanatomical and neurological substrates of memory

Memory is a brain function that classifies, encodes, stores and recovers different information relevant for the subject (Squire, 1986) and (Kandel, 2001). Memory can be divided into two major groups (**Figure 29, page 53**): declarative and non-declarative. Declarative (or explicit memory) is defined as the conscious memory for facts and events and is acquired with few exposures to the material to be learned. It can be classified into episodic memory (personal events) and semantic memory (general facts) (Squire, 1992; Squire and Zola, 1996). Declarative memory is mainly controlled by the medial temporal lobe, including the hippocampus, the entorhinal, perirhinal and parahippocampal cortices (Moscovitch et al., 2006). On the other hand, non-declarative (or implicit memory) involves different brain regions, it consists in procedural memory for habits or skills, and usually requires an extensive acquisition phase (Schacter and Cooper, 1993).

The time scale could also be used to classify memory (**Figure 29, page 53**). Short-term memory (STM) lasts from minutes to days in humans and from minutes to 3-4 hours in rodents and is susceptible to perturbations (Kumaran, 2008; Walker et al., 2003). Working memory is a particular form of STM, which permits to remember the current state of a plan that the subject is executing (Conway et al., 2001; Tetzlaff et al., 2012). STM depends on the context and the memory test, but the brain region mostly associated with this type of memory is the hippocampus. Long-term memory (LTM) is defined as a memory lasting from days to years in humans and from several hours to months in rodents. This memory is created with the contribution of different brain regions, where complex dynamic synaptic and plastic changes are required (Frankland and Bontempi, 2005; Xue et al., 2010; Ziv and Ahissar, 2009).

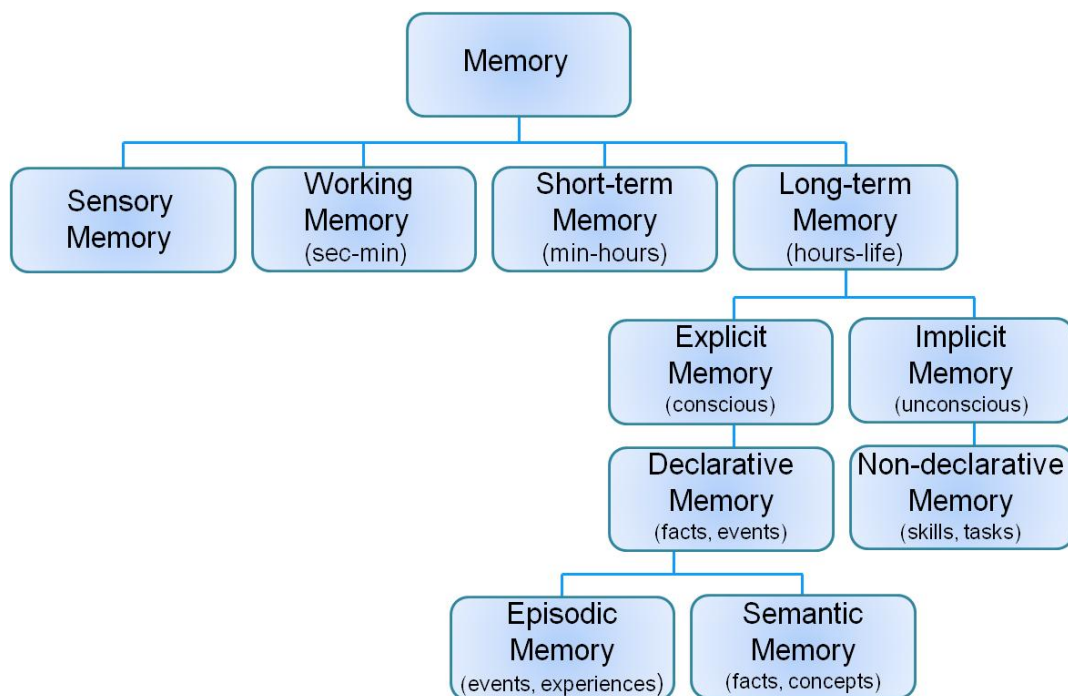


Figure 29: Schematic representation of the different types of memory.
The scheme shows the time scale of the different types.

The hippocampus is the major brain region involved in memory formation and plays a key role in the consolidation of information, especially in declarative memory. The dentate gyrus, CA3 and CA1 areas are the main areas of the hippocampus (**Figure 30, page 54**). The flow of information into the hippocampus starts in the entorhinal cortex, which represents its main input. Layer II neurons of the entorhinal cortex project mainly to the dentate gyrus and the CA3 via the perforant pathway, whereas layer III neurons send afferents directly to the CA1 and the subiculum. CA3 also projects to CA1 through the Schaffer collaterals (Witter and Amaral, 1991). The CA1 pyramidal neurons receive excitatory inputs through the perforant path (entorhinal cortex) and the Schaffer collaterals (CA3) (Amaral and Witter, 1989; Steward and Scoville, 1976). Both inputs to the pyramidal neurons are crucial for memory formation in the hippocampus (Remondes and Schuman, 2002, 2004) and undergo significant plasticity processes that is better described in Chapter II, part IV.

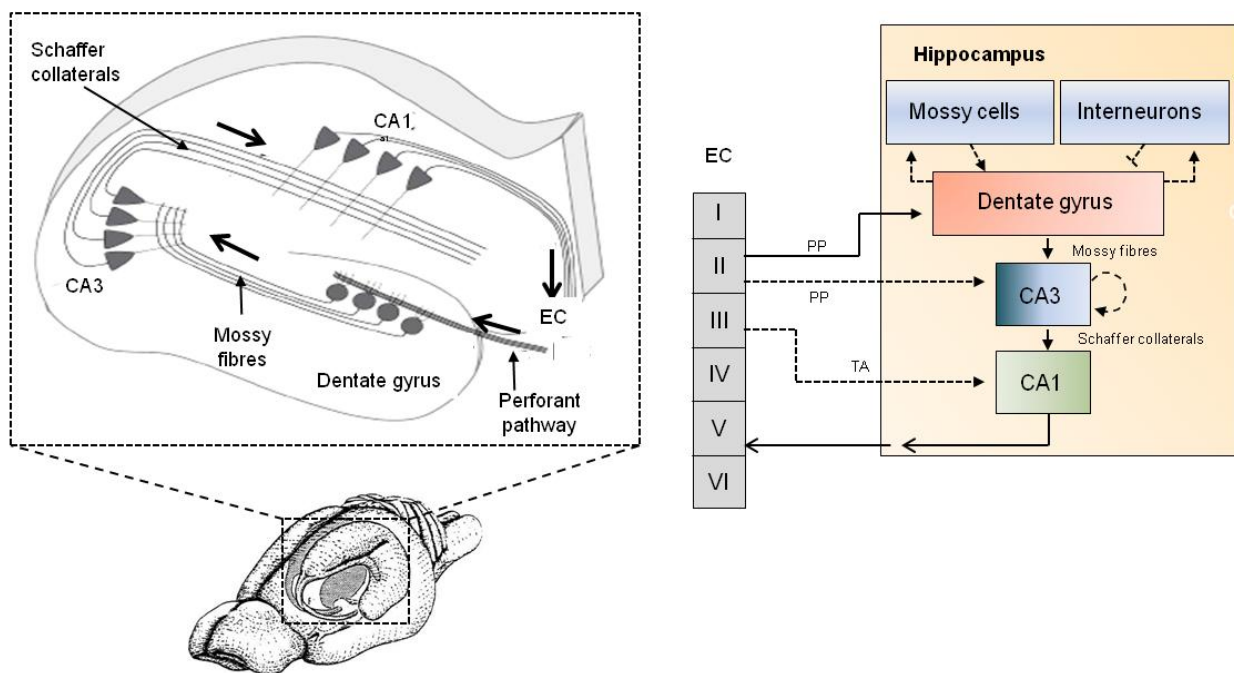


Figure 30 : Illustration of the hippocampal circuitry (A) and a diagram of the hippocampal neural network (B).

Solid arrows reflect the classic excitatory trisynaptic pathway. The axons of the entorhinal cortex project to the dentate gyrus through the perforant pathway (PP). The dentate gyrus sends projections to the pyramidal cells in CA3 through the mossy fibers. CA3 neurons send projections to CA1 pyramidal neuron through the Schaffer collateral. CA1 pyramidal neurons send back projections into the layer V of the entorhinal cortex. CA3 receives direct projections from the layer II of the entorhinal cortex through the PP. CA1 receives direct inputs from the layer III of the entorhinal cortex through the temporoammonic pathway (TA). Finally, the dentate granule cells project to the mossy cells and interneurons, which in turn send excitatory and inhibitory projections to the granule cells, respectively.

II. A model to study memory: The object recognition memory

Memory is a function extensively studied in animal models and different behavioral paradigms are available to evaluate memory performances in rodents. Memory is evaluated with different mazes and training schedule. Of course memory is a complex process that is difficult to be captured by single tests. Moreover, a major limitation of animal experiments is the fact that they exclusively rely on actions that the subjects display (we cannot ask a mouse if it remembers something). Therefore, potential confounding factors such as locomotion, motivation or anxiety are always to be taken into account in the interpretation of results.

The paradigm that I have mainly used during my Thesis work is the novel object recognition (NOR) task. This episodic memory task can be studied in human, monkeys and rodents (Squire et al., 2007) and can be used to study learning and memory functions with a good control of potential confounding variable (Ennaceur, 2010). This test is designed to evaluate the ability to judge whether something is novel or familiar. In animals, the main postulate behind this test is that subjects have an innate exploratory behavior and are attracted by novelty. Thus, they increase their exploration towards a novel object compared to an object they previously encountered (Ennaceur and Delacour, 1988). The NOR task can be configured to study different memory types such as STM, working memory or LTM (Antunes and Biala, 2012; Goulart et al., 2010).

Different forms of mazes can be used for NOR task. The general procedure of the task consists of 3 phases: habituation, acquisition and test phase. In the habituation phase, the animal is allowed to explore freely the maze without objects. During the acquisition phase, the animal is put back in the maze containing 2 identical objects. Finally, during the test phase, the animal is returned to the maze with 2 objects, of which one is identical to the ones presented during the acquisition phase and the other is completely novel (Ennaceur, 2010).

Observers measure the time of exploration of the objects, and the memory performance can be calculated through different indexes, including discrimination index, index of global habituation, or preference index depending on the aim of the study (Gaskin et al., 2010). Several factors might influence the performance in this task, including mouse strains, age and sex, the presence of spatial cues, types of objects, duration of the trials, interval period between acquisition and test, the dimension, shape, illumination of the apparatus (Antunes and Biala, 2012; Schimanski and Nguyen, 2004). In particular, the potential anxiogenic effect of the maze can be a powerful confounding factor. In this sense, whereas most NOR task experiments are generally conducted in open field environments, it was recently proposed that using an L-maze with the objects placed at the ends of each arm reduces anxiety and increases reliable object exploration. Indeed, experience from the laboratory showed that *CB₁-KO* mice explore objects much more in a L-maze than in an open field, likely due to their anxious phenotype (Haller et al., 2002; Moreira and Wotjak, 2010). Thus, the L-maze approach was chosen in the present work (**Figure 31, page 56** and Material and Methods Section for more details).

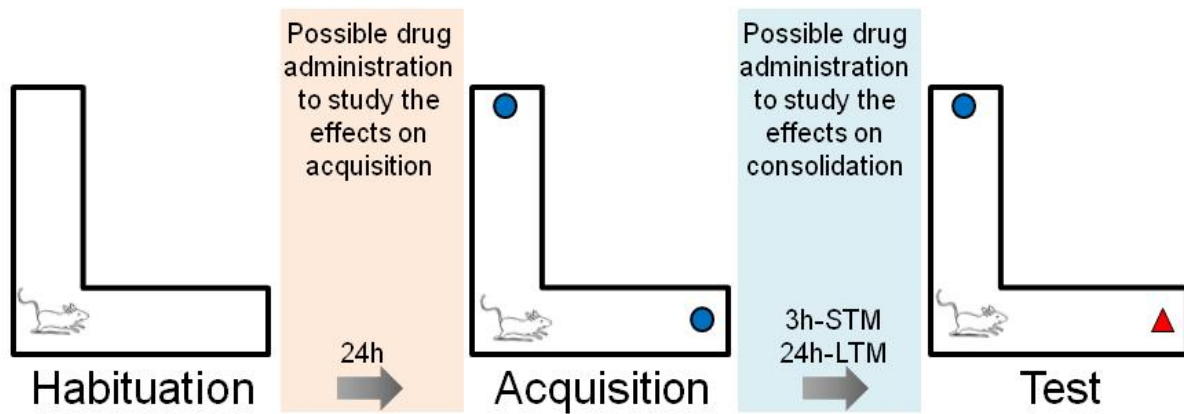


Figure 31 : Scheme of the Novel Object recognition (NOR) task in a L-maze.

The protocol consists in three different phases, habituation, acquisition and test, each phase lasts 9 minutes. Short-term and Long-term memory can be assessed depending on the retention time between acquisition and test session. Different memory phases can be studied (acquisition, consolidation and retrieval). The read-out is the exploration time of objects and the discrimination index is calculated during the test session ($DI = \frac{\text{Exploration novel object} - \text{exploration familiar object}}{\text{Total exploration of both objects}}$). Higher is the DI, better is the memory.

The NOR task has several technical advantages as compared to other behavioral tests for memory functions (Hebert-Chatelain et al., 2016; Puighermanal et al., 2012). First, as the acquisition happens in a single session, it allows to easily study the different phases of memory formation. Pre- or post-training pharmacological or pharmacogenetic manipulations [e.g. the Designer Receptor Exclusively Activated by Designer Drugs (DREADDs)] allow acute manipulation of the acquisition and consolidation phases, respectively. Furthermore, pharmacological manipulations during the pre-test session, allows the study of retrieval (Urban and Roth, 2015). Overall, the NOR task provides high reproducibility and low variability in the study of episodic memory formation.

III. Modulation of memory by the endocannabinoid system, NMDARs and astrocytes

A. ROLES OF THE ENDOCANNABINOID SYSTEM IN MEMORY

The presence of CB₁ receptors in key brain regions for memory functions such as the hippocampus and their involvement in synaptic plasticity support the role of the endocannabinoid system in the modulation of learning and memory (Busquets-Garcia et al., 2015).

Due to their very specific temporal and spatial constraints, the physiological actions of the endocannabinoid system in memory can be very different from the exogenous pharmacological activation of CB₁ receptors, lacking such spatio/temporal specificities.

Exogenous administration of CB₁ agonists has been shown to induce learning and memory impairments in both rodents and humans under different experimental conditions (Broyd et al., 2016). Interestingly, these effects have been shown to be mediated by the activation of CB₁ receptors in the CNS. Local hippocampal treatment with CB₁ receptor agonists has been shown to impair hippocampal memory whereas CB₁ receptor antagonists block these effects (Barna et al., 2007; Wise et al., 2009). The hippocampus contains high levels of the CB₁ receptor protein that is expressed at different levels in several cellular populations, such as glutamatergic pyramidal neurons, specific GABAergic inhibitory interneurons, astrocytes and others (Busquets-Garcia et al., 2015; Marsicano and Kuner, 2008). This differential expression accounts for specific effects of cannabinoids on memory. For instance, the disruptive effects of THC on NOR task have been shown to depend on activation of CB₁ receptors expressed on GABAergic interneurons (Puighermanal et al., 2009). As another example, acute THC administration has been shown to impair spatial working memory in mice through activation of CB₁ receptors located at hippocampal astrocytes (Han et al., 2012). Similarly, many studies combining genetic, pharmacological and electrophysiological techniques, have shown that pharmacological activation of CB₁ receptors in specific cell populations within the hippocampus can modulate synaptic plasticity and learning and memory (Busquets-Garcia et al., 2016).

Due to the complexity of expression of CB₁ receptors in different cell populations and even sub-cellular locations (e.g. pre- or post-synaptic, or plasma or mitochondrial membranes), the study of the physiological roles of the endocannabinoid system in the control of learning and memory require sophisticated tools to reach satisfying levels of detail. Generalized pharmacological blockade or global genetic deletion of CB₁ receptors provide the first indication of the endocannabinoid system involvement in specific memory tasks (Busquets-Garcia et al., 2015). However, the use of conditional KO mice lacking CB₁ receptors in selected brain cells revealed, in the last decade, that cell type-specific functions of the endocannabinoid system can be very different or even opposite. For example, freezing responses in fear conditioning memory protocols are enhanced (i.e. extinction of freezing is impairment) in global CB₁-KO mice and conditional mouse mutants lacking CB₁ in cortical glutamatergic neurons (Glu-CB₁-KO mice; (Kamprath et al., 2009; Marsicano et al., 2002; Metna-Laurent et al., 2012). However, deletion of the CB₁ receptor gene in GABAergic forebrain neurons (the large majority of CB₁ receptor protein in the brain) results in a drastic reduction of the freezing response in fear conditioning (Metna-Laurent et al., 2012). These results were interpreted as an indication of an opposite role of "glutamatergic" versus "GABAergic" CB₁ receptors in the control of fear responses. Independently of the primary behavioral mechanisms involved, however, these data underline the importance of cell type-specificity in analyzing the physiological impact of the endocannabinoid system in behavioral memory tasks (Busquets-Garcia et al., 2015).

As mentioned above, CB₁ receptors are not only expressed in different cell types, but they are also functionally located at different intracellular sites. For instance, they can be present at cellular plasma membranes, but also at mitochondrial membranes (mtCB₁). A recent study from our team showed that hippocampal mtCB₁ receptors are responsible for pharmacological cannabinoid-induced LTM impairments, by modulating cellular mitochondrial bioenergetics (Hebert-Chatelain et al., 2016). Interestingly, our published and unpublished studies showed that mtCB₁ receptors

are dispensable for physiological memory acquisition and expression in the NOR task (Hebert-Chatelain et al., 2016 and data not shown). Therefore, future studies will have to address the endogenous role of mtCB₁ receptors in memory functions.

In conclusion, the impact of exogenous or endogenous cannabinoids on learning and memory is under intense scrutiny. However, the mechanisms involved appear to be very complex and specific, implying that sophisticated tools are required to reach detailed levels of analysis. In this Thesis work, I aimed at dissecting the specific physiological role of astroglial ₁ receptors in NOR memory.

B. ROLES OF NMDARS IN MEMORY

NMDAR is the predominant molecular element in the control of synaptic plasticity and memory (Tsien, 2000). Indeed, the activation of post-synaptic NMDA receptors in most hippocampal pathways controls the induction of LTP (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995). LTP shares important properties with memory function and is considered to underlie the brain's ability to store information (Malenka, 1994; Miller and Mayford, 1999; Rison and Stanton, 1995). Moreover, overwhelming evidence indicates that genetic, pharmacological or functional manipulations of NMDA receptors impair or promote behavioral memory responses. In the following, I will present a brief summary of this evidence, with specific focus on recognition memory. However, for more detailed information concerning the numerous mechanistic implications of NMDARs in learning and memory, the reader is referred to important recent reviews on the subject.

i. Pharmacological blockade of NMDARs

The effects of NMDAR antagonists in rodents strongly suggest that a decreased in NMDAR functions can lead to memory and learning deficits. Indeed, NMDAR antagonists (competitive or non competitive) transiently impair spatial learning in rats (Morris, 1989; Morris et al., 1990) and object recognition memory (Puma et al., 1998). In certain conditions, memory deficits induced by NMDAR antagonists involve impairment in the acquisition or encoding of new information, rather than its retrieval from storage (McNamara and Skelton, 1993; Spangler et al., 1991; Walker and Gold, 1991) or an impairment in the consolidation from short-term to long-term memory (Kim et al., 1992). In non-human primates, ketamine, phencyclidine (PCP), and MK-801 (all NMDARs antagonists) have also been reported to induce impairments in learning and memory. Hippocampal NMDARs play a key role in many forms of memory as indicated by local infusions of antagonists (Buffalo et al., 1994; Thompson and Disterhoft, 1997; Thomson et al., 1985). These and other studies indicate that proper NMDAR functions are a *conditio sine qua non* for most experimentally observable forms of memory (Riedel et al., 2003).

ii. Genetic models

Studying transgenic and mutant mice has provided more evidence of the involvement of NMDARs in learning and memory. Mutant mice lacking the NMDAR subunit GluN2A have reduced hippocampal LTP and spatial learning (Sakimura et al., 1995). Also, transgenic mice lacking all subtypes of NMDA receptors in the CA1 region of the hippocampus show LTP impairments and deficits in spatial and non-spatial

learning (Shimizu et al., 2000; Tsien et al., 1996). On the other hand, genetic enhancement of NMDAR functions trigger better learning and memory performances. Thus, an interesting series of studies used the GluN2B transgenic (Tg) mouse line carrying overexpression of the GluN2B subunit in neurons of the forebrain and showed that mutant mice had better memory performances in various behavioral tasks (Philpot et al., 2001; Tang et al., 1999) In particular, the transgenic mice displayed much longer memory retention (up to 1 week versus 3 days) in a NOR task paradigm. These findings confirm and support the important role of the NMDA receptor in memory.

C. ROLES OF ASTROCYTES IN MEMORY

The astroglial modulation of neuronal plasticity has been shown to play a role in learning and memory (Adamsky and Goshen, 2018; Adamsky et al., 2018; Araque et al., 2014a; Oliveira da Cruz et al., 2016) and an increasing number of studies link the activity of the tripartite synapse with behavior.

i. Gliotransmission

One of the first pieces of evidence of a direct control of gliotransmission on synaptic plasticity was provided by Pascual et al., in 2005. These authors showed that following tetanic stimulation of CA1 afferents, astrocytes release ATP that can be metabolized into adenosine and act at the pre-synaptic adenosine A1 receptors to depress excitatory transmission (Pascual et al., 2005). Using the tetracycline-controlled transcriptional activation (tet on/off system) in mice, the same group genetically inhibited SNARE-dependent release of transmitters from astrocytes in dnSNARE mice (Halassa et al., 2009). As a consequence, dnSNARE mutant mice were resistant to both recognition memory deficits and impairment of hippocampal LTP induced by sleep deprivation. By coupling this genetic approach with pharmacological targeting of A1 receptors, the authors proposed that astrocytic ATP and adenosine A1 receptor activity contribute to the effects of sleep deprivation on memory (Florian et al., 2011).

As we described earlier, astrocytes directly participate to NMDA receptor-mediated LTP by releasing D-serine. Interestingly, inhibition of D-serine synthesis by knocking-out the serine racemase in mice leads to recognition memory impairments, whereas exogenous systemic administration of D-serine in mice facilitates memory performances (Bado et al., 2011; Labrie et al., 2009).

Other non-vesicular mechanisms for the release of gliotransmitters were suggested to play a role in the modulation of learning and memory (Bazargani and Attwell, 2016), such as the connexin hemichannels (Cheung et al., 2014). Indeed, when connexin 43 hemichannels are blocked during memory consolidation, long-term memory is impaired (Chever et al., 2014b). This memory loss is recovered after co-infusion of a mixture of molecules known to be released from astrocytes, including glutamate, glutamine, lactate, D-serine, glycine, and ATP (Stehberg et al., 2012).

ii. Metabolic functions of astrocytes

More recently, several studies suggested that astrocytes could control learning and memory at a different levels, such as metabolic support of neuronal functions. This was shown in chicks (Gibbs et al., 2006; Hertz and Gibbs, 2009) and rodents (Newman et al., 2011; Suzuki et al., 2011; Tadi et al., 2015). Using a passive avoidance task, Suzuki et al. (2011), demonstrated that pharmacological inhibition of glycogenolysis (the breakdown of glycogen into glucose typical of astrocytes) during the initial phase of memory storage prevents recall of the learned task. Moreover, extracellular lactate levels increase in the hippocampus immediately after learning. When glycogenolysis is blocked, administration of exogenous lactate rescues the memory impairment. The inhibition of the expression of astroglial and neuronal lactate transporters disrupts memory retention. However, only the blockade of neuronal transporters is rescued by lactate administration, suggesting that astrocyte–neuron lactate transport is required for long-term memory consolidation (Suzuki et al., 2011).

iii. New tool to study the role of astrocytes in memory

Transgenic mice expressing DREADDs in astrocytes have been in use for several years, and more recent studies employed viruses to achieve DREADD expression in astrocytes. In particular astroglial expression of the Gq-coupled hM3Dq DREADD in the hippocampus led to altered neuronal activity and memory performance (Adamsky et al., 2018; Bonder and McCarthy, 2014; Li et al., 2013; Scofield et al., 2015). Using transgenic mice with conditional adult astroglial expression of the Gs-coupled DREADD Rs1, Orr et al. (2015) showed that activation of astroglial Gs signaling during Morris water maze training did not impair memory acquisition, but it resulted in impaired recall 2 days later. Furthermore, when astrocytes were activated 1 day after acquisition (but not during training) recall was also impaired due to disturbed consolidation (Orr et al., 2015).

Finally, Martin-Fernandez et al. (2017) expressed the Gq-coupled hM3Dq DREADD selectively in astrocytes of the central amygdala (CeM) and exposed mice to a cued fear conditioning protocol (Martin-Fernandez et al., 2017). The injection of CNO during testing decreased the freezing response as compared to saline-injected animals, suggesting that the selective activation of astrocytes in the CeM is involved in the extinction of specific fear behaviors.

iv. Astroglial CB₁ receptors

Astrocytes were found to play an important role in spatial working memory impairment induced by exogenous cannabinoids. After acute exposure to exogenous cannabinoids, GFAP-CB₁-KO mice displayed no spatial working memory impairment and no cannabinoid-induced *in vivo* LTD of hippocampal synaptic transmission (CB-LTD). Also, blockade of NMDARs and synaptic trafficking of AMPARs abolished the effects of exogenous cannabinoids on spatial working memory and LTD, suggesting that the impairment of working memory is due to activation of astrocytic CB₁R and release of glutamate (Han et al., 2012). However, so far, it is the only study the direct role of astroglial CB₁ receptors in memory processes and further study should clarify its functions in different memory tasks.

The studies described in this section show the vast promises in researching the role of astrocytes in learning and memory but are limited due to the technical difficulties to specifically modulate astroglial activity without also affecting neuronal activity. Indeed, astrocytes share many of their receptors with neurons, making it difficult to alter their activity without influencing neuronal functions (Araque et al., 2014a; Bazargani and Attwell, 2016; Fiacco et al., 2007). Thus, the development of new tools and the use of genetic models show vast promises for the future of the study of astrocytes in memory and physiological functions.

RESEARCH GOALS

RESEARCH GOALS

Long-term synaptic plasticity is considered as a cellular substrate for learning and memory. The most studied long-term plasticity is the NMDAR-dependent LTP. In the hippocampus, at the CA3-CA1 synapses, we know that the astrocyte is a key partner for the induction of LTP by releasing D-serine, an essential co-agonist to activate synaptic NMDARs. Thus, it seems essential to decipher the mechanisms involved in the bio-availability of D-serine to better understand the learning and memory processes. The release of astroglial D-serine to activate synaptic NMDARs activity depends on intracellular astroglial Ca^{2+} concentration increase (Henneberger et al., 2010). However, the identity of the astroglial receptors controlling D-serine release and consequently NMDAR activity remains unknown. Recently, a functional interplay across CB_1 receptors, astrocytes and a specific form of long-term synaptic plasticity was suggested, possibly involving D-serine (Andrade-Talavera et al., 2016). Interestingly, in the hippocampus, exogenous stimulation of astroglial CB_1 receptors triggers intracellular Ca^{2+} increase coupled to gliotransmission, which leads to modulation of synaptic transmission, long-term synaptic plasticity and behavioral effects (Han et al., 2012). Moreover, in adult mice, AEA application on neocortex slices of adult mice triggers an increase of D-serine release (Rasooli-Nejad et al., 2014). However, whereas the roles of astroglial CB_1 receptors in the effects of exogenous administration of cannabinoid drugs started to be investigated, nothing was known before this work concerning the physiological role of this specific subpopulation of CB_1 receptors.

Thus, **this Thesis work mainly aims at understanding the physiological functions of astroglial CB_1 receptors in memory functions.**

For the past years, scientific studies suggested astroglial CB_1 receptors could be a good candidate in the modulation of D-serine release, NMDAR-dependent LTP and associated long-term memory.

Therefore, this present Thesis aims at answering 4 specific questions:

1. Does the deletion of hippocampal astroglial CB_1 receptors impair long-term memory and NMDAR-dependent LTP?
2. Does astroglial CB_1 receptor control D-serine levels at the synapse?
3. Does astroglial CB_1 receptor control NMDAR-dependent LTP through D-serine?
4. Are hippocampal astroglial CB_1 receptors necessary for long-term memory through D-serine?

To address these questions, we used a combination of genetic (conditional mutagenesis of CB_1 and virus delivery in mice aiming at specific CB_1 deletion in astrocytes) coupled with behavioral, pharmacological and *in vitro* and *in vivo* electrophysiological approaches to identify the impact of astroglial CB_1 receptors on physiological memory processes and to dissect the specific mechanisms involved.

RESULTS

Astroglial CB₁ Receptors Determine Synaptic D-Serine Availability to Enable Recognition Memory

Highlights

- Astrocytes are important for long-term object recognition memory
- Astroglial CB₁ receptors are coupled to D-serine availability at synapses
- Appropriate D-serine levels are required for NMDAR activity and LTP induction

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In Brief

Robin et al. show that astroglial CB₁ receptors in the hippocampus regulate D-serine supply to NMDA receptors, a process necessary for LTP induction and object recognition memory.

Astroglial CB₁ Receptors Determine Synaptic D-Serine Availability to Enable Recognition Memory

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SUMMARY

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 (NMDARs), but the physiological factors regulating its impact on memory processes are scantily known. We show that astroglial CB₁ receptors are key determinants of object recognition memory by determining the availability of D-serine at hippocampal synapses. Mutant mice lacking CB₁ receptors from astroglial cells (GFAP-CB₁-KO) displayed impaired object recognition memory and decreased *in vivo* and *in vitro* long-term potentiation (LTP) at CA3-CA1 hippocampal synapses. Activation of CB₁ receptors increased intracellular astroglial Ca²⁺ levels and extracellular levels of D-serine in hippocampal slices. Accordingly, GFAP-CB₁-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-CB₁-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 (i.e., endocannabinoids, eCB), and the enzymatic machinery responsible for their synthesis and degradation (Busquets-Garcia et al., 2018; Piomelli, 2003). The presence of type-1 cannabinoid receptors (CB₁) and the activity-dependent mobilization of endocannabinoids in 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 Lafenêtre, 2009). Moreover, brain CB₁ receptors are expressed in different neuronal types, including inhibitory gamma-aminobutyric acid (GABA)ergic and excitatory glutamatergic neurons, where their stimulation negatively regulates the release of neurotransmitters (Kano et al., 2009).

CB₁ receptors are also expressed in glial cells, particularly astrocytes (Andrade-Talavera et al., 2016; Han et al., 2012; Min and Nevean, 2012; Navarrete and Araque, 2008; Rasooli-Nejad et al., 2014). For more than a century, astrocytes were thought 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 peri-synaptic astroglial processes surrounding pre- and postsynaptic neuronal elements form the so-called “tripartite synapse,” where astrocytes actively contribute to information processing (Araque et al., 2014; Perea et al., 2009).

In vivo and *in vitro* studies showed that astroglial CB₁ receptor signaling indirectly modulates 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 disruptive effect of exogenous cannabinoids on short-term spatial working memory is mediated by astroglial CB₁ receptors through an N-methyl-D-aspartate receptor (NMDAR)-dependent mechanism in the hippocampus (Han et al., 2012). Yet, the role of astroglial CB₁ receptors in physiological long-term memory processes 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; Panatier et al., 2006; Papouin et al., 2012, 2017b; Shigetomi et al., 2013; Sultan et al., 2015). Although the direct source of the amino acid is still under debate (Araque et al., 2014; Papouin et al., 2017c; Wolosker et al., 2016), there is convergent consensus that its supply to synapses requires Ca²⁺-dependent astrocyte activity (Araque et al., 2014; Papouin et al., 2017c; Wolosker et al., 2016). However, whether astroglial CB₁ receptors control the synaptic availability of D-serine during memory processing is not known.

Using genetic, behavioral, electrophysiological, imaging, and biochemical experimental approaches, in this study we asked whether the physiological activity of astroglial CB₁ 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 CB₁ 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 CB₁ receptors contribute to the time- and space-specific synaptic actions of astrocytes to promote memory formation.

RESULTS

Deletion of Hippocampal Astroglial CB₁ Receptors Impairs Object Recognition Memory and *In Vivo* NMDAR-Dependent LTP

To study the physiological role of astroglial CB₁ receptors in memory, we tested conditional mutant mice lacking CB₁ receptors in glial fibrillary acidic protein (GFAP)-positive cells (GFAP-CB₁-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., 2009, 2013). GFAP-CB₁-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 NMDAR-dependent transmission is involved in many forms of memory (Kandel et al., 2002; Puighermanal et al., 2009; Warburton et al., 2013), yet the involvement of hippocampal NMDARs on NOR memory is still under debate, as it seems to depend on specific experimental conditions (Balderas et al., 2015; Warburton and Brown, 2015). To clarify this issue, we set to investigate where these receptors are required for NOR memory

formation in our behavioral paradigm. Intra-hippocampal administration of the NMDARs antagonist D-AP5 (15 μg/side; Figure S1C) fully abolished memory performance in wild-type (WT) mice when injected immediately after acquisition (Figure 1B; see also Figure S1D), but not 6 hr later (Figures S1F–S1H), with no alteration in total exploration time (Figure S1E). Thus, consolidation of long-term object recognition memory in the NOR task specifically requires astroglial CB₁ receptors and hippocampal NMDARs signaling.

Activity-dependent plastic changes of synaptic strength, such as NMDAR-dependent long-term potentiation (LTP), are considered cellular correlates of memory formation (Kandel et al., 2002; Whitlock et al., 2006). To study astroglial CB₁ receptor involvement in LTP, we recorded *in vivo* evoked field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA3-CA1 pathway of anesthetized WT and mutant mice. High-frequency stimulation (HFS) induced LTP in C57BL/6N mice (Figures 1C and 1D). The systemic administration of the NMDAR antagonist MK-801 (3 mg/kg, i.p.), which did not alter basal evoked fEPSPs (Figures S1I–S1K), fully blocked the induction of LTP (Figures 1C and 1D; Figures S1I–S1K), confirming its NMDAR dependency. Notably, this form of plasticity was abolished in GFAP-CB₁-KO mice as compared to their WT littermates (Figures 1E and 1F), showing that CB₁ receptors expressed in GFAP-positive cells are necessary for *in vivo* hippocampal NMDAR-dependent LTP induction. Altogether, these data demonstrate that astroglial CB₁ receptors are essential for hippocampal NMDAR-dependent object recognition memory and LTP.

Activation of CB₁ Receptors Increases Astroglial Ca²⁺ Levels and Extracellular D-Serine

Increase of astroglial intracellular Ca²⁺ 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 CB₁ receptors generate Ca²⁺ signals in astrocytes (Araque et al., 2014; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016), the impaired object recognition memory and synaptic plasticity in GFAP-CB₁-KO mice might result from alterations of astroglial Ca²⁺ regulation of specific hippocampal gliotransmitters.

First, we tested whether the CB₁ receptor-dependent modulation of intracellular Ca²⁺ levels (Gómez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010) depends on direct activation of astroglial CB₁ receptors. Local pressure application of the CB₁ receptor agonist WIN55,212-2 (WIN) induced a reliable increase of Ca²⁺ levels in somas and principal processes of hippocampal astrocytes in slices from GFAP-CB₁-WT mice (Figures 2A–2E). As expected (Gómez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010), this effect was fully blocked by the CB₁ receptor antagonist AM251 (2 μM; Figures 2B–2E). Notably, WIN had no effect in slices from GFAP-CB₁-KO littermates (Figures 2B–2E), clearly indicating the direct impact of astroglial CB₁ receptor activation on intracellular Ca²⁺ levels.

Via Ca²⁺-dependent mechanisms, astrocytes can promote the synaptic release of several signaling molecules known as gliotransmitters (Araque et al., 2014). One of them is D-serine, which

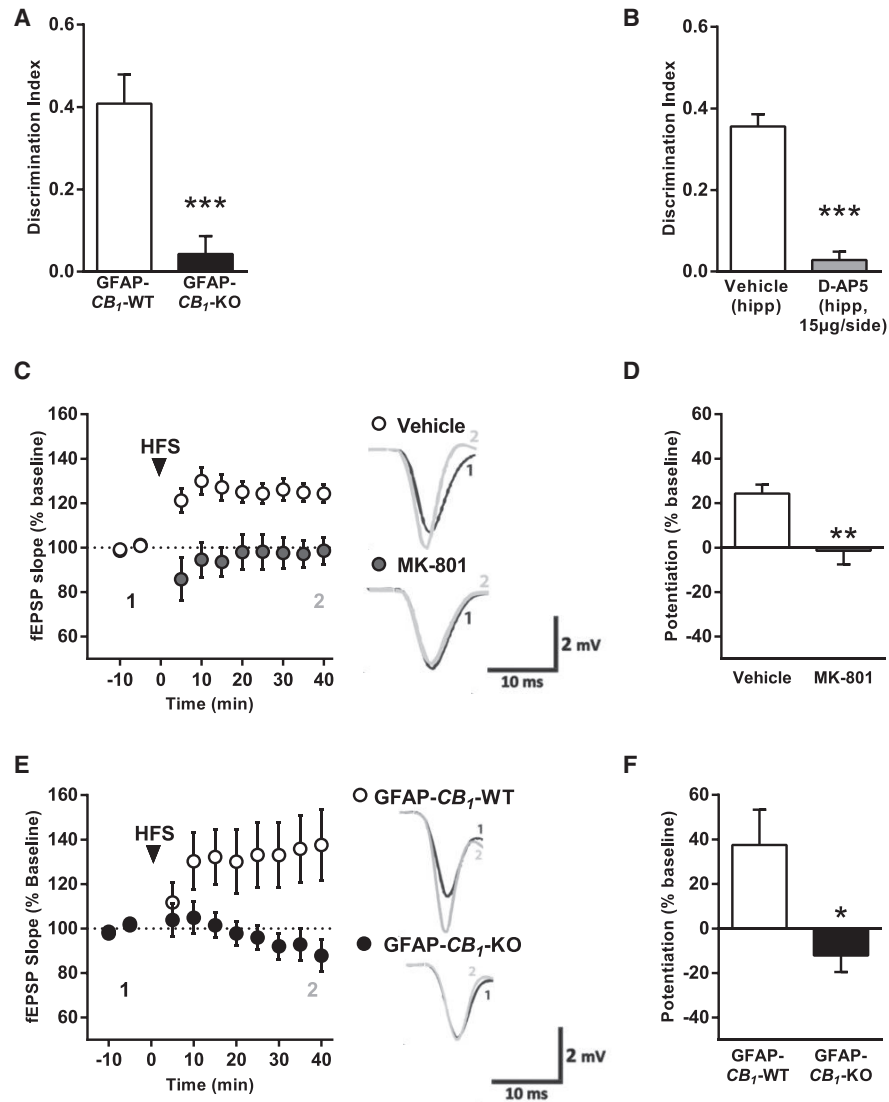


Figure 1. Hippocampal Astroglial CB₁ Receptors Are Necessary for NMDAR-Dependent Object Recognition Memory and *In Vivo* LTP

(A) Memory performance of GFAP-CB₁-WT mice (n = 10) and GFAP-CB₁-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 and 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 in (C), 40 min after HFS.

(E and F) *In vivo* LTP is absent in GFAP-CB₁-KO mice. (E) Summary plots of normalized fEPSPs in GFAP-CB₁-WT (n = 9) and GFAP-CB₁-KO (n = 6) littermates. (F) Bar histograms of normalized fEPSPs from experiment in (E), 40 min after HFS. Traces on the right side of the summary plots in (C) and (E) represent 150 superimposed evoked fEPSPs before (1, black) and after (2, gray) HFS.

Data, mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S1 and Table S1.

plays a key role in NMDAR signaling (Araque et al., 2014). Therefore, we asked whether activation of CB₁ receptors might modulate the release of this amino acid. Application of WIN (5 µM) to hippocampal slices did not alter the tissue levels of several amino acids (Figures S2A–S2D). However, the same treatment slightly but specifically increased the extracellular levels of D-serine (Figures 2F–2I), indicating that activation of astroglial CB₁ receptors can control the release of this signaling amino

acid, a process that depends on intracellular Ca²⁺ signaling (Bohmbach et al., 2018; Henneberger et al., 2010).

Astroglial CB₁ Receptor-Mediated D-Serine Supply Is Required for Hippocampal LTP

D-serine is the co-agonist of hippocampal synaptic NMDARs and its presence is necessary for LTP induction (Bohmbach et al., 2018; Henneberger et al., 2010; Papouin et al., 2012).

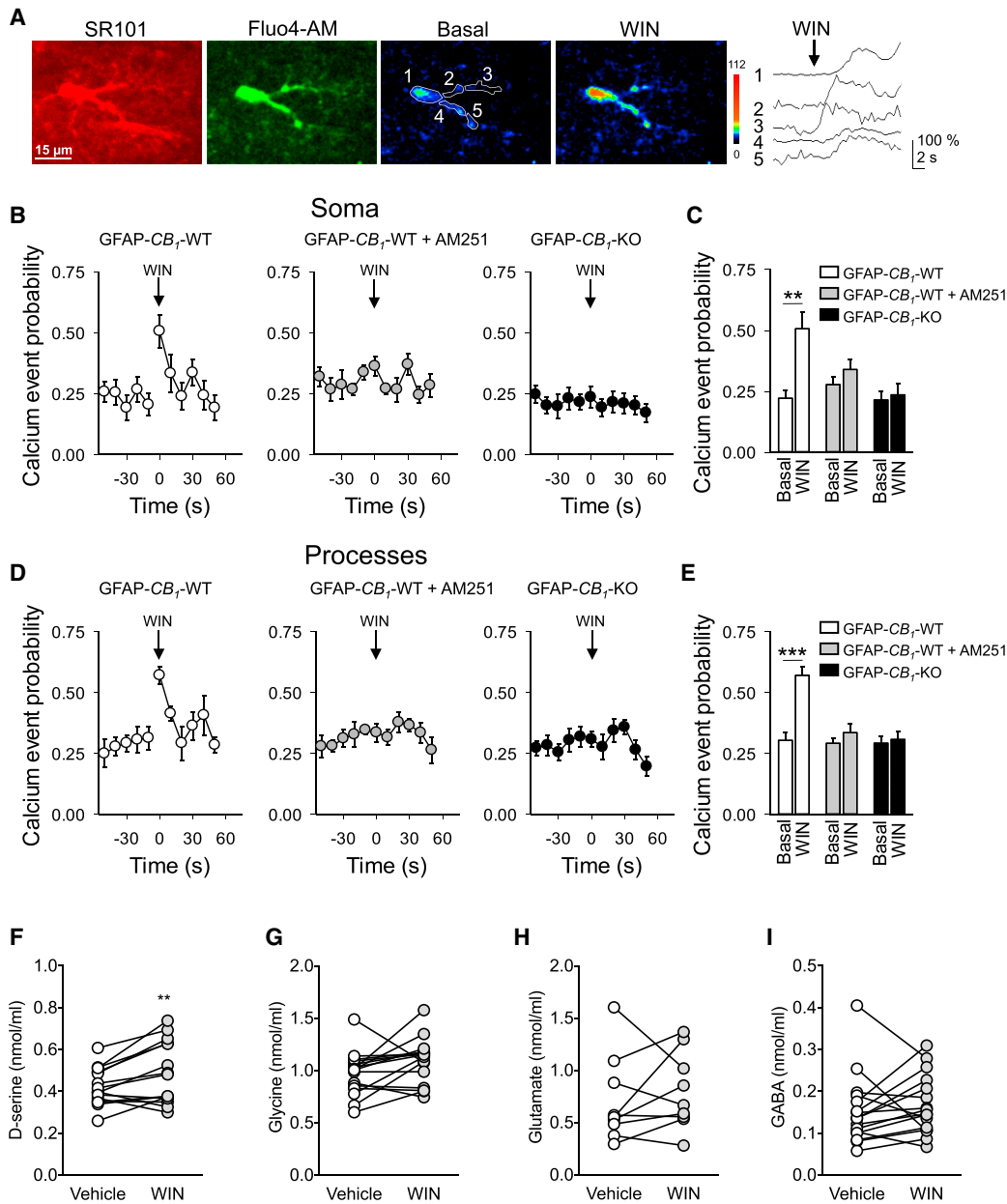


Figure 2. Activation of Astroglial CB₁ Receptors Enhances Intracellular Ca²⁺ Levels in Astrocytes and Extracellular D-Serine

(A) Representative image of a hippocampal astrocyte stained with SR101 and Fluo4 and pseudo-color images representing fluorescence intensities before and after WIN 515,212-2 (WIN) application, with the correspondent Ca²⁺ traces (numbers refer to different subcellular locations on the astrocyte).

(B) Somatic calcium event probability before and after WIN (at time = 0) in GFAP-*CB₁*-WT in control conditions (white), in the presence of AM251 (2 μ M; gray), and in GFAP-*CB₁*-KO mice (black).

(C) Somatic calcium event probability before and after WIN in GFAP-*CB₁*-WT in control conditions (white; n = 9 slices and 79 somas), in the presence of AM251 (gray; n = 12 slices and 159 somas), and in GFAP-*CB₁*-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-*CB₁*-WT in control conditions (white), in the presence of AM251 (2 μ M; gray), and in GFAP-*CB₁*-KO mice (black).

(E) Calcium event probability in the processes before and after WIN in GFAP-*CB₁*-WT in control conditions (white; n = 8 slices and 171 processes), in the presence of AM251 (gray; n = 8 slices and 140 processes), in GFAP-*CB₁*-KO mice (black; n = 10 slices and 189 processes).

(F–I) Determination of D-serine (F), glycine (G), glutamate (H), and GABA (I) as measured by capillary electrophoresis in extracellular solutions of acute mouse hippocampal slices treated with vehicle or WIN.

Data, mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

See also [Figure S2](#) and [Table S1](#).

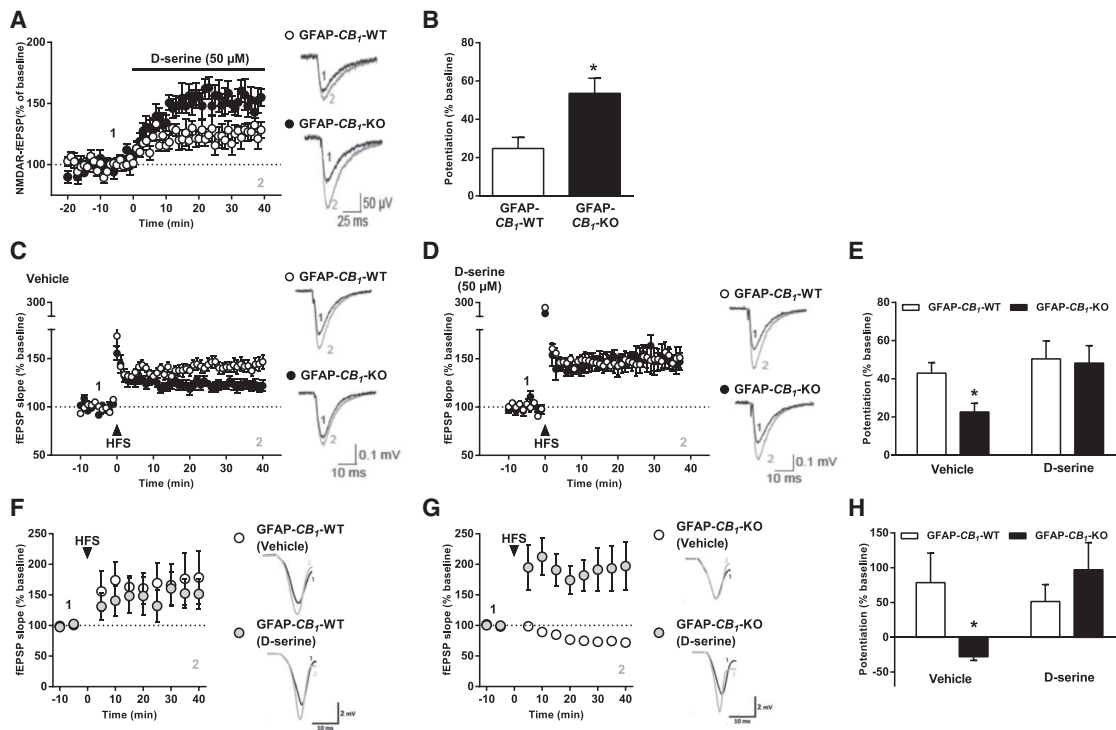


Figure 3. Astroglial CB₁ Receptors Control LTP Induction through D-Serine

(A) Summary plots showing the effect of D-serine application on NMDAR co-agonist binding site occupancy in slices from GFAP-CB₁-WT mice and GFAP-CB₁-KO littermates. Traces represent 60 superimposed NMDAR-fEPSPs before (1, black) and after (2, gray) D-serine application. (B) Bar histograms of normalized NMDAR-fEPSPs from experiment in (A) measured 20–40 min after D-serine application. (C) *In vitro* LTP is impaired in GFAP-CB₁-KO mice. Summary plots of normalized fEPSPs in slices from GFAP-CB₁-WT (n = 16) and GFAP-CB₁-KO (n = 12) mice before (1) and after (2) high-frequency stimulation (HFS). (D) D-serine application rescues LTP in slices from GFAP-CB₁-KO mice. Summary plots of fEPSPs showing the effect of D-serine (50 μM) on LTP in slices from GFAP-CB₁-WT (n = 8) and GFAP-CB₁-KO (n = 7) mice. In (C) and (D), traces represent 30 superimposed successive fEPSPs before (1, black) and after (2, gray) the HFS stimulation (arrow). (E) Bar histograms of fEPSPs from experiments in (C) and (D) measured 30–40 min after HFS. (F and G) Summary plots of normalized fEPSPs in GFAP-CB₁-WT (F) and GFAP-CB₁-KO (G) treated with vehicle (GFAP-CB₁-WT, n = 4; GFAP-CB₁-KO, n = 7) or D-serine (GFAP-CB₁-WT, n = 6; GFAP-CB₁-KO, n = 5). In (F) and (G), traces represent 150 superimposed successive fEPSPs before (1, black) and after (2, gray) the HFS stimulation (arrow). (H) Bar histograms of normalized fEPSPs from experiment in (F) and (G), 40 min after HFS.

Data, mean ± SEM. *p < 0.05.

See also [Figure S3](#) and [Table S1](#).

Thus, astroglial CB₁ receptors might control the activity of NMDARs and hippocampal LTP by regulating the synaptic levels of D-serine. Measurements of bulk extracellular amino acids as performed in [Figures 2F–2I](#) do not specifically address whether D-serine levels impact synaptic function. A direct way to evaluate the levels and functions of synaptic D-serine is to perform electrophysiological measurements to assess the occupancy of the NMDAR co-agonist binding site at CA3-CA1 synapses ([Papouin et al., 2012](#)). Thus, we measured the impact of exogenous applications of the D-serine on NMDAR-mediated fEPSPs at CA3-CA1 synapses in acute hippocampal slices ([Papouin et al., 2012](#)). Bath application of D-serine (50 μM) increased NMDAR-dependent synaptic responses in both GFAP-CB₁-WT and GFAP-CB₁-KO mice ([Figures 3A and 3B](#)). Strikingly, the effect of D-serine was twice more pronounced in the absence of astroglial CB₁ receptors ([Figures 3A and 3B](#)), indicating that these receptors are necessary to maintain appropriate concen-

trations of D-serine within the synaptic cleft and consequently ensuring a proper level of occupancy of the NMDAR co-agonist binding site.

Next, we asked whether astroglial CB₁ receptor-dependent release of D-serine controls synaptic plasticity by regulating NMDAR activity. First, *in vitro* electrophysiological recordings of fEPSPs at CA3-CA1 synapses in hippocampal slices revealed that GFAP-CB₁-WT and GFAP-CB₁-KO have comparable input-output relationships ([Figure S3A](#)), indicating that the deletion of astroglial CB₁ receptors did not alter basal glutamatergic synaptic transmission. HFS is known to induce endocannabinoid mobilization through the activation of mGluR1/5 receptors, eventually leading to long-term depression of inhibitory transmission (I-LTD) in the hippocampus ([Castillo et al., 2012](#); [Chevalleyre and Castillo, 2003](#)). Therefore, we asked whether mGluR1/5 receptors could be involved in HFS-induced LTP. Application of the mGluR1/5 antagonists LY367385 and MTEP,

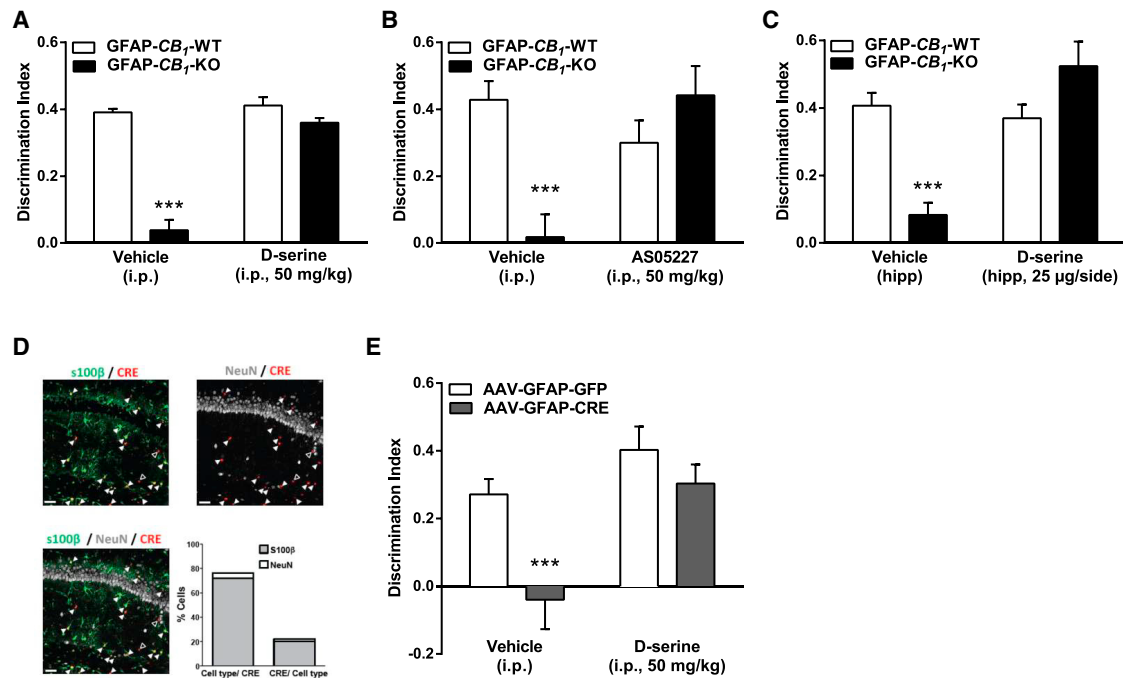


Figure 4. Hippocampal Astroglial CB₁ Receptors Are Necessary for Object Recognition Memory through D-Serine

(A) Memory performance of GFAP-CB₁-WT and GFAP-CB₁-KO mice injected with vehicle (n = 5 both groups) or D-serine (50 mg/kg; i.p.; GFAP-CB₁-WT, n = 4; GFAP-CB₁-KO, n = 5).

(B) Memory performance of GFAP-CB₁-WT and GFAP-CB₁-KO mice injected with vehicle (GFAP-CB₁-WT, n = 8; GFAP-CB₁-KO, n = 9) or AS052278 (50 mg/kg; i.p.; GFAP-CB₁-WT, n = 9; GFAP-CB₁-KO, n = 8).

(C) Memory performance of GFAP-CB₁-WT and GFAP-CB₁-KO mice intra-hippocampally injected with vehicle (GFAP-CB₁-WT, n = 5; GFAP-CB₁-KO, n = 7) or D-serine (25 µg/side; GFAP-CB₁-WT, n = 5; GFAP-CB₁-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, 50 µm. Bottom right: quantification of co-expression indicating the percentage of neurons (NeuN-positive) and astrocytes (s100β 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 CB₁-floxed mice intra-hippocampally injected with either an AAV-GFAP-GFP or an 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).

Data, mean ± SEM. ***p < 0.001.

See also Figure S4 and Table S1.

respectively, did not alter LTP (Figures S3B and S3C), suggesting that mGluR1/5 receptors are not involved in this process. Similarly to *in vivo* electrophysiological results, HFS-induced LTP was significantly reduced in GFAP-CB₁-KO mice as compared to GFAP-CB₁-WT (Figure 3C). Whereas the exogenous application of D-serine (50 µM) had no effect in slices from GFAP-CB₁-WT mice, it fully rescued *in vitro* LTP in GFAP-CB₁-KO littermates (Figures 3D and 3E). Importantly, the lack of *in vivo* LTP observed in GFAP-CB₁-KO was fully restored by the systemic administration of D-serine (50 mg/kg, i.p.; Figures 3F–3H).

Considering that activation of astroglial CB₁ receptors increases Ca²⁺ in astrocytes, we asked whether this subpopulation of cannabinoid receptors is involved in the HFS-induced regulation of astroglial Ca²⁺ dynamics (Perea and Araque, 2005; Porter and McCarthy, 1996; Sherwood et al., 2017). While the Ca²⁺ activity evoked in both soma and large processes of astrocytes during the HFS was the same, GFAP-CB₁-KO astro-

cytes displayed a reduction in the Ca²⁺ event probability after the HFS as compared to WT littermates (Figures S3D–S3J). Altogether, these results show that astroglial CB₁ receptors regulate Ca²⁺ dynamics in astrocytes and determine the synaptic levels of the NMDAR co-agonist D-serine necessary for NMDAR-dependent *in vitro* and *in vivo* LTP.

Astroglial CB₁ Receptors Determine NOR Memory via D-Serine

If, as shown above, astroglial CB₁ 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-CB₁-KO mice (Figure 4A; see also Figures S4B and S4C). This effect of D-serine in GFAP-CB₁-KO mice was not present when the injection occurred 1 hr after acquisition or immediately before test

(Figures S4D and S4E), 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-amino-acid oxidase AS057278 (50 mg/kg, i.p.), which increases endogenous D-serine levels *in vivo* (Adage et al., 2008), also rescued the phenotype of GFAP-*CB₁*-KO mice (Figure 4B; see also Figures S4G and S4H). Moreover, post-acquisition (i.e., after training phase) intra-hippocampal injections of D-serine (sub-effective dose of 25 μg/side; Figure S4I) also restored NOR memory performance in GFAP-*CB₁*-KO mice (Figure 4C; see also Figures S4J and S4K). This suggests that the hippocampus is the brain region where astroglial CB₁ receptors control NMDAR-dependent memory formation via D-serine signaling. GFAP-*CB₁*-KO mice, however, carry a deletion of the *CB₁* 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 CB₁ receptors elsewhere. To specifically delete the *CB₁* 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 “floxod” *CB₁* receptor gene (Marsicano et al., 2003) (Figure 4D). Mice injected with the CRE recombinase were impaired in NOR memory performance (Figure 4E; see also Figures S4L and S4M), and notably, the systemic injection of D-serine (50 mg/kg, i.p.) fully reversed this phenotype (Figure 1E; see also Figures S4L and S4M). Thus, hippocampal astroglial CB₁ receptors are required for NOR memory performance via the control of D-serine signaling during the initial phase 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 Ca²⁺-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 CB₁ receptors, astroglial control of NMDAR activity via the gliotransmitter D-serine, and synaptic plasticity, these data provide an unforeseen physiological mechanism underlying memory formation.

By showing that astroglial CB₁ receptors play a key role in the maintenance of the basal levels of D-serine in the synaptic cleft and thus in the control of NMDAR activity, these data shed light onto the pathway underpinning D-serine availability at synapses. Interestingly, it has been recently demonstrated that the amount of D-serine available during wakefulness depends on the activity of cholinergic fibers from the medial septum (Papouin et al., 2017b). Thus, synaptic D-serine levels are under the control of at least two sets of astroglial receptors, namely CB₁ (present data) and α7-nicotinic acetylcholine receptors (Papouin et al., 2017b).

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.,

2017a). 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, it is tempting to speculate that astroglial CB₁ 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 CB₁-dependent regulation of D-serine supply is a major mechanism determining how much D-serine each astrocyte contributes to NMDARs as a function of neuronal activity within its territory.

Astroglial CB₁ receptors have so far been described to impact synaptic plasticity in different ways. For instance, their activation by exogenous cannabinoids can promote NMDAR-dependent hippocampal LTD (Han et al., 2012), whereas their endogenous stimulation can, depending on experimental conditions, lead to heterosynaptic potentiation in the hippocampus, amygdala, and striatum (Martín et al., 2015; Martín-Fernández et al., 2017; Navarrete and Araque, 2008, 2010); spike-timing depression in the neocortex (Min and Nevian, 2012); or hippocampal LTP (present results). The conditions through which the activation of astroglial CB₁ receptors might lead to different synaptic effects are currently not known (Araque et al., 2017; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). Future studies will surely reveal novel functions of astroglial CB₁ receptors and will hopefully determine the physiological conditions and the cellular mechanisms leading to different forms of synaptic plasticity. In this context, the present data extend the value of astroglial CB₁ receptors to the processing of object recognition memory through the regulation of D-serine, a key astrocyte-dependent modulator of synaptic functions.

The direct release of D-serine by astrocytes has recently been 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; Papouin et al., 2017b, 2017c; Sherwood et al., 2017; Wolosker et al., 2016). Activation of hippocampal CB₁ receptors by the agonist WIN induces a slight but significant increase of extracellular D-serine levels, which is specific among different amino acids. No clear evidence is currently present to explain the relative low amplitude of this pharmacological effect. However, it is possible that bulk measurement of amino acids lacks the power to detect specific changes at synaptic level. Unfortunately, it is currently technically impossible to obtain synaptic extracellular samples to directly measure amino acids in these tiny volumes. For this reason, we implemented another direct and reliable measure of synaptic D-serine levels by assessing the occupancy of synaptic NMDAR co-agonist sites (Henneberger et al., 2010; Papouin et al., 2012). The results clearly show that the occupancy of synaptic NMDAR co-agonist sites by D-serine is strongly reduced (more than 50%) in GFAP-*CB₁*-KO mice. HFS-induced LTP is fully abolished in GFAP-*CB₁*-KO mice *in vivo* but only partially reduced in *ex vivo* hippocampal slices. These slight discrepancies are likely due to uncontrollable factors that are necessarily different between

in vivo and *ex vivo* experimental conditions (Andersen, 2007; Windels, 2006), such as, for instance, the inevitable disruption of astroglial networks during slicing procedures or others. Importantly, however, the exogenous application of D-serine at the same doses, respectively, restoring learning *in vivo* and revealing the decrease of NMDAR occupancy in slices rescues LTP in both experimental settings. Thus, independently of their direct source, synaptic D-serine levels are under the control of CB₁ receptors specifically expressed in astrocytes, whose activation increases astroglial Ca²⁺ 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 CB₁ receptors does not reliably reflect the highly temporally- and spatially-specific physiological functions of the endocannabinoid system (Busquets-Garcia et al., 2018). 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 CB₁ 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 CB₁ receptors, because it is known that CB₁ 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, 2018), and this is particularly true between neurons and astroglial cells (Busquets-Garcia et al., 2018; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). Indeed, global pharmacological activation, blockade, and genetic deletion of CB₁ receptors are not able to catch subtle but important effects of endocannabinoid signaling. For instance, recent data show that deletion of the CB₁ gene in hippocampal GABAergic or glutamatergic neurons induces decreased and increased *in vitro* LTP, respectively, as compared to WT mice (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 CB₁ receptors. Thus, the present results determine an unforeseen link between endogenous activation of astroglial CB₁ 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 CB₁ gene in our study is induced in adult mice by tamoxifen treatment of GFAP-CB₁-KO mice or local injection of AAV-Cre under the control of a GFAP promoter into the hippocampus of CB₁-flox mice. These procedures occur a few weeks before testing, excluding potential compensatory confounding events during pre- and post-natal development. Moreover, the phenotypes of GFAP-CB₁-KO mice in NOR and LTP are rescued by increasing D-serine-dependent NMDAR signaling at the moment of memory acquisition/early consolidation or electrophysiological analysis. In particular, the behavioral effects of D-serine were present when it was administered systemically or locally immediately after task acquisition, but not 1 hr later or at recall. Thus, considering pharmacokinetic studies showing that the extracellular levels of D-serine are increased after exog-

enous administration for about 100 min in the brain (Fukushima et al., 2004), it is reasonable to conclude that the control of synaptic NMDAR plasticity and of NOR memory by astroglial CB₁ 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 CB₁ 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, CB₁ 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 CB₁ receptors expressed in GFAP-positive cells in NOR appears to be limited to the early phases of memory processing, namely early consolidation. Indeed, whereas the injection of D-serine immediately after memory acquisition fully rescues the phenotype of GFAP-CB₁-KO mice in NOR, the same treatment as soon as 1 hr 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, in which the tight interaction between astrocytes and neurons required for the formation of object recognition memory is under the control of astroglial CB₁ receptors. Thus, by determining the physiological availability of D-serine at NMDARs, astroglial CB₁ receptors are key causal elements of spatial and temporal regulation of glia-neuron interactions underlying synaptic plasticity and cognitive processes in the brain.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at <https://doi.org/10.1016/j.neuron.2018.04.034>.

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AUTHOR CONTRIBUTIONS

L.M.R., J.F.O.d.C., and V.C.L. performed behavioral, *in vivo* electrophysiology, and *in vitro* electrophysiology experiments, respectively, and wrote the manuscript. M.M.-L., A.B.-G., L.B., M.V., and E.S.-G. contributed to behavioral experiments. A.A. and M.M.-F. provided calcium measurements. B.B., I.S., A.V.E., I.M., G.B., and I.B. contributed to the measurements of amino acids. F.D. supervised part of the work. T.P. and M.W.S. helped with *in vitro* electrophysiology. F.G. supervised *in vivo* electrophysiology. A.P. and S.H.R.O. supervised *in vitro* electrophysiology. G.M. conceived and supervised the whole project and wrote the manuscript. All authors edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-NeuN antibody	Millipore	Cat# MAB377; RRID: AB_2298772
Rabbit anti-s100 β antibody	Sigma-Aldrich	Cat# HPA015768; RRID: AB_1856538
Donkey anti-mouse Alexa 647	Thermo Fisher Scientific	Cat# 10226162; RRID: AB_895335
Donkey anti-rabbit Alexa 488	Thermo Fisher Scientific	Cat# 10424752; RRID: AB_1623506
Bacterial and Virus Strains		
Viral Vector: AAV-GFAP-GFP	This paper	Virus n52 lab stock
Viral Vector: AAV-GFAP-CRE-mCherry	UNC Vector Core	N/A
Chemicals, Peptides, and Recombinant Proteins		
2,3-Naphthalenedicarboxaldehyde (NDA)	Fluka	Cat# 70215, CAS: 7149-49-7
AM251	Tocris	Cat# 1117
AS057278	Sigma-Aldrich	Cat# 644927
D-AP5	Sigma-Aldrich	Cat# A8054
DMSO	Sigma-Aldrich	Cat# D5879-M
D-serine	Ascent Scientific	Cat# ab120048
Fluo-4 AM	Thermo Fisher Scientific	Cat# F14201
Glutamate	Sigma-Aldrich	Cat# 49621, CAS: 6106-04-3
Glycine	Sigma-Aldrich	Cat# G7126, CAS: 56-40-6
Isoflurane	Virbac	N/A
Ketamine (IMALGENE 500)	Merial	N/A
Lidocaine (Lurocaine)	Vetoquinol	N/A
LY367385	Abcam	Cat# ab120067
MK-801	Abcam	Cat# ab120027
MTEP	Tocris	Cat# 2921
NBQX	Tocris	Cat# 1044
Paraformaldehyde	Sigma-Aldrich	Cat# HT501128
SR101	Sigma-Aldrich	Cat# S7635
Tamoxifen	Sigma-Aldrich	Cat# T5648
TTX	Tocris	Cat# 1078
Xylazine (ROMPUN)	Bayer	N/A
WIN 515,212-2	Sigma-Aldrich	Cat# W102
γ -Aminobutyric acid (GABA)	Sigma-Aldrich	Cat# A2129, CAS: 56-12-2
Critical Commercial Assays		
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23225
Experimental Models: Organisms/Strains		
Mouse: C57BL/6N	JANVIER Labs	C57BL/6NRj
Mouse: CB ₁ -flox	Marsicano et al., 2003	N/A
Mouse: GFAP-CB ₁ -KO	Han et al., 2012	N/A
Software and Algorithms		
Axoclamp 900A	Molecular Devices	N/A
Axon pClamp10	Molecular Devices	N/A
Behavioral Scoring Panel	A. Dubreucq	N/A
GraphPad Prism 6.0	GraphPad Software	N/A
ImageJ	NIH	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IGOR Pro v.6.37	WaveMetrics	N/A
Karat 32 software v.8.0	Beckman Coulter	N/A
NeuroMatic v.2.8t	Rothman and Silver, 2018	N/A
Spike2	Cambridge Electronic Design	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources should be directed to the Lead Contact Giovanni Marsicano (giovanni.marsicano@inserm.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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 naive male C57BL/6N (JANVIER, France), *CB₁-flox* (mice carrying the “floxed” *CB₁* gene (*CB1^{f/f}*)) and male GFAP-*CB₁-KO* mutant mice and GFAP-*CB₁-WT* littermates were used. Animals were housed in groups under standard conditions in a day/night cycle of 12/12 hr (light on at 7 am). Behavioral experiments were conducted between 2 and 5 pm. *In vivo* electrophysiological experiments were conducted during the light phase. Mice undergoing surgery were housed individually after the procedure.

GFAP-*CB₁-KO* mice were generated using the CRE/loxP system as previously described ([Han et al., 2012](#)). Mice carrying the “floxed” *CB₁* gene (*CB1^{f/f}*) ([Marsicano et al., 2003](#)) were crossed with GFAP-CreERT2 mice ([Hirrlinger et al., 2006](#)), using a three-step backcrossing procedure to obtain *CB1^{f/f};GFAP-CreERT2* and *CB1^{f/f}* littermates, called GFAP-*CB₁-KO* and GFAP-*CB₁-WT*, respectively. As CreERT2 protein is inactive in the absence of tamoxifen treatment ([Hirrlinger et al., 2006](#)), deletion of the *CB₁* 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, France) for 8 days. Mice were used 3-5 weeks after the last tamoxifen injection ([Han et al., 2012](#)).

METHOD DETAILS

Drug Preparation and Administration

For behavioral experiments, D-serine (Ascent Scientific, United Kingdom) was dissolved 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 cerebrospinal fluid (aCSF). AS057278 (Sigma-Aldrich, France) was dissolved in 0.9% saline added with 2% DMSO, 10% ethanol. D-AP5 (Sigma-Aldrich, France) was dissolved in 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 for exceptions), except for AS057278, which was injected 2 hr 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 hr after the acquisition and right before the test session. D-AP5 was also injected intra-hippocampally 6 hr after the acquisition.

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 μ L at a rate of 0.3 μ L/min. After infusion, injectors were kept in place for 60 s to prevent outflow of injected solutions.

Intra-hippocampal Drug Delivery

Mice (8-12 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (100mg/kg, Imalgene 500, Merial, France) and Xylazine (10mg/kg, Rompun, Bayer, France) and placed into a stereotaxic apparatus (David Kopf Instruments, CA, USA) with mouse adaptor 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, ML \pm 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.

Viral Vectors, Intra-hippocampal Delivery, and Histological Verification

AAV-GFAP-GFP control virus was produced in the lab as previously described (Hebert-Chatelain et al., 2016; Soria-Gómez et al., 2014, 2015) by using pAAV-GFAP-EGFP plasmid (ADDGENE #50473) as vector backbone. AAV-GFAP-CRE-mCherry was acquired from UNC vector core (NC, USA). Both viral vectors were rAAV serotype 8 with titers 4.17×10^{11} for AAV-GFAP-GFP and 1.2×10^{12} for AAV-GFAP-CRE-mCherry, respectively. 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 μ L per injection site at a rate of 0.5 μ L 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 min before being slowly withdrawn from the brain. CB₁-flox mice were injected with AAV-GFAP-GFP (control) or AAV-GFAP-CRE (fused to mCherry, serotype 8, 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 paraformaldehyde (Sigma Aldrich, France) and their brains were sliced with a vibratome. 40 μ m hippocampal sections incubated with primary antibody directed against s100 β (Rabbit polyclonal, Sigma Aldrich, France) and NeuN (Mouse monoclonal, Millipore, France). Secondary antibodies incubation was performed in order to detect s100 β with Alexa 488 (Thermo Scientific, France) and NeuN with Alexa 647 (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), s100 β and NeuN expressing cells was performed with ImageJ software as previously described (Bolte and Cordelières, 2006). Briefly, after threshold subtraction and crosstalk correction, the number of cells co-expressing mCherry/s100 β or mCherry/NeuN was automatically obtained by the “particle analysis” tool of the same software. mCherry/s100 β co-expressing cells were expressed in percentage of CRE positive cells as well as percentage of total s100 β cells. On the other hand, mCherry/NeuN 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., 2011, 2013; Puighermanal et al., 2009, 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 CB₁ receptors (Puighermanal et al., 2009, 2013); (ii) the NOR test performed in a L-maze decrease variability and give strong and replicable results; (iii) 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 gray polyvinyl chloride shaped by two identical perpendicular arms (35 cm and 30 cm long respectively for external and internal L walls, 4.5 cm 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 of 3 sequential daily trials of 9 min 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 center 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 hr 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 ethanol (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 to the treatment and/or genotype of the animals.

In Vivo Electrophysiology

GFAP-CB₁-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, United Kingdom) 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 μ L of the local anesthetic Lurocaine (Vetoquinol, France) was injected in the scalp region. Surgical procedure started with a longitudinal incision of 1.5 cm in length aimed to expose Bregma and Lambda. After ensuring 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 M Ω) 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, 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, UK). Test pulses were generated through an Isolated Constant Current Stimulator (DS3, Digitimer, 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 s. 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 s each), separated by 20 s interval. fEPSP were then recorded for a period of 40 min. In the specific group of mice the following treatments were applied: 1) MK-801 (Abcam, United Kingdom; 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 (Ascent Scientific, United Kingdom; 50 mg/kg, i.p., dissolved in saline) approx. 2 hr before HFS or vehicle (saline, i.p.). At the end of each experiment, the positions of the electrodes were marked by iontophoretic infusion of the recording solution during 180 s at –20 μA and continuous current discharge over 20 s 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-*CB₁*-WT or GFAP-*CB₁*-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% O₂ and 5% CO₂. aCSF contained (in mM): 125 NaCl, 2.5 KCl, 1 Na₂HPO₄, 1.2 MgCl₂, 0.6 CaCl₂, 26 NaHCO₃ 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, Thermo Scientific, USA). Slices were hemisected and maintained at 33°C during 30 min in aCSF containing 2 mM MgCl₂ and 1 mM CaCl₂. Then, they were allowed to recover at room temperature for at least 1 hr.

Slices were transferred into a recording chamber perfused with aCSF (2.8 mL/min) containing 1.3 mM MgCl₂ and 2.5 mM CaCl₂, and maintained at 30°C. fEPSPs slope were recorded with a Multiclamp 700A amplifier (Molecular Devices, CA, USA), using pipettes (2–3 M Ω) 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 min, LTP was induced by applying a HFS protocol consisting of a 100 Hz train of stimuli for 1 s repeated three times at 20 s intervals. MTEP (500 nM; 500 μM stock in dH₂O; Tocris, United Kingdom) and LY367385 (100 μM ; 100 mM stock in 1.1e⁴ NaOH; Abcam, United Kingdom), or the vehicle control, were perfused after a stable baseline of at least 10 min. To inhibit mGluR1/5 during LTP induction, MTEP and LY367385 were perfused for 20 min prior to LTP induction and removed 2 min post-LTP induction. NMDAR-fEPSPs were recorded in low Mg²⁺ aCSF (0.2 mM) with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10 μM , Tocris, United Kingdom) to block AMPA/kainate receptors. At the end of each experiment, D-AP5 (50 μM , Sigma-Aldrich, France), 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 pClamp10 software (Molecular Devices, CA, USA).

Ca²⁺ Imaging

Ca²⁺ levels in astrocytes located in the *stratum radiatum* of the CA1 region of the hippocampus were monitored by fluorescence microscopy using the Ca²⁺ indicator fluo-4 AM (Thermo fisher scientific, MA, USA). 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 (Sigma-Aldrich, USA) staining. Astrocytes were imaged using a Leica SP5 multiphoton microscope and images were acquired at 1 to 2 Hz. Intracellular Ca²⁺ signals were monitored from astrocytic somas and processes and the signal was measured as fluorescence over baseline [(Fluorescence_t - Background fluorescence_t) - (Fluorescence₀ - Background fluorescence₀)] / (Fluorescence₀ - Background fluorescence₀) and cells were considered to display a Ca²⁺ event when the calcium signal increased three times the standard deviation of the baseline.

The astrocyte Ca²⁺ signal was quantified as the probability of occurrence of a Ca²⁺ event (calcium event probability). The Ca²⁺ 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 Ca²⁺ event. The calcium event

probability during 20 s before the WIN 515,212-2 (WIN, Sigma-Aldrich, USA) application (200 μ M, 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 μ L of the DMSO-WIN solution was diluted in 1 mL of aCSF, obtaining a final concentration of 200 μ M 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 μ M around the recorded cells (Navarrete and Araque, 2008). In some cases, experiments were performed in the presence of the CB₁ antagonist AM251 (2 μ M, Tocris, United Kingdom). Mean values were obtained from at least 5 slices and 2 mice in each condition.

Measurement of Amino Acids 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 μ m slices and incubated in 350 μ L oxygenated aCSF containing 0.5 μ M TTX (Tocris, United Kingdom) with either vehicle (1/4000 DMSO) or WIN (5 μ M in DMSO) during 30 min at 31°C. Extracellular medium was quickly removed, 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 naphthalene-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 tissue 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, USA) 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.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were expressed as mean \pm SEM or single data points and were analyzed with Prism 6.0 (Graphpad Software), using t test (paired or unpaired), Mann Whitney test or ANOVA (One- or Two-Way), where appropriate. Dunnet's, Holm-sidak (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](#).

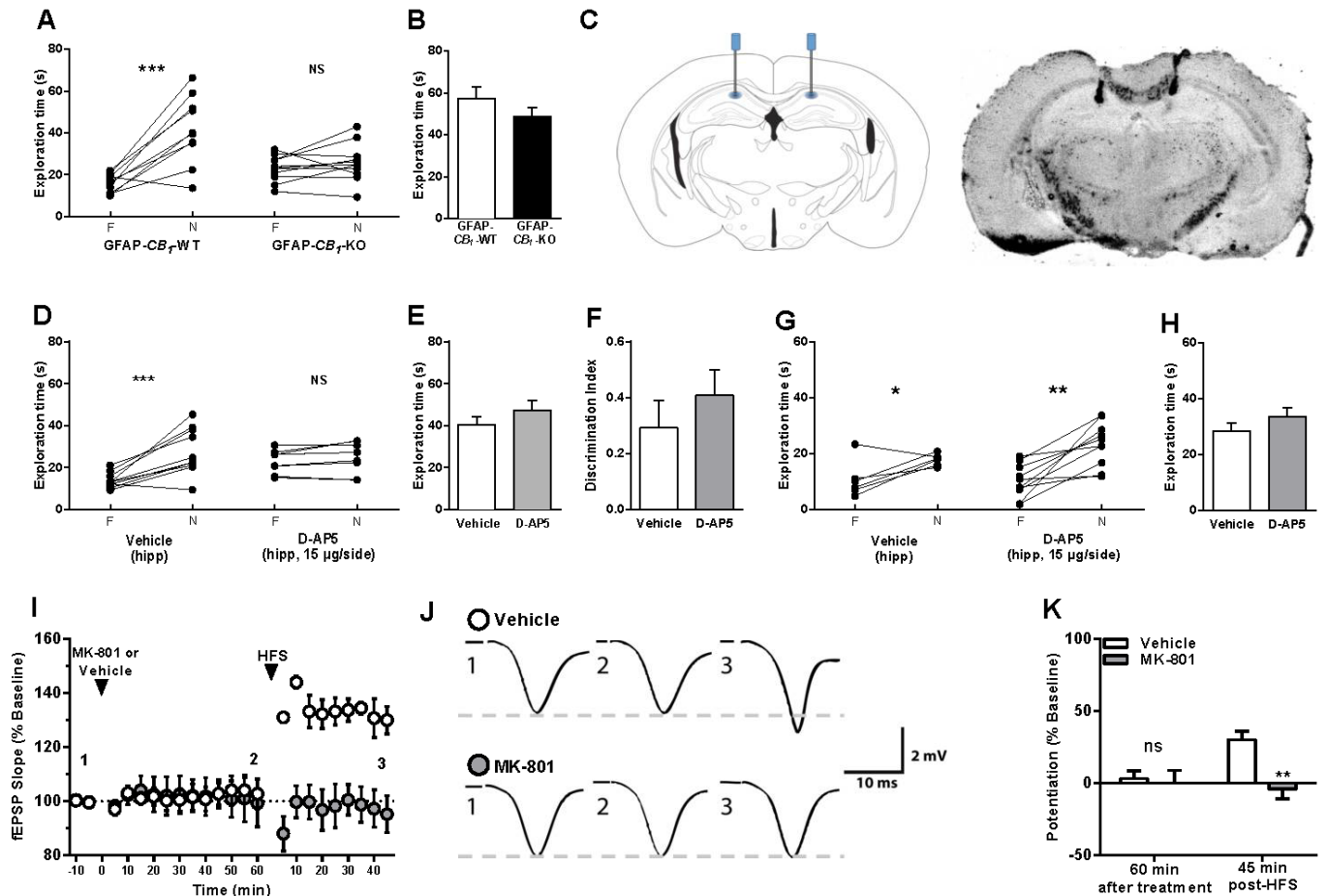


Figure S1. Related to Figure 1. (A) Exploration time of the familiar (F) and the novel object (N) of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates. (B) Total exploration time of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates. (C) Schematic drawing of local hippocampal injection (left) and representative image of injection sites (right). (D) Exploration time of the familiar (F) and the novel object (N) of C57BL/6N and GFAP-*CB₁*-WT mice intra-hippocampally injected with vehicle or D-AP5 (15 μ g/side) in the NOR task. (E) Total exploration time of C57BL/6N and GFAP-*CB₁*-WT mice intra-hippocampally injected with vehicle or D-AP5 (15 μ g/side). (F) Memory performance of WT mice treated with intra-hippocampal infusions of vehicle or D-AP5 6 hours post-acquisition. (G) Exploration time of the familiar (F) vs the novel object (N) and (H) total exploration time of WT mice intra-hippocampally injected with vehicle or D-AP5 6 hours post-acquisition. (I) Summary plots of normalized fEPSPs of WT treated with MK-801 or vehicle 60 minutes before LTP induction by HFS. (J) Representative traces (superimposed, 150 successive fEPSP) of baseline (1), 60 minutes post-vehicle (top) or MK-801 (3 mg/kg; i.p., bottom) injection and 45 minutes post-HFS (3). (K) Bar histograms of normalized fEPSPs from experiment (I). Data, mean \pm SEM. *, P < 0.05, **, P < 0.01; ***, P < 0.001, NS, not significant. See **Table S1** for detailed statistics.

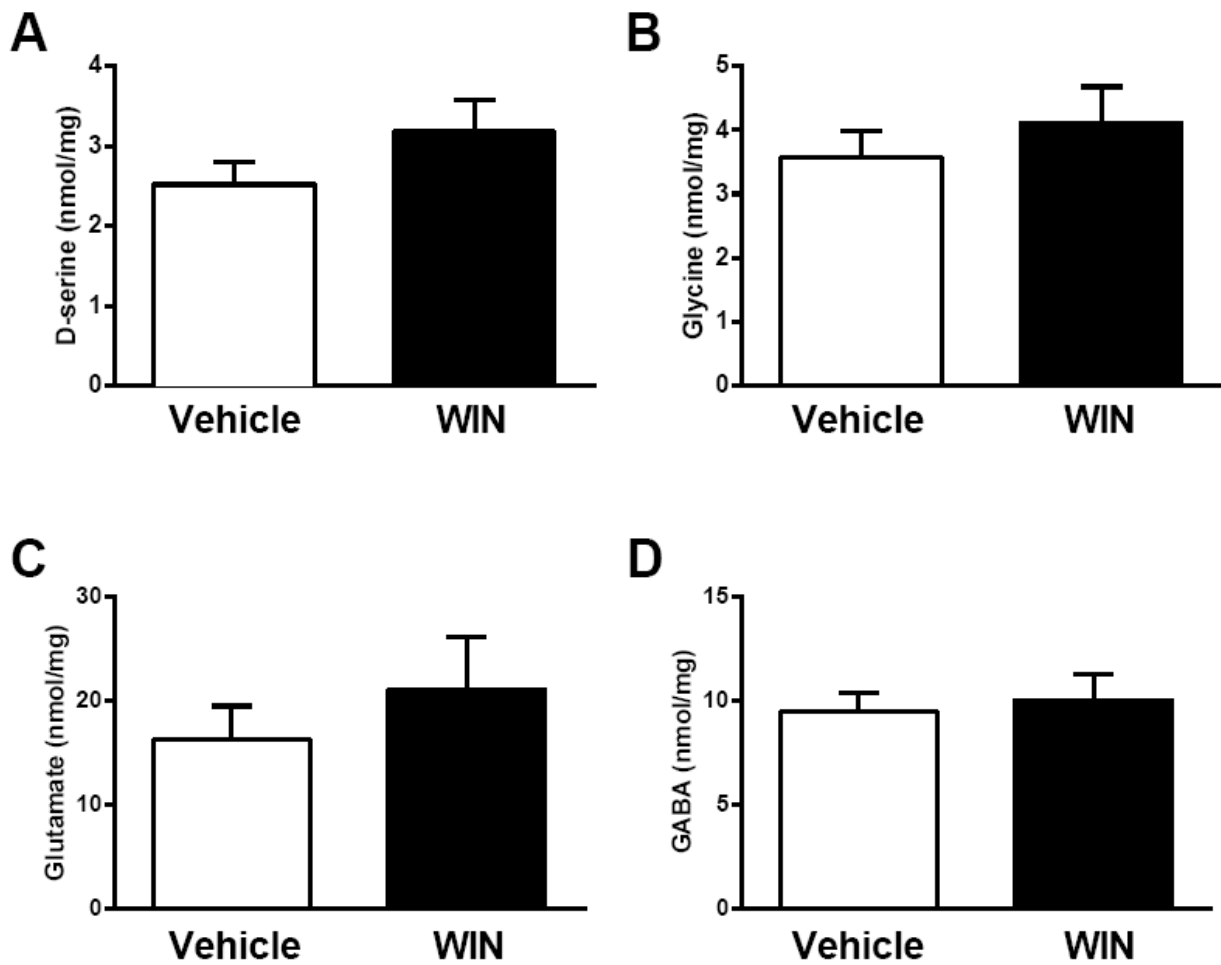


Figure S2. Related to Figure 2. Determination of tissue levels of D-serine (A), glycine (B), glutamate (C) and GABA (D) as measured by capillary electrophoresis in mouse hippocampal slices treated with vehicle or WIN (5µM). Data, mean ± SEM. See **Table S1** for detailed statistics.

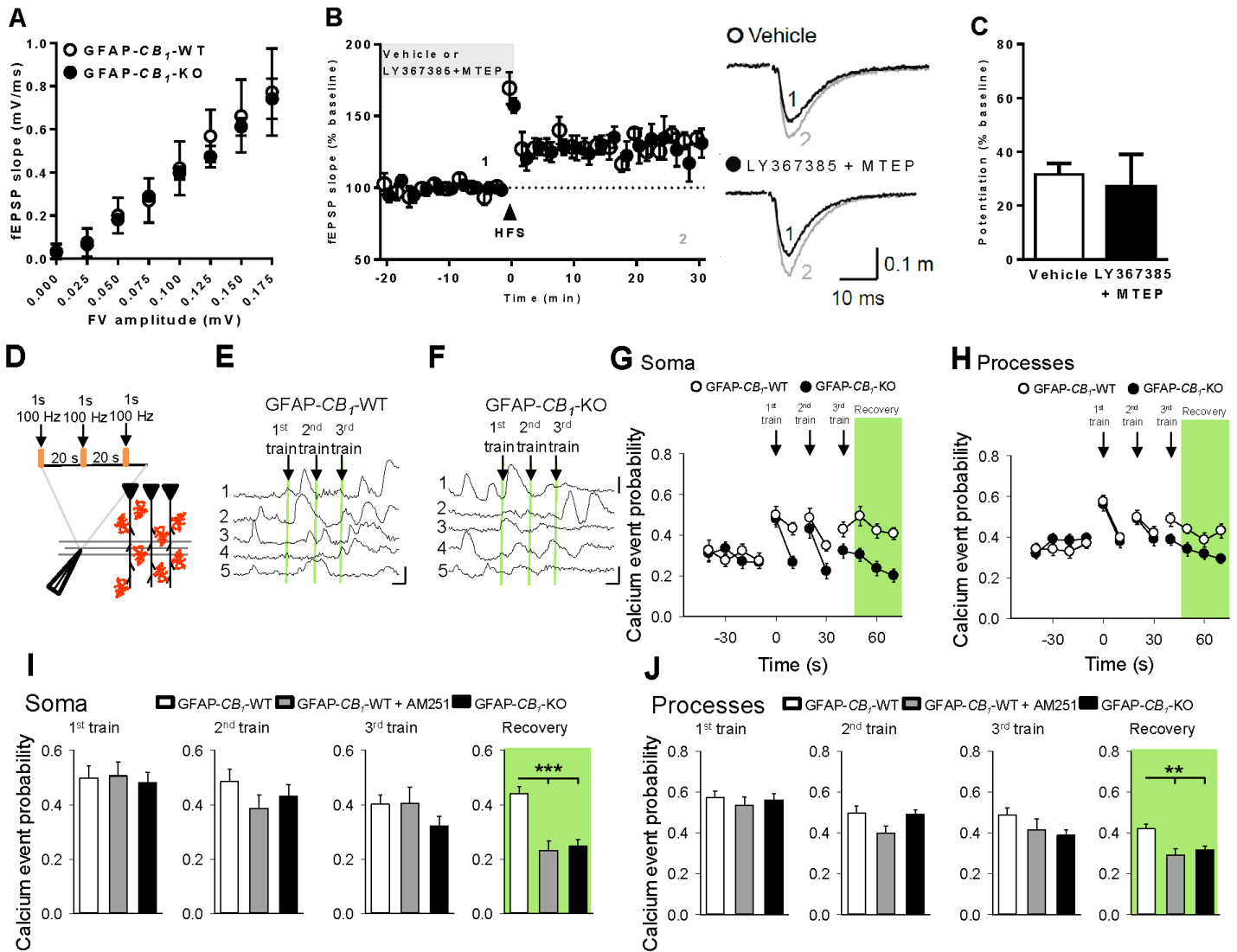


Figure S3. Related to Figure 3. (A) Input-output curves of fEPSPs in GFAP-*CB₁*-WT (n=8) and GFAP-*CB₁*-KO mice (n=7). (B) Summary plots showing the lack of effect of mGluR1/5 antagonists LY367385 and MTEP on LTP induction. Traces represent 30 superimposed successive fEPSPs before (1, black) and after (2, grey) the HFS stimulation (arrow). (C) Bar histograms of fEPSPs from experiments. Schaffer collateral HFS effect on astrocytic calcium. (D) Schematic representation of the experimental design. A stimulation electrode was placed in the *stratum radiatum* in CA1 region. The stimulus consist on 3 trains of 100 Hz (1 sec each), separated by 20 seconds interval. Astrocytes in the adjacent field of view were image and their calcium intensities measured. (E) Representative somatic calcium traces of 5 astrocytes of the same field of view before and after Schaffer collaterals stimulations (indicated by arrows and green lines) in GFAP-*CB₁*-WT mice. Scale bars: 5% and 10 s. (F) Representative somatic calcium traces of 5 astrocytes of the same field of view before and after Schaffer collaterals stimulations (indicated by arrows and green lines) in GFAP-*CB₁*-KO mice. Scale bars: 21% for top trace and 7% for bottom traces and 10 s. (G) Somatic

calcium event probability before and after Schaffer collaterals stimulation (at time=0, 20 and 40) in GFAP-*CB₁*-WT (white; 163 somas from n=17 slices) and GFAP-*CB₁*-KO mice (black; 146 somas from n=21 slices). **(H)** Processes calcium event probability before and after Schaffer collaterals stimulation (at time=0, 20 and 40) in GFAP-*CB₁*-WT (white; 319 processes from n=17 slices) and GFAP-*CB₁*-KO mice (black; 320 processes from n=21 slices). **(I)** Somatic calcium event probability in GFAP-*CB₁*-WT in control conditions (white, n=17) and in the presence of AM251 (grey, n=10) and in GFAP-*CB₁*-KO mice (black, n=21). No differences were observed between the different groups after the first stimulation train, second stimulation train or third stimulation trains. In contrast a difference was observe when comparing the post stimulus “recovery” period of GFAP-*CB₁*-WT mice in control conditions with GFAP-*CB₁*-WT mice in the presence of AM251 and with GFAP-*CB₁*-KO mice. **(J)** Processes calcium event probability in GFAP-*CB₁*-WT in control conditions (white, n=17) and in the presence of AM251 (grey, n=10) and in GFAP-*CB₁*-KO mice (black, n=21). No differences were observed between the different groups after the first stimulation train, second stimulation train or third stimulation trains. In contrast a difference was observe when comparing the post stimulus “recovery” period of GFAP-*CB₁*-WT mice in control conditions with GFAP-*CB₁*-WT mice in the presence of AM251 and with GFAP-*CB₁*-KO mice. Data, mean \pm SEM. **, P<0.01; ***, P<0.001. See **Table S1** for detailed statistics.

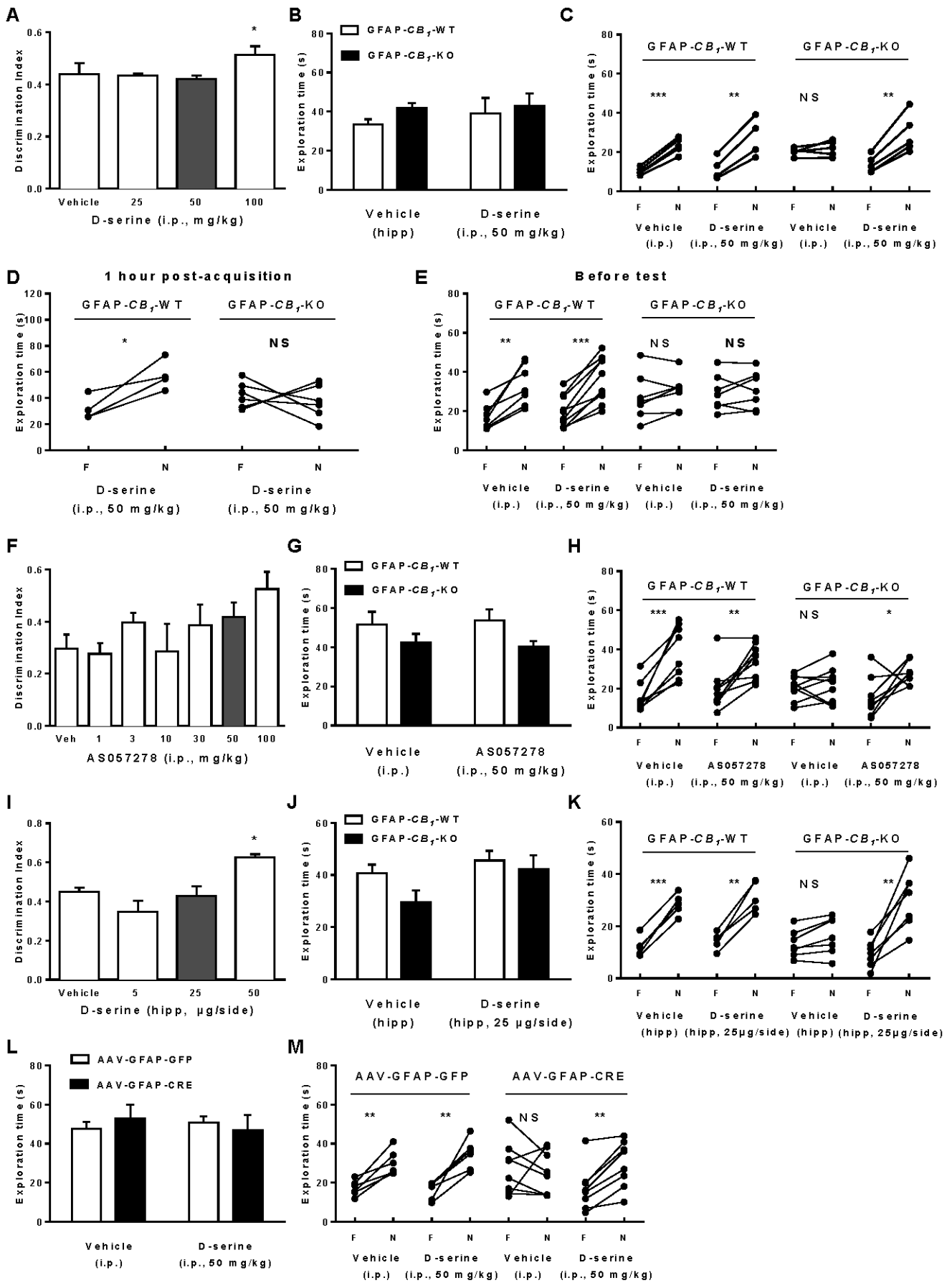


Figure S4. Related to Figure 4. Effects of D-serine and AS057278 on NOR task. **(A)** Effect of vehicle or different doses of D-serine (25, 50 or 100 mg/kg, i.p.) on memory performance in WT mice. Grey bar, sub-effective dose used in following experiments. **(B)** Total exploration time of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates injected with vehicle or D-serine (50 mg/kg, i.p.). **(C)** Exploration time of the familiar (F) and the novel (N) objects of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-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-*CB₁*-WT mice and GFAP-*CB₁*-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 AS057278 (1, 3, 10, 30, 50 or 100 mg/kg i.p.) on memory performance of WT mice. Grey bar, sub-effective dose used in following experiments. **(G)** Total exploration time of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates injected with vehicle or AS057278 (50 mg/kg, i.p.). **(H)** Exploration time of the familiar and the novel object of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates injected with vehicle or AS057278 (50 mg/kg, i.p.). **(I)** Effect of intra-hippocampal vehicle or different doses of D-serine (5, 25 or 50 μ g/side) on memory performances of WT mice. Grey bar, sub-effective dose used in following experiments. **(J)** Total exploration time of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates injected with intra-hippocampal vehicle or D-serine (25 μ g/side). **(K)** Exploration time of the familiar and the novel object of GFAP-*CB₁*-WT mice and GFAP-*CB₁*-KO littermates injected with intra-hippocampal vehicle or D-serine (25 μ g/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 *CB₁*-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 \pm SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, NS, not significant. See **Table S1** for detailed statistics.

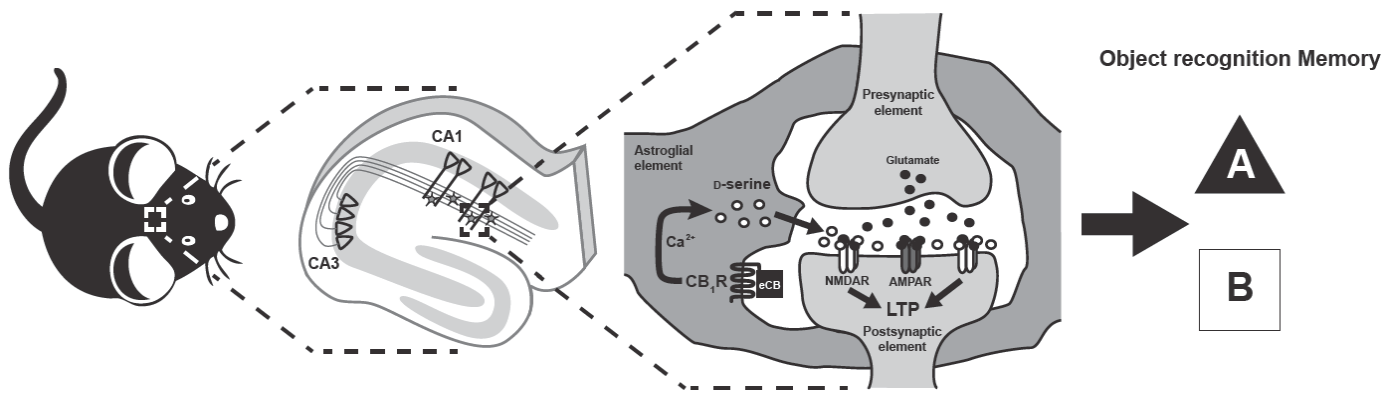


Figure S5. Related to Figure 2-4. Schematic summary of the results. In adult mice, at the hippocampal CA3-CA1 synapse, astroglial CB₁ receptors regulate cellular calcium (Ca²⁺) levels, synaptic D-serine availability and thus D-serine-dependent synaptic NMDAR gating. By this means, astroglial CB₁ receptors control synaptic NMDAR-dependent long-term potentiation (LTP) and object recognition memory. eCB; endocannabinoid.

Figure	Conditions	"n" (per group)	Analysis (post-hoc test reported in figures)	Factors analyzed	F-ratios	P values
1A	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO	10-11	Unpaired <i>t</i> -test			P = 0.0002
1B	Vehicle vs D-AP5	8-10	Unpaired <i>t</i> -test			P < 0.0001
1C	Vehicle vs MK-801 / Time	5-6	2-WAY ANOVA (Bonferroni)	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 MK-801	5-6	Unpaired <i>t</i> -test			P = 0.0054
1E	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO	6-9	Unpaired <i>t</i> -test			P = 0.0250
2C	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO basal vs treatment	8-10	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	Vehicle vs WIN	15	Paired <i>t</i> -test			P = 0.0092
2G	Vehicle vs WIN	16	Paired <i>t</i> -test			P = 0.0639
2H	Vehicle vs WIN	9	Paired <i>t</i> -test			P = 0.5075
2I	Vehicle vs WIN	16	Paired <i>t</i> -test			P = 0.4524
3A	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO	7	Unpaired <i>t</i> -test			P = 0.0121
3C	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO Vehicle vs AS057278	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- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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/S100 β 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
S1A	Exploration Novel object vs familiar					
	GFAP- <i>CB₁</i> -WT	10	Paired <i>t</i> -test			P = 0.0005
S1B	GFAP- <i>CB₁</i> -KO	11	Paired <i>t</i> -test			P = 0.2466
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO	10-11	Unpaired <i>t</i> -test			P = 0.2306
S1D	Exploration Novel object vs familiar					
	Vehicle	11	Paired <i>t</i> -test			P = 0.0005
S1E	AP5	8	Paired <i>t</i> -test			P = 0.1284
	Vehicle vs AP5	8-11	Unpaired <i>t</i> -test			P = 0.2637
S1F	Vehicle vs AP5	6-10	Unpaired <i>t</i> -test			P = 0.4145
S1G	Exploration Novel object vs familiar					
	Vehicle	6	Paired <i>t</i> -test			P = 0.0439
S2A	AP5	10	Paired <i>t</i> -test			P = 0.0012
	Vehicle vs WIN	8	Mann-Whitney test			P = 0.1975
S2B	Vehicle vs WIN	7	Mann-Whitney test			P = 0.5350
S2C	Vehicle vs WIN	5	Mann-Whitney test			P = 0.4206
S2D	Vehicle vs WIN	7	Mann-Whitney test			P = 1

S3A	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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
S3C	Vehicle vs LY367385 + MTEP	8, 7	Mann-Whitney test (Two-tailed)			P = 0.5358
S3I	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 1st train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,45) = 0.089	P = 0.91
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 2nd train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,45) = 0.9	P = 0.41
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 3rd train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,45) = 2.308	P = 0.11
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO recovery	10-21	1-WAY ANOVA (holm-Sidak)		F(2,45) = 19.105	P < 0.0001
S3J	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 1st train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,44) = 0.257	P = 0.77
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 2nd train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,44) = 2.267	P = 0.16
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO 3rd train	10-21	1-WAY ANOVA (holm-Sidak)		F(2,44) = 2.533	P = 0.091
	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO recovery	10-21	1-WAY ANOVA (holm-Sidak)		F(2,44) = 8.265	P < 0.0001
S4A	Dose of D-serine	5	1-WAY ANOVA (Dunnnett's)		F(3,16) = 2.194	P = 0.0128
S4B	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -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- <i>CB₁</i> -WT / Vehicle	5	Paired <i>t</i> -test			P = 0.0002
	GFAP- <i>CB₁</i> -WT / D-serine	4	Paired <i>t</i> -test			P = 0.0062
	GFAP- <i>CB₁</i> -KO / Vehicle	5	Paired <i>t</i> -test			P = 0.2666
S4AD	Exploration Novel object vs familiar					
	GFAP- <i>CB₁</i> -WT	4	Paired <i>t</i> -test			P = 0.0308
	GFAP- <i>CB₁</i> -KO	6	Paired <i>t</i> -test			P = 0.5701
	Exploration Novel object vs familiar					
S4E	GFAP- <i>CB₁</i> -WT / Vehicle	7	Paired <i>t</i> -test			P = 0.0032
	GFAP- <i>CB₁</i> -WT / D-serine	10	Paired <i>t</i> -test			P < 0.0001
	GFAP- <i>CB₁</i> -KO / Vehicle	7	Paired <i>t</i> -test			P = 0.2375
	GFAP- <i>CB₁</i> -KO / D-serine	7	Paired <i>t</i> -test			P = 0.5468
S4F	Dose of AS057278	5-7	1-WAY ANOVA (Dunnnett's)		F(6,37) = 2.117	P = 0.0744
S4G	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO Vehicle vs AS057278	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- <i>CB₁</i> -WT / Vehicle	8	Paired <i>t</i> -test			P = 0.001
	GFAP- <i>CB₁</i> -WT / AS057278	9	Paired <i>t</i> -test			P = 0.0031
	GFAP- <i>CB₁</i> -KO / Vehicle	9	Paired <i>t</i> -test			P = 0.5975
S4I	Exploration Novel object vs familiar					
	GFAP- <i>CB₁</i> -KO / AS057278	9	Paired <i>t</i> -test			P = 0.0392
	GFAP- <i>CB₁</i> -KO / D-serine	7	Paired <i>t</i> -test			P = 0.0017
	Dose of D-serine (hipp.)	4-10	1-WAY ANOVA (Dunnnett's)		F(3,23) = 5.043	P = 0.0079
S4J	GFAP- <i>CB₁</i> -WT vs GFAP- <i>CB₁</i> -KO Vehicle vs D-serine (hipp.)	5-7	2-WAY ANOVA (Bonferroni)	Genotype x Treatment	Genotype F(1,22) = 0.77	P = 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- <i>CB₁</i> -WT / Vehicle	5	Paired <i>t</i> -test			P = 0.0002
	GFAP- <i>CB₁</i> -WT / D-serine	5	Paired <i>t</i> -test			P = 0.0018
	GFAP- <i>CB₁</i> -KO / Vehicle	7	Paired <i>t</i> -test			P = 0.3847
S4L	Exploration Novel object vs familiar					
	GFAP- <i>CB₁</i> -KO / D-serine	7	Paired <i>t</i> -test			P = 0.0017
	AAV-GFAP-GFP vs AAV-GFAP-CRE Vehicle vs D-serine	6-8	2-WAY ANOVA (Bonferroni)		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 <i>t</i> -test			P = 0.0036
	AAV-GFAP-GFP / D-serine	7	Paired <i>t</i> -test			P = 0.0019
	AAV-GFAP-CRE / Vehicle	8	Paired <i>t</i> -test			P = 0.6639
	AAV-GFAP-CRE / D-serine	8	Paired <i>t</i> -test			P = 0.0013

Table S1. Statistical details. Related to Figures 1-4 and Figures S1-S4.

DISCUSSION

DISCUSSION

In the present work, we revealed specific physiological roles of hippocampal astroglial CB₁ receptors in novel object recognition (NOR) memory and long-term synaptic plasticity. The results presented in this Thesis work and published (Robin et al., 2018) suggest that the astroglial activity-dependent Ca²⁺ increase induced by CB₁ receptor activation provides proper synaptic D-serine levels to activate NMDARs in the CA1 region of the hippocampus. This mechanism plays a permissive role for the induction of NMDAR-dependent LTP and the consolidation of memory in the NOR task. These data provide an unforeseen physiological mechanism linking astroglial CB₁ receptors, the bioavailability of D-serine and NMDARs activity, ultimately underlying memory formation.

By showing the roles of astroglial CB₁ receptors in gliotransmission, in synaptic plasticity and in behavior, these data are in agreement with the idea that astrocytes can actively modulate synaptic plasticity and memory (Araque et al., 2014; Metna-Laurent et al., 2014; Oliveira et al., 2015). At the behavioral level, we show that hippocampal astroglial CB₁ receptors are necessary and sufficient to NOR memory. The NOR memory paradigm was first described when scientists reported that rodents naturally explore more novel than previously encountered familiar object (Berlyne et al., 1966). The task is non aversive and has many other advantages, such as the short acquisition which allows temporal definition of memory formation (Dere et al., 2007). These advantages contributed to the popularity of this paradigm. Classically, the task is performed in an open-field where the mice can freely explore the objects (Bevins and Besheer, 2006). However, this version of the task presents some important issues, such as the need of long habituation (several days) and several acquisition sessions (Dere et al., 2005), a certain difficulty in interpreting the behavioral performances (Sik et al., 2003) and the high variability in the time exploring the objects amongst subjects (Sik et al., 2003). The fact that open fields are generally considered as anxiogenic environments (Sturman et al., 2018) is likely explaining at least part of these issues. It was recently proposed that using an L-maze with the objects at the ends of each arm reduces anxiety and increases reliable object exploration. Indeed, experience from the laboratory showed that CB₁-KO mice explore objects much more in a L-maze than in an open-field, likely due to their anxious phenotype (Haller et al., 2002; Moreira et al., 2010). Interestingly, other groups have also used a Y-shaped maze to perform NOR in rats, with very similar results as in the mouse L-maze (Forwood et al., 2005; Winters et al., 2004).

Our starting observation that GFAP-CB₁-KO mice are unable to perform NOR is particularly interesting, because it represents the first demonstration of a physiological role of astroglial CB₁ receptors in behavior. Previous data from our laboratory actually showed that deletion of astroglial CB₁ receptors blocks the pharmacological effects of exogenous cannabinoids on spatial working memory. However, in that study, vehicle-treated GFAP-CB₁-KO displayed no behavioral impairments as compared to their littermate controls (Han et al., 2012), suggesting that astroglial CB₁ receptors are not involved in physiological short-term working memory. Interestingly, previous studies have shown that constitutive deletion of CB₁ receptors does not alter NOR memory in the same protocol we used in this study

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(Hebert-Chatelain et al., 2016; Puighermanal et al., 2009). Indeed, with global deletion of CB₁ receptors in all cells and at all developmental stages, compensatory mechanisms can occur, masking the roles of CB₁ receptors in specific cell-types (Eisener-Dorman et al., 2009). Moreover, CB₁ receptors often exert different or even opposite functions in different cell types, leaving the possibility that global deletion might hide specific roles (Busquets-Garcia et al., 2015). Thus, to fully understand the cell type-specific roles of CB₁ receptors, the use of different experimental models is necessary.

One way to bypass these issues is the use of conditional mutant mice. In our study, we used conditional mutagenesis by the Cre/LoxP system, in its tamoxifen-inducible version. This system allows temporal deletion of CB₁ receptors in adult mice to avoid compensatory developmental mechanisms (Hirrlinger et al., 2006). To specifically target astrocytes we used the Cre recombinase under the control of the GFAP promoter (Han et al., 2012). The use of inducible systems is particularly necessary when dealing with GFAP-positive cells, because the expression of this marker not only characterizes astroglial cells, but also neuronal progenitors during development (Kriegstein and Alvarez-Buylla, 2009). Thus, Cre expression at early embryonic stages in GFAP-positive cells would lead to deletion of CB₁ in many neuronal cells together with astrocytes. However, also adult neuronal precursor in the dentate gyrus and the subventricular zone express GFAP (Seri et al., 2001), leaving the possibility that deletion of CB₁ receptor in GFAP-CB₁-KO mice might occur in adult newborn neurons. CB₁ receptor signaling impacts on adult neurogenesis in mice (Galve-Roperh et al., 2006; Massa et al., 2011), although it is not yet clear whether this function is exerted *via* direct expression of the receptor in neuronal precursors or *via* indirect modulation of synaptic transmission impinging on them. Therefore, the deletion of CB₁ in adult neural progenitors, might represent a potential confounding factor of our results (Aguado et al., 2006; Massa et al., 2011). Indeed, D-serine has been shown to be necessary to synaptic integration of newborn neurons from the dentate gyrus in adults (Sultan et al., 2015). Consequently, the deletion of astroglial CB₁ receptors in newborns neurons in the dentate gyrus might contribute explaining the memory impairment of GFAP-CB₁-KO mice in the NOR task. Yet, this hypothesis is unlikely. Injections of a AAV-Cre under the control of the GFAP promoter in the CA1 region of CB₁-floxed mice induces the same impairments in NOR memory as in the GFAP-CB₁-KO mice, suggesting that NOR memory depends on the astroglial CB₁ receptors of the CA1. Moreover, unpublished preliminary data indicate that neurogenesis is not altered in GFAP-CB₁-KO mice. However, we still cannot definitely rule out a role of neurogenesis in the phenotype of GFAP-CB₁-KO mice.

Besides their roles in the modulation of NOR memory, other memory types could be modulated by CB₁ receptors expressed in GFAP-positive cells. For instance, contextual fear-conditioning has been recently shown to be associated, like NOR, with hippocampal D-serine availability, NMDAR activation and LTP induction (Basu et al., 2016). Moreover, it has been shown that D-serine enhances the extinction of amygdala-dependent fear expression in auditory-cued fear conditioning (Matsuda et al., 2010), as well as in inhibitory avoidance tasks in rodents (Bai et al., 2014). In this context, as CB₁ receptors modulate aversive long-term memory formation (Marsicano et al., 2002) and astrocytes play an important role in memory and D-serine release (Oliveira et al., 2015; Henneberger et al., 2010; Papouin et al., 2017), it is interesting to address the role of astroglial CB₁ receptors in this type of memory. Indeed, in

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collaboration with the group of Alfonso Araque, we recently addressed this issue and showed that astroglial CB₁ receptors in the central nucleus of amygdala control fear conditioning expression (Martin-Fernandez et al., 2017). However, the role of astroglial CB₁ receptors in memory cannot be generalized to all types of memory since spatial working memory performances are not impaired in GFAP-CB₁-KO (Han et al., 2012).

The role of the hippocampus in NOR memory is debated [(For review, see (Cohen and Stackman, 2015)). Although this stays true for the open field version of the task, we show that post-acquisition intra-CA1 injection of the potent NMDAR antagonist AP5 abolishes NOR memory performance, clearly indicating consolidation of NOR memory evaluated in a L-maze depends on NMDAR activity in this brain region. Interestingly, NMDAR-dependent LTP at the CA3-CA1 synapse is a key player for learning and memory (Henneberger et al., 2010; Whitlock et al., 2006). Various groups tried to understand the role of CB₁ receptors in LTP at the CA3-CA1 synapse but the role of the different subpopulations of CB₁ receptors is difficult to interpret because of their very diverse localizations and signaling properties. Genetic models suggest that glutamatergic CB₁ receptor inhibits LTP, while GABAergic CB₁ receptor favors it (Monory et al., 2015). Pharmacological approaches using CB₁ antagonists also linked CB₁ receptors and LTP (Carlson et al., 2002; De Oliveira Alvares et al., 2008). However, these studies suggest a neuronal CB₁ receptor-dependent decrease of the pre-synaptic release probability of glutamate, without involving modulation of post-synaptic NMDARs activity. In the present work, we report that astroglial CB₁ receptors modulate NMDAR-dependent LTP both *in vivo* and *in vitro* via the synaptic bioavailability of D-serine. This difference can be explained by the different approaches used in the various studies. Here, we studied the specific role of astroglial CB₁ receptors, which is not possible with pharmacological approaches.

Other forms of long-term synaptic plasticity could depend on astroglial CB₁ receptors. A recent study suggested that the activation of astroglial CB₁ receptors induces the release of D-serine at the CA3-CA1 synapse. However, they suggest that D-serine binds pre-synaptic NMDARs to induce t-LTD (Andrade-Talavera et al., 2016). This result is in agreement with our conclusions, except that D-serine would act pre-synaptically rather than at post-synaptic NMDARs. Thus, long-term synaptic plasticity at the CA3-CA1 synapse seems to depend on the modulation of D-serine bioavailability by astroglial CB₁ receptors. In the neocortex, two studies suggested a role of astroglial CB₁ receptors in LTP and t-LTD (Min and Nevian, 2012; Rasooli-Nejad et al., 2014). However, these forms of long-term plasticity are not modulated by D-serine, but by ATP and glutamate, respectively. Thus, gliotransmission induced by astroglial CB₁ receptors activity is necessary for long-term plasticity but the nature of the gliotransmitters involved seems to depend on the form of synaptic plasticity and brain structures.

Increase of astroglial intracellular Ca²⁺ modulates synaptic activity and plasticity via the release of gliotransmitters, whose identity depend on the brain region and the type of plasticity involved (Araque et al., 2014; Sherwood et al., 2017). In agreement with previous studies (Navarrete et al., 2008; Navarrete et al., 2010), our results indicate that astroglial CB₁ receptor activation induces intracellular Ca²⁺ increase in astrocytes. There is currently a debate about the Ca²⁺-dependent release of gliotransmitters. Some groups report PLC-IP3-dependent astroglial Ca²⁺-dependent

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gliotransmission while others report that Ca^{2+} activity in astrocytes do not impact gliotransmission and synaptic activity (Agulhon et al., 2012, Bazargani and Attwell, 2016, Henneberger et al., 2010). Astroglial CB_1 receptors have been proposed to be coupled to Gq-protein signaling (Navarrete et al., 2008), which is known to induce PLC activity with the generation of IP_3 and DAG (De Pittà et al., 2009), activation of IP_3R and Ca^{2+} release from the ER. Thus, it is tempting to speculate that D-serine released by astrocytes depends on this mechanism. However, several studies have demonstrated that knocking out $\text{IP}_3\text{R}2$ ($\text{IP}_3\text{R}2\text{-KO}$ mice), the main IP_3R in astrocytes does not impair synaptic plasticity nor memory (Agulhon et al., 2010). However, more recent data showed that astroglial Ca^{2+} signals can also depend on other IP_3R types (Sherwood et al., 2017). Other authors showed that initial studies using $\text{IP}_3\text{R}2\text{-KO}$ mice monitored only a small portion of the astrocytic Ca^{2+} signals but not the fast and local signals at the level of the thinner branches of astrocytes, the so-called gliapil (Srinivasan et al., 2015; Bindocci et al., 2017), which are the astroglial regions in close association with synapses (Chao et al., 2002 and (Volterra et al., 2014). At this level, Ca^{2+} signals appear to be mainly $\text{IP}_3\text{R}2$ -independent (Bazargani and Attwell, 2016; Kanemaru et al., 2014; Rungta et al., 2016) and depend on other sources of Ca^{2+} , such as the mitochondria (Bazargani and Attwell, 2016; Volterra et al., 2014). Understanding the Ca^{2+} -dependent gliotransmission requires extensive studies at the level of the thinner astroglial structures to be able to record the fast and small Ca^{2+} events (Bindocci et al., 2017; Di Castro et al., 2011; Panatier et al., 2011) and it will be interesting in the future to study the role of astroglial CB_1 receptors on Ca^{2+} signals in these sub-domains.

It is currently accepted that D-serine is a major player in the modulation of synaptic plasticity and memory (Henneberger et al., 2010), but the source of D-serine at the synapse remains controversial. Some studies suggest that D-serine is not synthesized or released by astrocytes, but by neurons (Wolosker et al., 2016). Part of the debate is due to the localization of SR in the brain (Wolosker et al., 2017). While the enzyme was first proposed to be present in astrocytes (Wolosker et al., 2009), it was later shown to be mostly expressed in neurons (Wolosker et al., 2017). Therefore, the hypothesis was proposed that astrocytes produce L-serine, which is then shuttled to neurons in order to be converted by SR into D-serine and released at the synapse to modulate NMDAR activity (Wolosker et al., 2016). However, a study shows that clamping internal Ca^{2+} in individual CA1 astrocytes blocks NMDAR-LTP induction by decreasing the occupancy of the co-agonist site of the NMDARs which is restored by exogenous D-serine (Henneberger et al., 2010). Moreover, one recent paper by Papouin et al., in 2017 shows that the inhibition of SNARE-mediated vesicular release, which depends on Ca^{2+} in astrocytes abolishes D-serine oscillations during the sleep-wake cycle, suggesting that D-serine is released by astrocytes. Our results demonstrate that astrocytes *in vivo* modulate synaptic plasticity *via* astroglial CB_1 receptors and indicate that astroglial modulation of D-serine bioavailability at the synapse is a key determinant in the modulation of NMDAR-dependent LTP. Although our work might support the idea that D-serine is released from astrocytes, we cannot exclude that our results could be due to the neuronal release of D-serine. Indeed, astroglial CB_1 receptor could also be involved in the astrocyte-to-neuron shuttle of L-serine. It is currently technically impossible to determine the origin of D-serine in *in vivo* models. However, to clarify the origin of D-serine in our model, it could be interesting to use the mice in which the astroglial SNARE-dependent release is inhibited in our version of the NOR task.

DISCUSSION

Astrocytes are closely associated with synapses thanks to their intricate cellular prolongations, the PAPs, which facilitate transmitter exchange (Dallérac et al., 2013; Ghézali et al., 2016). PAPs are highly mobile during induction of LTP and their motility can depend on the physiological state of the subject (Bernardinelli et al., 2014; Panatier et al., 2006; Theodosis et al., 2008). During the induction of LTP, PAPs' motility occurs mainly towards the active synapses, leading to their enhanced astrocytic coverage (Bernardinelli et al., 2014). Interestingly, CB₁ receptor signalling has been shown to interact with cytoskeleton proteins to change neuronal morphology (Roland et al., 2014). As CB₁ receptors are also expressed in astrocytes, we could suggest that astroglial CB₁ receptors do not only modulate gliotransmission but also PAPs motility by promoting synaptic PAPs coverage, that could have two consequences. First, by increasing PAPs coverage, the eCB could bind more easily the low expressed astroglial CB₁ receptors and trigger intracellular cascades. Second, it could favor the synaptic release of D-serine, which in turn activates the post-synaptic NMDARs to trigger NMDAR-dependent LTP and memory consolidation. In the close future, thanks to the development of high resolution live microscopy such as STED, studies of the role of astroglial CB₁ receptors at the nanoscale level will bring more evidence on this putative scenario.

In vitro electrophysiology is a powerful technique to investigate synaptic plasticity. However, slicing of the brain, washing and incubating solutions, temperature used for recordings and other parameters can have important impact on the results (Accardi et al., 2016). Moreover, astrocytes in brain slices are less complex than astrocytes *in vivo* (Verkhatsky et al., 2012). In our study, it is interesting to notice that NMDAR-dependent LTP is decreased by 50% in GFAP-CB₁-KO mice *in vitro* while it is totally blocked *in vivo*. This difference can be explained by the experimental conditions such as the slicing or the temperature. First, astrocytes are connected in a network through gap-junctions and communicate to modulate metabolism and synaptic plasticity (Adermark and Lovinger, 2008; Ball et al., 2007; Bazargani and Attwell, 2016; Blomstrand et al., 2004; D'Ambrosio et al., 1998; Giaume and Theis, 2010; Houades et al., 2008; Konietzko and Müller, 1994; Rouach et al., 2008). Thus, slicing the brain might disrupt the network and impair communication between astrocytes, thereby altering physiological synaptic processes. Secondly, *in vivo*, temperature was fixed at 37°C while it was 30°C *in vitro*. Interestingly, the motility of cellular membranes depends on the temperature. Thus, it is important to keep in mind this factor since, due to technical constraints, *in vitro* electrophysiology can only be performed below the physiological temperature. For instance, low temperatures might impair normal PAPs motility towards the active synapse, thereby decreasing the efficacy of synaptic transmission. Moreover, temperature also influences CB₁-dependent signaling by modulating the rate of synthesis and degradation of eCBs (Di Marzo et al., 1994; Kreitzer and Regehr, 2001). These different parameters could explain the different results obtained either *in vitro* or *in vivo*.

Endocannabinoids are thought to be produced and delivered “on demand”. But the nature of the eCB(s) involved in the modulation of different forms of synaptic plasticity in different brain regions remain poorly documented. For instance, whereas anandamide is suggested to mediate LTD_i in the amygdala (Azad et al., 2004; Marsicano et al., 2002) and LTD of excitatory transmission in the striatum (Gerdeman et al., 2002), on demand 2-AG mobilization seems to be required for I-LTD in the

DISCUSSION

hippocampus (Chevaleyre and Castillo, 2003) but there is no evidence concerning the identity of the eCB(s) involved in LTP at the CA3-CA1 synapse. Moreover, it is still unknown how eCBs act either on neuronal or astroglial CB₁ receptors and whether the nature of the eCB is different between the two cell types. One hypothesis could be that different eCBs bind at neuronal or astroglial CB₁ receptors. This is a likely possibility, because evidence indicates that different neuronal CB₁ receptor populations have different affinities for different endocannabinoids (Turu and Hunyady, 2010). Furthermore, neuronal CB₁ receptors are mainly coupled to Gi/o proteins while astroglial CB₁ receptors are mainly coupled to Gq. Interestingly, the identity of the eCBs involved in the activation of different CB₁ receptors population seems to depend on the G proteins activated (Turu and Hunyady, 2010). Thus, the nature of the eCB binding neuronal or astroglial CB₁ receptors could be different. We did not address this issue in our study, mainly because of technical reasons. Indeed, the direct quantitative measurement of eCBs at the level of the synapse and during behavior is currently impossible. However, the development of more sophisticated tools could hopefully help addressing this important question in the future.

By using a variety of approaches, our studies revealed a physiological role of astroglial CB₁ receptors in the hippocampus. Indeed, astroglial CB₁ receptors modulate the supply of D-serine to synaptic NMDARs, eventually regulating hippocampal NMDAR-dependent synaptic plasticity and object recognition memory. Thus, our data provide a cellular mechanisms by which astroglial CB₁ receptors modulates neuron-glia interactions to control learning and memory.

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ANNEX

Annex 1

RESEARCH ARTICLE

Localization of the cannabinoid type-1 receptor in subcellular astrocyte compartments of mutant mouse hippocampus

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Abstract

Astroglial type-1 cannabinoid (CB₁) receptors are involved in synaptic transmission, plasticity and behavior by interfering with the so-called tripartite synapse formed by pre- and post-synaptic neuronal elements and surrounding astrocyte processes. However, little is known concerning the subcellular distribution of astroglial CB₁ receptors. In particular, brain CB₁ receptors are mostly localized at cells' plasmalemma, but recent evidence indicates their functional presence in mitochondrial membranes. Whether CB₁ receptors are present in astroglial mitochondria has remained unknown. To investigate this issue, we included conditional knock-out mice lacking astroglial CB₁ receptor expression specifically in glial fibrillary acidic protein (GFAP)-containing astrocytes (GFAP-CB₁-KO mice) and also generated genetic rescue mice to re-express CB₁ receptors exclusively in astrocytes (GFAP-CB₁-RS). To better identify astroglial structures by immunoelectron microscopy, global CB₁ knock-out (CB₁-KO) mice and wild-type (CB₁-WT) littermates were intra-hippocampally injected with an adeno-associated virus expressing humanized renilla green fluorescent protein (hrGFP) under the control of human GFAP promoter to generate GFAPhrGFP-CB₁-KO and -WT mice, respectively. Furthermore, double immunogold (for CB₁) and immunoperoxidase (for GFAP or hrGFP) revealed that CB₁ receptors are present in astroglial mitochondria from different hippocampal regions of CB₁-WT, GFAP-CB₁-RS and GFAPhrGFP-CB₁-WT mice. Only non-specific gold particles were detected in mouse hippocampi lacking CB₁ receptors. Altogether, we demonstrated the existence of a precise molecular architecture of the CB₁ receptor in astrocytes that will have to be taken into account in evaluating the functional activity of cannabinergic signaling at the tripartite synapse.

**KEYWORDS**

cannabinoids, glia, immunoelectron microscopy, intracellular receptors, mitochondria, tripartite synapse

1 | INTRODUCTION

Glial cells constitute the most abundant cell population in the central nervous system. The astrocytes at the tripartite synapse establish bidirectional communication with neurons by both intricate morphological non-overlapping domains (Halassa, Fellin, Takano, Dong, & Haydon, 2007) and biochemical and signaling interactions (Araque et al., 2014; Bezzi & Volterra, 2011) that play important roles in brain metabolic processes (Magistretti & Allaman, 2015), in the maintenance and regulation of synaptic physiology (Araque et al., 2014; Perez-Alvarez, Navarrete, Covelo, Martin, & Araque, 2014) and in brain information processing (Volterra & Meldolesi, 2005).

The endocannabinoid (eCB) system is composed of the seven-transmembrane G protein coupled cannabinoid type-1 (CB₁) receptor and other receptors (including CB₂ receptors), their endogenous lipid ligands (endocannabinoids) and the proteins involved in synthesis, transport and degradation of the endocannabinoids (Katona & Freund, 2012; Lutz, Marsicano, Maldonado, & Hillard, 2015; Pertwee, 2015; Piomelli, 2003). This system is widely distributed in the central and peripheral nervous system (Katona & Freund, 2012; Lu & Mackie, 2016), and also in peripheral organs (Piazza, Cota, & Marsicano, 2017), where the CB₁ receptors are also localized in mitochondria of striated and heart muscles (Mendizabal-Zubiaga et al., 2016). The eCB system regulates brain functions by acting on different cell types and cellular compartments (Busquets-García, Bains, & Marsicano, 2018; Gutiérrez-Rodríguez et al., 2017; Katona & Freund, 2012; Lu & Mackie, 2016). The activation of CB₁ receptors in astrocytes promotes astroglial differentiation (Aguado et al., 2006) and mediates neuron-astrocyte communication that plays a role in synaptic plasticity, memory and behavior (Araque et al., 2014; Gómez-Gonzalo et al., 2015; Han et al., 2012; Metna-Laurent & Marsicano, 2015; Navarrete & Araque, 2008, 2010; Navarrete, Diez, & Araque, 2014; Oliveira da Cruz, Robin, Drago, Marsicano, & Metna-Laurent, 2015). Furthermore, CB₁ receptor activation is involved in energy supply to the brain through the control of leptin receptor expression in astrocytes (Bosier et al., 2013).

The CB₁ receptor-mediated astrocyte functions are highly dependent on the CB₁ receptor distribution in astrocytes relative to close neuronal compartments, particularly at synapses (Bonilla-Del Río et al., 2017). However, the low CB₁ receptor expression in astrocytes (Bosier et al., 2013; Han et al., 2012; Kovács et al., 2017; Rodríguez, Mackie, & Pickel, 2001) and mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014a; b; 2016) constrains a consolidated picture of the subcellular CB₁ receptor distribution in the astroglial compartments that holds the anatomical substrate for a functional interaction with the nearby synapses under normal or pathological conditions (Bonilla-Del Río et al., 2017). Yet, whether intracellular CB₁ receptors exist in astroglial mitochondria has remained unknown. In the hippocampus, mitochondrial

CB₁ (mtCB₁) receptor activation affects synaptic transmission and memory formation through reduced phosphorylation of specific subunits of the complex I electron transport system, and through decreased mitochondrial respiration and mobility (Hebert-Chatelain et al., 2016). These effects are due to intra-mitochondrial G α i protein activation by mtCB₁ receptors that leads to the inhibition of soluble adenylyl cyclase and, consequently, to the decrease in intra-mitochondrial protein kinase A (PKA) activity (Hebert-Chatelain et al., 2016). New tools based on genetic rescue strategies have proven to be useful to dissect the sufficiency of the CB₁ receptors expressed in specific cell types for a particular brain function (de Salas-Quiroga et al., 2015; Gutiérrez-Rodríguez et al., 2017; Lange et al., 2017; Remmers et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014). Importantly, knock-in mice with cell type-specific rescue of CB₁ receptors in dorsal telencephalic glutamatergic neurons (Glu-CB₁-RS) or GABAergic neurons (GABA-CB₁-RS) showed that the distribution pattern and the subcellular CB₁ receptor localization is maintained as it is observed in the wild-type hippocampus (Gutiérrez-Rodríguez et al., 2017; Remmers et al., 2017).

In this study, we hypothesized that intracellular CB₁ receptors are present in astroglial mitochondria as observed in neuronal and muscular mitochondria. The GFAP-CB₁-RS rescue mice expressing the CB₁ receptor gene exclusively in the astrocytes and the GFAPhrGFP-CB₁-WT mice are ideal genetic tools to test this hypothesis. Our results show that the subcellular CB₁ receptor distribution in astrocytes in the rescue mice completely matches the endogenous CB₁ receptor expression and localization in astrocytes of the wild-type mouse hippocampus. Moreover, our findings illustrate for the first time the localization of CB₁ receptors in astroglial mitochondria.

2 | MATERIALS AND METHODS

2.1 | Animal procedures

2.1.1 | Ethics statement

Experiments were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country UPV/EHU (CEIAB/2016/074, CEEA/M20/2016/073) and the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization number, A501350). All animals were used according to the European Community Council Directive 2010/63/UE and the Spanish and French legislation (RD 53/2013 and Ley 6/2013). Maximal efforts were made in order to minimize the number and the suffering of the animals used.

2.1.2 | Conventional and conditional CB₁-KO

CB₁-KO mice were generated and genotyped as previously described (Marsicano et al., 2002). In addition, conditional CB₁ receptor mutant

mice were obtained by crossing the respective Cre expressing mouse line with $CB_1^{f/f}$ mice (Marsicano et al., 2003), using a three-step breeding protocol (Monory et al., 2006). Specifically, transgenic mice expressing the inducible version of the Cre recombinase CreERT2 under the control of the human glial fibrillary acid protein promoter, i.e. GFAP-CreERT2 mice (Hirrlinger, Scheller, Braun, Hirrlinger, & Kirchhoff, 2006) were crossed with mice carrying CB_1 receptor "floxed" sequence (Marsicano et al., 2003). As a result, transgenic mice $CB_1^{f/f;GFAP-CreERT2}$ were obtained (Han et al., 2012).

2.1.3 | Generation of GFAP- CB_1 -RS

STOP- CB_1 mice were previously generated by inserting a loxP-flanked stop cassette into the 5'UTR of the coding exon of the CB_1 gene, 32 nucleotides upstream of the translational start codon (Ruehle et al., 2013). The STOP- CB_1 mice were crossed with GFAP-CreERT2 mice (Hirrlinger, Scheller, Braun, Hirrlinger, & Kirchhoff, 2006) to obtain $CB_1^{stop/stop;GFAP-CreERT2}$ mice.

Seven to nine-week-old $CB_1^{f/f;GFAP-CreERT2}$ and $CB_1^{f/f}$ littermates, as well as $CB_1^{stop/stop;GFAP-CreERT2}$ and $CB_1^{stop/stop}$ littermates were treated daily for eight consecutive days with 1 mg/kg (i.p.) of either tamoxifen or 4OH-tamoxifen synthesized as previously reported (Detsi, Koufaki, & Calogeropoulou, 2002; Yu & Forman, 2003) to induce the Cre-dependent astroglial deletion of CB_1 (GFAP- CB_1 -KO and GFAP- CB_1 -WT littermate mice) or its exclusive astroglial re-expression (rescue, GFAP- CB_1 -RS and STOP- CB_1 littermates). Mice were used for immunocytochemistry 3–5 weeks after the last day of tamoxifen or 4OH-tamoxifen injections.

2.1.4 | Generation of GFAPhrGFP- CB_1 -WT and GFAPhrGFP- CB_1 -KO mice

Intrahippocampal injection of a recombinant adeno associated virus expressing hrGFP under the control of the human GFAP promoter (von Jonquieres et al., 2013) were performed in CB_1 -WT and CB_1 -KO mice to generate GFAPhrGFP- CB_1 -WT and GFAPhrGFP- CB_1 -KO, respectively. The vector backbone was the pAAV-GFAP-hChr2(H134R)-EYFP kindly provided by Karl Deisseroth (Stanford University, CA, USA). We replaced the hChr2(H134R)-EYFP with the cDNA encoding for hrGFP by using standard molecular cloning techniques. The virus production and purification, as well as the injection procedure were performed as previously described (Chiarlone et al., 2014). Coordinates for intrahippocampal injections were: anteroposterior – 2.0 mm, medio-lateral \pm 1.5 mm, dorsoventral – 2 mm relative to bregma. Mice were allowed to recover for at least 4 weeks after surgery before their anatomical characterization.

2.1.5 | Tissue isolation

Mice were housed under standard conditions (*ad libitum* food and water; 12hr/12hr light/dark cycle). CB_1 -WT, GFAP- CB_1 -RS, GFAP- CB_1 -KO, CB_1 -KO, STOP- CB_1 , GFAPhrGFP- CB_1 -WT and GFAPhrGFP- CB_1 -KO mice (3 animals of each condition) were deeply anesthetized by intraperitoneal injection of ketamine/xylazine (80/10 mg/kg body weight) and transcardially perfused at room temperature (RT, 20°C–25°C) with phosphate buffered saline (.1 M PBS, pH 7.4) for 20 s,

followed by the fixative solution (4% formaldehyde freshly depolymerized from paraformaldehyde, .2% picric acid and .1% glutaraldehyde) in PBS (.1 M, pH 7.4) for 10–15 min. Brains were removed from the skull and post-fixed in the fixative solution for about 1 week at 4°C and stored at 4°C in 1:10 diluted fixative solution until use.

2.1.6 | Double pre-embedding immunogold and immunoperoxidase method for electron microscopy

Coronal hippocampal vibrosections were cut at 50 μ m and collected in phosphate buffer (.1 M PB, pH 7.4) with .1% sodium azide at RT. They were transferred and pre-incubated in a blocking solution of 10% bovine serum albumin (BSA), .1% sodium azide and .02% saponine prepared in Tris-hydrogen chloride buffered saline 1 \times (TBS), pH 7.4 for 30 min at RT. Then, the CB_1 -WT, GFAP- CB_1 -RS, GFAP- CB_1 -KO, CB_1 -KO and STOP- CB_1 tissue sections were incubated with the primary goat polyclonal anti- CB_1 receptor antibody (2 μ g/ml, #CB1-Go-Af450, Frontier Institute Co. Ltd, Ishikari, Hokkaido, Japan, RRID: AB_257130) and mouse monoclonal anti-GFAP antibody (1:1,000, #G3893, Sigma-Aldrich, St. Louis, MO, USA, RRID: AB_477010) diluted in 10% BSA/TBS containing .1% sodium azide and .004% saponine on a shaker for 2 days at 4°C. In parallel, GFAPhrGFP- CB_1 -WT and GFAPhrGFP- CB_1 -KO hippocampi were incubated with the same primary goat polyclonal anti- CB_1 receptor antibody as above and rabbit polyclonal anti-hrGFP antibody (1:500, #240142-51, Stratagene-Agilent, Santa Clara, CA, USA, RRID: AB_10598674) in 10% BSA/TBS with .1% sodium azide and .004% saponine for 2 days at 4°C.

The tissue was incubated after several washes in 1% BSA/TBS with the corresponding biotinylated secondary antibody (1:200 biotinylated anti-mouse, BA-2000; RRID:AB_2313581, and 1:200 biotinylated anti-rabbit BA-1000; RRID:AB_2313606, Vector Labs, Burlingame, CA, USA) in 1% BSA/TBS with .004% saponine for 3 hr at RT. The sections were washed in 1% BSA/TBS overnight on a shaker at 4°C, incubated with the secondary 1.4 nm gold-labeled immunoglobulin-G antibody (Fab' fragment, 1:100, Nanoprobes Inc., Yaphank, NY, USA) in 1% BSA/TBS with .004% saponine on a shaker for 3 hr at RT, washed in 1% BSA/TBS and subsequently incubated in the avidin-biotin complex (1:50; PK-7100, Vector Labs, Burlingame, CA, USA) diluted in the wash solution for 1.5 hr. After washing the sections in 1% BSA/TBS overnight at 4°C, they were post-fixed with 1% glutaraldehyde in TBS for 10 min and washed in double-distilled water. Then, the gold particles were silver-intensified with a HQ Silver kit (Nanoprobes Inc., Yaphank, NY, USA) for about 12 min in the dark, washed in .1 M PB (pH 7.4) and subsequently incubated in .05% diaminobenzidine (DAB) and .01% hydrogen peroxide prepared in .1 M PB for 3 min. Finally, the sections were osmicated (1% osmium tetroxide, in .1 M PB pH 7.4) for 20 min, washed in .1 M PB (pH 7.4), dehydrated in graded alcohols (50%–100%) to propylene oxide and plastic-embedded in Epon resin 812. 50–60 nm-ultrathin sections were cut with a diamond knife (Diatome USA), collected on nickel mesh grids or on formvar-coated single slot grids for serial sectioning, stained with 2.5% lead citrate, and examined with a Philips EM208S electron microscope. Tissue preparations were photographed by means of a digital camera coupled to the electron microscope. Minor adjustments in contrast and



brightness were made to the figures using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). GIMP (GNU Project) and Adobe Photoshop were used to blend the electron micrographs into the serial photocomposition.

2.1.7 | Semi-quantification of the CB₁ receptor immunogold and immunoperoxidase staining

The pre-embedding immunogold and immunoperoxidase methods were simultaneously applied and repeated three times on the sections obtained from each of the three individual CB₁-WT, GFAP-CB₁-RS, GFAP-CB₁-KO, CB₁-KO, STOP-CB₁, GFAPhrGFP-CB₁-WT and GFAPhrGFP-CB₁-KO animals studied. Immunogold-labeling was visualized on the hippocampal sections with a light microscope and portions of the CA1 stratum radiatum and the dentate molecular layer with good and consistent CB₁ receptor immunolabeling were identified and trimmed down for ultrathin sectioning. Three to four semi-thin sections (1 μm-thick) were then cut with a histo-diamond knife (Diatome USA) and stained with 1% toluidine blue. To further standardize the conditions, only the first 20 ultrathin sections (60 nm thick) were cut, collected onto the grids and photographed. The electron micrographs were taken at 18,000× with a Digital Morada Camera from Olympus (Hamburg, Germany). Sampling was always carefully and accurately carried out in the same way for all the animals studied and it was blinded to experimenters during CB₁ receptor quantification.

Positive astrocytic processes were identified by the presence of DAB immunodeposits. Positive CB₁ receptor labeling was considered if at least one immunoparticle was within ~30 nm of the plasmalemma or outer mitochondrial membranes. Furthermore, only particles on mitochondrial membrane segments far away from other astrocytic membranes (distance ≥80 nm) and well distinct from the astrocytic intermediate filaments or any other intracellular organelle membranes were taken into account for mitochondrial localization. Image-J software (NIH; RRID:SCR_003070) was used to measure the membrane length. Percentages of CB₁ receptor positive profiles (astrocytic processes and mitochondria), density (particles/μm membrane), the proportion of CB₁ receptor particles in astrocytes versus total CB₁ receptor expression and the proportion of CB₁ receptor particles in terminals versus total CB₁ receptor expression in plasmalemma, were analyzed and displayed as mean ± SEM using a statistical software package (GraphPad Prism 5, GraphPad Software Inc, San Diego, USA; RRID:SCR_002798). The normality test (Kolmogorov-Smirnov normality test) was applied before statistical tests and subsequently data were analyzed using nonparametric tests (Mann-Whitney *U* test when *k* = 2 or Kruskal-Wallis test when *k* > 2). Potential variability between animals of the same mutant mouse line was assessed statistically. Because no differences were detected, all data within each mouse line were pooled.

2.1.8 | Semi-quantification of the distance from the CB₁ receptor particles in astroglial mitochondria to the nearest synapse

Image-J software was used to measure the distance between the CB₁ receptor immunogold particles on the astrocytic mitochondria and the nearest synapse on single 60 nm-thick sections. Data were tabulated,

analyzed and displayed as mean ± SEM using GraphPad Prism 5 software.

3 | RESULTS

3.1 | Subcellular CB₁ receptor localization in the mutant mice

Astrocytes and their processes were identified by DAB immunodeposits of GFAP or hrGFP and the CB₁ receptor was detected by immunogold labeling. As expected, the CB₁ receptor was mainly localized on neuronal terminals, preterminal membranes and, to a lesser extent, on GFAP-labeled astrocytes. CB₁ receptor-immunopositive synaptic terminals followed in serial ultrathin sections obtained from the CA1 (Figure 1) and dentate molecular layer (Figure 2) of the CB₁-WT mouse could be found adjacent to double-labeled GFAP and CB₁ receptor-immunopositive astrocytic processes (Figures 1–3a and 4a) that also contained CB₁ receptor-immunopositive mitochondria (Figures 1–4). In GFAP-CB₁-RS hippocampus, the CB₁ receptor immunolabeling was restricted to the DAB-containing astrocytic elements and no labeling was found on axon boutons (Figures 3c and 4c). Conversely, the CB₁ receptor particles in the GFAP-CB₁-KO hippocampus were only on synaptic terminals but not in astrocytic processes (Figures 3d and 4d). Also, CB₁ receptor immunoparticles were found in neuronal mitochondria but not in mitochondria of astrocytes in the GFAP-CB₁-KO (Figure 3d). Finally, the subcellular distribution of the CB₁ receptor on synaptic terminals and astrocytic elements of the GFAPhrGFP-CB₁-WT resembled the CB₁-WT hippocampus (Figures 3f,g and 4f,g). Importantly, this CB₁ receptor staining pattern was absent in CB₁-KO (Figures 3b and 4b), STOP-CB₁ (Figures 3e and 4e) and GFAPhrGFP-CB₁-KO mice (Figures 3h and 4h).

3.2 | CB₁ receptor assessment in astrocytes of the CA1 stratum radiatum

The percentage of the CB₁ receptor immunopositive astrocytic processes in the CA1 stratum radiatum of the GFAP-CB₁-RS (37.12% ± 3.79%) was not statistically different (*p* > .05; Figure 5a) relative to the CB₁-WT mouse (42.06% ± 3.56%), however the proportion in the GFAPhrGFP-CB₁-WT was significantly higher (59.91% ± 3.29%; ****p* < .001; Figure 5a). Only background metal particles were found in CA1 astrocytes of the STOP-CB₁, GFAP-CB₁-KO, CB₁-KO and GFAPhrGFP-CB₁-KO mice (****p* < .001; Figure 5a).

The density of CB₁ receptor immunoparticles on astrocytic membranes (particles/μm) was also analyzed (Figure 5b). Similar densities were detected in the GFAP-CB₁-RS (.128 ± .020) and the CB₁-WT (.135 ± .019; *p* > .05; Figure 5b) however the density was much higher in the GFAPhrGFP-CB₁-WT (.384 ± .039; ****p* < .001; Figure 5b). Only residual non-specific particles were observed in the STOP-CB₁ (.005 ± .003), GFAP-CB₁-KO (.005 ± .003), CB₁-KO (.001 ± .001) and GFAPhrGFP-CB₁-KO mice (.004 ± .002; ****p* < .001; Figure 5b).

5.31% ± .84% of the total CB₁ receptor labeling in the CB₁-WT, 11.97% ± 2.17% in the GFAPhrGFP-CB₁-WT (*p* > .05; Figure 5c) and

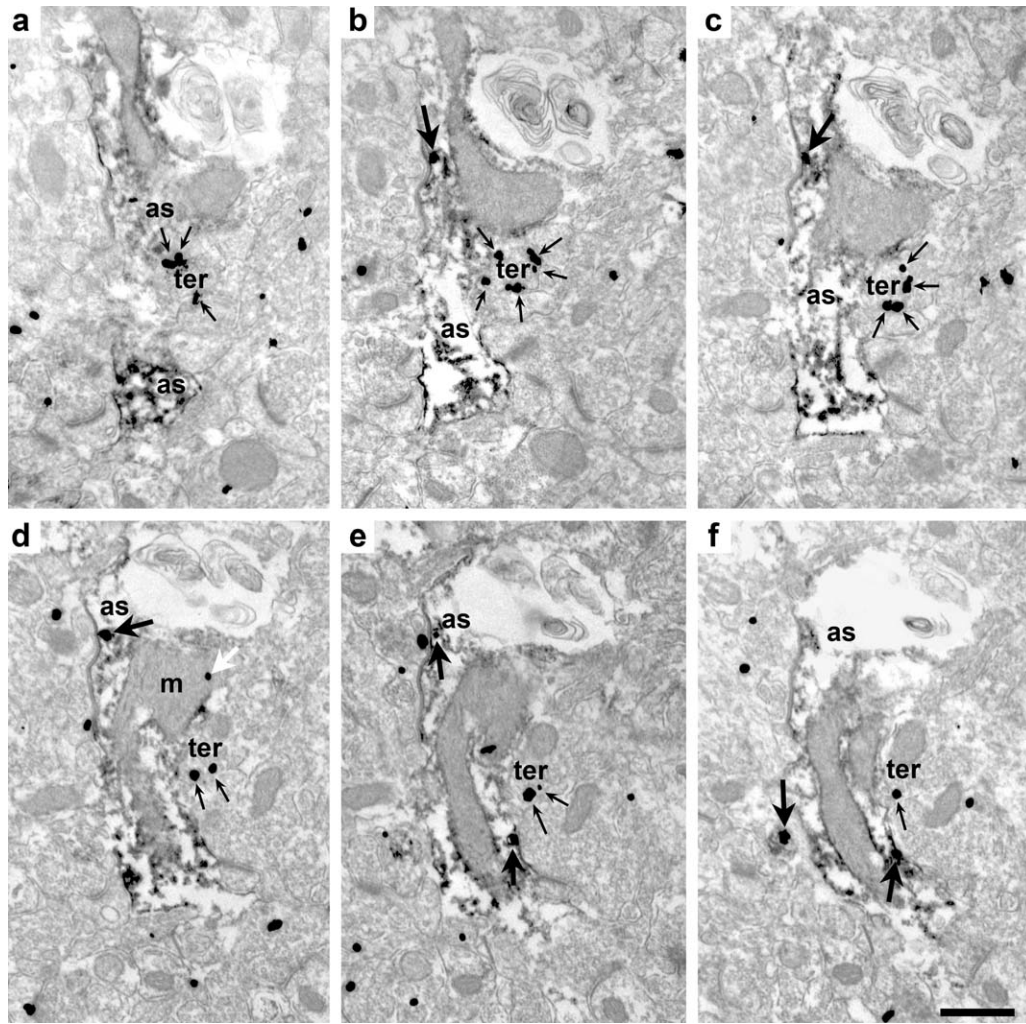


FIGURE 1 Follow up of a CB_1 receptor positive astrocytic process in the CA1 stratum radiatum of CB_1 -WT. Double pre-embedding immunogold (CB_1 receptor) and immunoperoxidase (GFAP) method for electron microscopy. Serial ultrathin sections showing a GFAP positive (DAB immunodeposits) astrocytic process (as) with a few CB_1 receptor immunoparticles on the astrocytic membrane throughout the reconstruction (b–f). In the astrocyte, CB_1 receptor labeling is also observed on the mitochondrial membrane (d). A CB_1 receptor-positive terminal (ter) is closely associated to the astrocytic process. Black thin arrows: neuronal CB_1 receptor labeling; black thick arrows: astrocytic CB_1 receptor labeling; white arrow: mitochondrial CB_1 receptor labeling in astrocyte; as: astrocytic process; ter: axon terminal; m: CB_1 receptor-positive mitochondria in astrocyte. Scale bar: 0.5 μ m

95.31% \pm 1.87% in the GFAP- CB_1 -RS were in astrocytic processes ($***p < .001$; Figure 5c). Only background immunoparticles were detected in astrocytic processes of the STOP- CB_1 , GFAP- CB_1 -KO, CB_1 -KO and GFAPhrGFP- CB_1 -KO ($***p < .001$; Figure 5c). As a comparison, 65.52% \pm 2.44% of the total CB_1 receptor gold particles in the CB_1 -WT, 75.13% \pm 4.06% in the GFAP- CB_1 -KO and 56.32% \pm 2.73% in the GFAPhrGFP- CB_1 -WT were distributed on synaptic terminals ($p > .05$; Figure 5d). Scattered metal particles were found in GFAP- CB_1 -RS, STOP- CB_1 , CB_1 -KO and GFAPhrGFP- CB_1 -KO mice ($***p < .001$; Figure 5d).

3.3 | CB_1 receptor assessment in astrocytes of the dentate molecular layer

The proportion of the CB_1 receptor immunopositive astrocytic processes in the GFAP- CB_1 -RS (39.84% \pm 3.50%) and the CB_1 -WT

(44.67% \pm 3.85%) was statistically similar ($p > .05$; Figure 6a), but it was significantly higher in the GFAPhrGFP- CB_1 -WT group (59.99% \pm 3.37%; $**p < .01$; Figure 6a). Particles were virtually undetectable in the STOP- CB_1 , GFAP- CB_1 -KO, CB_1 -KO and GFAPhrGFP- CB_1 -KO mice ($***p < .001$; Figure 6a).

The CB_1 receptor density (particles/ μ m) on astrocytic membranes did not differ statistically between the GFAP- CB_1 -RS (.138 \pm .016) and the CB_1 -WT (.112 \pm .011; $p > .05$; Figure 6b) but it was higher in the GFAPhrGFP- CB_1 -WT group (.334 \pm .033; $***p < .001$; Figure 6b). Negligible particle numbers were noticed in the STOP- CB_1 (.006 \pm .003), GFAP- CB_1 -KO (.006 \pm .003), CB_1 -KO (.004 \pm .002) and GFAPhrGFP- CB_1 -KO (.002 \pm .002; $***p < .001$; Figure 6b).

Of the total CB_1 receptor labeling, 5.35% \pm 1.00% in the CB_1 -WT, 13.13% \pm 2.60% in the GFAPhrGFP- CB_1 -WT ($P > .05$; Figure 6c) and 95.61% \pm 1.56% in the GFAP- CB_1 -RS was in astrocytes ($***P < .001$; Figure 6c). Non-specific CB_1 receptor immunoparticles were found on

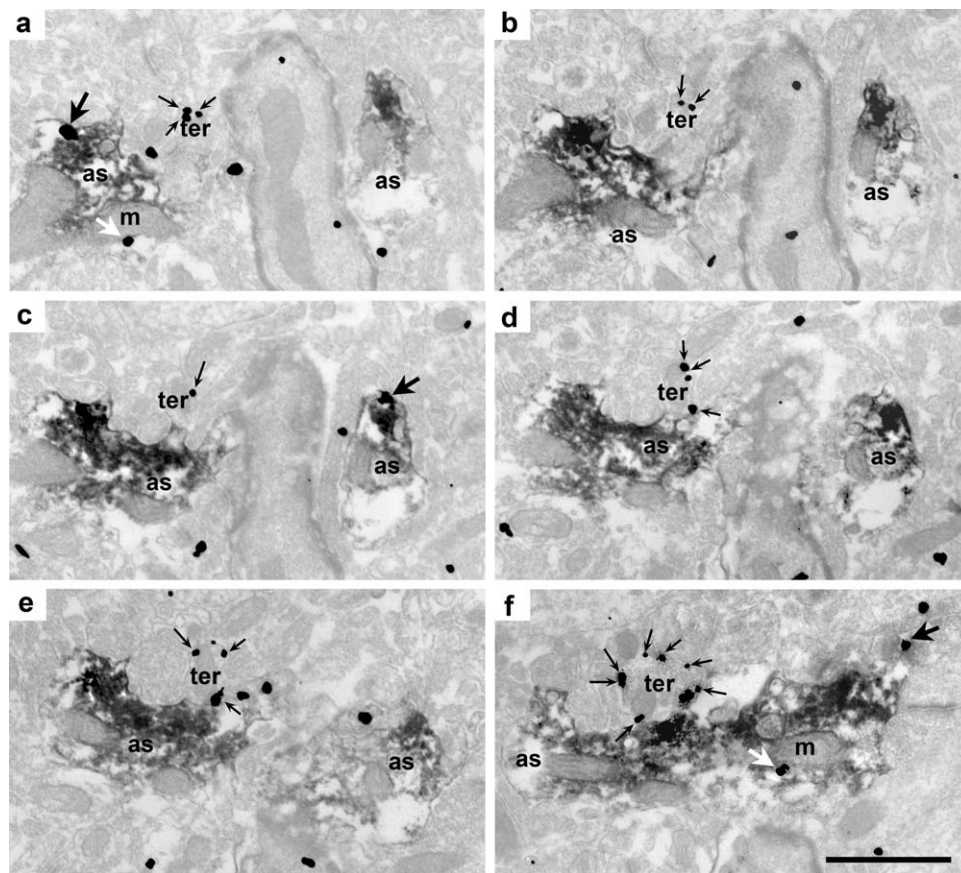


FIGURE 2 Follow up of a CB₁ receptor-positive astrocytic process in the dentate molecular layer of CB₁-WT. Double pre-embedding immunogold (CB₁ receptor) and immunoperoxidase (GFAP) method for electron microscopy. Serial ultrathin sections showing a GFAP positive (DAB immunodeposits) astrocytic process (as) with scattered CB₁ receptor immunoparticles on the astrocytic (a,c,f) and mitochondrial (a,f) membranes. A CB₁ receptor-positive synaptic terminal (ter) is related to the astrocytic process. Black thin arrows: neuronal CB₁ receptor labeling; black thick arrows: astrocytic CB₁ receptor labeling; white arrows: mitochondrial CB₁ receptor labeling in astrocyte; as: astrocytic process; ter: axon terminal; m: CB₁ receptor-positive mitochondria in astrocyte. Scale bar: 1 μm

astrocytic processes in the STOP-CB₁, GFAP-CB₁-KO, CB₁-KO and GFAPhrGFP-CB₁-KO mice (***P* < .001; Figure 6c). Conversely, 64.27% ± 2.88% of the total CB₁ receptor labeling in the CB₁-WT, 76.17% ± 4.70% in the GFAP-CB₁-KO and 57.17% ± 2.19% in the GFAPhrGFP-CB₁-WT was located on synaptic terminals (*P* > .05; Figure 6d). Residual metal particles were detected in the GFAP-CB₁-RS, STOP-CB₁, CB₁-KO and GFAPhrGFP-CB₁-KO (***P* < .001; Figure 6d).

3.4 | CB₁ receptor localization in astroglial mitochondria

CB₁ receptor labeling was observed in mitochondria (mtCB₁ receptors) of astrocytes distributed throughout the CA1 stratum radiatum (Figures 1d and 3a,c,g) and dentate molecular layer (Figures 2a,f and 4a,c,f,g). In CB₁-WT mice, 11.12% ± 1.80% of the astrocytic mitochondrial sections in the CA1 stratum radiatum and 11.56% ± 2.33% in the dentate molecular layer were CB₁ receptor immunopositive (Figures 7a,b). The percentage was roughly similar in GFAP-CB₁-RS (CA1 stratum radiatum: 12.39% ± 1.81% (*p* > .05; Figure 7a); dentate molecular layer: 11.48% ± 1.76% (*p* > .05; Figure 7b) and GFAPhrGFP-CB₁-WT (CA1 stratum radiatum: 13.12% ± 2.53% (*P* > .05; Figure 7a); dentate

molecular layer: 13.74% ± 3.20% (*p* > .05; Figure 7b). Non-specific mitochondrial particles were detected in STOP-CB₁ (CA1 stratum radiatum: 4.66% ± 1.55%, ***p* < .01; Figure 7a; dentate molecular layer: 5.38% ± 1.22%, **p* < .05; Figure 7b), GFAP-CB₁-KO (CA1 stratum radiatum: 3.97% ± 1.70%, ***p* < .01; Figure 7a; dentate molecular layer: 3.04% ± 1.04%, ***p* < .01; Figure 7b), CB₁-KO (CA1 stratum radiatum: 2.97% ± 1.15%, ****p* < .001; Figure 7a; dentate molecular layer: 2.49% ± .80%, ****p* < .001; Figure 7b) and GFAPhrGFP-CB₁-KO mice (CA1 stratum radiatum: .95% ± .95%, ****p* < .001; Figure 7a; dentate molecular layer: 1.98% ± .91%, ****p* < .001; Figure 7b).

3.5 | Distance from the astroglial mtCB₁ receptors to the nearest synapse

The distance between the astrocytic mtCB₁ receptor particles and the midpoint of the nearest synapse was assessed in CB₁-WT, GFAP-CB₁-RS and GFAPhrGFP-CB₁-WT hippocampi (Figure 8; table 1). In the CA1, 10.55% ± 4.01% of the total synapses analyzed were in a range of 0–400 nm from the astrocytic mtCB₁ receptor particles in CB₁-WT, 2.67% ± 2.67% in GFAP-CB₁-RS and 7.41% ± 3.70% in GFAPhrGFP-CB₁-WT. 38.54% ± 8.32% of the synapses were located between 400

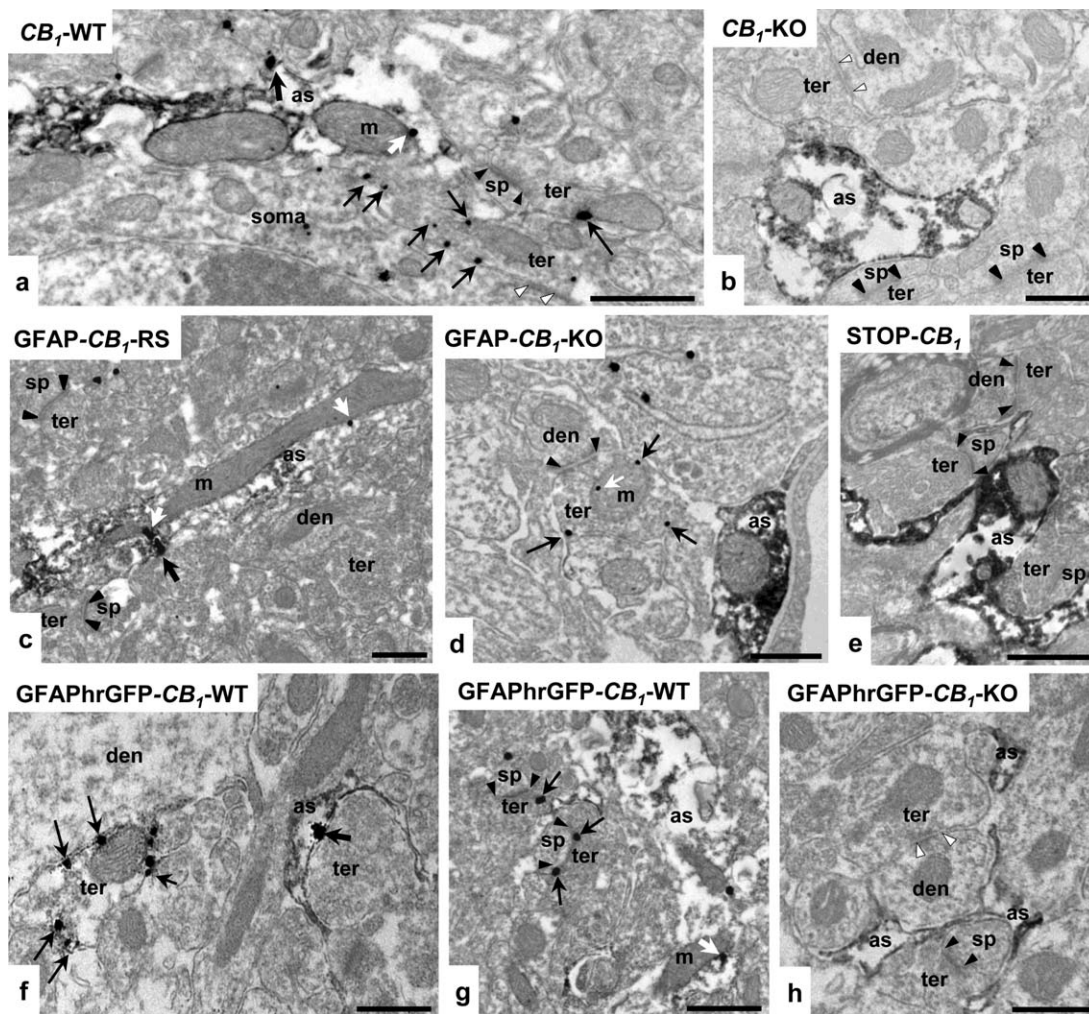


FIGURE 3 CB_1 receptor localization in identified astrocytes and astrocytic mitochondria in the CA1 stratum radiatum of mutant mice. Pre-embedding immunogold and immunoperoxidase method for electron microscopy. In CB_1 -WT (a), CB_1 receptor immunoparticles are localized on membranes of astrocytic processes. Mitochondrial CB_1 receptor labeling is also visualized in identified astrocytes of CB_1 -WT (a). As expected, CB_1 receptor immunoparticles are also on membranes of synaptic terminals and preterminals (a). No CB_1 receptor immunolabeling is detected in CB_1 -KO (b), confirming the specificity of the CB_1 receptor antibody. Astrocytic processes, but not axon terminals, are CB_1 receptor immunopositive in GFAP- CB_1 -RS (c). Note in this mutant, CB_1 receptor labeling on the outer membrane of an astrocytic mitochondrion (c). CB_1 receptor particles are found in synaptic terminals and neuronal mitochondria, but not in astrocytes and astrocytic mitochondria, of GFAP- CB_1 -KO (d). No CB_1 receptor immunoparticles are observed in STOP- CB_1 (e). In GFAPhrGFP- CB_1 -WT (f and g), presynaptic terminals and astrocytic processes are CB_1 receptor positive. Mitochondrial CB_1 receptor labeling is also visualized in identified astrocytes (g). No CB_1 receptor immunolabeling is detected in GFAPhrGFP- CB_1 -KO (h). Black arrowheads: excitatory synapses; white arrowheads: inhibitory synapses; black thin arrows: neuronal CB_1 receptor immunoparticles; black thick arrows: astrocytic CB_1 receptor immunoparticles; white thick arrows: mitochondrial CB_1 receptor labeling in astrocytes; white thin arrows: mitochondrial CB_1 receptor labeling in neurons; as: astrocytic processes; ter: terminal; den: dendrite; sp: dendritic spine; m: CB_1 receptor-positive astroglial/neuronal mitochondria. Scale bars: 0.5 μ m

and 800 nm in CB_1 -WT, $49.28\% \pm 2.87\%$ in GFAP- CB_1 -RS and $51.85\% \pm 3.70\%$ in GFAPhrGFP- CB_1 -WT. 29.51% $\pm 6.85\%$ of the synapses were detected between 800 and 1,200 nm in CB_1 -WT, $37.26\% \pm 2.02\%$ in GFAP- CB_1 -RS and $29.63\% \pm 7.41\%$ in GFAPhrGFP- CB_1 -WT. Finally, $21.40\% \pm 5.56\%$ of the synapses were found at more than 1,200 nm from the astrocytic mt CB_1 receptor in CB_1 -WT, $10.79\% \pm 2.94\%$ in GFAP- CB_1 -RS and $14.81\% \pm 7.41\%$ in GFAPhrGFP- CB_1 -WT (Figure 8; Table 1). In the dentate molecular layer, 11.11% $\pm 6.42\%$ of the total synapses analyzed were at 0–400 nm in CB_1 -WT, 2.82% $\pm 1.48\%$ in GFAP- CB_1 -RS and 1.52% \pm

1.52% in GFAPhrGFP- CB_1 -WT. 50% $\pm 3.21\%$ of the synapses were located at a distance of between 400 and 800 nm from the astrocytic mt CB_1 immunoparticle in CB_1 -WT, $47.57\% \pm 4.81\%$ in GFAP- CB_1 -RS and $57.37\% \pm 6.26\%$ in GFAPhrGFP- CB_1 -WT. 23.15% $\pm .93\%$ of them were located between 800 and 1,200 nm in CB_1 -WT, $43.79\% \pm 3.13\%$ in GFAP- CB_1 -RS and $35.86\% \pm 2.53\%$ in GFAPhrGFP- CB_1 -WT. Finally, 18.52% $\pm 3.70\%$ of the synapses in CB_1 -WT, 11.82% $\pm 3.51\%$ in GFAP- CB_1 -RS and 5.25% $\pm 2.72\%$ in GFAPhrGFP- CB_1 -WT were observed at more than 1,200 nm from the astrocytic mt CB_1 receptor particles (Figure 8; Table 1).

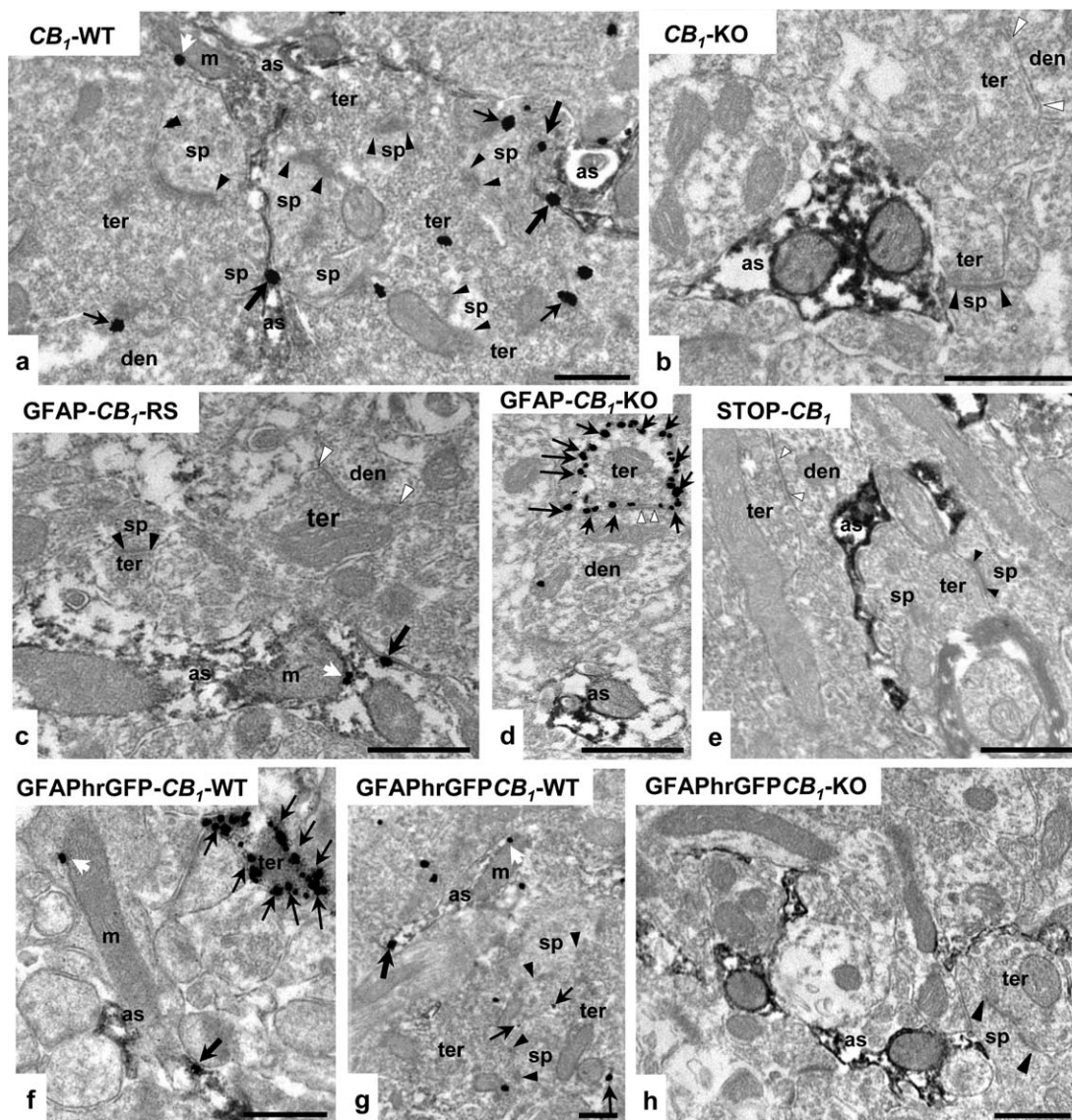


FIGURE 4 CB_1 receptor localization in identified astrocytes and astrocytic mitochondria in the dentate molecular layer of mutant mice. Pre-embedding immunogold and immunoperoxidase method for electron microscopy. In CB_1 -WT, CB_1 receptor immunoparticles are localized on membranes of presynaptic terminals, astrocytic processes as well as on mitochondrial membranes of identified astrocytes (a). Importantly, the CB_1 receptor labeling is absent in CB_1 -KO (b). In $GFAP-CB_1$ -RS, CB_1 receptor gold particles are only detected on astrocytes and astrocytic mitochondria but not on neuronal synaptic compartments (c). Conversely, CB_1 receptor immunolabeling is only present on synaptic boutons and not on astrocytes of $GFAP-CB_1$ -KO (d). The CB_1 receptor labeling is not observed in the $STOP-CB_1$ mouse (e). In $GFAPhrGFP-CB_1$ -WT, CB_1 receptor immunoparticles are localized on membranes of presynaptic terminals, astrocytic processes and astrocytic mitochondria (f and g). No CB_1 receptor immunolabeling is detected in $GFAPhrGFP-CB_1$ -KO (h). Black arrowheads: excitatory synapses; white arrowheads: inhibitory synapses; black thin arrows: neuronal CB_1 receptor immunoparticles; black thick arrows: astrocytic CB_1 receptor immunoparticles; white arrows: mitochondrial CB_1 receptor labeling in astrocytes; as: astrocytic processes; ter: terminal; den: dendrite; sp: dendritic spine; m: CB_1 receptor-positive astrocytic mitochondria. Scale bars: 0.5 μ m

4 | DISCUSSION

The high CB_1 receptor expression in the hippocampus is unevenly distributed between subcellular compartments of GABAergic and glutamatergic synaptic terminals, astrocytes and neuronal mitochondria (Bénard et al., 2012; Gutiérrez-Rodríguez et al., 2017; Han et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016; Katona & Freund, 2012; Lu & Mackie, 2016; Marsicano & Lutz, 1999; Steindel et al., 2013). However, no information is available to date whether the CB_1 receptor localizes

in astroglial mitochondria as it does in mitochondria of hippocampal GABAergic and glutamatergic neurons (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016). In order to address this, we used conditional CB_1 receptor rescue mice re-expressing the CB_1 receptor exclusively in astrocytic GFAP expressing cells ($GFAP-CB_1$ -RS), as well as CB_1 -WT and CB_1 -KO mice expressing hrGFP (De Francesco et al., 2015; Hadaczek et al., 2009; Kerr et al., 2015; Navarro-Galve et al., 2005; Ward & Cormier, 1979) under the control of the GFAP promoter ($GFAPhrGFP-CB_1$ -WT and $GFAPhrGFP-CB_1$ -KO, respectively). As a

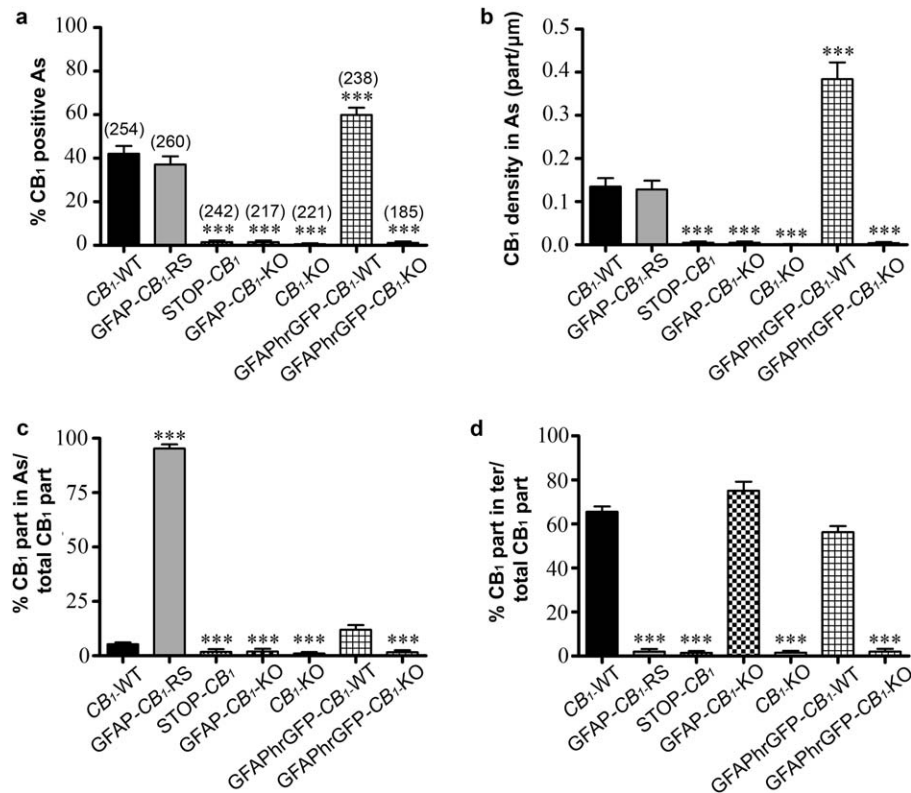


FIGURE 5 Statistical assessment of the CB₁ receptor distribution on astrocytes in the CA1 stratum radiatum of the mutant mice. (a). Percentages of CB₁ receptor immunopositive astrocytic processes in CB₁-WT (42.06% ± 3.56%) and GFAP-CB₁-RS (37.12% ± 3.79%) do not show statistical differences. The proportion of 59.91% ± 3.29% in GFAPhrGFP-CB₁-WT is statistically significant. Only residual background is found in STOP-CB₁ (1.46% ± .78%), GFAP-CB₁-KO (1.45% ± .77%), CB₁-KO (1.54% ± .39%) and GFAPhrGFP-CB₁-KO (1.16% ± .67%). The number of astrocytic processes examined is in parentheses on the top of each column. (b). CB₁ receptor immunoparticle density on membranes of astrocytic processes (particles/μm). Densities in CB₁-WT (.135 ± .019) and GFAP-CB₁-RS (.128 ± .020) are statistically similar, whereas a significant increase in particle density is found in GFAPhrGFP-CB₁-WT (.384 ± .039). Non-specific particles are detected in STOP-CB₁ (.005 ± .003), GFAP-CB₁-KO (.005 ± .003), CB₁-KO (.001 ± .001) and GFAPhrGFP-CB₁-KO (.004 ± .002). (c) Proportion of CB₁ receptor gold particles on astrocytic membranes versus total CB₁ receptor expression on plasmalemma: 5.31% ± .84% of the total CB₁ receptor immunoparticles are located in astrocytes of CB₁-WT and 95.31% ± 1.87% in astrocytes of GFAP-CB₁-RS. Only residual CB₁ immunoparticles are in astrocytic processes of STOP-CB₁ (1.76% ± 1.29%), GFAP-CB₁-KO (1.96% ± 1.28%), CB₁-KO (1.02% ± .72%) and GFAPhrGFP-CB₁-KO (1.62% ± .94%). (d) Proportion of immunogold particles on synaptic terminals versus total CB₁ receptor expression on plasmalemma: 65.52% ± 2.44% (CB₁-WT), 75.13% ± 4.06% (GFAP-CB₁-KO), and 56.32% ± 2.73% (GFAPhrGFP-CB₁-WT). Residual CB₁ receptor immunoparticles are in astrocytes of GFAP-CB₁-RS (2.02% ± 1.17%), STOP-CB₁ (1.47% ± .84%), CB₁-KO (1.52% ± .87%) and GFAPhrGFP-CB₁-KO (2.08% ± 1.19%). Data are expressed as mean ± SEM of three different animals. Data were analyzed by means of Kruskal-Wallis Test and the Dunn's Multiple Comparison Post-hoc test. ****p* < .001; ***p* < .01; **p* < .05. As: astrocytic processes; ter: terminal; part: immunoparticles

first step, we determined the CB₁ receptor expression and distribution in the conditional mutant mice in order to draw the level of agreement with the CB₁ receptor expression pattern in the CB₁-WT mice. The combined pre-embedding immunogold and immunoperoxidase method applied in this study has been previously proven to be an excellent approach for the localization of the CB₁ receptor in astrocytes (Bonilla-Del Río et al., 2017; Bosier et al., 2013; Han et al., 2012) and mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016). Specificity control experiments of the CB₁ receptor antibodies were carried out in CB₁-KO and STOP-CB₁ mice (carrying a loxP-flanked stop cassette inserted into the sequences of the 5'UTR of the CB₁ receptor). According to recent observations, we detected very low levels of metal particle deposits in STOP-CB₁ (Remmers et al., 2017) and scattered background particles in CB₁-KO.

The results showed that the proportion and density of the CB₁ receptor immunolabeling (particles/μm) of astrocytic processes in the hippocampus were not significantly different between GFAP-CB₁-RS and CB₁-WT. The percentage of immunopositive astrocytes in CA1 of CB₁-WT was in the range of the previous values reported by our group (Bonilla-Del Río et al., 2017; Han et al., 2012), and almost all of the CB₁ receptor labeling was expressed in astrocytic elements in GFAP-CB₁-RS. Furthermore, the proportion of astrocytic processes expressing CB₁ receptors and the density of receptor particles were about 34% and 64% higher, respectively, in the mutant mice targeted to express hrGFP in astroglial cells (GFAPhrGFP-CB₁-WT) than in GFAP-CB₁-RS. These results suggest that the CB₁ receptor expression in astrocytes could actually be higher than previously reported using the astrocytic GFAP marker (Bosier et al., 2013; Han et al., 2012), because the GFAP

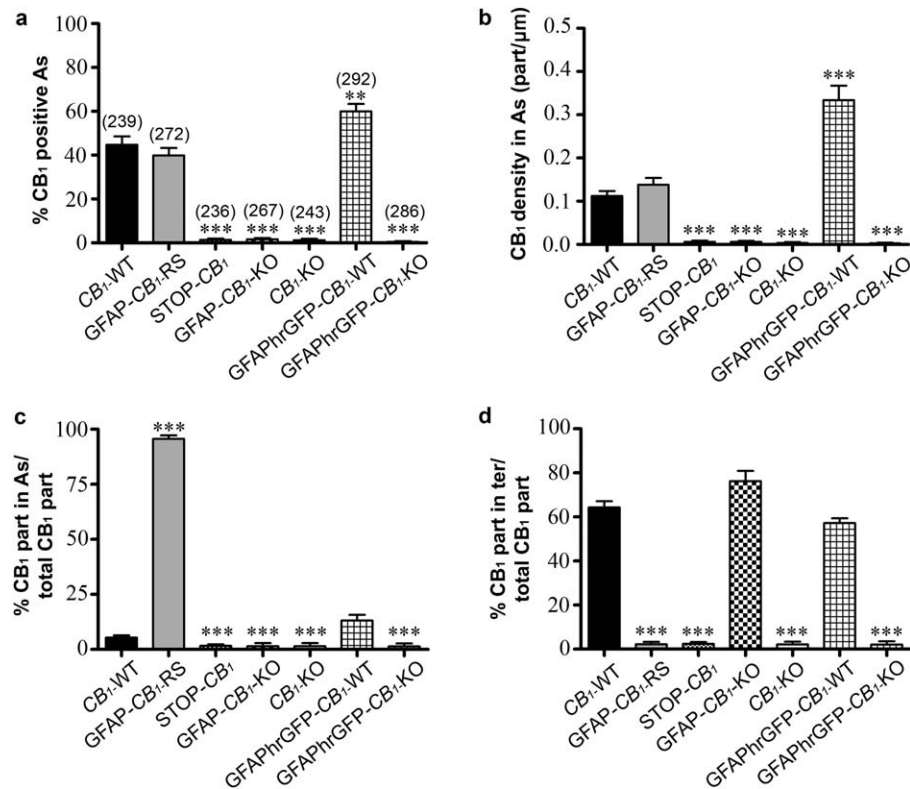


FIGURE 6 Statistical assessment of the CB₁ receptor distribution on astrocytes in the dentate molecular layer of the mutant mice. (a). Similar percentages of CB₁ receptor immunopositive astrocytic processes in CB₁-WT (44.67% ± 3.85%) and GFAP-CB₁-RS (39.84% ± 3.50%) are found. Statistical differences are obtained in GFAPhrGFP-CB₁-WT (59.99% ± 3.37%). Just residual particles are in: STOP-CB₁ (1.33% ± .64%), GFAP-CB₁-KO (1.59% ± .66%), CB₁-KO (1.19% ± .71%) and GFAPhrGFP-CB₁-KO (.47% ± .36%). The number of astrocytic processes studied is in parentheses on the top of each column. (b) Analysis of CB₁ receptor density (particles/μm) on astrocytic processes shows no statistical differences between CB₁-WT (.112 ± .011) and GFAP-CB₁-RS (.138 ± .016); however, the density on GFAPhrGFP-CB₁-WT (.334 ± .033) is statistically higher. Only residual background is counted in STOP-CB₁ (.006 ± .003), GFAP-CB₁-KO (.006 ± .003), CB₁-KO (.004 ± .002) and GFAPhrGFP-CB₁-KO (.002 ± .002). (c) Proportion of CB₁ receptor immunoparticles on astrocytic membranes versus total CB₁ receptor expression on plasmalemma: 5.35% ± 1.00% (CB₁-WT), 95.61% ± 1.56% (GFAP-CB₁-RS). Almost null non-specific immunoparticles are found in STOP-CB₁ (1.65% ± .66%), GFAP-CB₁-KO (1.45% ± 1.45%), CB₁-KO (1.43% ± 1.43%) and GFAPhrGFP-CB₁-KO (1.37% ± 1.37%). (d) Proportion of immunogold particles localized on synaptic terminals versus total CB₁ receptor expression on plasmalemma: 64.27% ± 2.88% (CB₁-WT), 76.17% ± 4.70% (GFAP-CB₁-KO), 57.17% ± 2.19% (GFAPhrGFP-CB₁-WT). Only background levels are in synaptic terminals of GFAP-CB₁-RS (2.19% ± 1.10%), STOP-CB₁ (2.36% ± .85%), CB₁-KO (2.14% ± 1.22%) and GFAPhrGFP-CB₁-KO (2.06% ± 1.52%). Data are expressed as mean ± SEM of three different animals. Data were analyzed by means of Kruskal-Wallis test and the Dunn's multiple comparison post-hoc test. ****p* < .001; ***p* < .01; **p* < .05. As: astrocytic processes; ter: terminal; part: immunoparticles

immunostaining, a cytoskeletal protein assembled in intermediate filament packets (Hol & Pekny, 2015), is mostly restricted to the main branches of the astrocyte. However, hrGFP is a diffusible protein extending into the delicate astrocytic processes that normally lack GFAP (Nolte et al., 2001), accomplishing better detection of the astrocyte processes. Finally, maybe there epigenetic mechanisms leading to the difference between GFAPhrGFP-CB₁-WT and GFAP-CB₁-RS, as in rescue mice re-expression was induced in the adult.

The rescue of CB₁ receptors in mice expressing the gene exclusively in dorsal telencephalic glutamatergic neurons (Glu-CB₁-RS) or in forebrain GABAergic neurons (GABA-CB₁-RS) (de Salas-Quiroga et al., 2015; Lange et al., 2017; Remmers et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014) has provided interesting insights into the sufficiency of the CB₁ receptor in these cells for specific brain functions and behaviors. Therefore, restoration of CB₁ receptor expression in

astrocytes and astroglial mitochondria could represent a new approach to assess the function of the tripartite synapse. CB₁ receptors in astrocytes play a key role in the two-way communication between neurons and astrocytes through rising calcium in astrocytes that modulates synaptic transmission and plasticity (Araque, Castillo, Manzoni, & Tonini, 2017; Gómez-Gonzalo et al., 2015; Martin-Fernandez et al., 2017; Navarrete & Araque, 2008, 2010; Navarrete et al., 2013; Navarrete, Diez, & Araque, 2014). Astroglial CB₁ receptor activation regulates astrocytic D-aspartate uptake (Shivachar, 2007) and might contribute to the brain's energy supply through the control of leptin receptors expression in astrocytes (Bosier et al., 2013). Furthermore, CB₁ receptor expression increases in astrocytes of the sclerotic hippocampus (Meng et al., 2014) and blockade of the astroglial CB₁ receptors modulates the intracellular calcium signaling dampening epileptiform activity (Coiret et al., 2012). In addition, a strong decrease in CB₁ receptors in

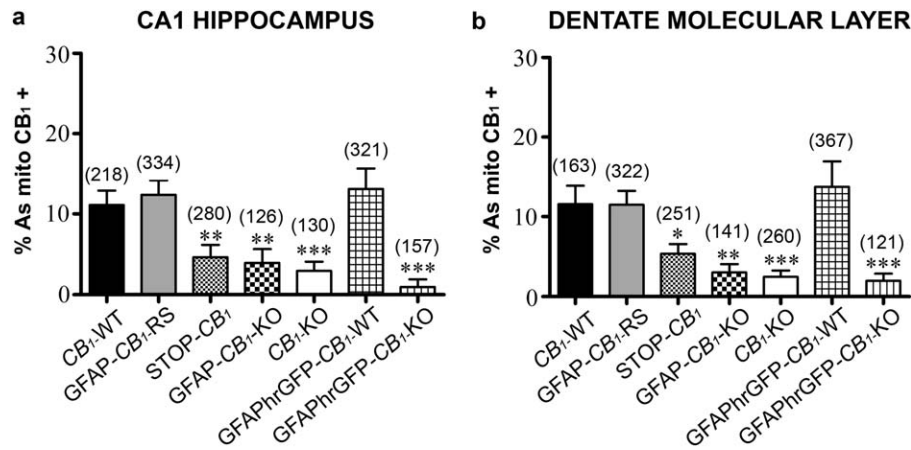


FIGURE 7 Proportion of CB₁ receptor immunopositive astrocytic mitochondria in the CA1 and dentate molecular layer of wild-type and mutant mice. (a) Values of the CB₁ receptor immunopositive astrocytic mitochondria in GFAP-CB₁-RS (12.39% ± 1.81%) and GFAPhrGFP-CB₁-WT (13.12% ± 2.53%) are closely similar to CB₁-WT (11.12% ± 1.79%) in the CA1 stratum radiatum. The background in astroglial mitochondria is: STOP-CB₁ (4.66% ± 1.55%), GFAP-CB₁-KO (3.97% ± 1.71%), CB₁-KO (2.97% ± 1.15%) and GFAPhrGFP-CB₁-KO (0.95% ± 0.95%). The number of total mitochondria examined is in parentheses on the top of each column. (b) In the dentate molecular layer, the values of CB₁ receptor immunopositive astrocytic mitochondria in GFAP-CB₁-RS (11.48% ± 1.76%) and GFAPhrGFP-CB₁-WT (13.74% ± 3.20%) are comparable to the CB₁-WT (11.56% ± 2.33%). Background in astroglial mitochondria is: STOP-CB₁ (5.38% ± 1.22%), GFAP-CB₁-KO (3.05% ± 1.04%), CB₁-KO (2.49% ± 0.80%), GFAPhrGFP-CB₁-KO (1.98% ± 0.91%). The number of total mitochondria examined is in parentheses on the top of each column. Data are expressed as mean ± SEM of three different animals. Data were analyzed by means of Kruskal-Wallis test and the Dunn's multiple comparison post-hoc test. ****p* < .001; ***p* < .01; **p* < .05. As: astrocytic processes; mito: mitochondria

adult mouse CA1 astrocytes has been recently observed after adolescent drinking-in-the-dark ethanol intake patterns (Bonilla-Del Río et al., 2017). Taken together, the subcellular compartmentalization of the CB₁ receptor in astrocytes suggests the existence of specific and precise distribution of the receptor that seems to be crucial for the functional role of the CB₁ receptor at the tripartite synapse (Araque et al., 2014; Araque, Castillo, Manzoni, & Tonini, 2017; Belluomo et al., 2015; Han et al., 2012; Metna-Laurent & Marsicano, 2015; Navarrete & Araque, 2008, 2010; Oliveira da Cruz et al., 2015; Perez-Alvarez et al., 2014).

4.1 | CB₁ receptors in astroglial mitochondria and potential functional implications

We estimated that 10%–15% of the total CB₁ receptor labeling in the hippocampus is localized at mitochondrial membranes (Bénard et al., 2012; Bonilla-Del Río et al., 2017; Hebert-Chatelain et al., 2016), and this percentage is increased in muscle and heart (Mendizabal-Zubiaga et al., 2016). Yet, about 22% of the mitochondrial sections in axon terminals and somatodendritic domains contain CB₁ receptors (Hebert-Chatelain et al., 2014a,b). In the present study, 11%–13% of the astrocytic mitochondrial sections were CB₁ receptor immunopositive,

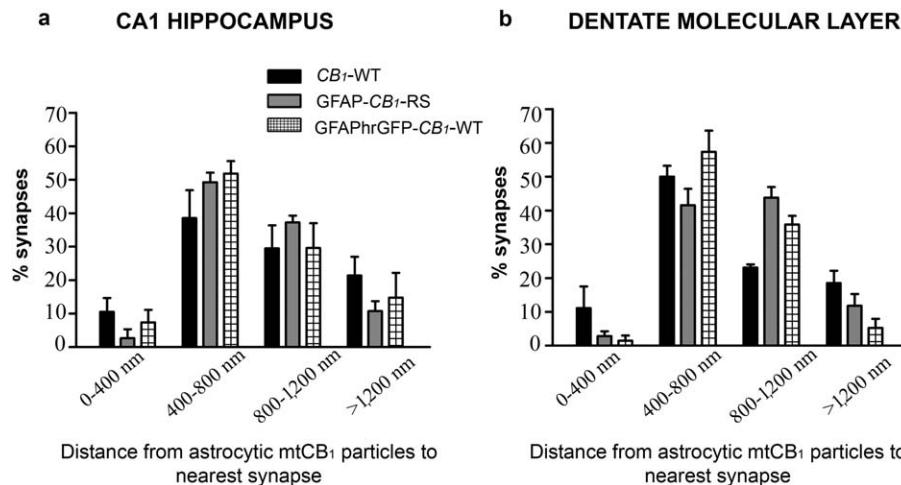


FIGURE 8 Distance from the mitochondrial CB₁ receptor particles in astrocytes to the synapses in the hippocampus. The distance between the CB₁ receptor particles on mitochondrial membranes in astrocytic processes and the midpoint of the nearest synapse surrounded by them was assessed in the CA1 (a) and dentate molecular layer (b) of CB₁-WT, GFAP-CB₁-RS and GFAPhrGFP-CB₁-WT (see Table 1 for values)

**TABLE 1** Proportion of synapses visualized in 400-nm-bit ranges from the CB₁ receptor labeling in astroglial mitochondria

CA1	CB ₁ -WT (1,790 μm ²)	GFAP-CB ₁ -RS (2,100 μm ²)	GFAPhrGFP-CB ₁ -WT (784 μm ²)
<400 nm	10.55% ± 4.01%	2.67% ± 2.67%	7.41% ± 3.70%
400–800 nm	38.54% ± 8.32%	49.28% ± 2.87%	51.85% ± 3.70%
800–1,200 nm	29.51% ± 6.85%	37.26% ± 2.02%	29.63% ± 7.41%
>1,200 nm	21.40% ± 5.56%	10.79% ± 2.94%	14.81% ± 7.41%
MDG	CB ₁ -WT (784 μm ²)	GFAP-CB ₁ -RS (1,708 μm ²)	GFAPhrGFP-CB ₁ -WT (1,512 μm ²)
<400 nm	11.11% ± 6.42%	2.82% ± 1.48%	1.52% ± 1.52%
400–800 nm	50.0% ± 3.21%	41.57% ± 4.81%	57.37% ± 6.26%
800–1,200 nm	23.15% ± .93%	43.79% ± 3.13%	35.86% ± 2.53%
>1,200 nm	18.52% ± 3.70%	11.82% ± 3.51%	5.25% ± 2.72%

indicating that mtCB₁ receptors in astrocytes might play important functional roles. Indeed, their activation may impact functions in which astroglial CB₁ receptors are involved, such as metabolic activity, neuroprotection, inflammatory responses, astrocyte development and survival, synaptic transmission, plasticity or memory formation (Aguado et al., 2006; Araque et al., 2014; Araque, Castillo, Manzoni, & Tonini, 2017; Bosier et al., 2013; Han et al., 2012; Metna-Laurent & Marsicano, 2015; Navarrete & Araque, 2008, 2010; Stella, 2010).

CB₁ receptors in astrocytes, but not in glutamatergic or GABAergic synaptic terminals, are responsible for long-term depression of synaptic efficacy at hippocampal CA3-CA1 synapses *in vivo* and the subsequent spatial working memory impairments induced by cannabinoid administration (Han et al., 2012). Outside of the hippocampus, endocannabinoids acting on astroglial CB₁ receptors in the central amygdala can regulate fear responses by selectively reducing excitatory transmission through synaptic A1 adenosine receptors and increasing inhibitory transmission by synaptic A2A receptors (Martin-Fernandez et al., 2017). Over the last decade, extensive study of mitochondrial CB₁ receptors has begun to establish their function and how their activity can modulate behaviors. The activation of mitochondrial CB₁ receptors leads to a remarkable decrease in mitochondrial respiration in brain mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016) and the cannabinoid shutdown of hippocampal mitochondrial activity produces a decrease in cellular and mitochondrial ATP, reduces mitochondrial mobility, CA3-CA1 excitatory synaptic transmission and abolishes discrimination of novel object recognition (Hebert-Chatelain et al., 2016). The potential involvement of astroglial mtCB₁ receptors in these effects is currently not known and future studies will address this interesting issue.

One open question is how the (endo)cannabinoids have access to the mtCB₁ receptors in astrocytes. With this aim in mind we took advantage of the enhanced detection of the astrocytic CB₁ receptors in the CA1 stratum radiatum and dentate molecular layer of GFAPhrGFP-CB₁-WT mice. Then, the gap between the mitochondrial CB₁ receptor particles and the nearest synapse was measured to understand the anatomical relationship of the receptor in the context of the functional tripartite synapse (Araque et al., 2014; Navarrete & Araque, 2008, 2010; Navarrete et al., 2013; Navarrete, Diez, & Araque,

2014). The most frequent distance of 400–800 nm spanning up to 1,200 nm suggests that the endocannabinoids generated on demand in the postsynaptic neurons would need to travel a significant distance in order to reach the CB₁ receptors localized on the astroglial mitochondria. However, astrocytes are able to produce endocannabinoids (Stella, 2010), contain the main enzymes for their synthesis and degradation (Suárez et al., 2010; Uchigashima et al., 2011) and brain mitochondria also contain these lipid signaling molecules (Bénard et al., 2012). Considering that endocannabinoids can signal in autocrine, paracrine or both manners (Metna-Laurent & Marsicano, 2015), it is possible that astrocytes or even astroglial mitochondria might produce “their own” endocannabinoids to specifically activate mtCB₁ receptors.

Altogether, activation of intracellular CB₁ receptors localized at mitochondria impacts cognition through the modulation of mitochondrial energy metabolism (Hebert-Chatelain et al., 2016). Whether mitochondrial CB₁ receptors also regulate the organelle's energy production in astrocytes and participate in high brain functions will be elucidated in future studies.

4.2 | Conditional CB₁ receptor mutants

Loss of function in mutant mice lacking CB₁ receptors in specific cell types allowed insights into their anatomical localization and a deeper understanding of their necessary role for several brain functions (Bénard et al., 2012; Han et al., 2012; Koch et al., 2015; Marsicano et al., 2003; Martín-García et al., 2016; Monory et al., 2006; Monory, Polack, Remus, Lutz, & Korte, 2015; Soria-Gómez et al., 2014). Conditional mutant mice lacking CB₁ receptors in astrocytes exhibit neither *in vivo* hippocampal long-term depression nor the impairment of spatial working memory typically observed following acute cannabinoid treatment (Han et al., 2012). The GFAP-CB₁-RS mouse expressing CB₁ receptors exclusively in astrocytes described here, together with the Glu-CB₁-RS rescue mouse expressing the receptor only in dorsal telencephalic glutamatergic neurons (de Salas-Quiroga et al., 2015; Lange et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014) and the GABA-CB₁-RS rescue mouse expressing the CB₁ receptor only in GABAergic neurons (de Salas-Quiroga et al., 2015; Lange et al., 2017) that were recently characterized anatomically (Gutiérrez-Rodríguez

et al., 2017; Remmers et al., 2017), suggest that the regulation of the CB₁ receptor expression in astrocytes, glutamatergic neurons and GABAergic neurons may be independent of each others. The present demonstration that the GFAP-CB₁-RS in the hippocampus maintains the normal CB₁ receptor expression and distribution in astrocytes make these mutants ideal suited for the study of the astroglial CB₁ receptor function, as shown for Glu-CB₁-RS (de Salas-Quiroga et al., 2015; Gutiérrez-Rodríguez et al., 2017; Lange et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014) and GABA-CB₁-RS mice (de Salas-Quiroga et al., 2015; Gutiérrez-Rodríguez et al., 2017; Lange et al., 2017; Remmers et al., 2017). In fact, these rescue strategies have the advantage of the restoration and visualization of existing CB₁ receptor levels in locations with sparse CB₁ receptors (as the astrocytes and astroglial mitochondria), allowing a more comprehensive functional characterization of the (endo)cannabinoid system based on the precise cellular and subcellular localization of the CB₁ receptor. At the same time, these strategies improve the fundamental knowledge for the development of innovative therapeutics in the struggle against brain diseases.

Altogether, our observations confirm the high specificity of the genetic CB₁ receptor rescue approach carried out in the astrocytes and that these mutant mice are emerging as excellent models for studying the contribution of the CB₁ receptors in astrocytes and astroglial mitochondria that, although scarce in expression as compared with their neuronal counterparts, are a constant feature and likely play a key role in brain function and dysfunction.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Annex 2

Synapse-specific astrocyte gating of amygdala-related behavior

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The amygdala plays key roles in fear and anxiety. Studies of the amygdala have largely focused on neuronal function and connectivity. Astrocytes functionally interact with neurons, but their role in the amygdala remains largely unknown. We show that astrocytes in the medial subdivision of the central amygdala (CeM) determine the synaptic and behavioral outputs of amygdala circuits. To investigate the role of astrocytes in amygdala-related behavior and identify the underlying synaptic mechanisms, we used exogenous or endogenous signaling to selectively activate CeM astrocytes. Astrocytes depressed excitatory synapses from basolateral amygdala via A₁ adenosine receptor activation and enhanced inhibitory synapses from the lateral subdivision of the central amygdala via A_{2A} receptor activation. Furthermore, astrocytic activation decreased the firing rate of CeM neurons and reduced fear expression in a fear-conditioning paradigm. Therefore, we conclude that astrocyte activity determines fear responses by selectively regulating specific synapses, which indicates that animal behavior results from the coordinated activity of neurons and astrocytes.

The amygdala, which encompasses several anatomical and functional subnuclei, plays critical roles in a variety of behavioral responses, including fear and anxiety¹. It is constituted primarily by the basolateral amygdala (BLA) and central amygdala (CeA)^{1–3}. The BLA contains a majority of spiny glutamatergic neurons⁴ and is the main input structure of the amygdala, receiving multimodal sensory information from thalamus⁵ and cortex⁶. The CeA contains a majority of GABAergic projecting neurons⁷ and can be divided into lateral (CeL) and medial (CeM) nuclei^{1–3,7}. The CeM, which receives excitatory and inhibitory inputs from the BLA and CeL, respectively, is the major output subnucleus projecting to the brainstem and hypothalamus to control autonomic and motor responses^{2,3,8,9}. Recently, great progress has been made in elucidating the role of the CeA and its neuronal populations in processing emotionally relevant information^{10–16}, but the role of glial cells in the CeA remains largely unknown. Elucidating the role of astrocytes in the amygdala may provide a deeper understanding of information processing that occurs in this area.

While they are already recognized for their classical metabolic, protective and supportive roles, astrocytes are now emerging as key determinants of synaptic function^{17–20}. They express receptors that are activated by neurotransmitters^{21–23} and release gliotransmitters that activate neuronal receptors^{17,24}. Through the release of gliotransmitters, astrocytes are able to regulate synaptic transmission^{17,22,25–27} and affect animal behavior^{28–31}. Important progress has been made toward defining the mechanisms of synaptic regulation by astrocytes^{17,20}, and behavioral effects have been observed after the disturbance of

astrocytic molecular events^{28–31}. Yet it remains unknown how physiological astrocyte activity regulates the synaptic and circuit functions that underlie specific behaviors. In the present study we aimed to fill the mechanistic gap between astrocyte-dependent regulation of synaptic function and behavior. The amygdala is an ideal structure for such an investigation because it is involved in well-characterized behaviors such as the expression of conditioned fear responses with a clear readout. Using endocannabinoids (eCBs) and designer receptors exclusively activated by designer drugs (DREADDs) as, respectively, endogenous and exogenous stimuli to activate astrocytes, we found that astrocytes regulated neurotransmission in specific synapses of the CeM through differential mechanisms. Astrocytes depressed excitatory synapses from the BLA via A₁ receptor activation, whereas they enhanced inhibitory synapses from the CeL via A_{2A} receptor activation. Consistent with these results, astrocytes decreased the CeM neuronal firing rate and influenced fear expression.

RESULTS

CeM astrocytes respond to endogenously mobilized endocannabinoids

To investigate the effects of astrocyte activation on synaptic transmission in the CeM, we recorded excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) evoked by the stimulation of BLA and CeL, respectively (**Supplementary Fig. 1a,b**), and stimulated astrocytes with either eCBs released by neurons, as an endogenous stimulus, or chemogenetic activation of Gq-protein-coupled DREADDs expressed in astrocytes, as a specific

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stimulus. First, we tested whether CeM astrocytes respond to eCBs^{32–34} released by CeM neurons during neuronal depolarization (ND; 0 mV, 10 s)^{35,36} by monitoring calcium levels in astrocytes (Fig. 1a), identified with SR101 (Supplementary Fig. 1c). ND increased the level of astrocytic calcium (Fig. 1b) and increased the calcium event probability (138 astrocytes from $n = 10$ slices; $P < 0.001$; Fig. 1c,d). This effect was abolished by the CB1R antagonist AM251 (2 μ M; 127 astrocytes from $n = 7$ slices; $P = 0.96$); in addition, it was absent in GFAP-CB1R-null mice (175 astrocytes from $n = 10$ slices; $P = 0.63$), which lack CB1 receptors specifically in astrocytes³⁰; present in wild-type littermates that expressed CB1 receptors (GFAP-CB1^{WT}; 97 astrocytes from $n = 9$ slices; $P = 0.006$); and absent in IP3R2-null mice, in which G-protein-mediated calcium elevation is selectively impaired in astrocytes^{33,37} (74 astrocytes from $n = 8$ slices; $P = 0.73$; Fig. 1d). Furthermore, our analysis of the ND-evoked calcium event probability in different conditions indicated that the observed increase in control was abolished in the presence of AM251 and in GFAP-CB1R-null and IP3R2-null mice (two-way analysis of variance (ANOVA) indicated a significant effect of ND ($P < 0.001$) and an interaction with the 'experimental condition' ($P < 0.001$); Supplementary Table 1; *post hoc* Holm–Sidak, $P = 0.004$, $P = 0.003$ and $P < 0.001$, respectively). In contrast, we did not observe any statistical differences when we compared the control condition with the GFAP-CB1^{WT} mice ($P = 0.421$; Fig. 1d). Taken together, these results indicate that eCBs released from CeM neurons activate astrocytic CB1Rs that increase calcium levels in astrocytes.

CB1R-dependent activation of astrocytes potentiates CeL–CeM inhibitory synaptic transmission

We then investigated whether astrocytes regulate synaptic transmission in CeM neurons. We obtained paired recordings^{33,38} of CeM neurons, depolarized one neuron (homoneuron) to induce the release of eCBs (which elevated astrocytic calcium), and recorded either CeL-evoked IPSCs or BLA-evoked EPSCs in the paired neuron (heteroneuron) to exclude direct presynaptic effects of eCBs³⁵ (Fig. 1e,i). We pharmacologically isolated IPSCs and EPSCs (Supplementary Fig. 1b) and adjusted the stimulus parameters to stimulate single or

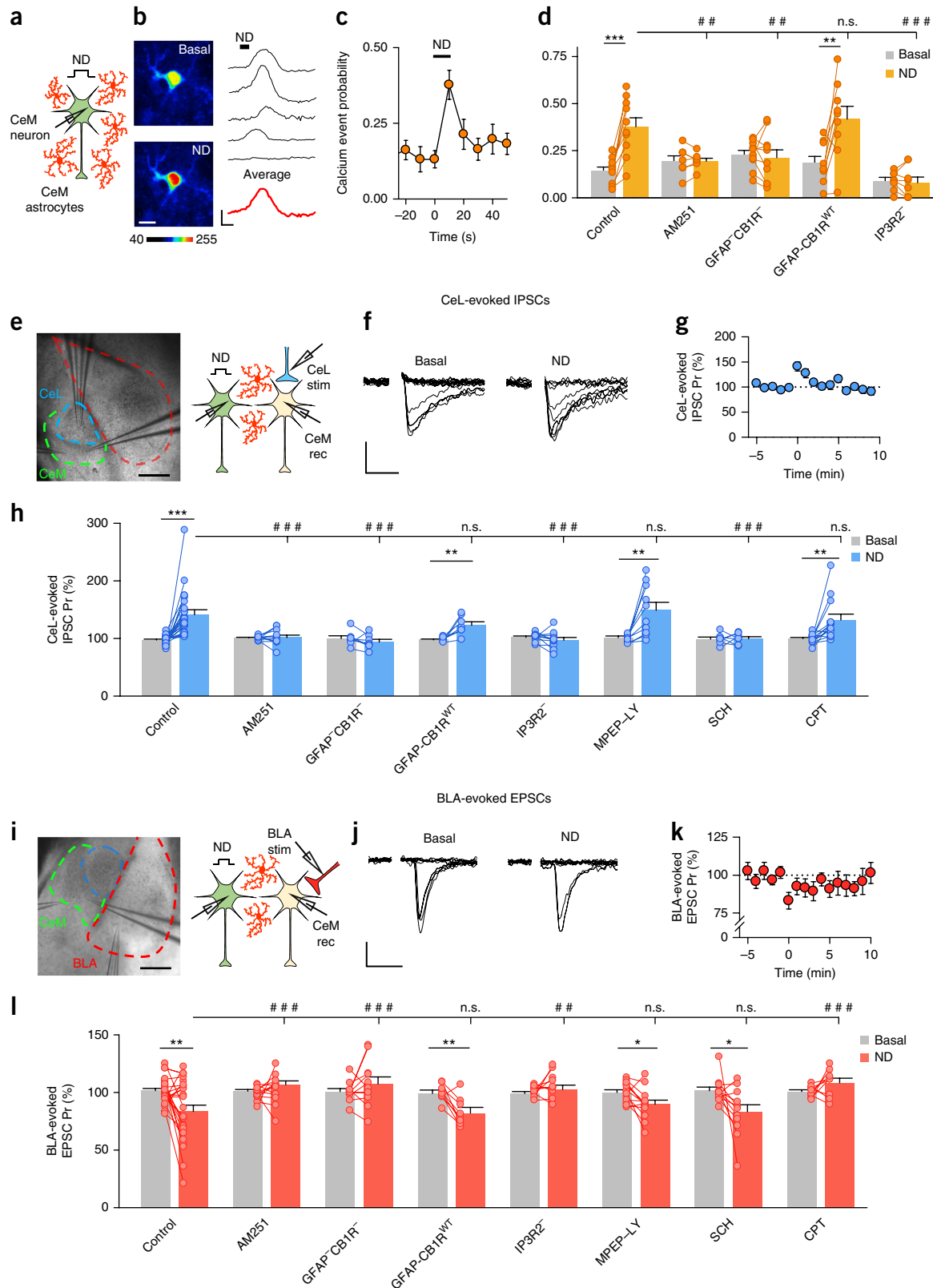
a few presynaptic fibers^{26,27,38,39} that induced failures or successes in synaptic responses. We quantified the probability of release (Pr; i.e., the proportion of successful responses) and the synaptic potency (i.e., the amplitude of the successful responses). ND induced a transient increase in the CeL-evoked IPSC Pr ($n = 22$; $P < 0.001$) recorded in the heteroneuron (Fig. 1f,g), with no changes in the synaptic potency ($n = 22$; $P = 0.88$; Supplementary Fig. 2a,b), suggesting a presynaptic mechanism. Consistent with this idea, the increase in the Pr was associated with a decrease in the paired pulse ratio (PPR; from 1.1 ± 0.02 to 1.04 ± 0.2 (mean \pm s.e.m.); $n = 17$; $P = 0.007$, paired *t*-test). The ND-induced increase in the CeL-evoked IPSC Pr was abolished by AM251 ($n = 11$; $P = 0.74$) and was absent in GFAP-CB1R-null mice ($n = 7$; $P = 0.21$) and IP3R2⁻ mice ($n = 10$; $P = 0.3$; Fig. 1h) but present in GFAP-CB1^{WT} littermates ($n = 7$; $P = 0.008$), indicating that the ND-evoked synaptic regulation was mediated by the activation of astrocytic CB1Rs and calcium mobilization. Astrocytic CB1R activation by eCBs stimulates the release of astrocytic glutamate in other brain regions, such as hippocampus, cortex and striatum^{33,34,38}. However, the ND-induced increase in the CeL-evoked Pr of IPSCs was unaffected by treatment with antagonists of group I metabotropic glutamate receptors (mGluRs) MPEP (50 μ M) and LY367385 (100 μ M; $n = 10$; $P = 0.0038$; Fig. 1h). Elevated calcium levels in astrocytes have been shown to trigger the release of ATP, which, after being converted to adenosine, may regulate synaptic transmission^{17,27}. The increase in the CeL-evoked IPSC Pr was abolished by the antagonist of adenosine A_{2A} receptors SCH 58261 (100 nM; $n = 7$; $P = 0.22$), but not by the antagonist of adenosine A₁ receptors CPT (5 μ M; $n = 13$; $P = 0.006$; Fig. 1h). Furthermore, the analysis of the Pr after ND indicated that ND-evoked Pr changes were prevented in the presence of AM251 and SCH, and in GFAP-CB1R-null and IP3R2-null mice (two-way ANOVA indicated a significant effect of ND ($P < 0.001$) and an interaction with the experimental condition ($P < 0.001$); Supplementary Table 1; *post hoc* Holm–Sidak, $P < 0.001$ for the four conditions), but were unaffected in the presence of antagonists of mGluRs (MPEP + LY) and A₁ receptors (CPT) and in GFAP-CB1^{WT} mice ($P = 0.35$, $P = 0.45$ and

Figure 1 Endogenously mobilized eCBs mediate CB1R-dependent increases in astrocytic calcium levels, enhance inhibitory synaptic transmission in CeL–CeM synapses and depress excitatory synaptic transmission in BLA–CeM synapses. **(a)** A schematic representation of the experimental design. **(b)** Left, pseudocolor images showing fluorescence intensities in CeM astrocytes before and after ND. Scale bar, 10 μ m. Right, astrocytic calcium levels before and after ND (black), and an averaged trace of astrocytes in the field of view (red). Scale bars, 50% and 10 s for the individual traces (black), and 20% and 10 s for the average trace (red). **(c)** Calcium event probability before and after ND in control conditions ($n = 10$; $P > 0.001$); in the presence of AM251 ($n = 7$; $P = 0.96$); and in GFAP-CB1R-null ($n = 9$; $P = 0.54$), GFAP-CB1R^{WT} ($n = 10$; $P = 0.006$) and IP3R2⁻ ($n = 8$; $P = 0.73$) mice. The increase observed in the control condition was abolished in the presence of AM251 ($P < 0.001$) and in GFAP-CB1R-null ($P = 0.004$) and IP3R2⁻ mice ($P = 0.003$), but not in the GFAP-CB1^{WT} mice ($P = 0.421$; two-way ANOVA, *post hoc* Holm–Sidak corrected for four comparisons). **(e)** Left, an infrared differential interference contrast microscopy (DIC) image showing the stimulation pipette in the CeL subnucleus and two recording pipettes in the CeM subnucleus. Scale bar, 250 μ m. Right, a scheme of the experimental approach for obtaining recordings (rec) in the CeM from the homoneuron (green) and the heteroneuron (yellow) and the stimulation (stim) of GABAergic inputs from the CeL (blue). **(f)** IPSCs evoked by CeL stimulation recorded in the CeM heteroneuron, in basal conditions and after CeM homoneuron ND. Scale bars, 10 pA and 25 ms. **(g)** CeL-evoked IPSC Pr before and after homoneuron ND (at time 0; $n = 22$). **(h)** CeL-evoked IPSC Pr before and after homoneuron ND in control conditions ($n = 22$; $P < 0.001$); in the presence of AM251 ($n = 11$; $P = 0.74$); in GFAP-CB1R-null ($n = 7$; $P = 0.21$), GFAP-CB1R^{WT} ($n = 7$; $P = 0.008$) and IP3R2⁻ ($n = 10$; $P = 0.03$) mice; and in the presence of MPEP + LY ($n = 10$; $P = 0.0038$), SCH ($n = 7$; $P = 0.22$) and CPT ($n = 13$; $P = 0.006$). The ND-evoked increase in Pr was prevented in the presence of AM251 ($P < 0.001$) or SCH ($P < 0.001$), and in GFAP-CB1R-null ($P < 0.001$) and IP3R2⁻ ($P < 0.001$) mice, but was unaffected in the presence of MPEP + LY ($P = 0.35$) or CPT ($P = 0.45$) and in GFAP-CB1^{WT} mice ($P = 0.18$; two-way ANOVA, *post hoc* Holm–Sidak corrected for seven comparisons). **(i)** Left, a DIC image showing the stimulation pipette in the BLA subnucleus and two recording pipettes in the CeM subnucleus. Scale bar, 250 μ m. Right, a scheme of the experimental approach for obtaining recordings in CeM from the homoneuron (green) and the heteroneuron (yellow) and the stimulation of excitatory inputs from BLA (red). **(j)** EPSCs evoked by BLA stimulation recorded in the CeM heteroneuron, in basal conditions and after homoneuron ND. Scale bars, 10 pA and 25 ms. **(k)** BLA-evoked EPSC Pr before and after homoneuron ND (at time 0; $n = 24$). **(l)** BLA-evoked EPSC Pr before and after homoneuron ND in control conditions ($n = 24$; $P = 0.004$); in the presence of AM251 ($n = 12$; $P = 0.66$); in GFAP-CB1R-null ($n = 9$; $P = 0.25$), GFAP-CB1R^{WT} ($n = 11$; $P = 0.003$) and IP3R2⁻ ($n = 10$; $P = 0.17$) mice; and in the presence of MPEP + LY ($n = 13$; $P = 0.01$), SCH ($n = 12$; $P = 0.04$) and CPT ($n = 9$; $P = 0.14$). The ND-evoked decrease in Pr was prevented in the presence of AM251 ($P < 0.001$) or CPT ($P < 0.001$) and in GFAP-CB1R-null ($P < 0.001$) and IP3R2⁻ ($P < 0.001$) mice, but was unaffected in the presence of MPEP + LY ($P = 0.46$) or SCH ($P = 0.96$) and in GFAP-CB1^{WT} mice ($P = 0.98$; two-way ANOVA, *post hoc* Holm–Sidak corrected for seven comparisons). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's paired *t*-test. ## $P < 0.01$, ### $P < 0.001$; two-way ANOVA with *post hoc* Holm–Sidak; n.s., nonsignificant ($P > 0.05$). Data in **c,d,g,h,k,l** are mean \pm s.e.m.

$P = 0.18$, respectively; **Fig. 1h**). To test whether Pr changes depend on the basal synaptic Pr, we compared the absolute basal Pr values in the different experimental conditions. We did not observe any significant differences between the basal Pr values of the different experimental conditions (one-way ANOVA, $P = 0.07$; **Supplementary**

Fig. 3a). Furthermore, we obtained similar results when we analyzed either absolute or normalized Pr values in the different conditions (**Supplementary Table 2a**).

Together, these results suggest that ND-induced astrocyte activation stimulates the release of ATP/adenosine that acts on



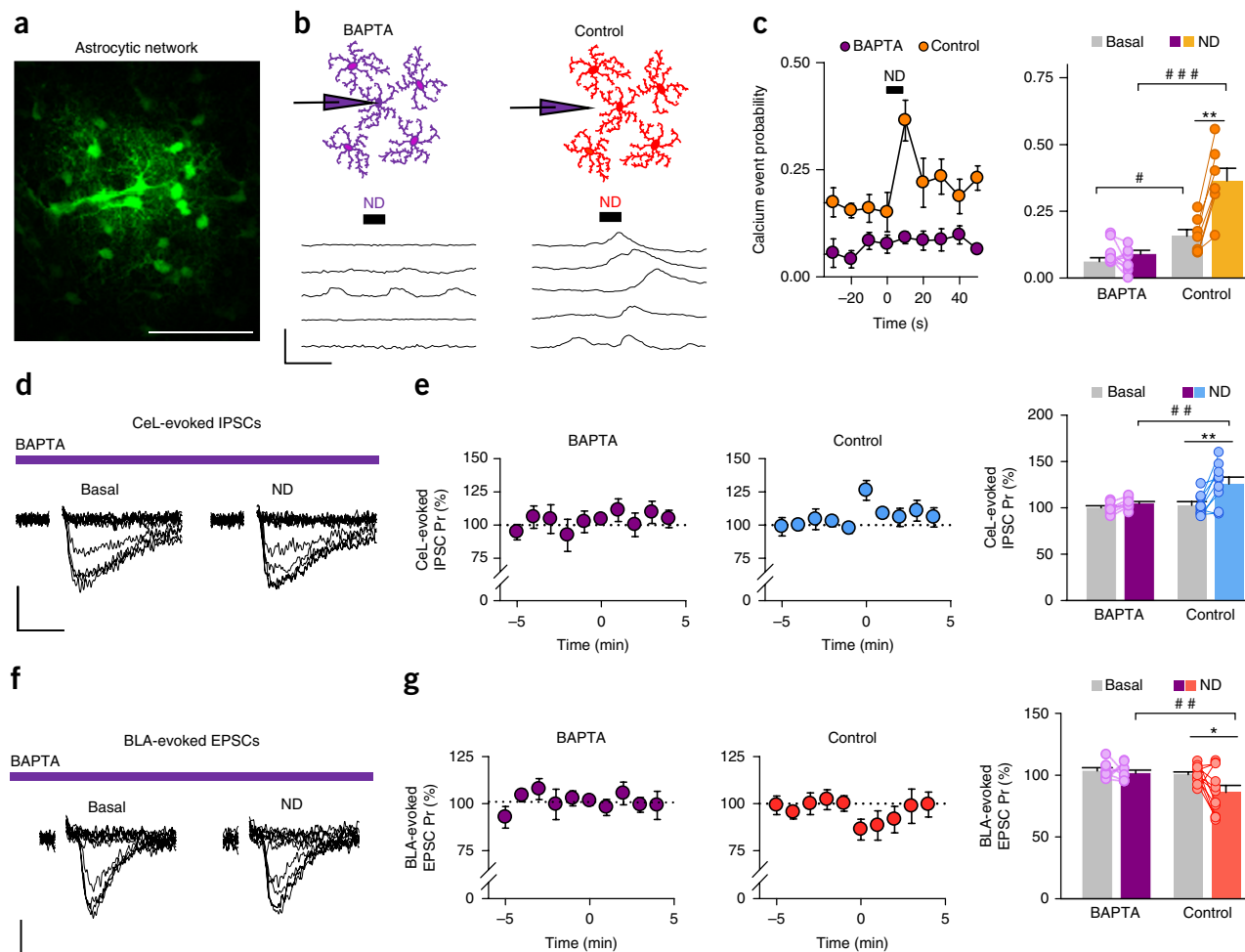


Figure 2 Astrocytic CB1R regulation of synaptic transmission relays on astrocytic calcium activity. **(a)** A network of coupled astrocytes after a single astrocyte was filled with biocytin. Scale bar, 70 μm . **(b)** Left, a schematic representation of the experimental condition: an astrocyte was filled with BAPTA-containing intracellular solution, and the astrocyte was kept patched long enough to allow the BAPTA to diffuse to neighboring astrocytes. The traces show the changes in calcium levels in response to ND in this condition. Scale bars, 20 s and 50%. Right, a schematic representation of the control condition: a pipette with BAPTA-containing intracellular solution was placed in the extracellular space. The traces show changes in calcium levels in response to ND in this condition. Scale bars, 20 s and 50%. **(c)** Left, calcium event probability before and after ND at time 0 in BAPTA ($n = 9$) and control ($n = 7$) conditions. Right, calcium event probability before and after ND in BAPTA ($n = 9$; $P = 0.16$) and control conditions ($n = 7$; $P < 0.001$). We observed a difference in the calcium event probability between control and BAPTA conditions both before and after ND (two-way ANOVA indicated a significant effect of ND ($P < 0.001$) and an interaction with the experimental condition ($P = 0.002$); *post hoc* Holm–Sidak corrected for two comparisons; difference between control and BAPTA before ND ($P = 0.016$) and after ND ($P < 0.001$)). **(d)** IPSCs evoked by CeL stimulation in the CeM heteroneuron in BAPTA conditions before and after CeM homoneuron ND. Scale bars, 9 pA and 25 ms. **(e)** CeL-evoked IPSC Pr before and after homoneuron ND (at time 0) in BAPTA ($n = 8$; $P = 0.16$) and control conditions ($n = 9$; $P = 0.003$). We observed a difference in the post-ND state between the BAPTA and control conditions (two-way ANOVA indicated a significant effect of ND ($P = 0.003$) and an interaction with the experimental condition ($P = 0.038$); *post hoc* Holm–Sidak $P = 0.002$). **(f)** EPSCs evoked by BLA stimulation in the CeM heteroneuron in the BAPTA condition before and after homoneuron ND. Scale bars, 5 pA and 25 ms. **(g)** BLA-evoked EPSC Pr before and after homoneuron ND (at time 0) in BAPTA ($n = 8$; $P = 0.63$) and control ($n = 11$; $P = 0.03$) conditions. We observed a difference in the post-ND state between the BAPTA and control conditions (two-way ANOVA indicated a significant effect of ND ($P = 0.037$) and interaction with the experimental condition ($P = 0.106$); *post hoc* Holm–Sidak $P = 0.007$). * $P < 0.05$, ** $P < 0.01$; Student's paired *t*-test. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$; two-way ANOVA, *post hoc* Holm–Sidak. Data in **c,e,g** are mean \pm s.e.m.

neuronal receptors to regulate inhibitory synaptic transmission. To test the idea that the adenosine-receptor activation occurs downstream from the astrocytic calcium activity, we analyzed the effects of A_{2A} and A_1 receptor antagonists on the ND-evoked astrocyte calcium signal. We observed that ND evoked an increase in the calcium event probability in the presence of SCH (from 0.25 ± 0.3 to 0.46 ± 0.6 ; 96 astrocytes from $n = 6$ slices; paired *t*-test, $P = 0.01$) and CPT (from 0.21 ± 0.1 to 0.47 ± 0.4 ; 115 astrocytes from $n = 7$ slices; paired *t*-test, $P = 0.01$).

Taken together, these results indicate that eCBs mobilized by CeM neurons increase calcium levels in astrocytes through the activation of CB1Rs, which leads to the activation of A_{2A} receptors, thus increasing the CeL-evoked IPSC Pr.

Astrocytic CB1R-dependent regulation of BLA–CeM excitatory synaptic transmission

We next investigated the effects of eCB signaling on the Pr of BLA-evoked EPSCs in CeM neurons. In a paired-neuronal-recording

approach, we recorded BLA-evoked EPSCs in the heteroneuron (Fig. 1i). In contrast to the effects on IPSCs, ND evoked a transient decrease in the EPSC Pr ($n = 24$ neurons; $P = 0.004$; Fig. 1j,k) without modifying the synaptic potency ($n = 24$ neurons; $P = 0.2$; Supplementary Fig. 2c,d). The PPR increased from 0.98 ± 0.03 to 1.12 ± 0.04 ($n = 12$; $P = 0.001$, paired t -test), suggesting a presynaptic mechanism. The ND-evoked depression of EPSCs was abolished by AM251 ($n = 12$; $P = 0.66$) and absent in GFAP-CB1R-null ($n = 11$; $P = 0.25$) and IP3R2-null mice ($n = 10$; $P = 0.17$), but present in the presence of mGluR antagonists MPEP and LY367385 ($100 \mu\text{M}$; $n = 13$; $P = 0.01$; Fig. 1l) and in GFAP-CB1^{WT} littermates ($n = 9$; $P = 0.003$; Fig. 1l). Moreover, the decrease in EPSC Pr was abolished by the A₁ adenosine-receptor antagonist CPT ($n = 9$; $P = 0.14$), but not by the A_{2A}-receptor antagonist SCH 58261 ($n = 12$; $P = 0.04$; Fig. 1l). CPT is known to enhance basal synaptic transmission in some brain regions, such as the hippocampal CA1 area, which is tonically inhibited by presynaptic adenosine receptors^{40,41}. However, this does not seem to be the case in the CeM, as similar Pr values were found in the absence and presence of CPT (EPSC Pr control, 0.47 ± 0.04 ($n = 24$); CPT, 0.37 ± 0.07 ($n = 9$); unpaired t -test, $P < 0.24$; IPSC Pr, 0.39 ± 0.04 and 0.47 ± 0.02 ; unpaired t -test, $P = 0.21$). Furthermore, although we cannot totally exclude the possibility that BLA stimulation affects synaptic transmission in the CeM indirectly through the CeL, this is unlikely, because BLA-evoked EPSCs were assessed in the presence of GABA-receptor antagonists.

Taken together, these results suggest that eCBs mobilized by ND increase of astrocyte calcium levels through the activation of CB1 receptors, thus resulting in the activation of A₁ presynaptic receptors and decreasing the BLA-evoked EPSC Pr (Fig. 1l). Furthermore, the combined statistical analysis indicated that the ND-evoked response observed in the control condition was absent in the presence of AM251 and CPT and in GFAP-CB1R- and IP3R2-null mice (two-way ANOVA indicated a significant effect of ND ($P < 0.001$) and an interaction with the experimental condition ($P < 0.001$); Supplementary Table 1; *post hoc* Holm–Sidak, $P < 0.001$, $P < 0.001$ and $P = 0.002$, respectively). We did not note any differences relative to the control in the presence of MPEP + LY and SCH or in GFAP-CB1^{WT} mice ($P = 0.46$, $P = 0.96$ and $P = 0.98$, respectively; Fig. 1l). In addition, we did not observe any statistical differences when we compared the absolute basal Pr values in the different experimental conditions (one-way ANOVA, $P = 0.073$; Supplementary Fig. 3b), which suggests that the effects of ND were independent of the basal Pr. Furthermore, we obtained similar statistical results when we compared either absolute or basal-normalized Pr values in different conditions (Supplementary Table 2b). Notably, we found similar basal Pr values in GFAP-CB1^{WT} and GFAP-CB1R-null mice (EPSC Pr, 0.5 ± 0.07 ($n = 11$) and 0.39 ± 0.07 ($n = 9$), respectively; unpaired t -test, $P = 0.28$; IPSC Pr, 0.55 ± 0.07 ($n = 7$) and 0.47 ± 0.06 ($n = 10$), respectively; unpaired t -test, $P = 0.4$), suggesting that eCBs do not tonically activate astrocytes, which are instead acutely activated by eCBs released on demand under neuronal stimulation.

Besides glutamate and ATP/adenosine, D-serine is another major gliotransmitter known to regulate synaptic transmission in other brain areas by acting as co-agonist of NMDA receptors (NMDARs)^{25,42}. A contribution of D-serine to the astrocyte-mediated regulation of inhibition here is unlikely because we isolated CeL-evoked IPSCs by recording in the presence of the NMDAR antagonist D-AP5. To investigate the involvement of D-serine in the regulation of BLA-evoked EPSCs, we tested the ND-evoked effects in the presence of D-AP5, which did not prevent the ND-dependent decrease of BLA-evoked EPSC Pr (96.9 ± 2.2 and 75.6 ± 5.5 before and after ND, respectively;

$n = 10$; paired t -test, $P = 0.003$). Therefore, although different synaptic regulatory mechanisms may be mediated by D-serine, the present results suggest that it is not involved in this phenomenon. Taken together, the present results indicate that eCBs differentially regulate inhibitory and excitatory synaptic transmission by stimulating astrocytes, which in turn leads to the activation of A_{2A} and A₁ adenosine receptors (Fig. 1h,l).

We then investigated whether these phenomena were present in the same CeM neuron (Supplementary Fig. 4a). First, we pharmacologically isolated CeL-evoked IPSCs and monitored the ND-evoked increase in IPSC Pr ($n = 6$; $P = 0.02$; Supplementary Fig. 4b,c); then we relocated the stimulation pipette in the BLA and, after washing out inhibitors of excitatory transmission, pharmacologically isolated EPSCs (Supplementary Fig. 4a,b). In these conditions, ND induced a decrease in EPSC Pr values recorded in the same neuron ($n = 6$; $P = 0.04$; Supplementary Fig. 4b,c), indicating that astrocyte activation by eCBs in the CeM differentially regulates excitatory and inhibitory synapses in the same neurons, affecting the excitatory/inhibitory balance of CeM neurons.

Increased astrocyte calcium is necessary for CB1R-dependent synaptic regulation

The results presented above show that both eCB-mediated excitatory and inhibitory synaptic regulation were absent in mice that lacked IP3R2 (Fig. 1h,l), which largely mediates G-protein-mediated calcium elevation in astrocytes, thus suggesting that synaptic regulation requires the elevation of calcium levels in astrocytes. Because other types of IP₃ receptors have recently been shown to contribute to astrocyte calcium mobilization⁴³, we further tested the astrocytic calcium dependency by loading astrocytes with the calcium chelator BAPTA, by whole-cell patch-clamping astrocytes with a solution containing 40 mM BAPTA. Astrocytes are known to be gap-junction coupled in different brain areas, which allows the diffusion of BAPTA in the astrocytic network from single recorded astrocytes^{44,45}. We confirmed that astrocytes in the CeM are also gap-junction coupled, as biocytin included in a single patch-clamped astrocyte diffused to neighboring astrocytes (Fig. 2a). Then, we either filled astrocytes with BAPTA or placed a BAPTA-containing pipette in the extracellular space as the control, to rule out potential effects of BAPTA leakage in the extracellular space (Fig. 2b). Although ND increased the astrocyte calcium event probability in the control conditions (i.e., when the BAPTA-containing pipette was located extracellularly (117 astrocytes from $n = 7$ slices; $P < 0.001$; Fig. 2b,c)), we did not observe any calcium changes in response to ND in astrocytes filled with BAPTA (131 astrocytes from $n = 9$ slices; $P = 0.16$; Fig. 2b,c), which indicated that loading astrocytes with BAPTA prevented ND-evoked astrocytic calcium responses.

We then tested the effects of astrocyte BAPTA-loading on CeL-evoked IPSCs and BLA-evoked EPSCs. In this condition, ND did not affect the CeL-evoked IPSC Pr ($n = 8$; $P = 0.16$; Fig. 2d,e) or the BLA-evoked EPSC Pr ($n = 8$; $P = 0.6$; Fig. 2f,g), whereas an increase in the CeL-evoked IPSC Pr ($n = 9$; $P = 0.003$; Fig. 2e) and a decrease in the BLA-evoked EPSC Pr ($n = 11$; $P = 0.03$; Fig. 2g) were observed in the control condition. Taken together, these results indicate that the observed synaptic regulation requires astrocyte calcium elevations (Fig. 2).

Chemogenetic astrocyte activation regulates CeM synaptic transmission

If synaptic regulation by astrocytic calcium elevations is a general phenomenon, astrocyte stimulation should be able to produce

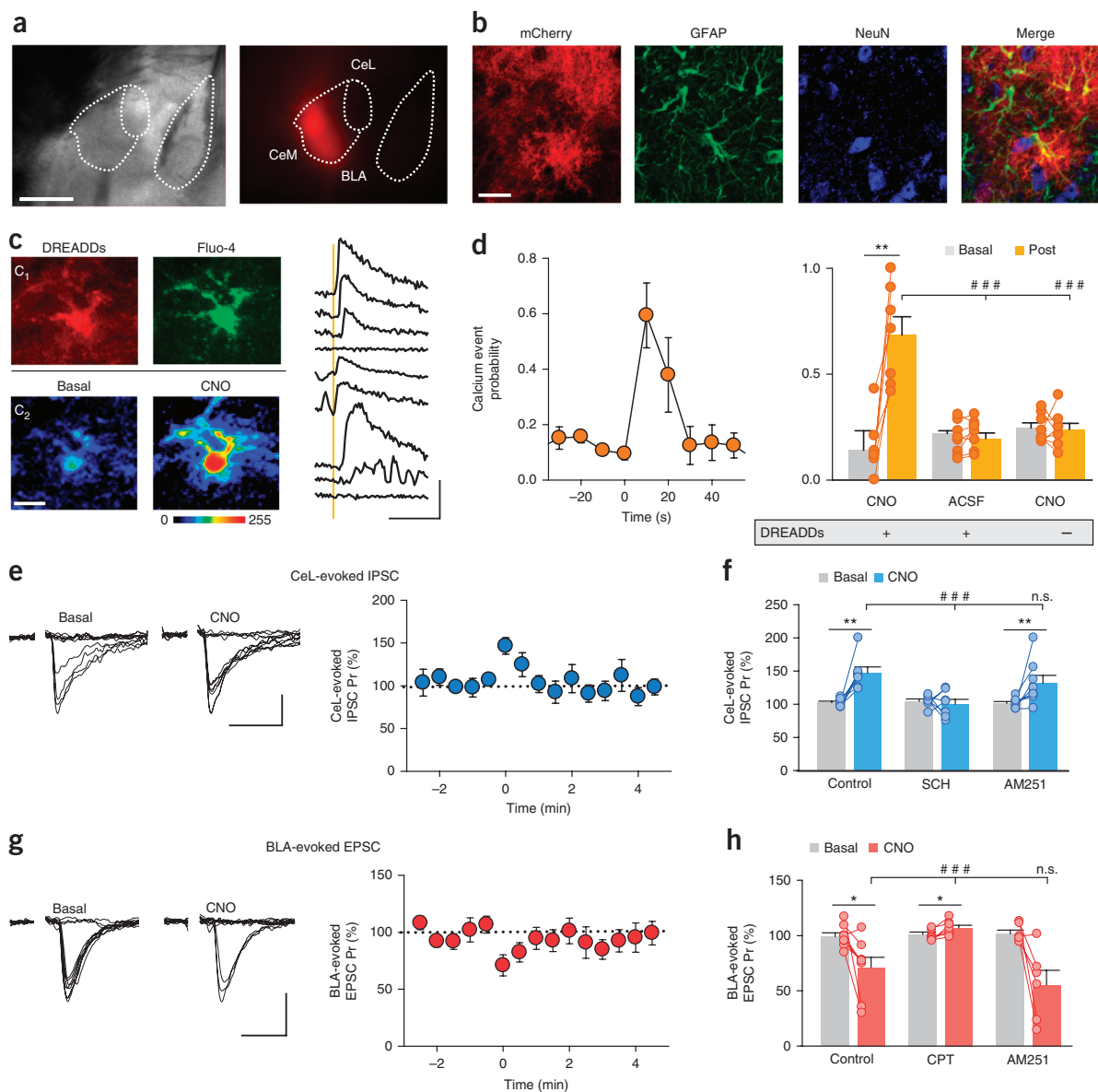


Figure 3 Selective expression and activation of astrocytic Gq-DREADDs in the CeM increases astrocytic calcium levels, increases inhibitory synaptic transmission at CeL–CeM synapses and depresses excitatory synaptic transmission at BLA–CeM synapses. **(a)** DIC and fluorescence images showing the localization of DREADDs in the CeM as reported by mCherry expression (red). Scale bar, 500 μm . **(b)** Confocal images of mCherry labeling; astrocytes are immunohistochemically labeled with the astrocytic marker GFAP, and neurons are labeled with the neuronal marker NeuN. Scale bar, 20 μm . **(c)** Left, images of CeM astrocytes. Top, C₁ fluorescence images showing mCherry and Fluo-4. Bottom, C₂ pseudocolor images of fluorescence intensities before and after local application of CNO. Scale bar, 5 μm . Right, astrocytic calcium levels before and after CNO application (vertical yellow bar). Scale bars, 50% and 30 s. **(d)** Left, calcium event probability in basal conditions and after CNO application at time 0 ($n = 7$). Right, calcium event probability before and after CNO application in DREADD-expressing slices ($n = 7$; $P = 0.0018$), before and after ACSF application in DREADD-expressing slices ($n = 8$; $P = 0.17$), and before and after CNO application in slices with no DREADD expression ($n = 8$; $P = 0.83$). The increase in calcium event probability observed after local application of CNO in DREADD-expressing animals was absent after local application of either ACSF or CNO in mice without DREADD expression (two-way ANOVA indicated a significant effect of CNO ($P < 0.001$) and interaction with the experimental condition ($P < 0.001$); *post hoc* Holm–Sidak-corrected for two comparisons; $P < 0.001$ in both cases). **(e)** Left, CeL-evoked IPSCs recorded in CeM neurons before and after CNO application. Scale bars, 10 pA and 25 ms. Right, CeL-evoked IPSC Pr before and after CNO application (time 0; $n = 7$). **(f)** CeL-evoked IPSC Pr before and after CNO application in control conditions ($n = 7$; $P = 0.004$) and in the presence of SCH ($n = 7$; $P = 0.96$) and AM251 ($n = 8$; $P = 0.003$). We observed a difference in the response to CNO between the control condition and the SCH condition (two-way ANOVA indicated a significant effect of CNO ($P < 0.001$) and interaction with the experimental condition ($P < 0.001$); *post hoc* Holm–Sidak-corrected for two comparisons ($P < 0.001$)) but not between control and AM251 conditions ($P = 0.12$). **(g)** Left, BLA-evoked EPSCs recorded in CeM neurons before and after CNO application. Scale bars, 20 pA and 25 ms. Right, BLA-evoked EPSC Pr before and after CNO application (time 0; $n = 8$). **(h)** BLA-evoked EPSC Pr before and after CNO application in control conditions ($n = 8$; $P = 0.02$) and in the presence of CPT ($n = 7$; $P = 0.3$) and AM251 ($n = 6$; $P = 0.02$). We observed a difference in the response to CNO between the control condition and the CPT condition (two-way ANOVA indicated a significant effect of CNO ($P < 0.001$) and interaction with the experimental condition ($P < 0.001$); *post hoc* Holm–Sidak-corrected for two comparisons ($P < 0.001$)) but not between control and AM251 conditions ($P = 0.1$). * $P < 0.05$, ** $P < 0.01$; Student's paired *t*-test. ### $P < 0.001$; two-way ANOVA, *post hoc* Holm–Sidak; n.s., nonsignificant ($P > 0.05$). Data in **d–h** are mean \pm s.e.m.

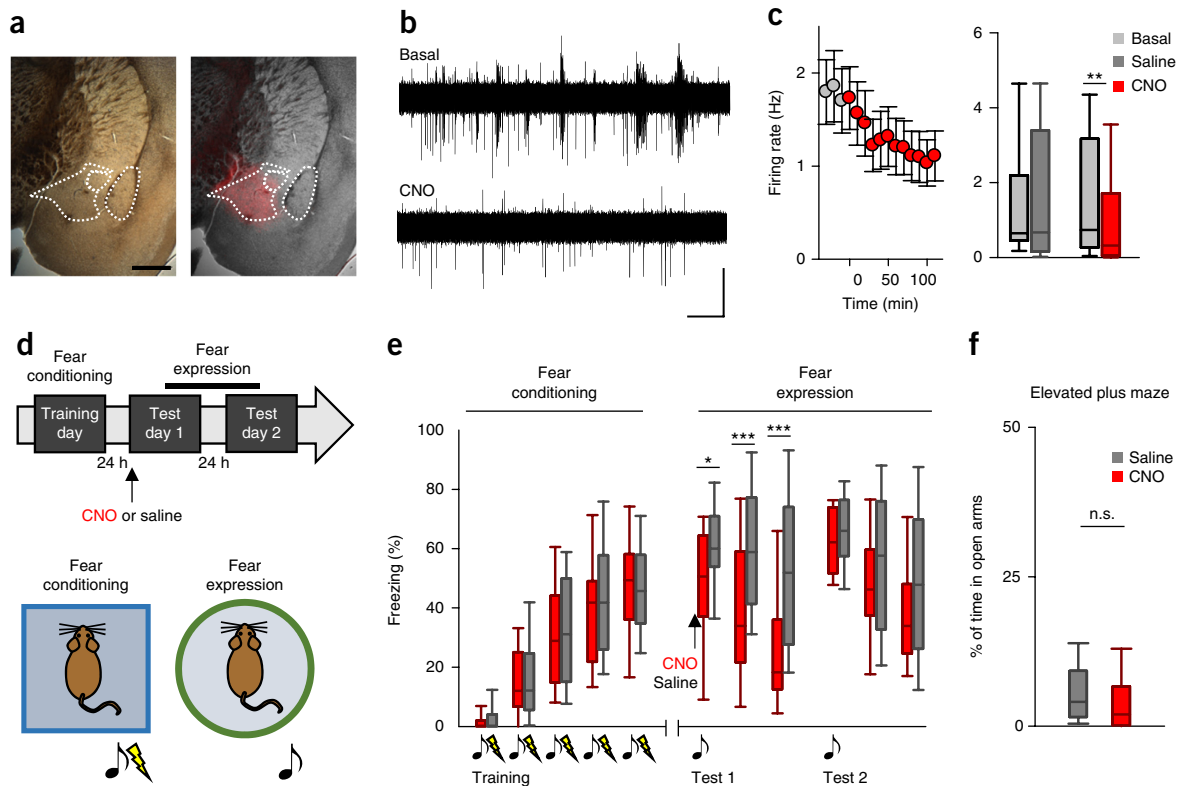


Figure 4 Selective activation of astrocytic DREADDs in CeM reduces the firing rate and decreases fear expression in a delayed fear conditioning paradigm. (a) Images showing DREADD expression in the CeM. Scale bar, 500 μ m. (b) Representative multi-unit activity recordings in the CeM before and after CNO i.p. injection. Scale bars, 50 μ V and 60 s. (c) Left, the mean CeM firing rate before (gray) and after CNO application (red; application at time 0). Data shown are mean \pm s.e.m. (red; application at time 0, $n = 28$). Right, the CeM firing rate in basal conditions and after saline application ($n = 23$; $P = 0.6$) or CNO application ($n = 28$; $P = 0.004$). (d) A schematic representing the delayed fear conditioning paradigm. Mice were fear conditioned on the training day in five trials consisting of a 15-s sound cue co-terminating with a 1-s foot shock. Fear retrieval was measured on test days 1 and 2, with either CNO or saline injected intraperitoneally 30 min before the first cue presentation only in test 1. (e) Left, fear response measured as the percentage of freezing during the 15-s cue presentation in CeM DREADD-expressing mice during fear conditioning. Right, fear response measured as the percentage of freezing during the 3 min of continuous cue presentation; data are depicted in 1-min time bins. One nonreinforced cue was presented in test 1 ($P = 0.037$, $P < 0.001$, $P < 0.001$) and in test 2 ($P = 0.23$, $P = 0.24$, $P = 0.066$; $n = 33$ mice injected with CNO (red) and 30 mice injected with saline (gray)). (f) The percentage of time spent in the open arms in the elevated plus maze test ($n = 30$ mice injected with CNO and 33 mice injected with saline). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's paired t -test (b) or unpaired t -test (e,f). In box-and-whisker plots, center lines indicate medians, box edges represent the interquartile range, and whiskers extend to the 10th and 90th percentiles of the distribution.

similar effects independent of eCB actions. To test this idea, we used an artificial but cell-specific stimulus to directly activate astrocytes. We injected mCherry-tagged adeno-associated virus (AAV8-GFAP-hM3D(Gq)-mCherry) into the CeM of mice to induce selective expression of the stimulatory Gq-DREADD hM3D in astrocytes (Fig. 3a,b; detailed information is provided in the Online Methods). Local application of the selective ligand clozapine-N-oxide (CNO; 1 mM) by pressure pulse (2 s) increased calcium levels and the calcium event probability in DREADD-expressing astrocytes (78 astrocytes from $n = 7$ slices; $P = 0.0018$; Fig. 3c,d). To confirm that these effects were selectively mediated by CNO activation of DREADDs, we locally applied either extracellular solution without CNO to DREADD-expressing astrocytes (105 astrocytes from $n = 8$ slices) or CNO in mice that lacked DREADD expression (109 astrocytes from $n = 8$ slices). In both cases, we observed no increases in calcium event probability ($P = 0.17$ and $P = 0.83$, respectively; Fig. 3d). In agreement with the effects produced by eCB-mediated astrocyte activation (Fig. 1h,i), selective stimulation of DREADD-expressing astrocytes by CNO increased the Pr of CeL-evoked IPSCs ($n = 7$; $P = 0.004$) and decreased the Pr of BLA-evoked EPSCs ($n = 8$; $P = 0.02$;

Fig. 3e-h), with no changes in synaptic potencies (IPSCs, $n = 7$, $P = 0.83$; EPSCs, $n = 8$, $P = 0.2$; Supplementary Fig. 5). Furthermore, the CNO-evoked increase in IPSC Pr was blocked by the A_{2A} receptor antagonist SCH58261 ($n = 7$; $P = 0.96$; Fig. 3f), and the CNO-evoked decrease in EPSC Pr was blocked by the A_1 receptor antagonist CPT ($n = 7$; $P = 0.3$; Fig. 3h). Therefore, direct activation of DREADD-expressing astrocytes produced similar synaptic effects as eCB-mediated activation of astrocytes by increasing astrocyte calcium levels and stimulating gliotransmitter release. To further test this idea, which suggested that the chemogenetic activation is independent of astrocytic CB1R activation, we applied CNO locally in the presence of the CB1R antagonist AM 251. In this condition, CNO increased the calcium event probability (from 0.21 ± 0.02 to 0.74 ± 0.1 ; 69 astrocytes from $n = 6$ slices; $P = 0.004$, paired t -test), increased the Pr of CeL-evoked IPSCs ($n = 8$; $P = 0.003$) and decreased the Pr of BLA-evoked EPSCs ($n = 6$; $P = 0.02$; Fig. 3f,h), with no changes observed in synaptic potencies (IPSCs, $n = 8$, $P = 0.87$; EPSCs, $n = 6$, $P = 0.77$; Supplementary Fig. 5a,b). These results indicate that selective activation of DREADD-expressing astrocytes mimics the effects of eCBs as endogenous stimuli: both induced elevations in astrocyte calcium

levels that led to an increase in IPSC Pr and a decrease in EPSC Pr. Thus, astrocyte stimulation by the activation of endogenous receptors (CB1Rs stimulated by eCBs mobilized from neurons) or exogenous but selective receptors (Gq-DREADDs activated by CNO) differentially regulate inhibitory and excitatory synapses in CeM neurons.

Next, we investigated the effects of sustained application of CNO (10 μ M). Perfusion of the agonist induced a persistent increase in the calcium oscillation frequency ($n = 74$ astrocytes, $n = 6$ slices; $P = 0.009$; **Supplementary Fig. 6a,b**), a tonic increase in the Pr of CeL-evoked IPSCs ($n = 9$; $P = 0.001$; **Supplementary Fig. 6c,d**), and a tonic decrease in the Pr of BLA-evoked EPSCs ($n = 6$; $P = 0.0001$; **Supplementary Fig. 6e,f**). Consistent with observations after the acute application of CNO, the effects on IPSCs and EPSCs were not accompanied by changes in the synaptic potency (IPSCs, $n = 9$, $P = 0.27$; EPSCs, $n = 6$, $P = 0.59$; **Supplementary Fig. 5c,d**) and were reversed by the A_{2A} receptor antagonist SCH58261 ($n = 3$; $P = 0.88$; **Supplementary Fig. 6c,d**) and by the A_1 receptor antagonist CPT ($n = 4$; $P = 0.23$; **Supplementary Fig. 6e,f**), respectively. Taken together, these results suggest that persistent application of CNO induces a tonic activation of astrocytes and a tonic regulation of both BLA–CeM excitatory and CeL–CeM inhibitory synaptic inputs.

***In vivo* functional consequences of astrocytic activation**

We then asked whether the astrocytic differential synaptic regulation observed in acute brain slices would alter the firing rate of CeM neurons *in vivo*. For this purpose, we injected DREADDs into CeM, which allowed us to locally activate a population of astrocytes (**Fig. 4a**) during *in vivo* electrophysiological recording of a neural population within the same CeM in anesthetized animals. We obtained basal electrophysiological recordings of multi-unit activity under control conditions (over 30 min) and after an intraperitoneal (i.p.) injection of CNO (2 mg/kg body weight). In mice expressing Gq-DREADDs in CeM astrocytes after injection with AAV8-GFAP-hM3D(Gq)-mCherry, CNO decreased the CeM firing rate ($n = 28$ neurons from 7 mice; $P = 0.004$; **Fig. 4b,c**), whereas no changes were observed after saline injection ($n = 23$ neurons from 6 mice; $P = 0.6$; **Fig. 4c**). This relative silencing of CeM neural activity is consistent with the increased inhibitory synaptic rate and decreased rate of excitatory synaptic inputs (**Fig. 3f,h**).

Finally, we studied the consequences of selective activation of CeM astrocytes on amygdala-related behavior by using the delayed auditory fear conditioning paradigm (**Fig. 4a,d**). Three weeks after receiving virus injections to induce DREADD expression in CeM astrocytes, mice underwent cued fear conditioning. On test day 1, 24 h after training, mice received i.p. injections of either CNO ($n = 33$) or saline ($n = 30$) 30 min before presentation of the first non-reinforced cue, at which point the freezing response was recorded (**Fig. 4d**). In these conditions, saline-injected mice did not show any reduction of freezing during the 3 min of cue presentation (i.e., no within-session extinction), whereas in test 1, animals injected with CNO showed a clear extinction of the freezing response and a decreased fear response to the cue compared with saline-injected animals ($P = 0.037$, $P < 0.001$, $P < 0.001$; **Fig. 4e**). Notably, 24 h after CNO or saline injection, on test day 2, no differences were observed between the freezing responses of the two animal cohorts ($P = 0.23$, $P = 0.24$, $P = 0.066$; **Fig. 4e**), indicating that CNO produced an acute effect in test 1 that was not present 24 h after the CNO application, in test 2. We also tested the effects of astrocytic activation in the elevated plus maze, a behavioral paradigm associated with anxiety behavior. We did not observe any differences in the percentage of time spent in the open arms of the maze ($P = 0.44$; **Fig. 4f**). Furthermore, CNO did not produce

any behavioral effects in mice that lacked DREADD expression (**Supplementary Fig. 7**). These results indicate that selective activation of astrocytes in the CeM specifically enhances within-session extinction and reduces the expression of an acquired fear response, without altering long-term extinction of the same behavior or anxiety-like behavior. Rather than acting in a broad, unspecific manner, astrocytes influence certain specific behaviors, which is consistent with specific synaptic regulation.

DISCUSSION

A growing body of evidence suggests that astrocyte–neuron interactions are crucial elements in the control of synaptic physiology^{26,27} and neuronal networks^{33,46}. Our results show that astrocytes differentially regulate both excitatory and inhibitory synaptic transmission in the CeM in a synapse-specific manner, thus resulting in the regulation of neuronal activity and influencing the behavioral output of the brain region (**Supplementary Fig. 8**).

The present results indicate that astrocytes in the central amygdala are functional components of the eCB system. In agreement with reports of other brain areas, eCBs regulate synaptic transmission through the activation of CB1Rs in astrocytes, calcium mobilization and the stimulation of gliotransmitter release^{32,34,38,47}. In addition to the well-known regulation of synaptic transmission and plasticity by eCBs through direct activation of neuronal CB1Rs^{48,49} (**Supplementary Fig. 9**), the present results add to the accumulating evidence indicating that eCBs may have additional synaptic regulatory effects by activating astrocytes, which can expand the signal range and regulate synapses relatively distant from the eCB source, a phenomenon termed lateral regulation of synaptic transmission⁵⁰. These complementary mechanisms of neuron- and astrocyte-driven signaling provide a high degree of complexity to the functional consequences of eCB signaling.

Astrocytes are able to release different neuroactive substances. Among them, glutamate ATP/adenosine and D-serine are the major gliotransmitters identified as regulators of synaptic transmission in several brain areas¹⁷. Our results indicate that the synaptic regulation observed in our experimental conditions depends on astrocyte calcium activity that stimulates the release of ATP/adenosine, which, acting as a gliotransmitter, activates neuronal adenosine receptors in CeM synapses. The astrocyte-mediated synaptic regulation of both CeL-evoked IPSCs and BLA-evoked EPSCs was insensitive to mGluR antagonists, which suggests that the gliotransmitter glutamate is not involved. Similarly, the insensitivity of the synaptic regulation to D-AP5 suggests that D-serine, which acts as a co-agonist of NMDARs^{25,42}, is not implicated. Therefore, although these gliotransmitters might have other potential effects, their involvement in the reported phenomena is unlikely. In contrast, our results show that synaptic regulation of CeL-evoked IPSCs and BLA-evoked EPSCs was prevented by A_{2A} and A_1 receptor antagonists, respectively, suggesting that ATP/adenosine is the gliotransmitter responsible for the phenomena (**Supplementary Fig. 8**).

The selective signaling of astrocytes to specific synapses belonging to specific pathways has been reported recently in basal ganglia circuits³³. The synapse specificity of astrocytic signaling is further supported by the present results, which show that adenosine derived from astrocytes differentially regulates excitatory and inhibitory synaptic transmission in the CeM by activating specific adenosine receptors. Therefore, rather than triggering broad, unspecific effects, astrocytes exert their regulatory actions through selective interaction with specific synapses via the activation of specific signaling pathways. In addition, here we show that the synapse specificity of synaptic

regulation by astrocytes has important consequences for network function and animal behavior.

Our results identify a functional role of astrocytes in the amygdala and reveal that bidirectional astrocyte–neuron communication is relevant in amygdala physiology, regulating the amygdala's functional connectivity and its behavioral outcome. Therefore, these results suggest that brain functions and their behavioral consequences result from synapse-specific signaling and the coordinated activity of astrocytes and neurons.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.M.-F. and A.A. conceived the study. M.M.-F., J.A., G.M. and A.A. wrote the manuscript. M.M.-F. performed and analyzed electrophysiological and calcium imaging experiments and analyzed the data. M.M.-F., E.D.M., J.A. and A.A. designed *in vivo* electrophysiological experiments. S.J. performed immunohistochemistry techniques. M.M.-F., M.A.B., G.M. and A.A. designed the behavioral experiments. M.A.B., L.M.R. and Z.Z. performed behavioral experiments. All the authors read and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Ethics statement. All of the procedures for handling and killing animals were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Animals. Mice were housed under a 12-h/12-h light/dark cycle with up to five animals per cage. Male C57BL/6J mice (14–21 d old) were used for slice electrophysiology. For specific experiments, slices were obtained from male GFAP-CB1R-null and GFAP-CB1R^{WT} mice (12–20 weeks old) and from male IP3R2⁻ mice (14–21 d old), which were generously donated by Dr. G. Marsicano and Dr. J. Chen, respectively^{51,52}. For DREADD (AAV8-GFAP-hM3D-mCherry) activation experiments, 9–20-week-old male C57BL/6J mice were used for slice electrophysiology and *in vivo* electrophysiology, and 9–12-week-old male C57BL/6J mice were used for the delayed fear conditioning and elevated plus maze experiments.

Mice carrying the ‘floxed’ CB1R-expressing gene (*Cnr1^{fl/fl}*) were crossed with GFAP-CreERT2 mice⁵³ via a three-step backcrossing procedure to produce *Cnr1^{fl/fl};GFAP-CreERT2* and *Cnr1^{fl/fl}* littermates, referred to here as GFAP-CB1R-null and GFAP-CB1R^{WT} mice, respectively. CreERT2 protein is inactive in the absence of tamoxifen treatment; *Cnr1* was ‘deleted’ in adult mice (8 weeks old) by eight daily injections of tamoxifen (1 mg i.p.), dissolved in 90% sunflower oil, 10% ethanol to a final concentration of 10 mg/ml (ref. 53). The animals were used at least 4 weeks after tamoxifen treatment.

Amygdala slice preparation. To obtain brain slices containing the amygdaloid complex, we decapitated animals and then rapidly removed their brains and placed the brains in ice-cold artificial cerebrospinal fluid (ACSF). Slices (350 μ m thick) were incubated for 1 h at room temperature (21–24 °C) in ACSF that contained 2.69 mM NaCl, 1.25 mM KH₂PO₄, 2 mM MgSO₄, 26 mM NaHCO₃, 2 mM CaCl₂ and 10 mM glucose and was gassed with 95% O₂, 5% CO₂, pH 7.3. Slices were then transferred to an immersion recording chamber and superfused at 2 ml/min. The chamber volume was replaced in 8–12 min with gassed ACSF. The amygdaloid complex and its different subnuclei were easily identified by transillumination with a 4 \times objective and use of the Allen Brain Atlas as a reference. We confirmed the location of the CeM nucleus on the basis of the neuronal electrical properties^{12,54}, observing low-threshold bursting (19 out of 35 recorded neurons; 54.3%), regular spiking (10 out of 35 neurons; 28.5%), late-firing (5 out of 35 neurons; 14.3%) and stuttering neurons (1 out of 35 neurons; 2.9%).

Electrophysiology. Neurons were identified by infrared differential interference contrast microscopy. Simultaneous electrophysiological recordings from CeM neurons were obtained via the whole-cell patch-clamp technique. Patch electrodes had resistances of 3–10 M Ω when filled with an internal solution that contained 135 mM KMeSO₄, 10 mM KCl, 10 mM HEPES-K, 5 mM NaCl, 2.5 mM ATP-Mg²⁺, and 0.3 mM GTP-Na⁺, pH 7.3. The BAPTA-containing intracellular solution contained 40 mM BAPTA-K₄, 2 mM ATP-Na₂, 10 mM HEPES, 1 mM MgCl₂ and 8 mM NaCl, pH 7.3. To reveal the astrocyte network, we also included biocytin (0.1%) in this solution; slices were fixed and biocytin was revealed by Alexa Fluor 488–streptavidin. Recordings were obtained with PC-ONE amplifiers (Dagan Instruments, Minneapolis, MN). Fast and slow whole-cell capacitances were neutralized and series resistance was compensated (~70%), and the membrane potential was held in a range from –70 mV to –80 mV. Electrophysiological properties were monitored during the experiments, and recordings were considered stable when the series and input resistances, resting membrane and stimulus artifact duration did not change by more than 20%. Cells that did not meet these criteria were discarded. Signals were fed to a Pentium-based PC through a DigiData 1440A interface board. Signals were filtered at 1 kHz and acquired at a 10-kHz sampling rate. The pCLAMP 10.2 (Axon Instruments) software was used for stimulus generation, data display, acquisition and storage. The distance between the somas of the paired recorded neurons was 70–150 μ m.

Synaptic stimulation. Theta capillaries (2–5- μ m tip) filled with ACSF were used for bipolar local stimulation. The electrodes were connected to an S-910 stimulator through an isolation unit. GABAergic IPSCs in CeM neurons were evoked by local electrical stimulation through an extracellular stimulation electrode located in the CeL, and isolated in the presence of AMPAR and NMDAR antagonists

(CNQX 20 μ M and D-AP5 50 μ M). Glutamatergic EPSCs in CeM neurons were evoked by local electrical stimulation through an extracellular stimulation electrode located in the BLA, and isolated in the presence of GABA_AR and GABA_BR blockers (Picrotoxin 0.05 mM and CGP 5 μ M, respectively). The synaptic responses showed failures and successes in neurotransmitter release^{26,38,39,55}. The stimulus parameters were adjusted to meet the conditions of putative single or very few presynaptic fibers, and remained unchanged during the experiment. Synapses that did not meet the criteria were discarded. A response was considered a success if the amplitude of the current was >3 times the s.d. of the baseline current and was verified by visual inspection. We quantified the Pr as the ratio between successes and failures in evoked synaptic transmission, and the synaptic potency as the amplitude of the successful responses. Paired pulses (250- μ s duration and 50-ms interval) were continuously delivered at 0.33 Hz. The paired-pulse ratio was estimated as PPR = second EPSC/first EPSC or second IPSC/first IPSC. The average of the successes and failures was used as the amplitude of the EPSC or IPSC for this calculation.

Basal synaptic parameters were considered to be the parameters during the 5 min before the application of the stimulus. The stimulus to induce eCB release was a 10-s ND to 0 mV (ref. 35). The ND was applied 2.5 s after the last basal delivered pulse, and no pulses were presented during the ND. Immediately after the ND was finished, the 0.33-Hz pulse protocol was started again. For acute application of CNO, a micropipette was filled with 1 mM CNO solution and placed 100–150 μ m away from the recording neuron, and a pressure pulse was applied for 2 s. The absence of mechanical movement of the tissue was confirmed in every case. In the text, data are expressed as a percentage relative to the basal 5 min. Results were compared by two-tailed Student’s paired *t*-test unless otherwise stated.

Ca²⁺ imaging. Ca²⁺ levels in astrocytes located in the CeM were monitored by fluorescence microscopy with the Ca²⁺ indicator fluo-4 (Molecular Probes, Eugene, OR). Slices were incubated with fluo-4 AM (2 μ l of 2 mM dye were dropped over the amygdaloid complex, yielding a final concentration of 2 μ M and 0.01% 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^{33,56}. SR101 was intraperitoneally injected (100 mg/kg) and the animal was left in the cage for ~30–45 min until intense coloration was observed in paws and ears, as reported⁵⁷. With this staining procedure SR101 stains specifically astrocytes^{56–58} (but see ref. 59). Astrocytes were imaged either with a CCD (charge-coupled device) camera (Retiga EX, Qimaging, Canada) attached to the Olympus microscope or in a multiphoton scope Leica SP5. Cells were illuminated for 100 ms with an LED at 488 nm, and images were acquired every 1 s. Intracellular Ca²⁺ signals were monitored from CeM astrocytes, and Ca²⁺ variations were recorded at the soma and proximal processes. The signal was measured as fluorescence over baseline ($\Delta F/F_0$), and cells were considered to have displayed a calcium event when the $\Delta F/F_0$ of the calcium signal increased by three times the s.d. of the baseline for at least two consecutive images.

The astrocyte Ca²⁺ signal was quantified as the probability of occurrence of a Ca²⁺ event (calcium event probability). The Ca²⁺ event probability was calculated as the number of astrocytes starting a calcium event per time bin in a field of view, divided by the number of astrocytes in that field of view (10–20 astrocytes). The calcium event probability was calculated in each slice, and for statistical analysis the sample size corresponded to the number of slices, because different slices were considered as independent variables. Events were grouped in 10-s time bins. The time of occurrence of an event was considered to be at the onset of the Ca²⁺ event. To test the effects of the different stimuli, we compared the respective mean basal calcium event probability with the calcium event probability in the time bin after the stimulus. Mean values were obtained from at least four slices in each condition. For the CNO perfusion, the Ca²⁺ signal was quantified as a calcium event frequency; thus it was calculated as the number of calcium events each astrocyte displayed per minute in a field of view. The calcium event frequency was grouped in time bins of 1 min. To test the effect of CNO perfusion, we compared the basal calcium event frequency to the calcium event frequency 4 and 5 min after the initial CNO application.

Virus delivery of DREADDs and confirmation of virus expression location. AAV8-GFAP-hM3D-mCherry (adenovirus serotype 8, 2 \times 10¹² virus molecules per ml; Gene Therapy Vector Core at University of North Carolina) was used.

Stereotaxic bilateral injections (300–500 nl at 100 nl min⁻¹) were made into the CeM (anterior–posterior, –1 mm; medial–lateral, ±2.75 mm; dorsal–ventral, 5.15 mm; from bregma) of C57BL/6J mice at 6–9 weeks of age. Three weeks after the virus injection, the location of the virus was confirmed on the basis of mCherry expression. Only animals in which the expression was located mainly in the CeM, with no major leak into other subnuclei, were used. Animals in which the expression did not meet these location criteria were discarded.

Immunohistochemistry. Anesthetized C57BL/6J mice transfected with AAV8-GFAP-hM3D-mCherry were perfused intracardially with 0.1 M PBS followed by 4% paraformaldehyde ($n = 6$ mice). Brains were extracted and post-fixed in paraformaldehyde overnight. Each brain was sectioned into 50- μ m slices that were then blocked in 10% normal goat serum with 0.1% Triton X-100 in PBS (1 h, room temperature) and stained for rabbit GFAP-specific antibody (1:1,000; Sigma; G9269), mouse NeuN-specific antibody (1:500; Millipore; MAB377), rabbit NeuN-specific antibody⁶⁰ (1:500; Millipore; MABN140), mouse NG2-specific antibody⁶¹ (1:500; Millipore; AB5320), rabbit Iba1 antibody⁶² (1:500; Dako; 019-19741), and mouse CC1-specific antibody⁶³ (1:500; Calbiochem; OP80) overnight (4 °C). This was followed by a 3-h incubation in Alexa Fluor 488 goat anti-rabbit (1:500; Invitrogen; A11034), Alexa Fluor 405 goat anti-mouse (1:500; Invitrogen; A31553) and Cy3 goat anti-mouse (1:500) before being mounted on a glass slide with Vectashield Hardset mounting media (Vector Labs). Detailed information regarding antibody validation is included in the **Life Sciences Reporting Summary**. The slides were imaged with a Leica SP5 multiphoton confocal microscope. The cellular specificity of DREADD expression was tested by immunohistochemical analysis of randomly selected areas of CeM. Out of 790 DREADD-expressing cells (assessed by mCherry fluorescence), 785 cells (99.36%) were identified as astrocytes on the basis of their colocalization with GFAP, 3 cells (0.37%) were neurons (identified by colocalization with NeuN), 2 cells (0.25%) were oligodendrocytes (identified by colocalization with CC1), and none (0.0%) were microglia (identified by colocalization with Iba1) or oligodendrocyte precursor cells (identified by colocalization with NG2). Moreover, 88.1% of astrocytes identified by GFAP (785 out of 891 astrocytes; 15 slices; 6 mice), 1.1% of oligodendrocytes identified by CC1 (2 out of 173 oligodendrocytes; 6 slices; 2 mice), 0.11% of neurons identified by NeuN (3 out of 2,596; 19 slices; 6 mice), 0.0% of microglia identified by Iba1 (0 out of 178; 9 slices; 2 mice) and 0.0% oligodendrocyte precursor cells identified by NG2 (0 out of 100; 9 slices; 3 mice) expressed DREADDs (monitored by mCherry expression). These results indicate that the number of cells other than astrocytes that expressed DREADDs was negligible (0.6%) and that a vast amount (88.1%) of CeM astrocytes expressed DREADDs.

The above-described selective GFAP-driven DREADD expression in CeM astrocytes supports the specific deletion of astrocytic CB1R in GFAP-CB1R-null mice. To directly test this idea, we analyzed the functional expression of CB1R. Neuronal expression of CB1R was assessed on the basis of the depolarization-induced suppression of inhibition (DSI), a well-characterized purely neuronal phenomenon dependent on presynaptic CB1R^{35,36,64}, and astrocyte expression of CB1R was assessed on the basis of CB1R-mediated Ca²⁺ elevations evoked by neuronal depolarization^{32,38}. We found that in wild-type mice neuronal depolarization evoked both DSI and increases in amounts of astrocyte Ca²⁺, whereas in GFAP-CB1R-null mice the DSI was still present but the increase in astrocyte Ca²⁺ was absent (**Supplementary Fig. 9**). These results show that in GFAP⁻CB1R⁻ mice, CB1R-mediated signaling was selectively abolished in astrocytes, whereas CB1R-mediated signaling was preserved in neurons, indicating the specific deletion of CB1R in astrocytes.

For astrocytic network labeling, after biocytin filling, slices were fixed in 4% PFA in 0.1 PBS, pH 7.4, at 4 °C. Biocytin was visualized with Alexa Fluor 488–streptavidin (RRID [AB_2315383](https://identifiers.org/AB_2315383); 1:500).

In vivo electrophysiological recordings. Mice were anesthetized (urethane, 1.8 g/kg i.p.) and placed in a stereotaxic frame (ASI Instruments). Their body temperature was maintained at 37 ± 1 °C with a heating blanket, and breathing rates were constantly monitored. A tungsten electrode (5-M Ω impedance at 1,000 Hz) for electrophysiological recordings of multi-unit activity was located stereotaxically in the same coordinates as for virus injection for each animal (anterior–posterior, –1 mm; medial–lateral, ±2.75 mm; dorsal–ventral, –5.15 mm; from bregma). The signal was amplified and filtered (300–3,000 Hz) with a differential amplifier

(Model 3000 AC/DC, AM System). Signals were digitalized at 10 KHz with an A/D converter (DigiData 1550A, Axons Instruments) and stored in a PC for posterior analysis with the software pCLAMP 10.2 (Axon Instruments). Spikes were detected in offline analysis with the following criteria: a voltage threshold was located at the level of the average of background noise plus three times the s.d. (obtained during long silent periods) and verified by visual inspection. In every mouse, spikes were grouped in clusters on the basis of spike amplitude. A scalp vein set was filled with either saline or CNO (2 mg/kg) and was placed intraperitoneally before the recording started. After 30 min of baseline recording, either CNO or saline was applied.

Delayed fear conditioning. This associative learning task involved measuring a fear response (i.e., time spent freezing) to a conditioned stimulus (cue) that was predictive of an unconditioned stimulus (mild foot shock) presented during training trials. Data collection and analysis were semi-automated via a video-monitoring fear-conditioning apparatus (Med Associates, Inc.). On the conditioning day (training day), mice were exposed to a series (five pairings; 60-s intertrial interval) of cue (80-dB white noise tone and light) presentations (15 s in duration) that co-terminated with a mild foot shock (0.7 mA, 1 s in duration). Twenty-four hours later mice were injected with either CNO (2 mg/kg i.p.) or saline 30 min before the first cued fear test (test day 1). Cued fear testing took place in a test chamber with altered contextual elements (floor, wall and odor) and consisted of a 3-min baseline (nonspecific freezing behavior) and a 3-min cue exposure (cued fear) period. This cued fear test was then repeated 24 h later (test day 2) without any CNO exposure. Freezing response was assessed during the various procedural components of both the conditioning (conditioned stimulus and intertrial interval) and testing (baseline and cue) sessions. For the memory tests, we broke freezing down further into 1-min time bins within each session to investigate within-session changes.

Elevated plus maze. Subjects were tested on an elevated plus maze (EPM) apparatus (Med Associates, Inc.). Testing was done under dim lighting conditions, with low-intensity LED lights over the open arms generating ~50 lx of brightness at the end of the arms. Tests were 5 min in duration, and movement was tracked and analyzed with ANY-maze software (Stoelting Co.). Open arm time (as a percentage of total arm exploration), open and closed arm entries and total distance traveled were determined by the software. Mice were injected with either CNO (2 mg/kg i.p.) or saline 30 min before the test. The same subjects were used in the EPM testing as were used for fear conditioning. The EPM test was performed 1 week after fear testing, and the CNO/saline treatments were assigned randomly, irrespective of previous exposure.

Drugs and chemicals. *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385 (LY)), and (2S)-3-[[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphonic acid hydrochloride (CGP 55845) were purchased from Tocris Cookson (Bristol, UK); Fluo-4 AM (Eugene, OR) and picrotoxin were from Indofine Chemical Company (Hillsborough, NJ). BAPTA tetrapotassium salt was from Thermo Fisher Scientific (Waltham, MA). All other drugs were purchased from Sigma.

Statistical analysis. The normality and equal variance tests were performed before the application of statistical comparisons, which were made by parametric Student's *t*-test unless otherwise stated. Data are expressed as mean ± s.e.m. unless otherwise stated. To analyze the effects of the stimulus in the same synapse, we used paired Student's *t*-test to compare values before and after the stimulus. To analyze the effects of different treatments and conditions, we carried out multiple comparison testing between the different groups. Therefore, results were compared by either a two-tailed Student's *t*-test ($\alpha = 0.05$) or a two-way ANOVA using the 'basal' and the 'post-stimulus' situations as factor 1 and the different experimental conditions as factor 2. The *post hoc* test used was Holm–Sidak, versus control comparisons, corrected for multiple comparisons, always using the 'basal' situation and the 'control' condition as the controls to compare. Statistical differences were established with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for Student's *t*-test or with # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ for the *post hoc* Holm–Sidak test. For detailed information see **Supplementary Tables 1 and 3–5**. No animals

or data points were excluded from the analysis. Data collection and analysis were not performed with blinding to the condition of the experiments, but the same criteria were applied to all allocated groups for comparisons. Randomization was not used. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those generally used in the field^{26,36,64}.

Data availability. The data that support the findings of this study are available from the corresponding author on reasonable request.

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Experimental design

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Sample size

Describe how sample size was determined.

No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those generally employed in the field.

Data exclusions

Describe any data exclusions.

No animals or data points were excluded from the analysis.

Replication

Describe whether the experimental findings were reliably reproduced.

The experimental findings were reliably reproduced.

Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Randomization was not employed.

Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Data collection and analysis were not performed blind to the condition of the experiments, but the same criteria was applied to all allocated groups for comparisons.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

The software used for electrophysiology was Clampfit 10.4. Images were analyzed with ImageJ. Data collected with those softwares was further analyzed with Microsoft Excell and Sigma Plot.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

1. rabbit GFAP-specific antibody: Sigma, cat # G9269, Anti-Glial Fibrillary Acidic Protein, lot # O83M4830, "Anti-Glial Fibrillary Acidic Protein antibody produced in rabbit is suitable for immunofluorescence using brain section from mice. The product reacts specifically with GFAP and labels astrocytes in immunohistochemical staining." <http://www.sigmaaldrich.com/catalog/product/sigma/g9269?lang=en®ion=US>.
2. mouse NeuN-specific antibody: Millipore, cat # MAB377, Neuronal Nuclei, lot # 2562102, Anti-NeuN antibody has a species reactivity to mouse and "specifically recognizes the DNA-binding, neuron-specific protein NeuN, which is present in most CNS neuronal cell types of all vertebrates tested." http://www.emdmillipore.com/US/en/product/Anti-NeuN-Antibody%2C-clone-A60,MM_NF-MAB377.
3. rabbit NeuN-specific antibody: Millipore, cat # MABN140, clone 27-4, lot # 2858898, Anti-NeuN antibody has a species cross-reactivity to mouse and "is found exclusively in the nuclei of neuronal cells." Ref: Kim, K. K., et al. (2009). *J. Biol. Chem.* 284(45):31052-31061.
4. mouse NG2-specific antibody: Millipore, cat # AB5320, Anti-NG2 Chondroitin Sulfate Proteoglycan, lot # 2834672, Anti-NG2 Chondroitin Sulfate Proteoglycan antibody has a species cross-reactivity to mouse and "is found on the surfaces of glial cells within the developing and mature central nervous system that have the properties of oligodendrocyte precursor cells (i.e., O-2A progenitor cells)." Ref: Rolls, Asya, et al. (2007). *Nat Cell Biol.* 9:1081-8.
5. rabbit Iba1 antibody: Wako, cat # 019-19741, Anti-Iba1, lot # LKR1186, Anti-Iba1 is "specific to microglia and macrophage, but not cross-reactive with neuron and astrocyte. Reactive with mouse Iba1." Ref: Imai, Y., Ibatata, I., Ito, D., Ohsawa, K. and Kohsaka, S.: *Biochem. Biophys. Res. Commun.*, 224, 855 (1996).
6. mouse CC1-specific antibody: Calbiochem, cat # OP80-100UG, Anti-APC, lot # D00172565, Anti-APC (Ab-7) Mouse mAb (CC-1) has a species reactivity to mouse and is "well suited for immunohistochemistry and immunofluorescence studies of oligodendrocytes and optic nerves." Ref: Bhat, R.V., et al. 1996. *Glia* 17, 169.

10. Eukaryotic cell lines

- State the source of each eukaryotic cell line used.
- Describe the method of cell line authentication used.
- Report whether the cell lines were tested for mycoplasma contamination.
- If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No eukaryotic cell lines were used.

No eukaryotic cell lines were used.

No eukaryotic cell lines were used.

No eukaryotic cell lines were used.

▶ **Animals and human research participants**

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

- Provide details on animals and/or animal-derived materials used in the study.

Mice were housed under 12/12-h light/dark cycle and up to five animals per cage. Male C57BL/6J mice (14-21 days old) were used for slice electrophysiology. For specific experiments, slices were obtained from male GFAP-CB1R^{-/-} and GFAP-CB1R^{WT} (12-20 week old) and from male IP3R2^{-/-} (14-21 days old) generously donated by Dr. G. Marsicano and Dr. J Chen respectively^{1,2}. Regarding DREADDs (AAV8-GFAP-hM3D-mCherry) activation experiments: 9-20 week old male C57BL/6J were used for slice electrophysiology as well as for in vivo electrophysiology and 9-12 week old male C57BL/6J were used for the delay fear conditioning and the elevated plus maze experiments.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

- Describe the covariate-relevant population characteristics of the human research participants.

The study did not involved human participants.

Annex 3

REVIEW

ASTROGLIAL TYPE-1 CANNABINOID RECEPTOR (CB₁): 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 (CB₁) receptors. CB₁ receptors are abundant at neuron terminals where their stimulation inhibits neurotransmitter release. However, CB₁ 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 CB₁ receptors control synaptic transmission and plasticity. The recent involvement of astroglial CB₁ 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.

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

INTRODUCTION

The discovery of the endocannabinoid system (ECS) as the endogenous target of the main active compound of the plant *cannabis sativa* (Marijuana), Δ⁹-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 CB₁ and CB₂), 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 CB₁ and CB₂ receptors are present in the central nervous system (CNS) and their activity directly affects glial functions (Stella, 2010). However, CB₁ 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 CB₁ receptors and we refer the reader to recent reviews describing the role of CB₂ receptors and other elements of the ECS in glial cells (Walter and Stella, 2004; Stella, 2009, 2010).

CB₁ 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 CB₁ 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-isoxazole 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, Δ⁹-tetrahydrocannabinol; t-LTD, spike-timing-dependent depression; VGCC, voltage-activated Ca²⁺ channels; WM, working memory.

(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 CB₁ 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 CB₁ 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 Ca²⁺ release from the endoplasmic reticulum (Verkhratsky et al., 2012). For instance, glutamate can increase astroglial intracellular Ca²⁺ via metabotropic glutamate receptors (mGluRs) (Araque et al., 2014). At a finer resolution, recent studies using high-resolution Ca²⁺ imaging of hippocampal slices demonstrated that even astrocytic thin processes are able to respond to activity of single synapses with local Ca²⁺ elevations (Di Castro et al., 2011; Panatier et al., 2011).

The functional meaning of Ca²⁺ 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 Ca²⁺ 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

CB₁ 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, CB₁ 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 CB₁ receptor expression levels can vary between different populations, it is important to state that differential expression of CB₁ receptors is not directly linked with equivalent functional relevance (Marsicano and Kuner, 2008; Bellocchio et al., 2010). This is particularly true when considering astroglial CB₁ receptors. Indeed, the presence of CB₁ 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 (Verkhatsky 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 CB₁ 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 CB₁ 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 Ca²⁺- and ATP-dependent pathways (Stella, 2010). Interestingly, recent data indicate that astroglial CB₁ 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 CB₁ receptors.

Another interesting aspect of astroglial CB₁ receptors refers to the mechanism of intracellular signaling. Classically, neuronal CB₁ receptors are believed to exert an inhibitory effect through the activation of G_{i/o} proteins. Thus, the activation of neuronal CB₁ 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 Ca²⁺ 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 Ca²⁺, thus leading to increases of intracellular Ca²⁺ levels (Navarrete and Araque, 2008; Perea et al., 2014). Thus, the intracellular consequences of CB₁ receptor signaling appears to emerge from its cellular localization, rather than being an intrinsic property of the protein. Moreover, CB₁ 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 CB₁ 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 CB₁ 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 (Chevalleyre 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 CB₁ 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 Ca²⁺ increase in neighboring astrocytes through the activation of astroglial CB₁ receptors (Navarrete and Araque, 2008). The authors also showed that this Ca²⁺ increase depends on phospholipase C (PLC) activation and it is not abolished in the presence of pertussis toxin, thus suggesting that astroglial CB₁ 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 Ca²⁺ 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 Ca²⁺ 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 CB₁ 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 CB₁ receptors (Navarrete and Araque, 2010). Mechanistically, the authors reported that

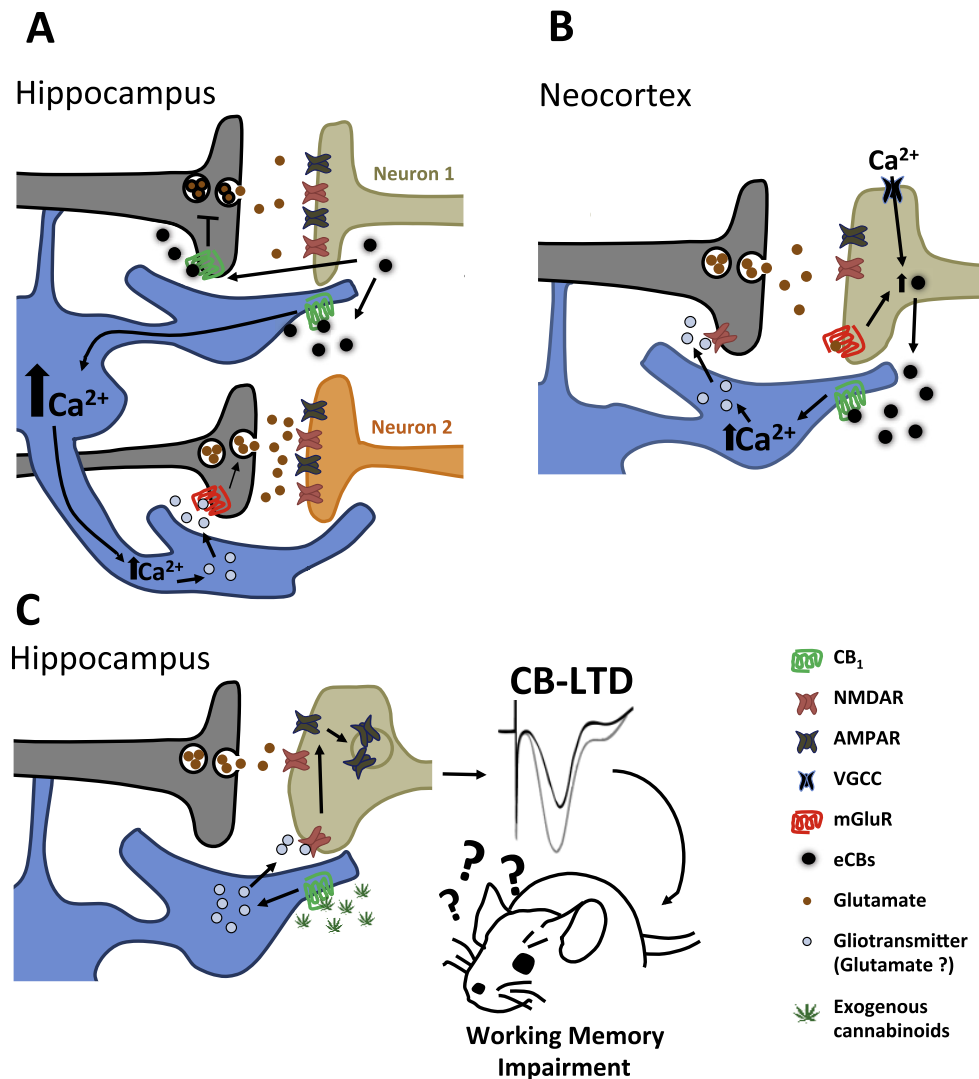


Fig. 1. ECS modulates synaptic function through astroglial CB₁ receptor-dependent signaling. (A) Activity-dependent production of eCB elicits a decrease of neurotransmitter release through the activation of presynaptic CB₁ receptor. In parallel, astroglial CB₁ receptor activation generates an intracellular Ca²⁺ 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 CB₁ receptor-dependent transient Ca²⁺ increase in astrocytes. Both postsynaptic mGluR activation and VGCC-dependent Ca²⁺ influx induced by a back propagating action potential are involved in the synthesis of eCBs. In astrocytes, the Ca²⁺ 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 Nevejan, 2012). (C) Exogenous cannabinoid administration elicits an astroglial CB₁ receptor-dependent *in vivo* long-term depression of synaptic transmission (CB-LTD). Astroglial CB₁ 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).

Ca²⁺ increases elicited by astroglial CB₁ 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 CB₁-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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ receptor modulatory role in bridging neuron–astrocyte interactions but also by proposing a new, different, non-canonical astroglial CB₁ receptor-mediated intracellular mechanism of action (Fig. 1A).

The astroglial CB₁ 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 Nevejan, 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 CB₁ receptors and presynaptic NMDA receptor activations (Sjostrom et al., 2003). Recently, Min and Nevejan (2012) provided functional evidence that astroglial CB₁ receptor activation mediates the t-LTD in the somatosensory neocortical L4 to L2/3 synapses (Min and Nevejan, 2012). The authors reported that eCBs released through post-before-presynaptic activity stimulate astroglial CB₁ receptors and induce an intracellular Ca²⁺ 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 Nevejan, 2012) (Fig. 1B).

Also LTP can be modulated by astroglial CB₁ 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 CB₁ receptors. The mechanism, similarly to what have been described previously (Navarrete and Araque, 2010), relies on an astroglial CB₁ receptor-dependent Ca²⁺ 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 CB₁-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 CB₁ receptors from astrocytes abolishes this cannabinoid-induced LTD (CB-LTD), whereas the genetic deletion of neuronal CB₁ 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 (AMPA) internalization (Han et al., 2012). These results not only constitute the first *in vivo* direct evidence regarding the astroglial CB₁ receptor modulation of synaptic transmission but also

support the validity of previous functional studies (Fig. 1C).

Although astroglial CB₁ 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 CB₁ 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 CB₁ receptors, thereby promoting a potential “fine-tuned” mechanism responsible for the observed LTP. Nevertheless, further investigations are needed to elucidate whether astroglial CB₁ receptor activation produces different effects depending on the nature of the agonists (e.g. endogenous versus exogenous cannabinoids), if astroglial CB₁ receptors behave *in vivo* differently from the main currently used models *in vitro*, or even the functional role of the astroglial CB₁ receptor-dependent signaling on the coordination of neuro-glial networks.

ROLE OF ASTROGLIAL CB₁ 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, CB₁ 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 CB₁ 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-CB₁-KO mouse line, bearing a specific deletion of the CB₁ receptor gene in astrocytes (Han et al., 2012), is a unique model for studying the role of astroglial CB₁ 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-CB₁^{-/-} 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 CB₁ 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 CB₁ receptors mutant mice (i.e. lacking the *CB₁* gene in cortical glutamatergic or forebrain GABAergic neurons), strengthening that astroglial, but not neuronal, CB₁ 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 CB₁ 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-CB₁-KO mice in other behavioral paradigms will likely provide information regarding the endogenous role of astroglial CB₁ 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). CB₁ 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). CB₁ 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 CB₁ 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 CB₁ 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 CB₁ receptors form homodimers or heterodimers? Is CB₁ present in astrocytic organelles such as mitochondria?
- (2) Are CB₁ 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 CB₁ 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 CB₁ 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 CB₁ activation modulate astrocytic mobility? Does it impact synaptic plasticity?
- (6) Calcium is an internal integration signal of astrocytic information (Araque et al., 2014). Astroglial CB₁ activation induces synaptic plasticity through the increase of astrocytic calcium (Navarrete and Araque, 2008, 2010). Can astroglial CB₁ receptor-dependent calcium modulation impact at network level with consequent modulation of neuronal circuits and behavioral functions? Is the calcium rise induced by CB₁ receptors different in nature and consequences as compared to the one induced by other mechanisms?
- (7) Does astroglial CB₁ receptor control other cognitive or behavioral functions than cannabinoid-induced working memory impairment?

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