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The role of Astroglial Type 1 Cannabinoid Receptor in Memory Functions

José Fernando Oliveira da Cruz

DEPARTAMENTO DE CIÊNCIAS DA VIDA

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José Fernando Oliveira da Cruz

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Em memória de Armindo Neves Oliveira,

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"Deus escreve direito por linhas tortas"

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Abstract

The endocannabinoid system is an important modulatory system, which is involved in the regulation of many physiological processes including learning and memory. The type 1 cannabinoid receptor (CB1) is abundant in the brain where it is mainly expressed on neurons. Moreover, it has also been shown that CB1 receptor is also present in astrocytes. Although astrocytes were long thought to be mainly supportive cells, it is now recognized that they play key roles in bidirectional communication with neurons, thereby modulating important aspects of synaptic transmission and plasticity. Recent key studies show that *exogenous* activation of hippocampal astroglial CB1 by natural or synthetic CB1 agonists impairs working memory in mice and induces alterations of synaptic plasticity. However, the *endogenous* role of astroglial CB1 receptors in vivo is unknown. Unpublished data from the host laboratory indicate that CB1 receptor in astrocytes is necessary for object recognition memory likely through a mechanism involving hippocampal D-serine, a co-agonist of the NMDA receptor. However, the neuronal mechanism by which astroglial CB1 receptor control this memory function is unknown. As long term changes in hippocampal synaptic plasticity are thought to participate in object recognition memory, we proposed to assess the role of CB1 receptor located in astrocytes in hippocampal synaptic plasticity. We used *in vivo* electrophysiology combined with conditional mutant mice lacking type-1 cannabinoid receptors and pharmacological approaches to investigate the role of astroglial CB1 receptor on synaptic functionality of the CA3-CA1 Schaffer Collateral Pathway in the hippocampus. Our results indicate that astroglial CB1 receptor is crucial for induction and maintenance of hippocampal NMDAR-dependent long term potentiation in the CA3-CA1 Shaffer Collateral pathway. We demonstrated that systemically administration of D-serine is also able to rescue the impairment in hippocampal NMDAR-dependent long term potentiation exhibited in mice lacking astroglial CB1 receptors. Altogether these results boost the growing concept that endocannabinoid system is crucial for neuronal-glial bidirectional communication, participating in the astrocytic-dependent regulation of synaptic plasticity in hippocampal glutamatergic synapses.

Keywords: Astroglial CB1 Receptor – D-Serine – Synaptic Plasticity – Learning and Memory – in vivo Electrophysiology

Resumo

O Sistema Endocanabinóide é um importante sistema modulador envolvido na regulação de funções fisiológicas como a aprendizagem e a memória. O receptor canabinóide tipo 1 (CB 1) encontra-se abundantemente expresso no encéfalo primariamente em neurónios. Recentemente, a sua presença foi demonstrada em astrócitos. Os astrócitos, aos quais se atribuem classicamente funções de suporte neuronal, participam intrinsecamente na comunicação bidireccional com neurónios exercendo deste modo uma modulação em fenómenos de transmissão e plasticidade sináptica. Estudos recentes mostram que a activação *exógena* de CB1 em astrócitos do hipocampo, pela acção de agonistas naturais e sintéticos, prejudica a memória de trabalho em ratinhos e induz ainda alterações na plasticidade sináptica. Contudo, a função endógena deste receptor em astrócitos *in vivo* é desconhecida. Dados não publicados do laboratório de acolhimento indicam que o receptor CB1 em astrócitos é necessário para memória de reconhecimento de objectos possivelmente através de um mecanismo envolvendo D-Serina, um co-agonista dos receptores de NMDA. Contudo, é ainda desconhecido o mecanismo envolvendo os CB1 em astrócitos e a formação deste tipo de memória. Devido ao facto das alterações de longo prazo na plasticidade sináptica no hipocampo serem assumidas como um potencial mecanismo de formação memória de reconhecimento de objectos, tencionamos desvendar o papel do CB1 em astrócitos nesses mesmos fenómenos de plasticidade sináptica. Para alcançar os nossos objectivos, usamos electrofisiologia *in vivo* combinada com tratamentos farmacológicos em ratinhos mutantes que não expressam o CB1 em astrócitos de modo a investigar o papel deste receptor na funcionalidade sináptica da região CA3-CA1 Schaffer Collateral no hipocampo. Os nossos resultados indicam que os receptores CB1 em astrócitos são cruciais para a indução e a manutenção de uma potenciação de longa duração que é dependente dos receptores NMDA. Demonstramos ainda que a administração sistémica de D-Serina é capaz de recuperar a perda da capacidade de potenciação exibida ratinhos que não expressam CB1 em astrócitos. Em suma, os nossos resultados reforçam o crescente conceito de que o sistema Endocanabinóide é essencial para a comunicação bidireccional entre neurónios e glia, participando na regulação da plasticidade sináptica dependente de astrócitos nas sinapses glutamatérgicas do hipocampo.

Palavras-chave: Receptor CB1 em astrócitos – D-Serina – Plasticidade Sináptica – Aprendizagem e Memória – Electrofisiologia *in vivo*

List of abbreviations

2-AG	2-Arachidonoylglycerol
AC	Adenylate Cyclase
AEA	N-arachidonoylethanolamine
AMPAR	(±)-α-Amino-3-hydroxy-5-Methylisoxazole-4-Propionic Acid hydrate Receptors
AP-5	DL-2-Amino-5-Phosphonovaleric acid
ATP	Adenosine-5'-Triphosphate
BBB	Blood Brain Barrier
cAMP	Cyclic Adenosine Monophosphate
CaMKII	Ca^{2+}/Calmodulin-Dependent Protein Kinase
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CNS	Central Nervous System
CCK	Cholecystokinin
CREB	cAMP Response Element-Binding Protein
DAGL	sn-1-Diacylglycerol lipase
DI	Discrimination Index
DNA	Deoxyribonucleic acid
DSE	Depolarization-induced Suppression of Excitation
DSI	Depolarization-induced Suppression of Inhibition
eCB	Endocannabinoids
ECS	Endocannabinoid System
FAAH	Fatty Acid Amide Hydrolase
fEPSP	field Excitatory Postsynaptic Potentials
FLAT	FAAH-1-like Anandamide Transporter
GFAP	Glial Fibrillary Acidic Protein
GPCR	G Protein-Coupled Peceptor
HFS	High Frequency Stimulation

LTD	Long Term Depression
LTP	Long Term Potentiation
MAPK	Mitogen-Activated Protein Kinases
MAGL	Monoacylglycerol Lipase
MCT	Mono Carboxylate Transporter
mGluR	Metabotropic Glutamate Receptors
mRNA	Messenger Ribonucleic Acid
mtCB1	Mitochondrial Cannabinoid Receptor 1
NAPE-PLD	N-Acylphosphatidylethanolamine-specific Phospholipase D
NArPE	N-Arachidonoyl Phosphatidylethanolamine
NAT	N-Acetyltransferase
NMDAR	N-Methyl-D-Aspartate-type Receptors
NO	Nitric Oxide
OAE	O-Arachidonoyl-Ethanolamine
ORM	Object Recognition Memory
PA	Phosphatidic Acid
PKMζ	Protein Kinase M ζ
PLC	Phospholipase C
PSD	Post Synaptic Density
THC	Δ9-Tetrahydrocannabinol
t-LTD	spike timing-dependent Long Term Depression
TNF-α	Tumor Necrosis Factor-Alpha
TRPV1	Transient Receptor Potential Vanilloid Type-1
VGCC	Voltage Gated Calcium Channel
VHC	Ventral Hippocampal Commissural
ZIP	Myristoylated Zeta-Pseudo Substrate Inhibitory Peptide

Chapter I – Introduction

Part I – The endocannabinoid system and the brain

Part I | Section 1 – Historical considerations and general characterization

Cannabis sativa, commonly known by Marijuana, is known to exert a huge array of physiological and psychotropic effects recognized and described by the humankind since, at least, 2000 BC. Throughout the history archaeological evidence shows that Cannabis diffused broadly in China, India and Arabia even reaching Africa and South America (Mechoulam and Parker, 2012). Its introduction in Europe dates the 19th century probably by the Napoleonic Armies that returned from Mesopotamian territories. After this period in which Cannabis became quite known to Europeans, O'Shaughnessy and Moreau did important studies on the subject. The first, a medical officer of the British Army in Calcutta, described therapeutic and narcotic properties assed in humans. The second, a psychiatrist, described in a book called "Hashish and Mental Illness" many effects of the administration of Cannabis to human test subjects (Mechoulam and Parker, 2012). These effects, that could random from feeling of happiness, excitement, illusions and hallucinations, helped to provide important medical data on the effects of cannabis consumption in humans.

Cannabis consumption is frequently associated with recreational purposes and has never been clearly assumed to be harmful to health in the long term usage (Murray et al., 2007). Although these apparent non harmful effects of cannabis, its consumption properties are known to cause several physiological and behavioral alterations such as cognitive impairments (i.e., working memory impairments), discoordination, sleepiness, mood alterations (such as euphoria or dysphoria) among other effects (Childers and Breivogel, 2000). Despite cannabis effects on human physiology, medical research on the subject was very poor and in the beginning of the 20th century its usage, both medical and recreational, was very low both in Europe and North America (Mechoulam and Parker, 2013). Moreover, anti-cannabis laws that were created from 1940 to 1960 made this plant almost inaccessible to research groups in North America. Paradoxically, at the same time its recreational usage exploded in

Europe and North America, making it again an interesting subject of study (Mechoulam and Parker, 2013).

The first breakthrough in understanding Cannabis effects came in 1964 with the identification and characterization of *Cannabis sativa*'s main active compound: Δ9-tetrahidrocanabinol (THC) (Gaoni and Mechoulam, 1964). This milestone, achieved by Gaoni and Mecholuam, and the subsequent artificial synthesis of THC (Mechoulam et al., 1967) for research purposes opened a new window into cannabinoid research. Interestingly, although the existence of more than 60 cannabis constituents with very similar structural and physical properties only THC shows major mood-altering effects (Mechoulam and Parker, 2013).

This availability of THC for research purposes allowed more precise quantitative studies of its effects in animals. Soon many of the physiological and psychoactive effects of cannabis were successfully replicated in THC studies allowing the correlation between Cannabis consumption and, for example, hypolocomotion, hypothermia, analgesia, catalepsy (these last four effects, together, are called tetrad), stress reactivity (Maldonado et al., 2011), anticonvulsant effects (Wallace et al., 2001), regulation of food intake (Di Marzo and Matias, 2005).

The discovery of THC and its lipophilic structure suggested that its effects were through a nonspecific membrane associated mechanism (Mechoulam and Parker, 2012). The first data suggesting a different model, a more specific one, appeared only in 1984. In this year, Howlett and colleagues reported that cannabinoids decreased cyclic Adenosine Monophosphate (cAMP) in neuroblastoma cell cultures H thus suggesting a mechanism dependent on the inhibition of the Adenylate Cyclase (AC) through a G protein. Later in 1988, through immunochemical and radio ligand binding procedures, the same research group led by Howlett reported the existence of binding sites in the brain (Devane et al., 1988). Two year later the Cannabinoid Receptor 1 (CB1) is finally cloned (Matsuda et al., 1990) and the research on CB1 knows a huge explosion. In 1993, Munro *et al.* described a second cannabinoid receptor (CB2), this time in the spleen (Munro et *al.*, 1993) and although CB1 was mainly found in the brain, the second receptor was mainly found the immune system (Kano et al., 2009). The discovery of the cannabinoid receptors propelled a new search for its purpose. The receptors, as a natural part of the body's physiology, were thought to respond to some kind of molecule that could be produced endogenously. This hypothesis led to the discovery of endogenous agonists of these receptors: the endocannabinoids. From these new signaling molecules,

the very first to be reported was N-arachidonoylethanolamine (AEA, Anandamide) (Devane *et al, 1992)* and the second was 2-Arachidonoylglycerol (2-AG) (Mechoulam et al., 1995). Due to their lipophilicity their effects were thought to be mediated locally and strictly dependent on the location of the receptors and/or production sites (Di Marzo, 2011). After the discovery of the endocannabinoids in the early 90s, the biochemical pathways for their biosynthesis and degradation were reported later, in 1994, by Di Marzo and colleagues. Altogether, the cannabinoid receptors, the endogenous agonists and the catabolic/anabolic pathways form a new modulatory system: the endocannabinoid system.

Part I | Section 2 - The Cannabinoid Receptors

Section 2.1 – Characterization and pharmacology

The endocannabinoid system comprises two main well identified and characterized receptors: CB1 and CB2 (Figure 1). Both belong to the Class 11 rhodopsin-like family of G-protein coupled receptors (GPCR) (Emmanuel et al., 2006) (Figure 1) and both are composed by 7-transmembrane domains. These receptors, the most abundant G-protein coupled receptors in the body (Herkenham et al. 1990), exert their molecular effect mainly through a G protein inhibitory component which leads to: 1) the inhibition of the activity of Adenylate Cyclase; 2) the stimulation of the Mitogen-Activated Protein Kinases (MAPK) pathway; 3) the inhibition of the N and Q voltage-activated calcium channels; 4) the stimulation of the potassium inwardly rectifying channels (Emmanuel et al., 2006; Howlett, 2002; McAllister et al., 2002).

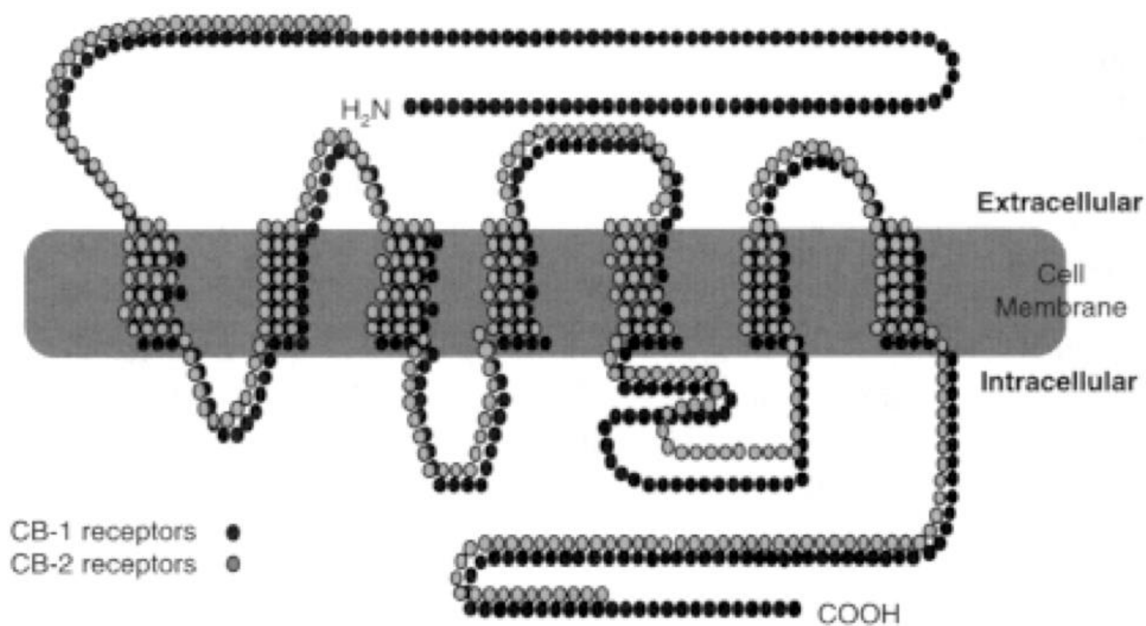

FIGURE 1 – GRAPHICAL REPRESENTATION OF CANNABINOID RECEPTOR 1 AND 2 (adapted from http://druglibrary.org/Schaffer/Library/studies/iom/iom2.htm - last access in 24/07/2013).

Both receptors show an overall amino acid similarity close to 48% (Mechoulam and Parker, 2013) where CB1 has more 72 additional amino acids in N-terminal region, 15 more residues in the third extracellular loop and 13 additional residues in the C-terminal region. Additionally, the highest difference between the structures occurs in the transmembrane regions TM2, TM3, TM5 and TM6 (Svízenská et al., 2008).

Although CB1 is mainly expressed in Central Nervous System (CNS) it was also reported in other several areas, such in the cardiovascular system, reproductive system and the gastrointestinal tract (Kano et al., 2009). Furthermore, it was estimated that in a population of CB1 receptors, 70% occur in an inactive state where the remaining 30% are in the active state (Onaivi et al, 2005). Although broadly expressed throughout the body, it's preferentially present in the brain and the spinal cord (Kano et al., 2009).

In the other hand, CB2 doesn't have its main expression in the brain. This receptor, firstly described in the macrophages from the marginal zone of spleen (Munro et al., 1993), has also been reported in the skin, the tonsil, the spleen and the thymus. Concerning the brain and more recently, CB2 was observed in microglial cells with a putative role in a general brain protective system (Mechoulam and Parker, 2012).

Although the classical cannabinoid receptors (CB1 and CB2) are accepted as the main drivers of the cannabinoid system, they are not the only ones responding to endocannabinoids. In experiments carried out on brain cells from transgenic mice lacking both CB1 and CB2 receptors, Anandamide could still induce a pharmacological effect leading to the idea of the existence of other putative cannabinoid receptors (Breivogel et al., 2001). After these findings, further research proposed the Transient Receptor Potential Vanilloid type-1 (TRPV1) as the best non-CB1/CB2 receptor for AEA (reviewed in Starowicz et al., 2007 and Petrocellis et al., 2009). The TRPV1 is a calcium channel that is gated by capsaicin, low pH, and endogenous cannabinoid agonists (Szallasi and Blumberg, 1999; Starowicz et al., 2007; Wong et al., 2009). TRPV1 brain expression is observed in areas such as the hippocampus, cortex, cerebellum, olfactory bulb, mesencephalon and the hindbrain (Tóth et al., 2005). Also two other GPCRs, the G protein-coupled receptor 55 (GPR55) and the G protein-coupled receptor 119 (GPR119), emerged as novel potential cannabinoid receptors (Brown, 2007). The GPR55 couples a $G_{\alpha13}$ and has affinity for endogenous, plant and synthetic cannabinoids. It is expressed in the body (mainly in the spleen, adrenal glands and gastrointestinal tract) and in the brain,

especially in the caudate nucleus and putamen with minor a presence in hippocampus, thalamus, pons, cerebellum and frontal cortex (Kano et al., 2009). In the other hand, the GPR119 (also coupled to G_{α}) share some similarities to the classical cannabinoid receptors. They are more present in the pancreas and also in the gastrointestinal tract both in humans and in mice. Their putative physiological role involves the control of glucose homeostasis through modulation of insulin secretion (Kano et al., 2009).

The design of several molecules that could interact specifically with the ECS was a key event to study the specific roles of the several components concerning the complex function of this system (Figure 2). Among the effects and potential therapeutic uses, some of these synthetic molecules are being use as anti-obesity and anti-tumor agents and as chronic pain reducing agents (Di Marzo et al., 2004). HU-210, an enantiomerically pure THC analogue, is one of these synthetic analogues that gave powerful insights on THC's mechanism of action. It further labeling to generate tritium (HU-245) and the synthesis of CP-55.245 led to the identification of the cannabinoid receptors (Di Marzo et al., 2004). In the other hand, compounds that are able to inhibit or reverse the effect of endocannabinoids are also important to the cannabinoid receptor's function. Examples are the SR 141716A, commonly known as Rimonabant (Rinaldi-Carmona et al., 1994), the AM251 (Gatley et al., 1996) and the AM281 (Lan et al., 1999). Other important compounds, such as URB-597, do not act directly in the receptors but on the availability of endocannabinoids levels through the inhibition of their degradation (Di Marzo et al., 2004). Altogether these pharmacological tools are of the most importance to understand the physiological function of the ECS.

De Petrocellis et al., (2009), describe the endocannabinoid system as a pleiotropic and locally acting pro-homeostatic signaling system activated 'on demand' following perturbation of cell homeostasis. It's a system that is constantly being updated since new molecules, potential receptors, new enzymes and more intracellular cascades are being discovered. The new molecular tools, along with new synthetic compounds that are able to play a specific role in the pathways, offer the possibility to investigate even further new possibilities and gather important insight that can boost medical research on the subject.

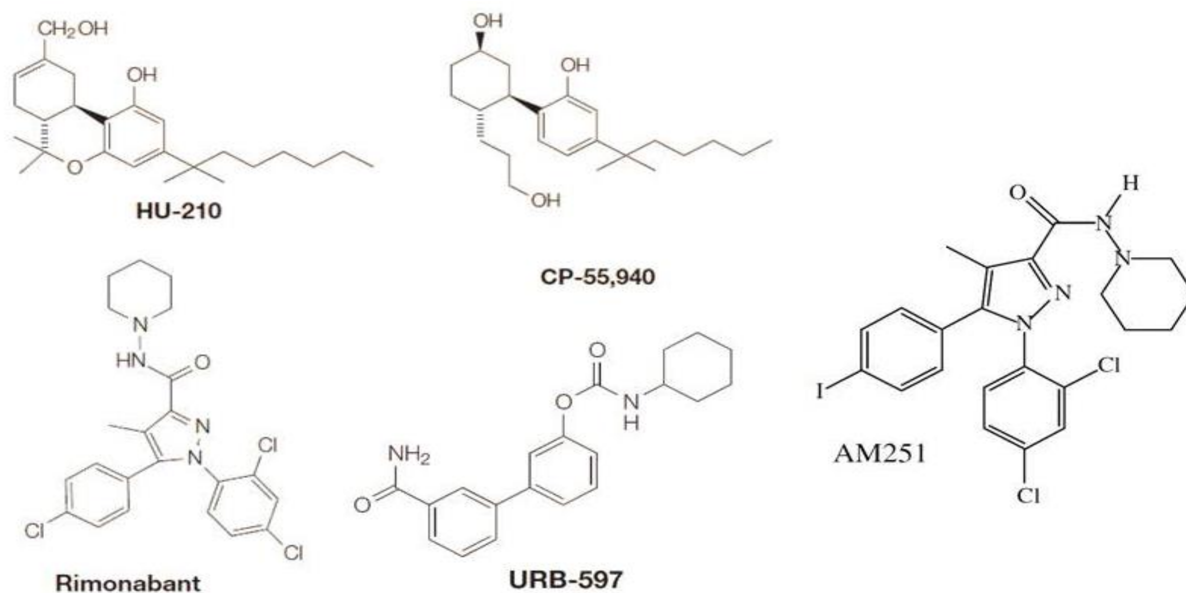

FIGURE 2 - CHEMICAL STRUCTURES OF DRUGS ACTING ON ENDOCANNABINOID SYSTEM (Adapted from De Petrocellis et al., 2004; Di Marzo et al., 2004; Di Marzo, 2008)

Section 2.2 – Distribution of the CB1 in the brain

The CB1 can be found mainly, but not only, in the neurons of the CNS. CB1 proteins and mRNA transcripts are present in brain areas which are responsible for several vital and important functions such as learning and memory, pain perception, reward, energy and metabolism, among others (Köfalvi, 2008). Due to the nature of this introduction and research objectives present in this thesis, the aspects of the CB1 distribution will be focused mainly in the hippocampus (Figure 3A and 3C).

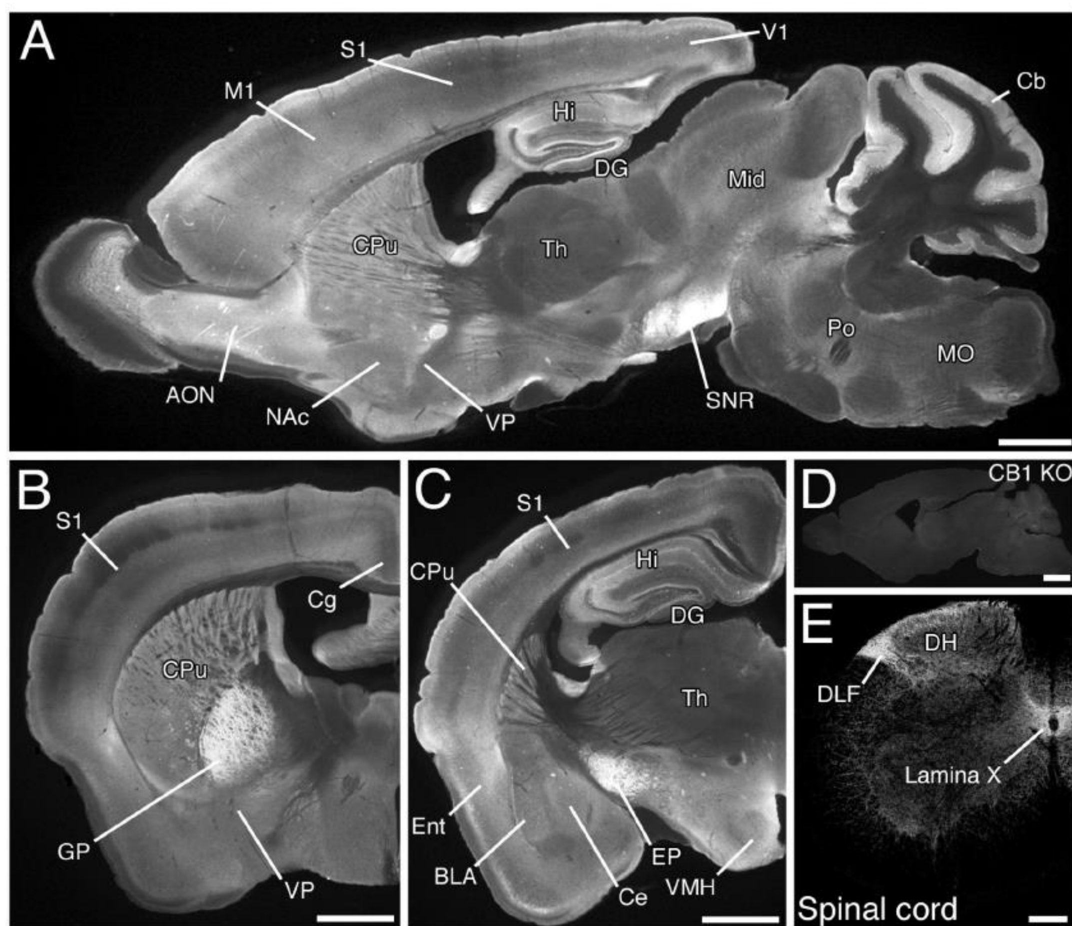

FIGURE 3 - DISTRIBUTION OF CB1 RECEPTORS IN THE CENTRAL NERVOUS SYSTEM OF ADULT MICE. CB1 distribution pattern through immunolabeling in a parasagittal (A) and coronal (B,C) wild-type and parasagittal CB1-KO (D) mice. High CB1 immunoreactivity, among other areas, is presented in structures belonging to the Medial Temporal Lobe such as: hippocampus (Hi), dentate gyrus (DG) and entorhinal cortex (Ent). High CB1 immunoreactivity can be also found in the anterior olfactory nucleus (AON, A), neocortex (A-C), caudate putamen (CPu, A-C), thalamus (Th, A,C) basolateral (BLA) and central (Ce) amygdaloid nuclei (C), cerebellum (Cb, A) and spinal cord (E). M1, primary motor cortex; S1, primary somatosensory cortex; V1, primary visual cortex; Cg, cingulate cortex; Ent, entorhinal cortex; DG, dentate gyrus; NAc, nucleus accumbens, GP, globus pallidus; VP, ventral pallidum; Mid, midbrain; SNR, substantia nigra pars reticulata; PO, pons; MO, medulla oblongata; EP, entopedoncular nucleus; VMH, ventromedial hypothalamus; DH, dorsal horn; DLF, dorsolateral funiculus. Scale bars: 1 mm (A-E), 200 µm (Adapted from KANO et al, 2009).

The CB1 mRNA displays a general brain distribution. It was described in major neuronal populations in the striatum, thalamus, hypothalamus, cerebellum, and lower brain stem and the protein mainly in presynaptic elements (Kano et al., 2009). In the hippocampus, the CB1 mRNA is abundantly expressed at high levels in several subsets of interneurons (Kano et al., 2009), neurons that co-express Glutamic Acid Decarboxylase 65 and Cholecystokinin (CCK) (Marsicano and Lutz, 1999). There is also data showing that CB1 is expressed in CCK-positive basket cells although it could not be detected in Parvalbumin-positive basket cells. CB1 receptors are not only present in the inhibitory interneurons but also in the excitatory glutamatergic pyramidal neurons (Freund et al., 2003). Evidence confirming this point was difficult to obtain since CB1 receptors are expressed in significantly lower levels when compared with GABAergic interneurons (Marsicano and Lutz, 1999; Marsicano et al., 2003). CB1 receptor is not restricted to this neuronal populations and it has been reported, for instance, on serotonin-releasing neurons and noradrenergic terminals.

The hydrophobic properties of the endocannabinoid molecules make them apparently able to diffuse freely between plasma membranes and interact on an intracellular level (Di Marzo et al., 2004). Mitochondria play a central role in the energy production in eukaryotic cells. They synthetize Adenosine-5'-triphosphate (ATP) from the breakdown of carbohydrates and fatty acids through a process called oxidative phosphorylation. They also play a key role in programmed cell death through the intrinsic apoptotic pathway (Lodish et al., 2000). In 2010, Whyte et al., under the previous hypothesis that cannabinoids could disturb mitochondrial function, tested mitochondrial O_2 consumption in human oral cancer cells (Tu 183). They found that Δ9-THC or Δ8-THC are potent inhibitors of Tu 183 cellular respiration and thereby highly toxic. In 2012, Bénard and colleagues showed the localization of CB1 receptors in the mitochondrial membrane from mice hippocampal neurons. They also demonstrate that it can control directly cellular respiration and energy production. These mitochondrial receptors (mtCB1) also display decreased activity in the complex 1 enzymatic activity, in PKA activity and in the levels of cAMP. Moreover, the striking feature is their capacity to contribute for endocannabinoid-dependent depolarization-induced suppression of inhibition in the hippocampus (Bénard et al., 2012).

Part I | Section 2 - Endocannabinoid molecules – Synthesis, Transport and Degradation

The discovery of the cannabinoid receptors was followed by the discovery of their endogenous agonists: the endocannabinoids. Inside this group of endogenous molecules that bind specifically to the cannabinoid receptors, those who are better characterized as specific ligands for the CB1 and CB2 are Anandamide and 2-AG (figure 4). These two molecules are structurally based on the Arachidonic Acid as well as other long chain polyunsaturated acids.

Anandamide (from the Sanskrit word 'ananda' meaning 'bliss') (Di Marzo et al., 1998) was first suggested as a natural ligand for the CB1 by Devane et al. (1992) after observing that it was able to inhibit the specific binding of a radiolabeled cannabinoid probe in synaptosomal membranes, acting as a competitive ligand and inducing psychotropic effect of the cannabinoids. This endogenous molecule acts as a partial agonist of both CB1 and CB2 receptors (Onaivi et al., 2005).

The discovery of a second endocannabinoid molecule came from the fact the, although AEA was specific to the receptor, its presence in the brain was very low. 2-AG, an Arachidonic-acid-containing Monoacylglycerol, was first described by Sugiura et al. (1995). After several binding essays they postulated that this molecule could act as an endogenous ligand for endocannabinoid system in the brain. In 1997, Stella et al. reported that a cannabinoid ligand isolated from intestinal tissue which levels were about 170-fold greater than AEA in brain. They also reported the biosynthesis of 2 AG in hippocampal slices after the stimulation of Schaffer Collaterals fibers. Altogether, these results lead them to propose 2-AG as the second endogenous cannabinoid ligand in the CNS, also suggesting 2-AG could as the main effector of the CB1-mediated regulation in central synapses (Hashimotodani et al., 2007; Tanimura et al., 2010).

Although Anandamide and 2-AG are the most well-known endocannabinoids, other molecules with physiological effects have been described as putative cannabinoid receptor agonists (Figure 4): 1) O-arachidonoyl-ethanolamine (OAE, Virodhamine); 2) 2-Arachidonyl glyceryl ether (Noladin Ether) and 3) N-arachidonoyl-dopamine. The first one acts as a selective agonist for CB1, the second as a partial agonist for CB2 and CB1 antagonist and the third as a selective agonist for CB 1 and as a potent agonist for TRPV1 (Huang et al., 2002).

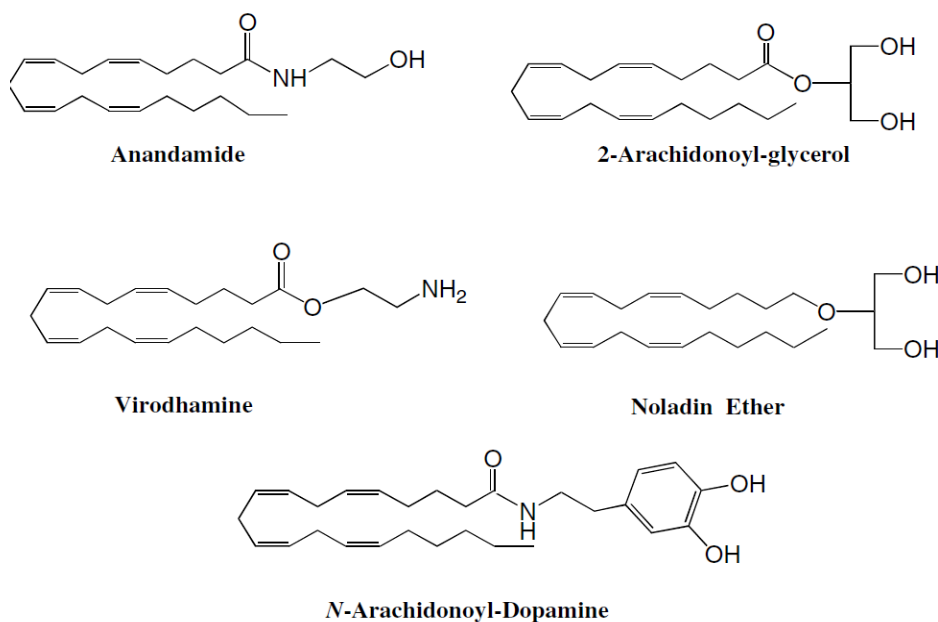

FIGURE 4 – CHEMICAL STRUCTURES OF ESTABLISHED AND PROPOSED ENDOCANNABINOIDS (Adapted from De Petrocellis et al., et al).

Due to the lipophilic nature of endocannabinoids and the apparent incapacity for storage inside the aqueous medium of synaptic vesicles, the process of synthesis is likely to happen "on demand" through a tightly regulated mechanism (Mechoulam et al., 1998). In 1994, Di Marzo et al., reported that Anandamide could be produced and released in a calcium dependent way after stimulation with membrane-depolarizing agents demonstrating that this molecule could, indeed, be produced "on demand". The initiation of this complex process in hippocampus seems to be potentiated by the activation of postsynaptic type 1 glutamate metabotropic receptors and by muscarinic acetylcholine receptors (Varma et al., 2001; Kim et al., 2002).

The biosynthesis of these two major endocannabinoid molecules involves several hydrolytic enzymes that regulate and process the synthesis/degradation of these compounds. Anandamide is produced from the precursor N-arachidonoyl Phosphatidylethanolamine (NArPE). This compound is synthetized by the enzyme N-Acetyltransferase (NAT) from Glycerophospholipids and Phosphatidylethanolamine. NArPE will then be directly converted into Anandamide by the enzyme N-Acylphosphatidylethanolamine-specific phospholipase D (NAPE- PLD). 2-AG is synthetized from the precursor sn-1-acyl-2-arachidonoylglycerol. These precursors can be produced either from the hydrolysis of Phosphatidic acid (PA) by the PA phosphohydrolase or from phosphoinositides (PI) after catalyzation by a PI-selective phospholipase C (PLC) (De Petrocellis et al., 2004). This AArG will be converted into 2-AG by the enzyme sn-1-diacylglycerol lipase (DAGL) (Di Marzo et al., 2004; Di Marzo, 2008). Although it is only shown here one pathway for each endocannabinoid, there are several new addictions that include new pathways, new enzymes and new intermediate molecules (Further reviewed in Di Marzo et al., 2004; De Petrocellis et al., 2004; Di Marzo, 2008).

After the cellular uptake, the degradations pathway is processed mainly by hydrolytic enzymes. Fatty Acid Amide Hydrolase (FAAH) is able to catalyze the conversion of Anandamide with the production of Arachidonic Acid and Ethanolamine. Another very important enzyme is the Monoacylglycerol Lipase (MAGL). This enzyme is able degrade 2-AG into Arachidonic acid and glycerol. Recombinant FAAH was also shown to be able not only to hydrolyze 2-AG in a much efficient way than Anandamide but also Oleamide and Methyl Arachidonate (Goparaju et al., 1998). Although the effectiveness of FAAH on degradation of 2-AG, it has been shown by Blankman et al., (2007) that the main enzyme responsible for its degradation is MAGL. The correct endocannabinoid degradation is very important for the optimal endocannabinoid signaling at glutamate synapses (Schlosburg *et al.* 2010). The whole synthesis, transport and degradation process is summarized in figure 5.

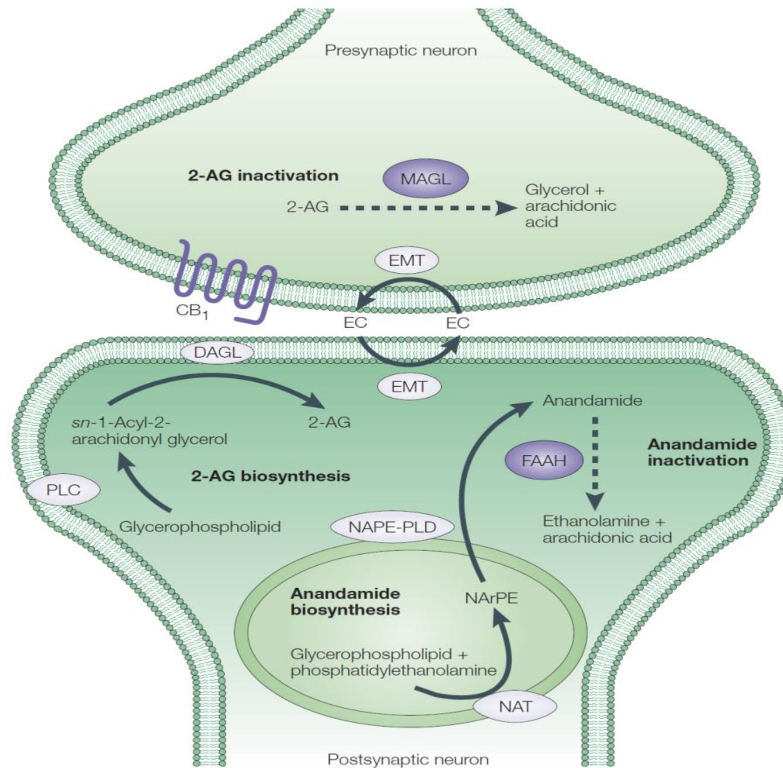

FIGURE 5 – MAIN ESTABLISHED MECHANISMS FOR ENDOCANNABINOID SYNTHESIS, TRANSPORT AND DEGRADATION IN THE BRAIN (Adapted from Di Marzo et al., 2004).

The degradation/inactivation pathways of these molecules must be very efficient and tightly regulate. With classical neurotransmitter molecules (such as glutamate or GABA), the inactivation after action can be done by uptake (for further degradation) from synapse by glial cells (such as astrocytes) or by neurons. The major question that remains is how are endocannabinoids transported through the intracellular or extracellular aqueous media? In 2011, Fu et al., reported that a Fatty Acid Amide Hydrolase -1 enzyme variant (FAAH-1) is able to act a specific transported of AEA in neurons and other cells types. Named FLAT by the authors (from FAAH-1–like Anandamide Transporter), this enzyme was shown to be able to specifically transport AEA and no other endocannabinoid molecules such as 2-AG. This was the first evidence of a possible first-in-class specific transporter of Anandamide (Marsicano and Chaouloff, 2012). After these interesting findings, a new question arises concerning 2-AG: can it be transported in a similar way?

Part I | Section 3 - Endocannabinoid Signaling and Synaptic function

The endocannabinoid system exerts an important regulatory and modulatory function and the main mechanism of regulation of synaptic plasticity is through retrograde signaling mechanism acting in the presynaptic terminals leading to changes in the whole synapse (Figure 6A). Synaptic plasticity can result from active-dependent changes that lead to the adaptation of the synapse and has a major importance in the neuronal functions (Further discussed in Part III).

Molecular changes triggered by the action of neurotransmitters (either changes in quantity or efficiency in their action at their receptors) have been demonstrated to be influenced by the endocannabinoid system, inducing several forms of synaptic plasticity either short and/or long term changes (Gerrow and Triller, 2010; Chevaleyre et al., 2006). As this system is present throughout excitatory and inhibitory synapses, interpretations about their specific role are very tricky. Furthermore, there is also growing evidence of a mechanism of action through a non-retrograde signaling cascade involving other potential receptors that respond to endocannabinoid (such as the TRPV1 receptors) leading to a non-classical non-retrograde signaling cascade (Figure 6B). These many connections with other neuromodulatory systems draw to a picture even more complex underling the general physiological and behavioral aspects of the endocannabinoid system.

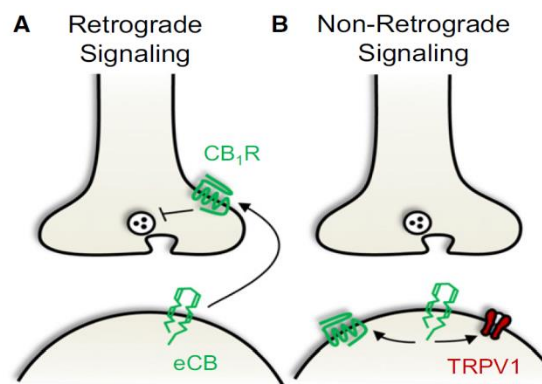

FIGURE 6 – ENDOCANNABINOID NEURONAL SIGNALING PATHWAYS (adapted from Castillo et al., 2012)

Section 3.1 – CB1-mediated intracellular signaling pathway

The cannabinoid receptors are known to be the most abundant G protein coupled receptors in the brain (Herkenham et al., 1990; Di Marzo et al., 2004) and it is known that the endocannabinoids can be released, probably "on demand", from the postsynaptic to the presynaptic terminal. When the CB1 receptors are activated, they will exert an inhibitory effect through a G protein-dependent mechanism coupled to the Adenylate Cyclase that will cause a decrease of cAMP production. This decrease of cAMP will lead to a decrease of cAMP-dependent protein kinase phosphorylated pathway, the Protein Kinase A. CB1 will also stimulate the MAPK pathway and it is known that this pathway controls the expression of several genes that can lead to long lasting cellular effects (Onaivi et al., 2005). CB1 is also coupled to the inhibition of N-type and P/Q-type of Voltage Activated Calcium Channels (thus decreasing Ca^{2+} levels) and the stimulation of inwardly rectifying potassium channels in the presynaptic terminal (Figure 7). Altogether these events will produce a hyperpolarization in the presynaptic terminal leading to the suppression of neurotransmitter release (Di Marzo et al., 2004; Svízenská et al., 2008).

Another interesting property regarding CB1 is desensitization. When CB1 is stimulated for a persistent period, it may lose its capacity to respond with the same efficacy to subsequent stimulations. Observations showed that THC treatment causes both region-specific decreases in CB1 receptors and G-protein coupling in the brain leading to a possible mechanism of tolerance development (Daigle et al., 2008).

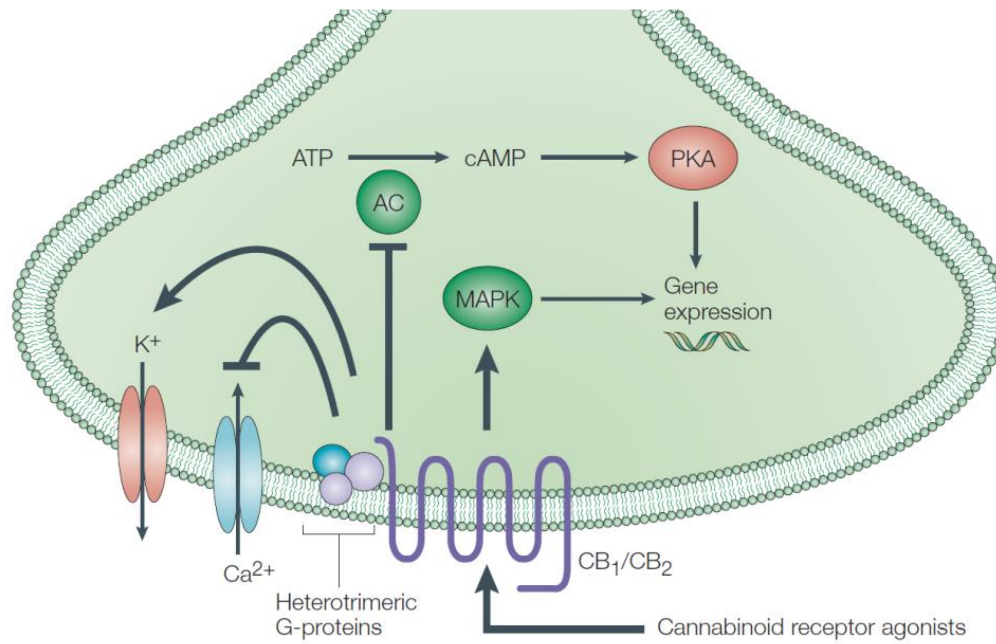

FIGURE 7 – ESTABLISHED INTRACELLULAR MECHANISMS UNDERLYING RETROGRADE ENDOCANNABINOID SIGNALING (Adapted from Di Marzo et al. 2004)

Section 3.2 – Endocannabinoids as mediators of short-term synaptic plasticity events

As stated before, one of the main consequences of CB1 action is the suppression of neurotransmitter release. After the depolarization in the postsynaptic membrane, a transient suppression neurotransmitter release is elicited in the presynaptic membrane. This suppression can cause both suppression of inhibition or excitation, depending if it occurs, respectively, on GABAergic or Glutamatergic neurons (Wilson and Nicoll, 2002). These two events, Depolarization-induced Suppression of Inhibition (DSI) and Depolarization-induced Suppression of Excitation (DSE), are characterized by events in the postsynaptic and presynaptic membrane. These events are both correlated with influx and increase in the calcium concentrations in the postsynaptic neuron. The first evidence that DSI was related with retrograde signaling was reported by Wilson and Nicoll (2001). They showed that DSI on hippocampus (where CB1 is expressed mainly in GABA-mediated inhibitory interneurons and clusters in the axon terminal) could be blocked by a CB1 antagonist and in CB1-KO mice (Wilson and Nicoll, 2001). In the same year, Kreitzer and Regehr (2001) showed that postsynaptic release of endocannabinoid molecules, in the cerebellum, can also inhibit the excitatory

synaptic response in Purkinje cells. The calcium increase in the postsynaptic terminal can be due the opening of voltage-gated calcium channels (Wilson and Nicoll, 2002) and there is evidence that calcium chelators, when applied postsynaptically, are able to block DSE and DSI (Pitler and Alger, 1992; Kreitzer and Regehr, 2001). AG-2 seems to be the principal endocannabinoid required to the events concerning the activity-dependent retrograde signaling although the relation between the Anandamide and the 2-AG in general effects is debatable (Castillo et al., 2012). In this model for short term plasticity, the eCBs released from the postsynaptic terminal, are synthetized possibly after the calcium increase via Voltage Gated Calcium Channel (VGCC) that stimulates the Ca^{2+} sensitive enzymes. Another possible mechanism is the production of 2-AG via PLCβ after the activation of post synaptic type I metabotropic glutamate receptors (I mGluR) through glutamate released from the presynaptic terminal (Castillo et al., 2012). The proposed mechanism is a cascade that will converge and thus leading to the production of endocannabinoids (Figure 8).

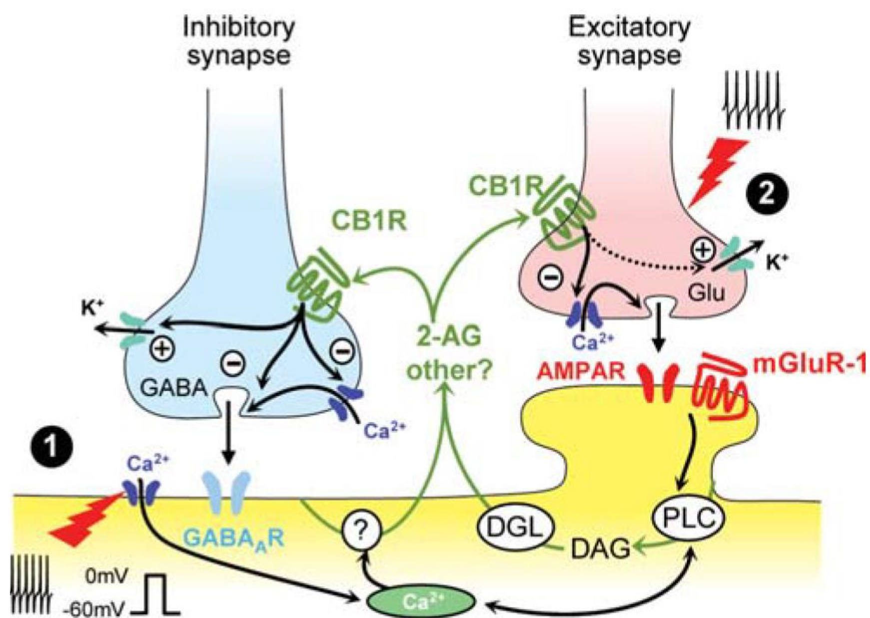

FIGURE 8 – ILLUSTRATIVE DIAGRAM OF ENDOCANNABINOID INDUCED SHORT TERM SYNAPTIC PLASTICITY (Adapted from Chevaleyre et al., 2006)

Section 3.3 – Endocannabinoids as mediators of long-term synaptic plasticity

It is known that besides the effects on short term plasticity, the ECS is involved in many aspects concerning long term plasticity. It has been shown that the stimulation of the CB1 receptors can lead, for instance, to the induction of forms of long term depression (LTD) known as endocannabinoid-mediated LTD (eCB-LTD) in brain areas such as the hippocampus, amygdala, striatum and neocortex (Chevaleyre et al., 2006). Unlike the short term induced synaptic plasticity, the continuous activation the presynaptic CB1 is thought to be crucial to the eCB-LTD (although after the induction, this activation is not required to sustain the LTD) (Chevaleyre and Castillo 2003; Chevaleyre et al., 2006).

This novel form of activity-dependent long-term depression at the hippocampal inhibitory synapses (known as I-LTD) on CA1 pyramidal cells area is known to be mediated by endocannabinoids. The induction of this type of synaptic plasticity shares some common features with the molecular mechanisms of the short term effects such as the activation of presynaptic CB1 receptors (Chevaleyre et al., 2006). Presynaptically released glutamate activates the type I mGluR coupled to the PLCβ and DGLα that allows the production and release of 2-AG. This 2-AG will act homosynaptically inhibiting the release of excitatory neurotransmitters and heterosynpatically in an inhibitory terminal leading to an Inhibitory LTD. The mechanism underlying the LTD in the excitatory terminals is the one previously described before and the mechanism underlying the effect in inhibitory terminals is basically the same although involving also Calcineurin (CaN). There is a putative involvement of an unidentified T target, a RIM1 protein and Rab3B that is also necessary for this inhibitory form of LTD (Castillo et al., 2012) (Figure 9).

The broadly distribution and increasing evidence of the regulation of synaptic plasticity indicates a possible correlation between the ECS and complex forms of behavior such as stress and depression, memory and learning, regulation of the energy metabolism, sexual behaviors (Pagotto, 2005; Cota et al., 2006; Hill et al., 2010).

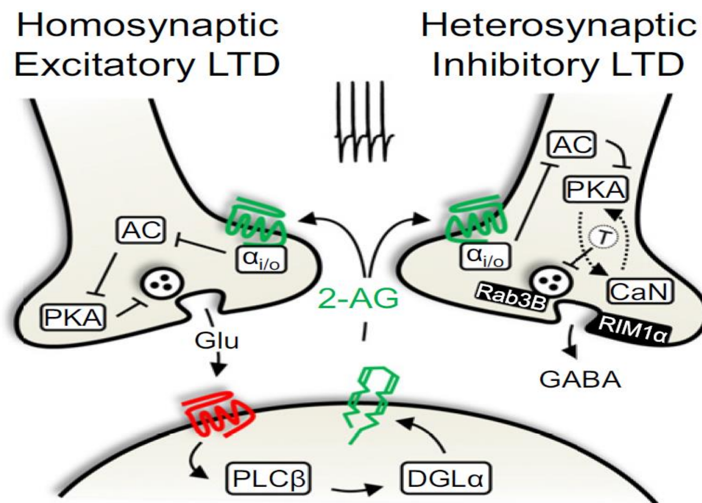

FIGURE 9 – MOLECULAR MECHANISMS UNDERLYING ENDOCANNABINOID MEDIATED LONG TERM SYNAPTIC PLASTICITY (Adapted from Castillo et al., 2012)

Section 3.4 – Knock-out mice models to study CB1 physiology

Studies in the physiology of the biological systems are based mainly in pharmacological approaches using natural and synthetic compounds (agonists, antagonists and inverse agonists) and null CB1 mutant lines which are characterized by a complete deletion of the CB1 in the whole specimen. Pharmacological approaches, despite of its advantages, display overall limitation such as the lack of specificity, drug delivery problems and spatial target inaccuracy (Picciotto and Wickman, 1998). Because CB1 have an inhibitory effect over the release of glutamate and GABA and the expression in GABAergic neurons is much higher than in the Glutamatergic neurons (Kano et al., 2009), the phenotypes obtained from a pharmacological approach are mainly attributed to direct regulation of GABAergic neurotransmission masking other possible mechanisms. In the other hand, a general full genetic deletion can also generate a compensation phenomenon in which the body is able to re-adapt itself throughout it development to compensate in some extent the unavailability of the deleted protein (Picciotto and Wickman, 1998).

This general compensation of a full deletion can be minimized by restricting CB1 to specific cell types. A way to assess specific function of a specific localization of the CB1 is through the creation of cell-type specific knockouts through the Cre/LoxP System.

The Cre recombinase system is a genetic approached that allows the excision or inversion of loxP-flaked Deoxyribonucleic Acid (DNA) segments and the creation of an intermolecular recombination between different DNA molecules (Nagy, 2000). The Cre recombinase is a 38kD protein that catalyzes a recombination between two of its recognition sites: the loxP sites. These sites are a 34 bp consensus sequence which orientation is defined by the asymmetric core sequence (Nagy, 2000). Since this sequence flanks only the region of interest and does not alter normal gene expression, the transgenic mice originated with the flanked gene are considered as wild type mice. To conceive a transgenic mouse line with the required knockout target, the mouse containing the floxed gene must be crossed with another mouse line expressing Cre Recombinase under the control of a cell type specific promoter. The Cre enzyme will then act between the spacer areas of the loxP sites creating the new specific transgenic lines without the flanked gene (Nagy, 2000).

In the endocannabinoid system, in order to create a cell type-specific CB1-KO, the first step is the creation of a transgenic line in which the gene of interest (i.e. the CB1 gene) is flanked by a loxP sequence. Subsequently these lines will be crossed with another transgenic line expressing Cre recombinase under the control of a specific promoter to a particular cell type (Figure 10). There are several knockout mice lines created with this procedure. For instance, in 2003, Marsicano and colleagues obtained the (Ca^{2+}/calmodulin-dependent protein kinase) CaMKIIα-CB1-KO mice from a CB1-floxed (Marsicano et al., 2002) and the CaMKIIα-Cre mice (Casanova et al., 2001). This promoter allowed the Cre recombinase expression and consequent excision of the CB1 receptor in the principal forebrain neurons.

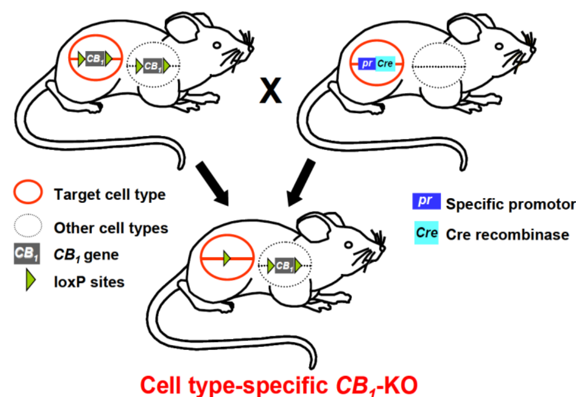

FIGURE 10 – CONDITIONAL DELETION OF CB1 GENE THROUGH CRE/LOXP SYSTEM (Adapted from Metna-Laurent, 2012)

Although targeting specific neuronal cells is quite easy, the same does not occurs when it comes to astrocytes. As the most widely used astrocyte specific promoter, Glial Fibrillary Acidic Protein (GFAP), is also active in embryonic radial glial that have neurogenic potential, when a conditional knockout beginning in embryogenic state using Cre Recombinase is attempted, gene deletion occurs also in neurons thereby creating an ambiguous and insufficient transgenic model (Hirrlinger et al., 2006).

In 2006, Petra Hirrlinger and colleges, from Frank Kirchhoff lab, developed a method in which a different Cre Recombinase system was used: the Tamoxifen-inducible Cre/loxP system (Figure 11). They generated a transgenic mutant mice line in which the Cre Recombinase protein was coupled to a mutated ligand binding domain of the estrogen receptor: the CreERT2. In this system, the activation of the Cre protein is achieved with i.p. injection of Tamoxifen (a selective estrogen response modifier). This leads to the complete deletion of the protein of interest reliably in all brain regions allowing the functional study of glial proteins in postnatal and adult brain (Hirrlinger et al., 2006).

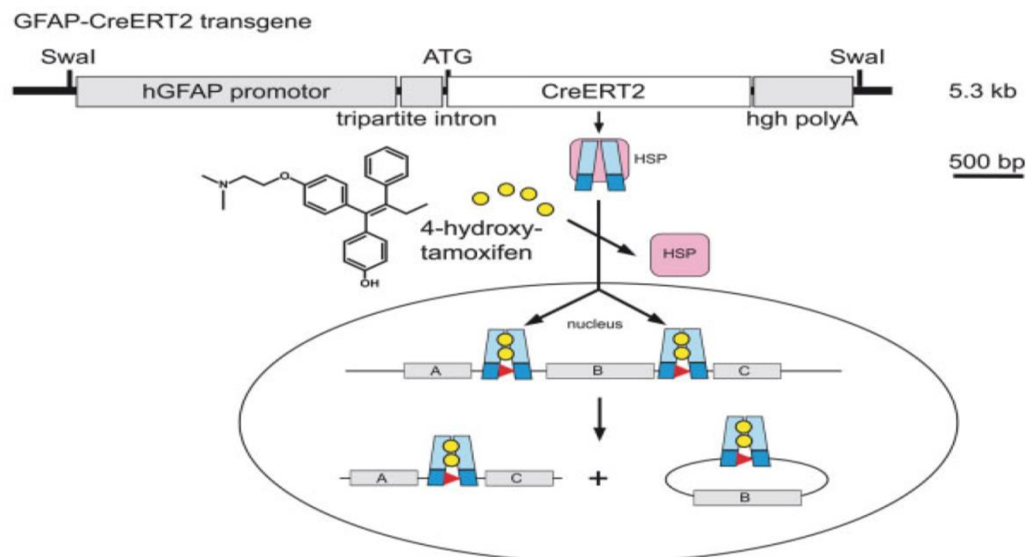

FIGURE 11 – SCHEMATIC REPRESENTATION OF TAMOXIFEN-INDUCIBLE CRE/LOXP SYSTEM (Adapted from Hirrlinger et al., 2006).

In 2012 and using the same the Tamoxifen-inducible Cre/loxP system described above, Han et al., generated a conditional mouse line in which they induced the deletion of the CB1 receptor in astrocytes. This mouse line, referred as GFAP.CB1.KO, expresses CreERT2 under the GFAP

promoter allowing its expression only in astrocytes. Three weeks after the injection of Tamoxifen, the line shows complete recombination and deletion of the astrocytic CB1 further confirmed by electron microscopy (figure 12) (Han et al., 2012).

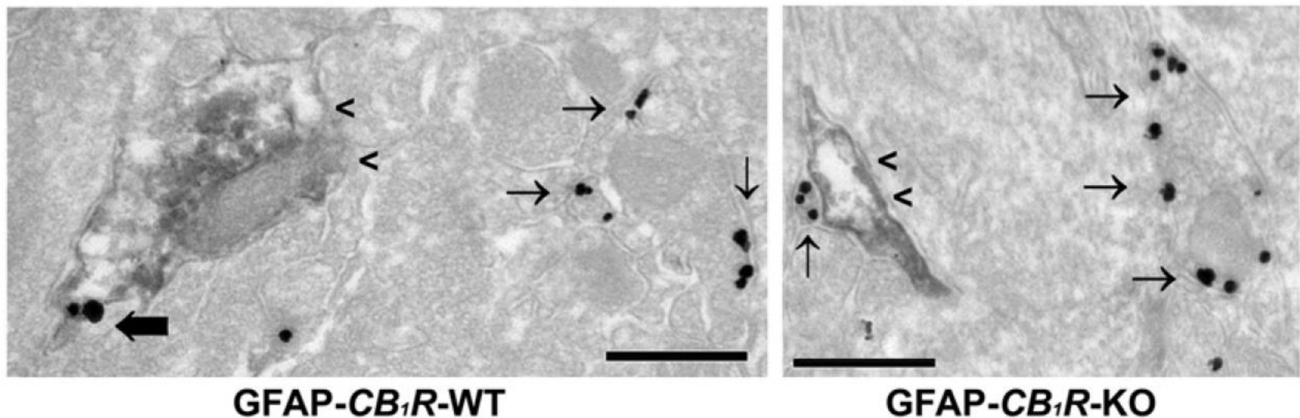

FIGURE 12 - AN ELECTRON MICROSCOPIC IMAGE SHOWING THE ABSENCE OF CB1 RECEPTOR IN ASTROCYTES AND AXONS. CB1 in axons and terminals (small arrows), CB1 in astrocytes (large arrow). The scale bars represent 500 nm (Han et al., 2012)

Part II – Astrocytes and the Endocannabinoid System

From the Greek *glia* (meaning glue), glial cells are generally smaller in length than neurons, possess multiple complex processes that emerge from their cell bodies, lack axons and dendrites, are established in complex networks and do not exhibit electrical properties (Purves et al., 2001; Giaume et al., 2010). Among the known glial cells, the best described are the oligodendroglia, microglia and astroglia (Araque and Navarrete, 2010).

All glial cells have complex and important functions such as the role of oligodendrocytes in the myelination of axons and the role of microglia in mechanism of brain defense and debris removal (Volterra and Meldolesi, 2005). The more dubious player in this concept of glial cell is the astroglia. For a long time astroglia was thought to act just on a supportive way contrasting a recent model in which it is believed that it participates in more complex processes such as neurotransmission and neuromodulation, development, maintenance and recovery of synapses (Auld and Robitaille, 2003).

From just supporting functions, new perspectives arrive concerning astrocytes and the control of synapse establishment and function, adult neurogenesis and brain vascular tone (Volterra and Meldolesi, 2005). In truth, they are vital for the survival and the correct function of the brain (Eroglu and Barres, 2010; Zangh and Barres, 2010).

Part II | Section 1 – Biology of Astrocytes

Section 1.0 – General Description, Functions and Properties

The Astrocytes derive from the same neural stem cells that give origin to oligodendrocytes although differently from the microglia which are originated from the immune system lineages (Eroglu and Barres, 2010). Astrocytes development tends to occur after the beginning of neuron development. Still, early they start to participate in striking functions in brain development such as guiding the migration of axons and certain neuroblasts (Sofroniew and Vinters, 2010). They participate in the correct foundation of the CNS being a key piece in the general process of differentiation.

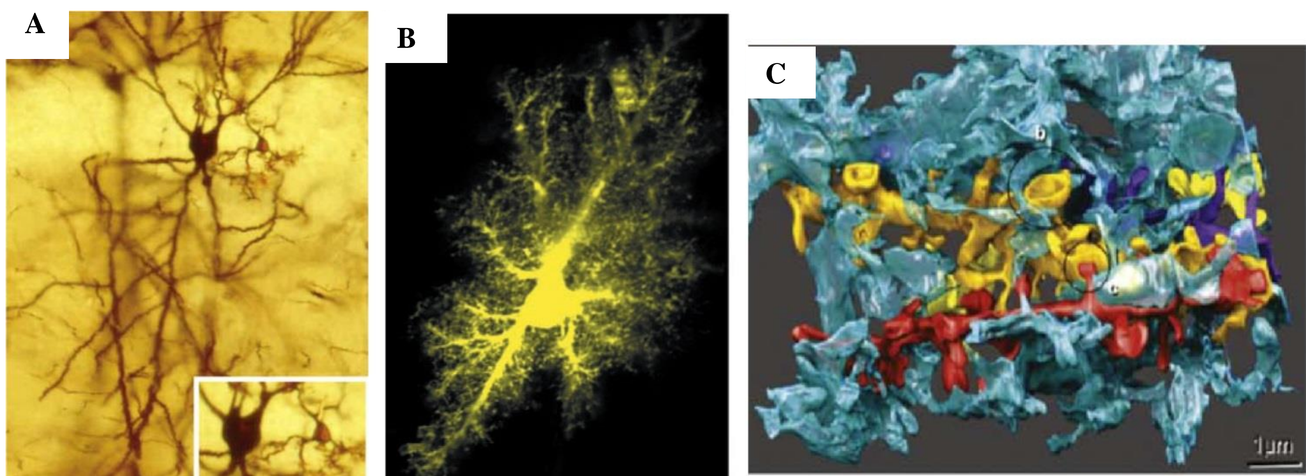

FIGURE 13 – ASTROCYTES. (**A**) Representation of a neuron and an astrocyte stained with the Golgi method from rat hippocampus. The astrocyte cell body is focused in the small bottom right square. (**B**) Protoplasmatic astrocyte from the hippocampus CA1 stratum radiatum. (**C**) 3D Representation of an astrocytic process (Blue) interacting with several synapses (red, yellow and violet) (A and C, adapted from Perea et al., 2009; B adapted from Bushong et al., 2002).

According to morphological and anatomical locations, in rodents, they can be divided in two main classes: the protoplasmatic astrocytes and the fibrous astrocytes. The protoplasmatic astrocytes, which can be found in all gray matter, are highly ramified, contact with blood vessels and are capable

of ensheathing synapses (Eroglu and Barres, 2010). The second class can be found mainly in all white matter and contact mainly with Ranvier nodes of axons (Eroglu and Barres, 2010). This division may not be adequate to separate the different astrocytes classes because they possess many different populations and they display distinct properties in different brain areas. It is also known that astrocytes are different among species, for instance, it is known that the human brain contains a higher diversity of astrocytes populations, more ramified and bigger that rodents (Eroglu and Barres, 2010). In 2009, Oberheim and her colleagues describe that, for example, human cortical astrocytes are both larger nd structurally both more complex and more diverse than those of rodents. Nevertheless this model of description still maintains its validity (Figure 13) (Sofroniew and Vinters, 2010).

The astrocytes retain in their structure an intermediate filament protein which is commonly used as a marker for astrocytes, the Glial Fibrillary Acid Protein (GFAP). This protein is more expressed upon brain damage or during CNS degeneration and is known to be highly expressed in the aged brain (Middeldorp and Hol, 2011). It is very important for cell support since it can maintain the cytoskeleton structure and it is associated with the transduction of molecular signals within these cells. Studies about this protein showed it importance in the processes of reactive astrogliosis and glial scar formation yet not for the normal function of the cell. Evidence obtained from double staining of astrocytes showed that some healthy astrocytes do not produce detectable molecule and it is also important to notice that GFAP is not present across all cell body but only in main stem branches (Figure 13) (Sofroniew and Vinters, 2010). Thereby, this cell marker is mainly used just to distinguish visually and genetically neurons from astrocytes (Middeldorp and Hol, 2011). Other proteins like the intermediate filament Vimentin, calcium binding protein beta (S100β) or the astroglial glutamate transporters EAAT1/GLAST and EAAT2/GTL-1 can be targeted specifically yet it is important to

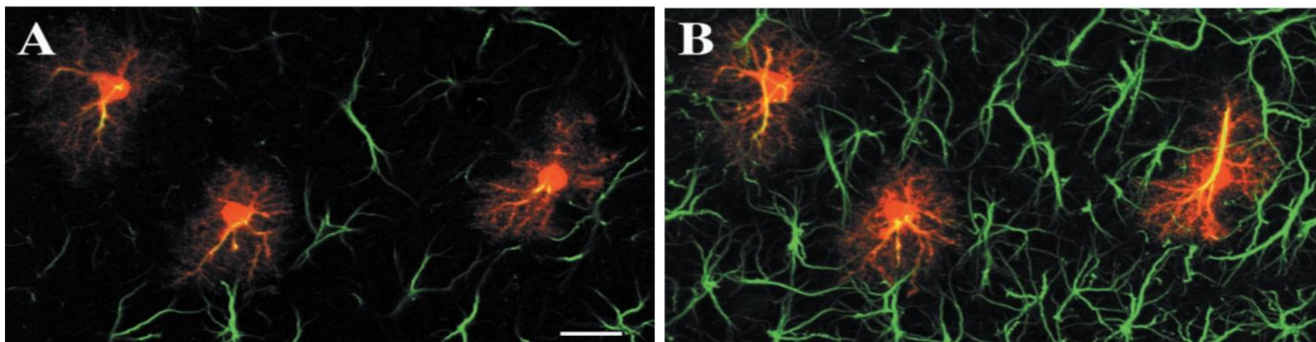

FIGURE 12 - GFAP IMMUNOLABELING OF LUCIFER YELLOW-FILLED ASTROCYTES OF CA1 STRATUM RADIATUM FROM RAT HIPPOCAMPUS A) Astrocytes labeled with LY display in their structure GFAP in their skeleton. B) Demonstrates the limited extent of GFAP throughout individual astrocytes which seems to be restricted to main branches not covering all the astrocyte's surface (Adapted from Bushong et al., 2002)

state that they are region-specific and do not permit enough astrocytic delimitation required for effective co-localization studies (Middeldorp and Hol, 2011). It is important to make clear that so far, no exclusive marker has been identified in mature astrocytes (McGann et al., 2012).

The astrocytes are known to take part in several functions that are essential to the correct physiology of the brain. They affect directly brain metabolism, neurotransmitter uptake, neuroinflammation, neurogenesis and more recently and less accepted, direct influence over synaptic plasticity (Figure 15). The evidence of the major importance of the astrocytes starts in the developing CNS. It is known that astrocytes are able to take a part in guiding neuritis thanks to their derived guidance molecules (Powell and Geller, 1999). They are also suggested to be essential to the formation of functional synapses through the release of molecular signals such as thrombospondin (Christopherson et al., 2005). They can also control fluid volume in the extracellular space, the regulation of ion concentrations and the uptake of neurotransmitter that are released during neuronal activity. Focusing on the regulation of the fluid levels, when for some reason there is an edema (due to pathological conditions). This control on fluid levels, ion, pH, and transmitter homeostasis (Sofroniew and Vinters, 2010) is important to the maintenance of correct synapse function. Potassium, due to its important effect over the neuronal membrane potential, must be under tight regulation because impairments on the concentration can lead to hyperexitability with pathological consequences.

Neurotransmitter uptake and homeostasis are also important to maintain a correct functional synaptic activity. Astrocytes express in their membranes transporters that are able to uptake, for example, glutamate. The excess of stimulation due to high concentrations of glutamate is also known to cause excitotoxicity (Purves et al., 2001) with severe consequences to neuron viability (On example is the glutamate spillover that it's associated with epileptic crisis). This regulation exhibits the indirect involvement of the astrocyte in the correct functioning the synapses (Haydon and Halassa, 2010). CB1 has been shown to protect against the excitotoxicity (Marsicano et al., 2003) and more recently, in 2010, Loría and colleagues showed that endocannabinoid tone leads to neuroprotection against (±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate receptor (AMPAR)-induced excitotoxicity (Loría et al., 2010).

Astrocytes are also known to contact and ensheathe directly blood vessels, controlling the molecules that diffuse into the brain through a close association named blood brain barrier (BBB). The BBB is formed by endothelial cells, perivascular pericytes and by the astrocytes. Astrocytes can also release molecules such as prostaglandins and nitric oxide that underlie the regulation of the blood flow, thus controlling the amount of blood that can transverse the brain during a certain event.

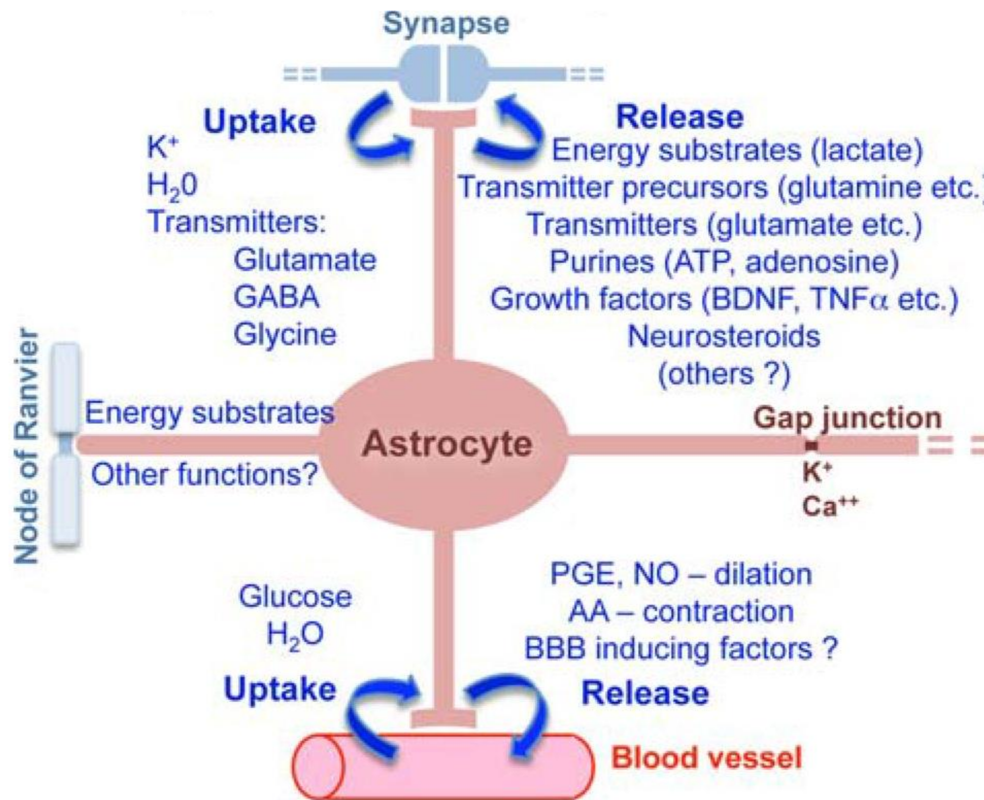

FIGURE 15 – GLOBAL, ESTABLISHED AND PUTATIVE, FUNCTIONS OF ASTROCYTES IN BRAIN PHYSIOLOGY. (Adapted from Sofroniew and Vinters, 2010)

Another function that is attributed to the astrocytes is their role in the regulation of brain inflammation induced by several insults such as infections (Sofroniew and Vinters, 2010). Astrocytes, when activated, are able to release immune mediators that can stimulate both neuroprotective and neurotoxic effects in a process called reactive astrogliosis (Sofroniew and Vinters, 2010). This complex phenomenon, which is a pathological land mark to scan disease in the CNS, does not have a concrete definition. Sofroniew and Vinters (2010), describe reactive astrogliosis in four categories that include: physiological changes of astrocytes in response to perturbations and injuries against the CNS;

these changes vary with the degree of the insult with progressive alterations in molecular and structural properties (including proliferation and scar formation), the events are context-specific and can be seen in variations of inter- and intracellular signaling molecules; molecular changes during this process can lead to loss or gain in astrocytic functions that can affect both positively and negatively the connections with surrounding neuronal cohorts (Sofroniew and Vinters, 2010). The endocannabinoid system has been shown to inhibit the release of inflammatory cytokines from reactive astrocytes, thereby exerting anti-inflammatory properties.

Astrocytes are also coupled to metabolic functions. It is known that the brain, although only representing 2% of the body weight, can consume about 20% of the body total energy. The astrocyte's processes involve tightly the blood vessels making them a platform to retrieve glucose from the blood vessels to supply neurons with brain metabolites. Astrocytes possess an energy reserve of glycogen that can be used to maintain neuronal activity even in times of hypoglycemia or high brain activity (Sofroniew and Vinters, 2010). Another important subtract is lactate. Lactate has been shown to be used by neurons as a principal energy subtract (Bouzier-Sore et al., 2003) and it was also shown that astrocyte-neuron lactate transport shuttle is required for long-term memory formation (Suzuki et al., 2011). Neurons can also use energy from fatty acids when they are the unique available source of energy (Figure 15) (Sofroniew and Vinters, 2010).

The astrocytes exist in a close relation with neurons and also among themselves. They are established in networks and they are able to communicate among themselves through gap junctions (Figure 16A and C). These gap junctions, which are composed by connexins, form channels in the plasma membrane that allow the traffic of molecules between astrocytes (Giaume et al., 2010). Astrocytes can be found associated with neurons, other astrocytes and the vascular system (Paixão and Klein, 2010). This new concept of astrocytic communication reveals important insights when comparing with the general picture of the system. The astrocytes are able to exhibit in vitro (Navarrete et al., 2013) (figure 16B) and in vivo calcium excitability (Kuga et al., 2011). This property was shown to be correlated with neuronal activity in awake behaving mice (Paixão and Klein, 2010). In 2009, Nimmerjahn his colleagues, described in awake and behaving mice that Bergmann glial cells could exhibit forms of calcium excitation. In one of the experiments, during locomotor activity, calcium excitation was observed in networks of hundreds of Bergmann glial cells extending across several

hundred micrometers. This excitability promoted by specific behavior could even lead to changes in brain dynamics and blood flow (Nimmerjahn et al., 2009).

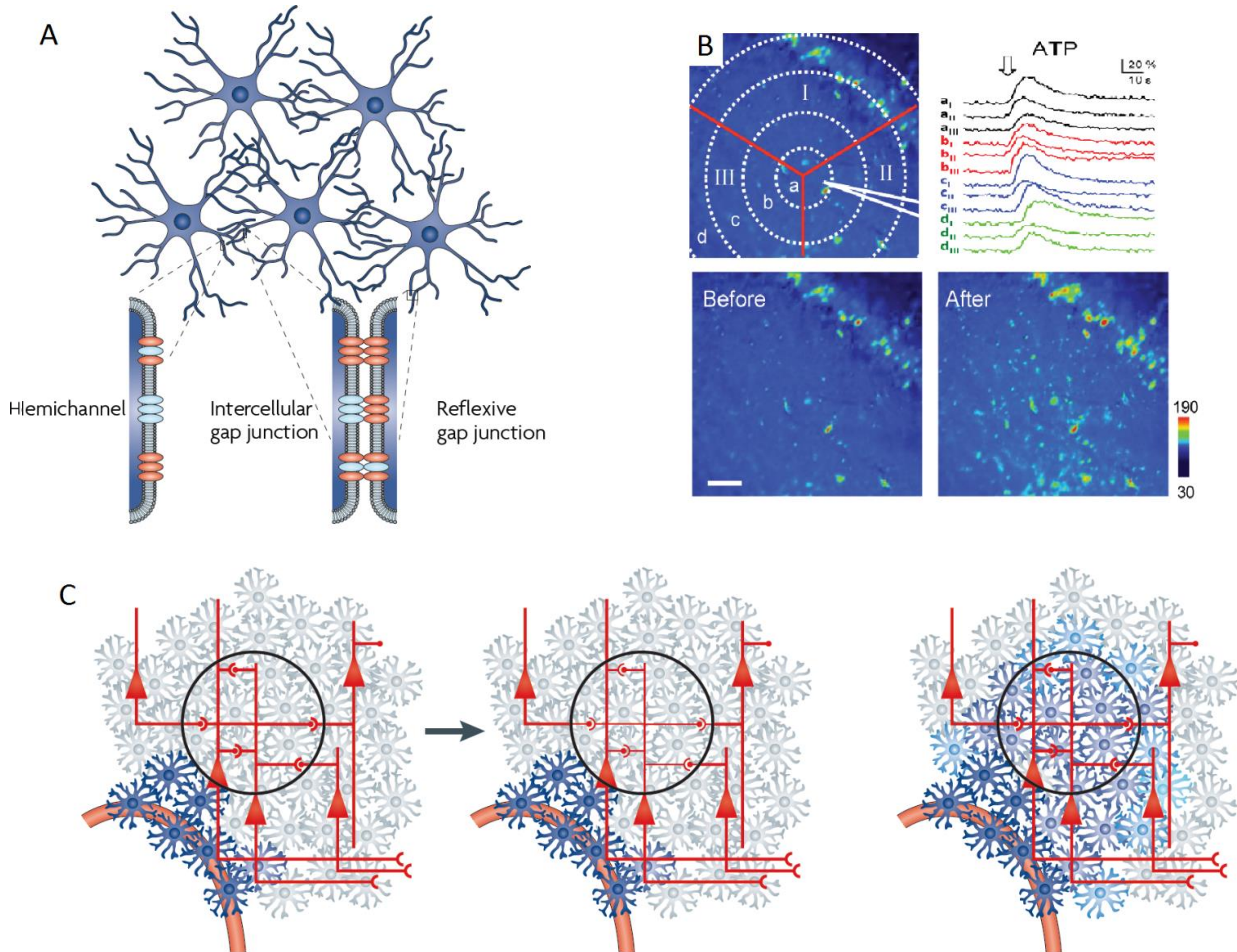

FIGURE 16 – ASTROCYTIC NETWORK PROPERTIES. A) Astrocytes communicate among themselves through gap junctions and hemi channels. Each one is responsible for its own domain of influence that is not overlapped by neighbor astrocytes. **B)** When stimulated with ATP, human hippocampal astrocytes exhibit increase in intracellular Calcium concentrations that can travel throughout the network (Navarrete et al., 2013). **C**) Astrocytes (in light blue) can sustain neuronal activity through the networks. In the presence of neuronal activity, an intercellular route (dark blue) is established to allow that trafficking of metabolic subtracts from blood vessels directly to areas of high synaptic activity (red arrows) (Giaume et al., 2010). (**A, C** adapted from Giaume et al., 2010; **B** adapted from Navarrete et al., 2013)

Part II | Section 2 – Astrocytes and Synaptic Plasticity

Section 2.1 – Neuronal-glial signaling: the tripartite synapse

Astrocytes were proposed as cellular processors of synaptic information in a concept called the tripartite synapse (Figure 17). This concept, originated in 1999 by Araque and colleagues, is based on evidence supporting bidirectional communication between astrocytes and neurons. Astrocytes are capable to display cellular ion excitability (through the intracellular calcium waves), show specificity in the response to specific synaptic inputs (Perea et al., 2009), display nonlinear input-output relationships and show cell intrinsic properties (for instance, the calcium signal is not non linearly modulated by simultaneously exogenous application of different neurotransmitters) (Perea and Araque, 2009).

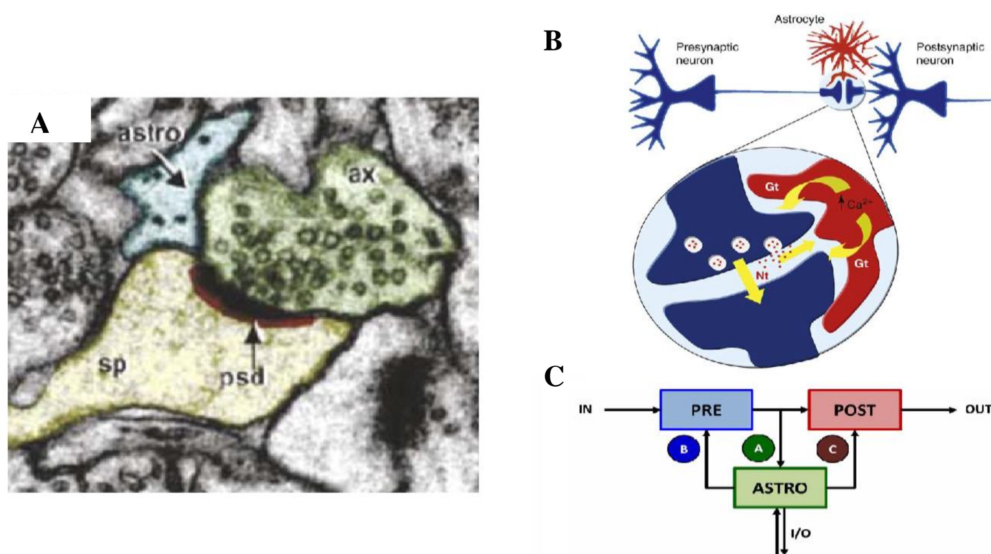

FIGURE 17 – TRIPARTITE SYNAPSE. (**A**) The Electron microscopy image shows an astrocytic process that ensheathes a synaptic terminal. (**B**) In a simple view, neurotransmitters released from the pre synaptic terminal, besides the action on the postsynaptic terminal, can act on the astrocytic process mediating the release of gliotransmitters through a putative calcium mediated mechanism (Perea et al., 2009). This release of gliotransmitters can affect the presynaptic terminal and/or the post synaptic terminal (**C**). Astrocytes can also receive an input or send an output to/from a neighbor astrocyte bellowing to the astrocytic network. Post synaptic density (psd); Spine (sp); Astrocyte (astro); Axon (ax); Gliotransmitters (Gt); Neurotransmitters (Nt); Pre synaptic terminal (Pre); Postsynaptic terminal (Post); Input (In); Output (Out) (**A** and **B** Adapted from Perea et al., 2009; **C** adapted from DePittà et al., 2012).

The astrocytes networks were proposed to coordinate the activity of local groups of synapses through, for example, the release of gliotransmitters that can affect directly neuronal activity (Giaume et al., 2010) and there is evidence showing that astrocytes can release, among molecules: Glutamate, D-serine, ATP, Adenosine and Tumor necrosis factor-alpha (TNF-α), among others (Paixão and Klein, 2010). For example, it has been shown that following tetanic stimulation of CA1 afferents, astrocytes can release ATP that can be converted into adenosine, acting this way on the presynaptic membrane adenosine receptor A1 which will exerted its effects by depressing the excitatory transmission. This active interaction can lead to the regulation of synaptic transmission with important consequences to processing, transfer and storage of information by the central nervous system (Perea et al, 2009).

The mechanisms and the role of gliotransmission are still not clear. Mechanisms proposed for gliotransmission may include: calcium-dependent vesicle exocytosis, through amino acid transporters, connexin/pannexin hemi channels, pore-forming P2X7 receptors and the swelling-induced activation of volume anion channels (Perea et al., 2009) (Figure 17B). Recently, concerning glutamate, an interesting mechanism of glutamate release through GPCR was described. This mechanism proposes the glutamate release through: 1) a fast way through activation of $G_{\alpha i}$, dissociation of $G_{\beta\gamma}$, and subsequent opening of glutamate-permeable, by the two-pore domain potassium channel TREK-1 and 2) a slow way by calcium dependent mechanism that requires the Gαq activation and opening of glutamate-permeable, Ca2+- activated anion channel Best1 (Woo et al., 2012). Another example in which astrocytes can influence synaptic plasticity events is through the release of D-Serine. This amino acid, which is an enantiomer of L-Serine, is present in high levels in brain and among other areas, in the hippocampus. D-Serine, which is a N-methyl-D-aspartate-type glutamate receptor (NMDAR) co-agonist, is able to bind to the glycine binding site and thereby regulate NMDAR transmission during synaptic plasticity events. Several pieces of data show that D-Serine is predominantly localized in astrocytes (Schell et al., 1995) and it can be released through a calcium dependent mechanism (Henneberger et a., 2010). Nevertheless it has been described that D-Serine can also be released from neurons (Kartvelishvily et al., 2006; Ehmsen et al., 2013). In vitro electrophysiological approach showed that D-Serine can be released from astrocytes in calcium-depend way and a single astrocyte can control NMDAR-dependent plasticity in many thousands of excitatory synapses from its own non overlapping domain (Henneberger et al., 2010).

Another molecule released from astrocytes that have been shown to be important for synaptic plasticity is the lactate. In 2011, Suzuki and colleagues reported that, in the rat hippocampus, lactate derived from glycogen and consequent released by astrocytes is essential for long term memory formation and maintenance of long-term potentiation in vivo. In this study they showed that by disrupting the expression of the astrocytic lactate transporters Mono Carboxylate Transporter (MCT4) or MCT1 is possible to cause amnesia as well as LTP impairments that can be rescued by L-Lactate and not by glucose. The disruption of the neuronal MCT2 leads also to amnesia that cannot be reverse by the administration of L-lactate or glucose. The lactate importation through MCT2 is important and necessary for the expression of Arc and for the phosphorylation of (cAMP response element-binding protein) CREB and cofilin (Bezzi and Volterra, 2011). Altogether these events make this "astrocyte-neuron lactate shuttle" required for gene expression, protein synthesis, and formation of new synaptic connections that underlies long term memory formation (Bezzi and Volterra, 2011; Suzuki et al., 2011).

Nevertheless, the mechanisms that underlie gliotransmission remain largely unclear mostly due to the techniques that do not allow enough precision and specificity to identify correctly glial components.

Part II | Section 3 – Astrocytes and the CB1 receptors

Section 3.1 – CB1 receptor is present in astrocytes

The presence of the CB1 on this type of glial cells started with evidence showing that mRNA from CB1 is present in primary cultures of rat astrocytes (Bouaboula et al., 1995). Since those results, successive attempts were tried to give clear evidence of the presence of the protein in these cells, not only in primary cultures but also in vivo models. It is known that astrocytes in primary cultures do not develop a normal structural and anatomical shape when comparing with the ones found in vivo. The simple fact can interfere with a correct way to evaluate the presence of these receptors in these cell types (Verkhatsky et al., 2011). The evidence of functional the astroglial CB1 control in bidirectional interactions between neurons and astrocytes came with functional studies from Navarrete and Araque (2008, 2010). Nevertheless it was in 2012, with Han et al., (2012) that the first conclusive in vivo evidence of this receptor was presented. In this study the authors generated a conditional mutant in which they delete specifically CB1 on astrocytes (described in Part I, Section 3.4 and further discussed in Part II, Section 3.2).

Section 3.2 – Astroglial CB1 and the Tripartite Synapse

Although there is evidence showing that astrocytes can express CB1 on their membrane in vivo (Han et al., 2012) and that they exhibit the machinery necessary to produce 2-AG and Anandamide in culture (Stella, 2009), it is not clear their influence in synaptic plasticity events.

Functional evidence of Astroglial CB1 involvement in endocannabinoid-mediated communication between neurons and glia in synaptic function came from two important studies. The first, from Navarrete and Araque (2008), shows that postsynaptic production of endocannabinoids by hippocampal CA1 pyramidal neurons can act on a putative cannabinoid receptors in astrocytes coupled to $G_{q/11}$ that is coupled to the phospholipase C, leading to an increase of intracellular calcium levels. This calcium increase is thought to

underlie glutamate release to the synapse and consequent activation of NMDAR transmission (Navarrete and Araque, 2008). Later on in 2010, by the same group of Araque, It was shown that Glutamate release through the same mechanism described earlier is able to act on presynaptic glutamate metabotropic receptors (mGuR1) leading to the potentiation of synaptic transmission and to a short-term facilitation of the neurotransmitter release (Navarrete and Araque, 2010). Altogether, the mechanism of eCB action on short term plasticity would be through the Depolarization Suppression of Neurotransmitter release through presynaptic CB1 receptors and to indirect facilitate the neurotransmitter release through astroglial CB1 (Figure 18A) (Castillo et al., 2012).

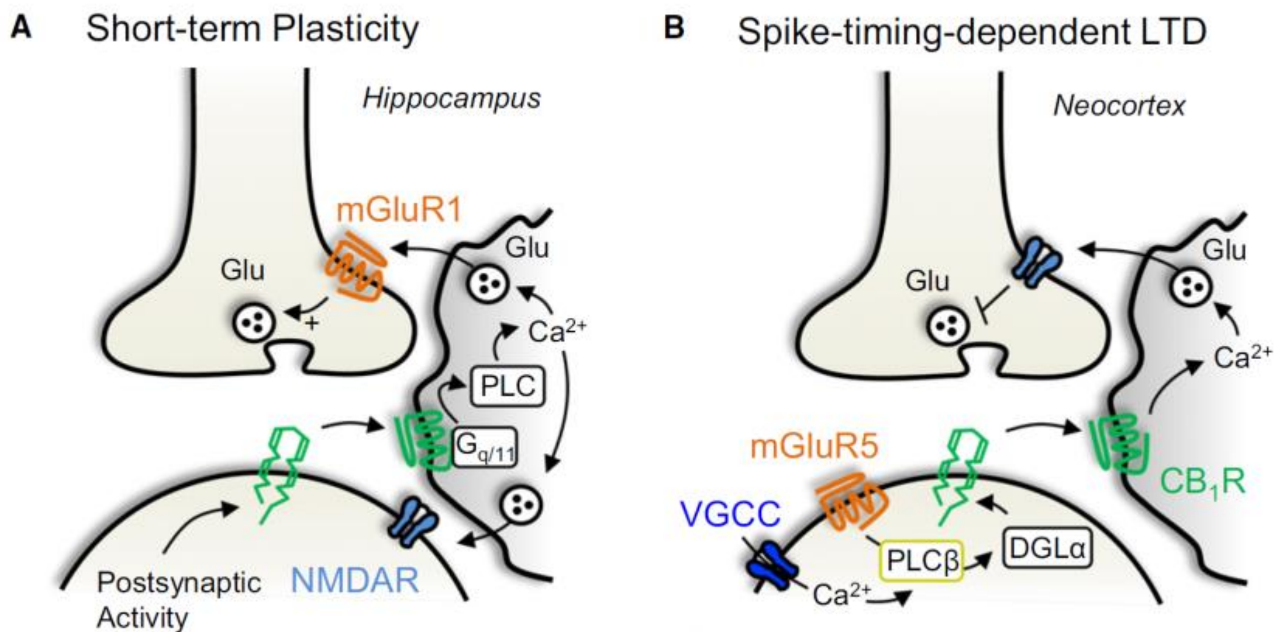

FIGURE 18 - ASTROGLIAL CB1 MEDIATED SHORT TERM AND LONG TERM SYNAPTIC PLASTICITY. (Adapted from Castillo et al., 2012)

Endocannabinoids have been also implied in mechanisms of long term synaptic plasticity. The repetitive pairings of post-before-presynaptic activity is able to induce Spike timing-dependent LTD (tLTD) between neocortical pyramidal neurons (Figure 18B). The neuronal endocannabinoids produced under the repetitive pairings will act on the astrocytic CB1, triggering glutamate release that will act on the presynaptic NMDAR leading to a depression on neurotransmitters release (Castillo et al., 2012). Another interesting model of long term plasticity involving the astroglial CB1 receptor is the in vivo LTD production at CA3-CA1 synapses and subsequent working memory impairment following THC administration (Han et al., 2012). In this model, THC

administration is able to activate astroglial CB1 and the postsynaptic GluN2B-containing NMDAR (possibly by the putative release of glutamate from astrocytes following THC binding to the astroglial CB1) that triggers an AMPAR endocytosis mediated expression of in vivo LTD at CA3-CA1 synapses. Furthermore the authors related the this LTD induced by THC administration with a working memory impairment observed in glutamatergic and GABAergic-specific CB1 knockout mice but not in astroglial CB1 knockout mice (Figure 19). Still, the endogenous role of endocannabinoid signaling remains highly unclear.

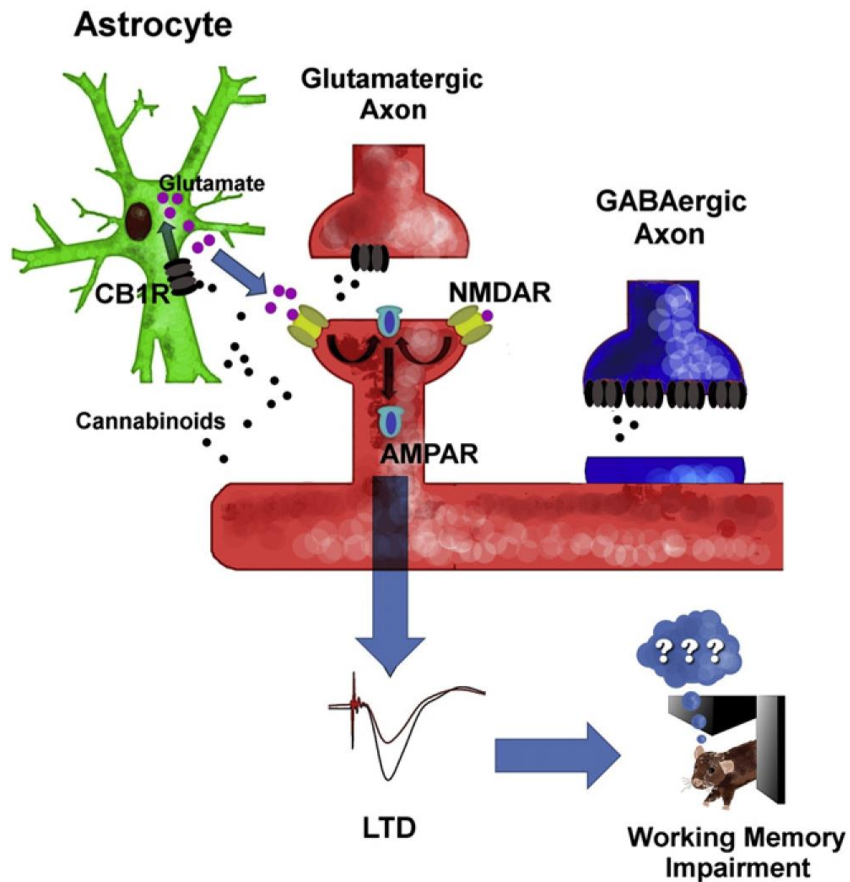

FIGURE 19 – PROPOSED IN VIVO LTD PRODUCTION AT CA3-CA1 SCHAFFER COLLATERAL SYNAPSES FOLLOWING THC ADMINISTRATION (From Han et al., 2012).

Part III – CB1 Receptors and memory

Part III | Section 1 – Memory Systems

Memory was once described by Milner and colleagues (1998) as ability the by which individuals were able to retain and recall past experiences based on the processes of learning, retention and retrieval. This complex ability requires disparate brain regions thereby originating different partially independent memory systems that can operate in parallel (Henke, 2010). This allows the survival, adaptation, well-being and adaptation of complex organisms (Tronson and Taylor, 2007).

Studies performed on patients shows that some of them are accessible to consciousness and other aren't. This fact suggested the existence of two different systems of long term memory: declarative versus non-declarative memory (Figure 20) (Henke, 2010). The declarative memory referred above can be characterized by the recollection of memories such as autobiographical events (episodic memory) or even general knowledge (semantic memory). On the other hand, the non-declarative memory consists in procedural learning of sensorimotor and cognitive skills such as tying a shoe or reading (Squire, 2009).

The memory formation and the brain areas involved are still in great debate. Concerning declarative memory system, the medial temporal lobe is the group that has been shown to be primarily responsible for this function (Squire and Zola-Morgan, 1991). This area is composed by the hippocampal complex (CA fields, the dentate gyrus and the subicular complex) together with the adjacent entorhinal, perihinal and parahippocampal cortices and are responsible for essential phenomena involving memory formation and consolidation after a learning period (Squire, 2009).

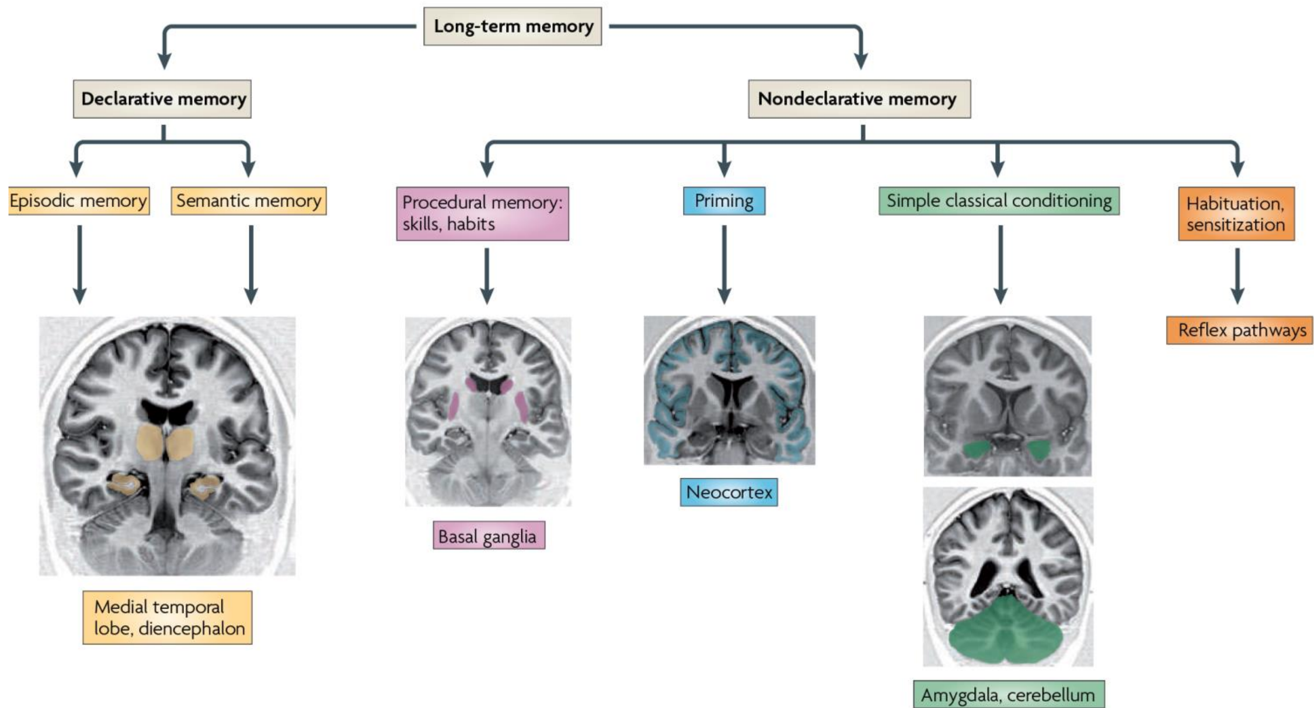

FIGURE 20 – DECLARATIVE AND NON-DECLARATIVE SYSTEMS OF LONG TERM MEMORY (From Henke, 2010).

The formation of memory in traditional view starts with the event (Figure 21). This event will be encoded and the memory starts to be established. During this encoding, immediately a new phase starts: the consolidation. In this phase the memory becomes permanent and stored (Nadel et al., 2012). In the traditional view of memory formation, once a memory is permanent no changes can occur in its form.

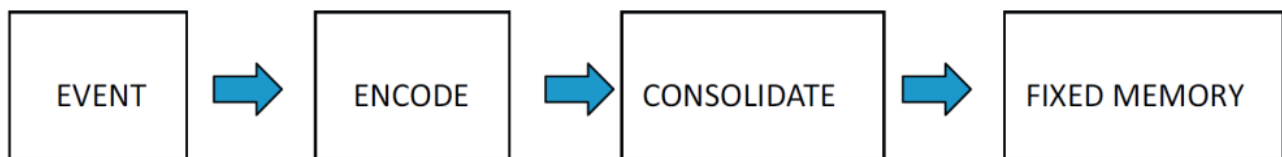

FIGURE 21 – TRADITIONAL VIEW OVER THE PROCESS OF MEMORY FORMATION (Adapted from Nadel et al., 2012).

However recently, a new theory challenges this very concept of memory formation. The concept of reconsolidation, shown in figure 22, supports the fact that when a memory suffers retrieval,

it became plastic and can be vulnerable to modifications making thereby the final memory not a reliable picture of what happened by more like a painting that small by small loses its colorful appearance (Squire, 2009). The retrieval process can put the memory in a labile state, making it prone to destabilization. For example, through protein synthesis inhibitors (disrupting the memory) or through a novel experience that updates the previous memory (Nadel et al., 2012).

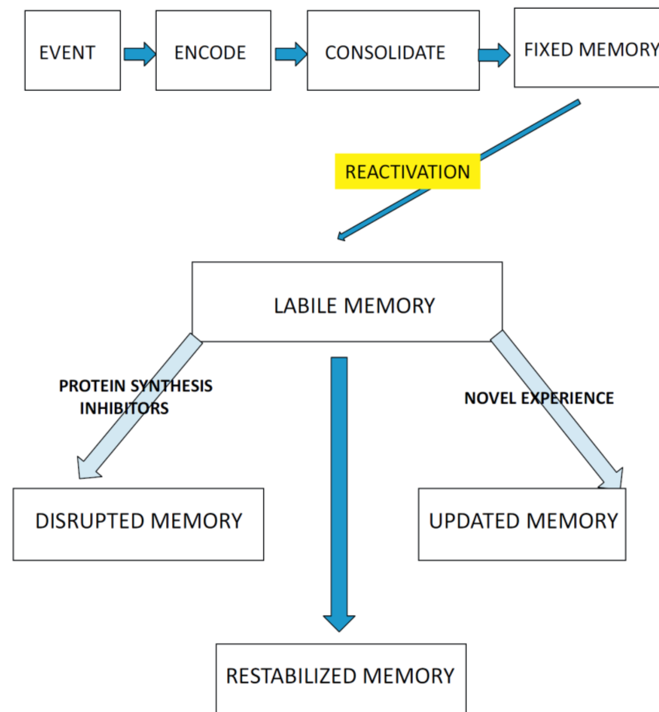

FIGURE 22 – RECONSOLIDATION OF A FIXED MEMORY. (Adapted from Nadel et al., 2012).

Memory functions are incredible diverse and the whole field is too vast to be covered in some paragraphs. More concerning to the objective of this introduction, a specific type of declarative memory will be introduced. Nevertheless, there are much more mechanism and types of memory that are widely covered in literature (for further information on: Tulving, 2002; Wood et al., 2012)

Part III | Section 2 – Molecular Mechanisms Underlying Memory

Understanding how memory is formed and what kind of changes occurs in the brain is quite an intensive hot topic in brain research. Evidence provided from lesions in hippocampus and memory deficits concerning episodic memory indicate a major role of hippocampus in memory formation and storage (Squire et al., 2009). In the past decades, the hippocampus has been extensively studied for molecular changes in synaptic composition and many theories were drawn from the acquired data.

Synaptic plasticity, which refers to activity dependent changes in the synapse form and composition, has been extensively studied in the hippocampus and it is believed to underlie learning and memory. These studies aimed to identify molecular mechanisms that could induce changes in the synapses. The mechanisms vary from identifying cell biological processes, the synaptic release and recycling of neurotransmitters, neurotransmitters receptors trafficking, cell adhesion, stimulus-induced changes in gene expression in neurons, among others (Ho et al., 2011). All this processes have the power to shape in a long term the synapse in experience directed changes which together forms the foundation of the mechanism that are believed to underlie learning and memory.

Long term changes in synaptic plasticity can be induced by several and distinct mechanisms. Due to the nature of this introduction the one focused is the NMDAR-dependent long term synaptic plasticity.

Section 2.1 – NMDAR dependent long term synaptic plasticity

The anatomy of the hippocampus makes this structure suitable for electrophysiological approaches that are useful to study mechanisms of synaptic function. There are three main sequential pathways and each of them encloses specific cell types and projections. In electrophysiology studies either in vivo or in vitro, the stimulation of the axons coming from the CA3 neurons with a specific frequency stimulus is able to induce changes in the synapses from the stratum radiatum of CA1 (Figure 23). This CA3-CA1 pathway is commonly known as Schaffer Collateral Pathway. High frequency stimulation is able to induce long term potentiation of the synapses (LTP) and low frequency stimulation is able to induce long term depression of the synaptic strength (LTD) both lasting from

hours to months (Lamprecht and LeDoux, 2004). It is also known that in CA1 synapses, specific stimulation protocols are able to induce and long term potentiation dependent on NMDA receptors (Ho et al., 2011). NMDAR have been shown to be important for several types of memory and they are important to the induction of long term synaptic plasticity in hippocampal synapses (Nakazawa et al., 2004).

This induction of long term changes in synaptic plasticity in excitatory glutamatergic synapses starts with the release of the main brain excitatory neurotransmitter: glutamate (Hu et al., 2011). This neurotransmitter is release from the presynaptic cell in a rapid and highly regulated vesicular exocytosis mechanism (Figure 24A). These vesicles are readily docked in the active zone of release of the presynaptic terminal and when an action potential arrives to this area, the increase of intracellular calcium (mainly through VGCC) leads to the fusion of the vesicles with the release of the neurotransmitter in the intermediary area called synaptic cleft. When glutamate is release in the cleft, it will bind to the AMPAR. These receptors which are important for the expression and maintenance of LTP can be activated by binding of glutamate at resting membrane potential allowing the influx of calcium. This depolarization of the membrane will allow the activation of NMDAR. NMDAR are

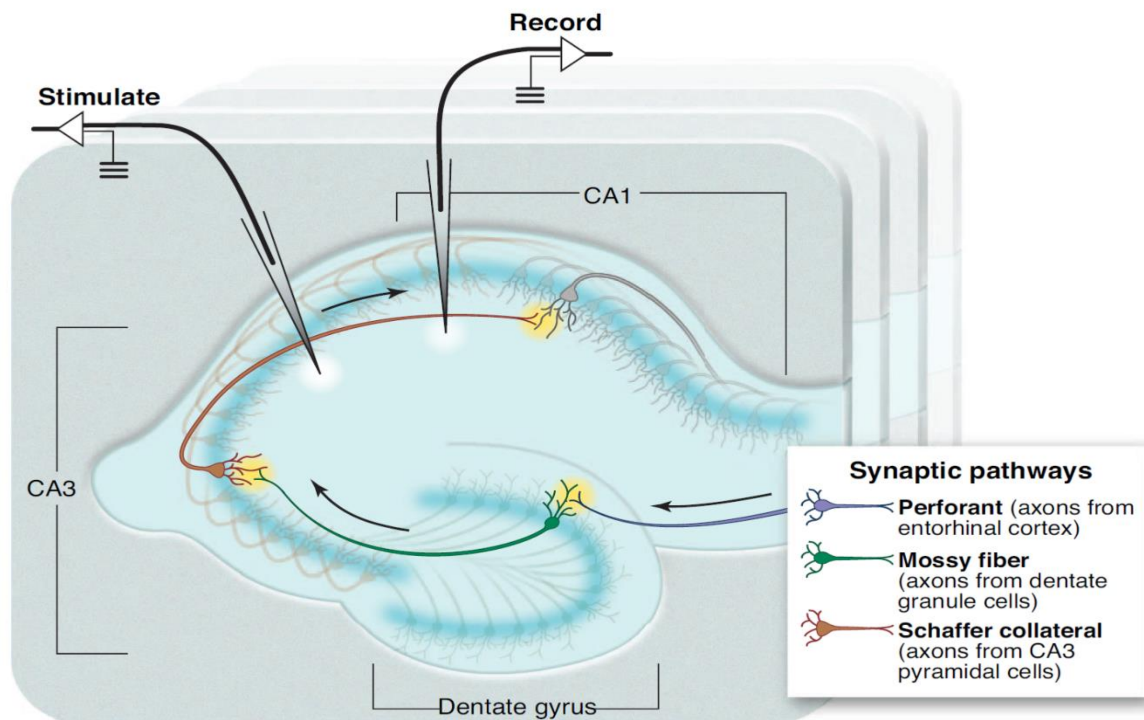

FIGURE 23 – SCHEMATIC REPRESENTATION OF AN HIPPOCAMPAL TRANSVERSE SLICE. In this example is possible to distinguish three well preserved ipsilateral synaptic pathways: Perforant pathway that concerns axons from entorhinal cortex that synapse with dendrites of dentate granule cells, Mossy fibers that comprises the axons from dentate granule cells that synapse with CA3 pyramidal neuronal dendrites and Schaffer Collateral which concerns the axons deriving from CA3 that synapse in CA1 pyramidal neuronal dendrites (from Ho et al., 2011).

present in the postsynaptic membrane and are constitutively blocked by Mg^{2+}. The depolarization induced by AMPAR will release the NMDAR from the block allowing the binding of glutamate and co agonist which is usually represented as glycine. This binding will allow the activation of the NMDAR and will allow the calcium influx to the postsynaptic membrane. The postsynaptic increase of calcium concentration will allow the expression of long term changes in the synaptic plasticity where LTP requires large calcium influx and LTP low influx. The LTP in CA1 is known to be dependent on the activation of Ca2+/Calmodulin-dependent protein kinase (CaMKII). Neuronal activity leads to the translocation of this enzyme to the PSD where it can phosphorylate many other postsynaptic density (PSD) proteins. The autophosphorylation is believed to be important to the LTP induction and maintenance (Hu et al., 2011). These changes can be characterized by morphological alterations through cytoskeleton regulation, transcription of RNA in the nucleus through modulation of transcription factors, increase of protein synthesis either on the cell body either in the dendrite (Santos et al., 2010), neurotransmitter release quantity, increasing in presynaptic vesicles, postsynaptic ribosomes and changes in the number and spine's morphology (Lamprecht and LeDoux, 2004) (Figure 23B).

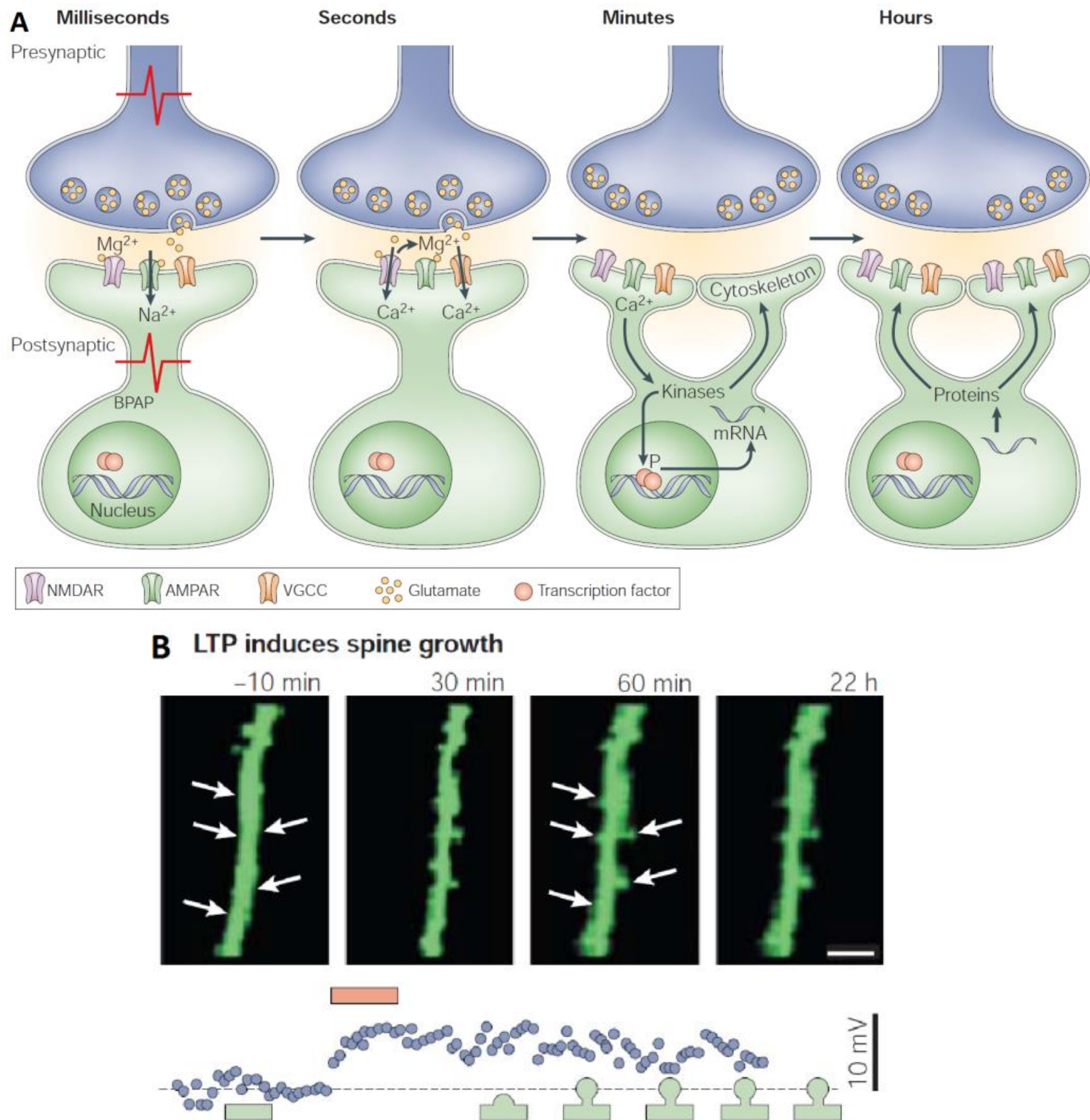

FIGURE 24 – (A) MOLECULAR MECHANISMS UNDERLYING INITIATION AND MAINTENANCE OF LONG TERM POTENTIATION AND (B) MORPHOLOGICAL CHANGES IN SPINES AFTER LTP (Adapted from Lamprecht and LeDoux, 2004).

Section 2.2 – NMDA Receptors

NMDAR exist in di-heteromeric or tri-heteromeric forms that confer them unique properties that are thought to be involved in their localization and function (Cull-Candy et al., 2001). The several subunits are: GluN1, GluN2A–D, and GluN3A and B (Figure 25A and B).

From these seven subunits the one that is indispensable for the correct functioning of the NDMAR, being thereby always present in the heteromers, is the subunit GluN1 (Nakazawa et al., 2004). Inhibition of the GluN1 either by direct inhibition with a specific NMDAR antagonist DL-2-Amino-5-phosphonovaleric acid (AP-5) or by the selective deletion of GluN1 gene show an inhibition on LTP induction in the neurons that lack this protein (Nakazawa et al., 2004). It is known that NMDAR can have different functions depending if their expression is synaptic or extrasynaptic. Literature currently available indicate that the NMDAR present in the synaptic membrane are able to act primarily through nuclear Ca^{2+} signaling, leading to the build-up of a neuroprotective action and the stimulation of NMDARs in the extra-synaptic area can promote cell death (Hardingham and Bading, 2010). There is evidence indicating the composition of these receptors it's not the same concerning the subunits.

To the activation of the NMDAR, it is needed cooperation between two molecules: Glycine, a co-agonist that binds to residues located in the GluN1 subunits, and glutamate which binds to residues located in GluN2 subunits. Not only Glycine is important to the activation of this molecule since D-serine can also act as co-agonist (further discussed below). Recently, it has been proposed that different NMDAR, synaptic and extrasynaptic, are gated by different endogenous co-agonists. In this study, from Stephane Oliet's group, they reported that synaptic and extra synaptic NMDAR are gated differentially by D-Serine and Glycine, respectively. There is a regionalization of each of the co agonist and this availability can be related with long term synaptic plasticity events. It was shown that D-Serine availability in the synapse is essential to the induction of LTP and neurotoxicity effect whereas the availability of both D-Serine synaptically and Glycine extrasynaptically is essential for the induction of LTD and neuroprotection (Papouin et al., 2012). They also show that NMDAR composition present in the synapse is rich in heteromers containing GluN2A subunits and NMDAR composition in the extrasynaptic space is rich in Glu2B subunit heteromers (Papouin et al., 2012).

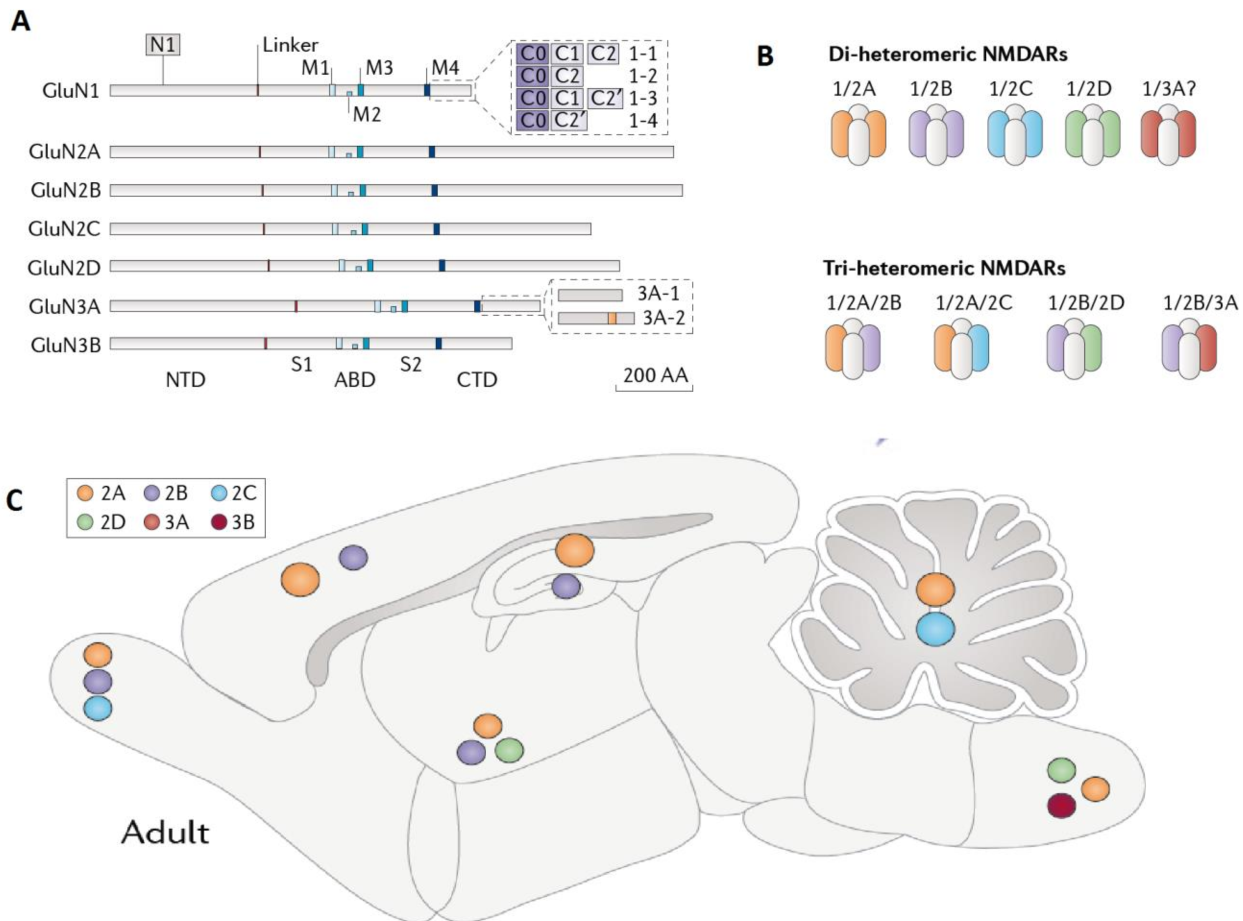

FIGURE 25 – NMDA RECEPTORS: DIVERSITY, STRUCTURE AND EXPRESSION PATTERN. A) Seven subunits of NMDA receptors have been identified: GluN1, GluN2A–D, and GluN3A and B. M1-4 indicate membrane segments. **B**) NMDAR can be found in Di-heteromeric or Tri-heteromeric forms. **C**) Patterns of NMDAR subunit expression in adult mouse brain (Adapted from Paoletti et al., 2013).

Section 2.3 – D-Serine and NMDA transmission

D-Serine is a NMDAR co-agonist acting on the Glycine binding site that is thought to play a major role in NMDAR dependent synaptic plasticity. D-Serine is an enantiomer of L-serine converted through the action of Serine Racemase. This enzyme, was once thought to be inexistent in organisms besides bacteria or insects, was described in the brain in 1999 after a period where it was not clear how could D-Serine levels be so high in brain tissues (Wolosker et al., 1999). D-Serine shows particularly

high levels in the hippocampus (Henneberger et al., 2012), more specifically in secretory vesicles inside astrocytes (Schell et al., 1995).

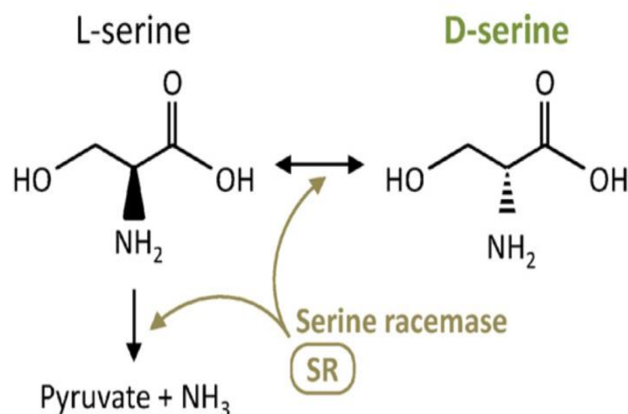

FIGURE 26 – MECHANISM OF D-SERINE PRODUCTION. Serine Racemase is able to convert L-Serine into D-Serine or break down into Pyruvate and Ammonia (From Henneberger et al., 2012).

The discovery of D-Serine's agonist properties of NMDAR triggered questions concerning it biological functions. Experimental evidence shows that D-Serine produced by Serine Racemase, stored and release by astrocytes is important for the modulation of NMDAR transmission mediated changes in synaptic plasticity (Henneberger et al., 2012). One of the experiments that showed the importance of D-Serine release by astrocytes was carried out in the hypothalamic nucleus where astrocytic processes retract from neurons during the physiological onset of lactation (Henneberger et al., 2012). In this study, Panatier and colleagues (2006) showed that during lactation, where this retraction occurred, the NMDAR mediated synaptic plasticity decreased and LTP was mainly suppressed. These events where fully reversed by D-Serine exogenous administration. These results imply that the astrocytic coverage contributes for affecting availability of NMDAR for activation and thus the activity dependence of long-term synaptic changes (Panatier et al., 2006). In another interesting work, in 2010, Henneberger and colleagues reported a mechanism in which a calcium dependent release of astrocytic D-Serine controls NMDAR-dependent plasticity in thousands of excitatory synapses (Henneberger et al., 2010).

Altogether this results fuel the idea that astrocytes are important for overall synaptic plasticity events and that gliotransmission can play an important role in NMDAR mediated long term changes.

Section 2.4 – Synaptic plasticity and Memory

Long term synaptic changes, such as LTP, are thought to underlie learning and memory. In this context the experimental model that could be used to test this hypothesis would be a trial in which the inhibition of the long term potentiation, for example, in the hippocampus could impair memory without changing other parameters (Neves et al., 2008). One of the possible approaches used is the local infusion of a NMDA blocker like AP-5 (2-amino-5-phosphonovaleric acid). In an experiment performed by Morris and colleagues in 1986 (Morris et al., 1986) the infusion of this drug is able to strongly impair the learning and memory formation in the Morris Water Maze without changing basal transmission. Furthermore, in another recently interesting experiment, it has been showed that the inhibition of the active form of the Protein kinase M ζ (PKMζ) by ZIP (myristoylated zeta-pseudo substrate inhibitory peptide) intra hippocampal infusion is able impair spatial memory and LTP even when administered several days after the memory formation (Neves et al., 2008). Nevertheless, it is important to have caution when analyzing such striking results. Recently two studies published in the beginning of the year 2013 and using genetic deletion of PKMζ, show that ZIP reverses late LTP and long-term memory in PKMζ knockout mice maybe through an unspecific PKMζ independent mechanism (Lee et al., 2013; Volk et al., 2013). Other approaches focused in the deletion of the GluN1 subunit of NMDAR in the CA1 subfield of the hippocampus. This subunit is known to be necessary for LTP induction and in this KO model mice showed a suppression of both LTP and Spatial Learning. Nevertheless it is not possible to correlated an LTP impairment with memory deficits because other mechanism dependent on GluN1, such as LTD, are also impaired (Neves et al., 2008).

Is important to focus on the fact that, based on current literature, it is not possible to direct correlate LTP/LTD impairment with memory impairment since it is not possible to check directly this condition. LTP-like synaptic plasticity events are likely to mediate hippocampal learning and memory but it is important to keep in mind that it can be through other long term changes in several hippocampal areas or even phenomena concerning metaplasticity.

Part III | Section 3 – Object Recognition Memory

Recognition memory refers the capacity of recognizing someone or something that was presented previously. This is a subcategory that belongs to the declarative memory and is a very important feature that is widely viewed as composed by two components: recollection and familiarity (Squire et al., 2007). These two components, according to Brown and Aggleton (2001), are thought to be dependent on two different brain structures. The recollection, which is defined as the capacity to remember specific contextual details about a previous learning episode is proposed to be dependent on hippocampus and on other hand, familiarity, the simple ability to remember the previous presented item without any specific info additional information about that learning episode (without recollection), is proposed to be dependent on the adjacent perihinal cortex (Squire et l., 2007; Brown and Aggleton, 2001).

Although the controversial in the field regarding which of the areas is important for a specific functions, hippocampus is mainly accepted as of most importance in recognition memory (Squire et al., 2007).

In the experimental context, depending on the organism of study, several memory tests can be used to access this kind of episodic memory. For instance, in rodents, based on the fact that these animals prefer novelty over to familiarity (a fact that is considered as existence of memory of a previous encountered item) it is possible to perform an object recognition task. In this task, animals will explore a field environment with two identical objects. After a delay from minutes to hours, the same environment is re-presented with a novel object substituting one of the previous objects. The preference of the novel object will be traduced in more time exploring that novel object without differences in the total exploration of both objects. With this time measurements it is possible to calculate a discrimination index (DI) that is close to one when there is a good performance (high exploration of the novel object) or close to zero when there is a bad performance (low exploration of the novel object) (Figure 27).

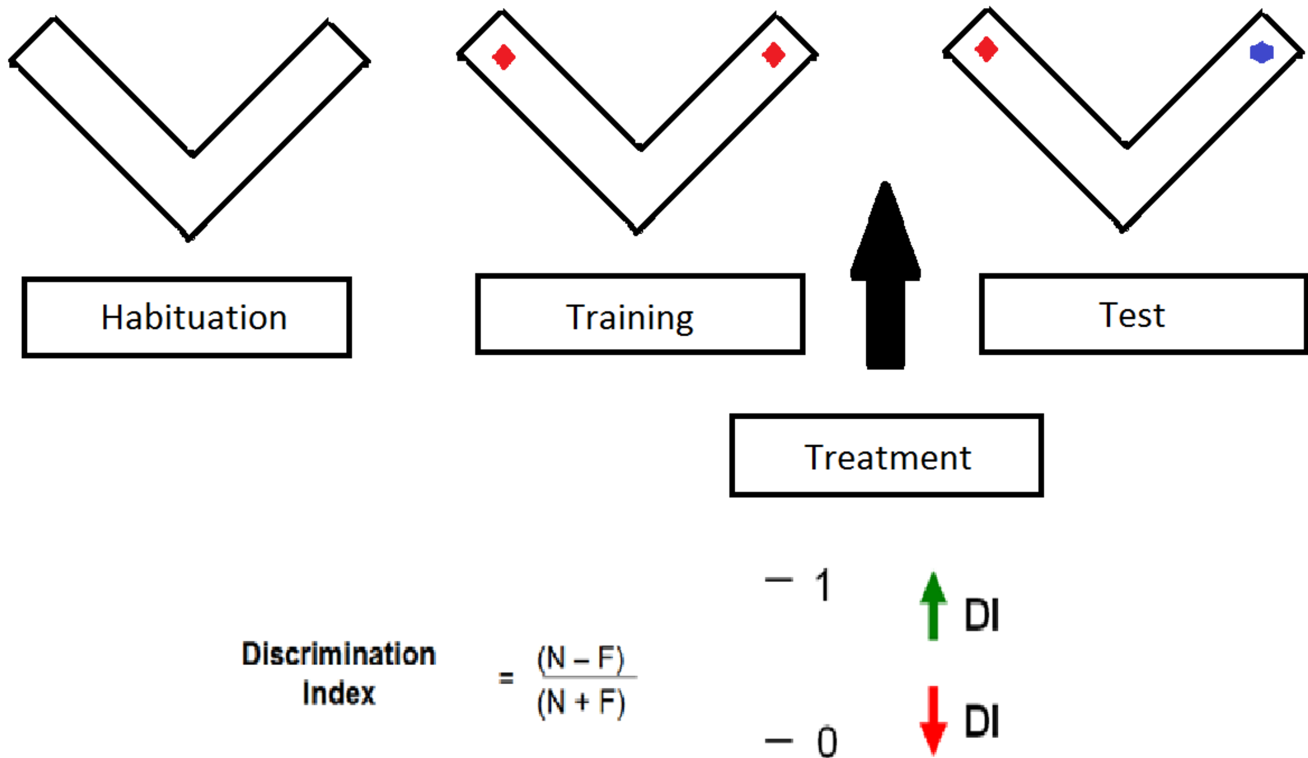

FIGURE 27 – SCHEMATIC REPRESENTATION OF THE OBJECT RECOGNITION TASK. After a period of habituation, usually 24 hours before the training phase, the mice will be exposed to two identical objects. After this phase, one of the objects will be changed and the time spent exploring the new object will be evaluated by the discrimination index (DI). Another parameter that must be taken in consideration is the total exploration time of the objects that must remain the same for both training and test. Novel object (N); Familiar Object (F).

Part III | Section 3 – Astroglial CB1 and Object Recognition Memory

The actual expression of CB1 receptor on brain astrocytes was a great matter of debate until very recently (Navarrete and Araque, 2008, 2010; Han et al., 2012). The role of the Astroglial CB1 receptor in brain physiology remains largely unknown. Although it has been implied in the synaptic physiology and metabolism in the hippocampus through pharmacological approaches (Duarte et al., 2012; Navarrete and Araque, 2008, 2010, Min and Nevian, 2012) only recently, through the generation of a conditional knockout model, It was possible to anatomically detect its presence in astrocytes through electron microscopy (Han et al., 2012). The generation of this conditional Knockout model by the host laboratory was used by Han and colleagues to show that exogenous cannabinoids impair working memory through astroglial CB1 receptor possible by the induction of a hippocampal cannabinoid mediated-LTD (Han et al., 2012). Notably, this study uncovered a first role of astroglial CB1 receptor in behavioral, cognitive functions, underlining the importance of this genetic tool to study its role in vivo, which, however, still remains largely unknown. In particular, Han and colleagues described a memory-related mechanism that is induced by exogenous activation of astroglial CB1. Nevertheless, the endogenous role of astroglial CB1 receptor in memory processing is not known so far.

Interestingly, unpublished results from the host laboratory show that the Astroglial CB1 receptor is necessary for object recognition memory because mice lacking the astroglial CB1 display strong recognition memory impairment (Metna-Laurent, Unpublished Data).

Intracellular Astroglial CB1R action mechanism is proposed to be through a G_q coupled to PLC that mobilize and increases calcium levels in hippocampal astrocytes (Navarrete and Araque, 2008). This increase is thought to mediate the release of gliotransmitters that can act in the synapse leading to the modulation of both short and long term synaptic plasticity. Long term synaptic changes are strongly associated with memory functions. As it was discussed previously during the introduction (Part III, Section 2), NMDAR-dependent transmission is one of the molecular mechanisms proposed to underlie object recognition memory in hippocampus. The further involvement of the NMDAR transmission in Object Recognition memory was tested with the injection of AP-5 in the dorsal hippocampus of C57BL/6N. This treatment is able to impair object recognition memory, confirming

that NMDAR transmission in hippocampus is necessary for intact Object Recognition Memory (Robin et al., Unpublished Data).

One of the molecules that are known to potentiate specifically NMDAR-dependent synaptic changes is D-Serine. This molecule, as it was widely spoken during the introduction, is thought to be released by astrocytes during synaptic activity maybe in a calcium-dependent way (Henneberger et al., 2010). Taking in consideration the putative calcium-dependent release of gliotransmitters by astrocytes, it is possible to speculate that endogenous Astroglial CB1 could control object recognition memory by favoring D-Serine availability in the synapse. Strikingly, the injection of D-Serine either i.p or directly in the hippocampus is able to fully and specifically rescue the object recognition impairment of the GFAP-CB1-KO mice. Altogether, these preliminary results indicate that endogenous astroglial CB1 receptor signaling controls object recognition memory through a mechanism that depends on hippocampal NMDAR transmission and that can be mimicked by exogenous administration of D-Serine (Robin et al., Unpublished results).

After these behavioral experiments, the host laboratory decided to investigate putative molecular mechanisms underlying this memory impairment. In vitro electrophysiology recording from hippocampal slices, the Astroglial CB1 receptors deletion does not change neither long term potentiation neither long term depression at CA3-CA1 synapses (Langlais et al., unpublished data). Nevertheless it is important to keep in mind that the aggressive procedure used to prepare the slices may damage or impair astrocyte function in these experiments (Aston-jones and Siggins, 2000) Nevertheless, the role of the astrocytic CB1 receptor must be further investigated in vivo experiments to seek for mechanisms that may underlie object recognition memory.

Part IV – Research Objectives

Long term changes in synaptic plasticity are believed to underlie complex functions in behavior such as learning and memory (Neves et al., 2008). The endocannabinoid system is a major modulator of several neural functions and behavior by through direct influence on the synaptic functions (Castillo et al., 2012). The cannabinoid receptor 1 is present across the whole brain (Kano et al., 2009). In the hippocampal formation, CB1 receptor is widely distributed, being present in much higher level in GABAergic interneurons than in glutamatergic pyramidal neurons (Kano et al., 2009). Although the presence of CB1 in astrocytes has been suggested through functional studies (Navarrete and Araque, 2008; 2010), only recently it has been observed and quantified through immunoelectron microscopy (Han et al., 2012). Evidence from the past decade established that astrocytes play a pivotal role in the control of synaptic plasticity. The evidence connecting the ECS, the astrocytes and the regulation of changes in synaptic plasticity and physiology in hippocampal networks may be of the most importance for the regulation of complex brain functions.

Recently it has been shown that astroglial CB1 is necessary for object recognition memory (Metna-Laurent et al., unpublished data) and that this impairment is rescued by either systemically and local injection of the NMDAR co-agonist, D-Serine. Object recognition memory depends on NMDAR transmission since the intra hippocampal administration of NMDAR specific antagonist AP-5 can elicit e ORM impairment (Robin et al., unpublished data). Altogether these findings suggested that astroglial CB1 receptor deletion impairs NMDAR through the decreased occupancy of their co-agonist-binding site (Robin et al., unpublished data).

The general objective of this thesis work is to uncover cellular mechanisms by which Astroglial CB1 receptor could control object recognition memory.

To achieve my objective I used *in vivo* electrophysiology combined with genetic and pharmacological approaches to investigate the synaptic functions of CB1 receptor on astrocytes in the CA3-CA1 Schaffer Collateral Pathway in the hippocampus.

In the first part of my project, I searched for a protocol that could induce a stable and consistence long term potentiation in wild type mice. In the second part, we used the selected protocol

to study the consequence of the absence of astroglial CB1 receptor on in vivo long term potentiation in mice were Astroglial CB1 receptors were absent.

Conclusions from this work show that deletion of the astroglial CB1 receptor leads to an impairment of long term potentiation in a NMDAR transmission -dependent manner. Furthermore, my results show that the same dose of exogenous D-Serine that can rescue the memory impairment in mice lacking the astroglial CB1 receptors is also sufficient to rescue the in vivo long term potentiation impairment induced by astroglial CB1 deletion. Altogether these results fuel the growing concept that Astroglial transmission through endocannabinoid signaling is crucial for synaptic plasticity phenomena ultimately underlying complex brain functions such as learning and memory.

Chapter II – Material and Methods

Animal Procedure Approval

Experiments were approved by the Comité d'Éthique pour l'Expérimentation Animale Bordeaux under the responsibility of the Comité National de Réflexion Ethique sur l´Expérimentation Animale of France (authorization number 50120117-A).

Animals

The GFAP.CB1 conditional mutants used were aged between 12 and 14 weeks old and were bred in the animal facilities of NeuroCentre Magendie (Bordeaux). They were maintained in rooms at 22–23°C with a light/dark cycle from (lights on from 7:00 A.M. to 7:00 P.M). They were housed individually one week before the experimental period where water and food was available ad libitum.

Adult male GFAP.CB1.KO transgenic mice were generated as described in Han et al., 2012. Due to the fact that GFAP.CB1 are a conditional mutant mouse line in which the CreERT2 protein is inactive in the absence of Tamoxifen treatment, the deletion of CB1 receptors in mice (7-8 weeks-old) is achieved by an eight days treatment of Tamoxifen (1 mg; i.p.) dissolved in 90% sesame oil and 10% ethanol to a final concentration of 10 mg/ml (Hirrlinger et al., 2006). The animals were used after a period of 3 to 4 weeks from the end of the treatment. The absence of the CB1 protein in GFAP expressing cells in hippocampus from GFAP.CB1.KO was accessed by double immunohistochemistry for electron microscopy (Han et al., 2012). All experimental animals were littermates. Each animal was genotyped before and after the experimental procedure from a tail biopsy by genomic PCR as described before (Marsicano et al. 2003). This procedure is achieved by the genotyping facility of NeuroCentre Magendie (Bordeaux).

In Vivo Electrophysiology in Anesthetized Mice

GFAP.CB1.WT and GFAP.CB1.KO mice were anesthetized in a box containing 5% Isoflurane before being placed in a stereotaxic frame with their nose in an anesthetic mask, in which 1, 5% of Isoflurane was continuously supply during the complete duration of the experiments. The mice's body temperature was maintained using at 37°C using a temperature control system.

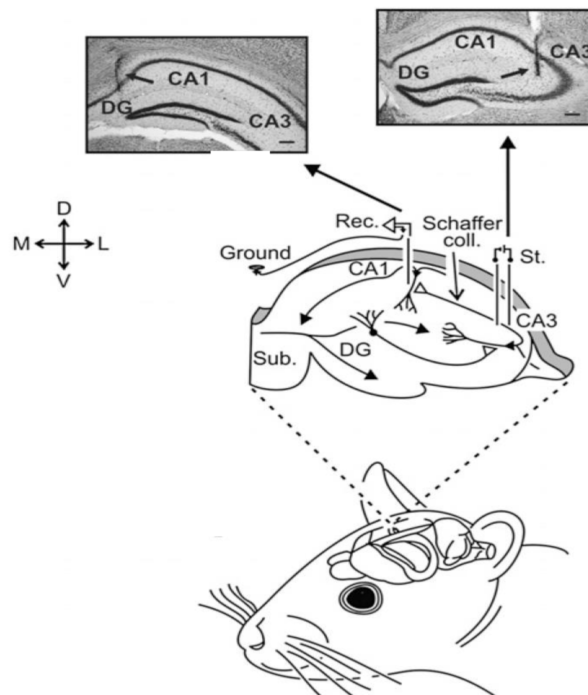

FIGURE 28 – SCHEMATIC REPRESENTATION OF IN VIVO STIMULATION AND RECORDING FROM THE IPSILATERAL CA3-CA1 SCHAFFER COLLATERAL PATHWAY. Dorsal (D), Medial (M), Lateral (L), Ventral (V), Recording Electrode (Rec), stimulation electrode (St), Subiculum (Sub) (From Clarke et al., 2010).

A longitudinal incision of 1.5 cm in length was made in the midline of the scalp, followed first by exposing the skull, including the Lambda and Bregma. After drilling two holes in the skull, one recording electrode was inserted in the CA1 region and one stimulation electrode was inserted either in Ventral Hippocampal Commissural (VHC) or CA3 regions with the following coordinates: 1) CA1 stratum radiatum: A/P -1.5 mm, M/L -1.0 mm, DV 1.20 mm; 2)VHC: A/P -0.5 mm, M/L 0.3, DV 2.8; CA3: A/P -2.5 mm, M/L -2.8, D/V -2.0 mm. A general representation is shown in figure 28. The recording electrode was a glass recording electrode (tip diameter = 2–3 µm, 4–6 MΩ) filled with a 2%

pontamine sky blue solution in 0.5 M sodium acetate. At first the recording electrode location was placed by hand until it reached the surface of the brain and then by automatic micrometer to ensure accuracy. The stimulation electrode was placed in the correct place only by hand. Both electrodes were adjusted to find the correct area. In vivo recordings of field excitatory postsynaptic potentials (fEPSPs) were amplified 10 times by the Axoclamp2B amplifier were further amplified 100 times and filtered (low-pass filter at 300 Hz) via a differential AC amplifier (model 1700; A-M Systems, Carlsborg, WA). fEPSPs were digitized and collected on-line using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design). Test pulses (500 □s duration) were administered using a square pulse stimulator and stimulus isolator (DS3; Digitimer, Hertfordshire, UK), and collected every 5 s at a 10 kHz sampling frequency and then averaged every 200 sec. Basal stimulation intensity was adjusted to 30-40% of the current intensity that evoked a maximum field response. The slope of the fEPSP was used to generate an input-output curve (I/O) relationship ranging from sub threshold to maximal response. All responses were expressed as percent change from the average responses recorded during the 10 min before high frequency stimulation. High frequency stimulation was induced by applying 1 train or 3 trains of 100 Hz (1 sec each), (the 3 trains being separated by 20 seconds interval). fEPSP were recorded for a period of 40 minutes. In another group of mice, the following treatments were applied: 1) MK 801 injection (Abcam, Cambridge, UK; 3.0 mg/kg i.p. dissolved in saline, one hour before HFS) or vehicle (physiological saline, i.p., 2-3 hours before HFS) 2) D-Serine injection (Abcam, Cambridge, UK; 50.0 mg/kg i.p. dissolved in physiological saline) 2-3 hours before HFS or vehicle (physiological saline, i.p., 2-3 hours before HFS).

Histology

At the end of each recording experiment, the electrode placement was marked with an iontophoretic deposit of pontamine sky blue dye (-20 μA, continuous current for 12-15 min). To mark electrical stimulation sites, 10 μA of positive current was passed through the stimulation electrode for 1 minute. After the experimental procedures, the animals were deeply anesthetized with Isoflurane (5%) and decapitated. Brains were removed and snap-frozen in a solution of Isopentane at -70°C. The brains were sectioned into 70 μm-thick coronal slices for histological determination of recording and stimulating electrode sites.

Statistical Analysis

Results are expressed throughout as means ± SEM. In multiple comparisons, values were subjected to a two-way ANOVA followed by Bonferroni post-hoc test. Statistical significance was set at $p < 0.05$.

Chapter III – Results

Preliminary results

Long term synaptic changes such as long term potentiation are thought to underlie complex brain functions such as learning and memory (Hu et al., 2011). In the hippocampus, the long term potentiation induced in the CA3-CA1 Schaffer Collateral pathway is one of the most well studied phenomena concerning synaptic plasticity. In this model, the stimulation of CA3 axons with a certain intensity and frequency can induce long term changes in the CA1 synapses. As it was discussed previously in the introduction, the astroglial CB1 receptor deletion causes a strong recognition memory impairment that is dependent on NMDAR transmission and that can be reversed by either systemically or hippocampal administration of D-Serine, a NMDAR co-agonist. In attempt to determine the mechanisms underlying these behavioral data, we decided to use in vivo electrophysiology to evaluate the synapse functionality of the Schaffer Collateral pathway after deletion of astroglial CB1 receptors in the GFAP.CB1.KO mice.

First, we decided to search for an effective HFS protocol that could induce a stable LTP in GFAP.CB1 mice. It is known that CA3 pyramidal neurons can synapse with themselves, ipsilaterally with CA1 and contralaterally with CA1 via the Ventral hippocampal Commissure (VHC) (Witter, 2007). It is known that LTP can be induced at CA1 synapses by directly stimulating the contralateral VHC instead of the CA3 (Vouimba et a., 2013). We thus applied this protocol which was also more convenient in our experimental setting than both stimulating and recording at the same brain hemisphere (i.e. bigger gap between both electrodes).

In this preliminary experiment, we searched for an optimal protocol for LTP induction. We used a classical HFS protocol of 1 train of 100 Hz (1s) that is known to induce long term potentiation in CA3-CA1 pathway (Ris et al., 2007). As we can see in Figure 29A and B, 40 minutes after HFS, the protocol used is not able to persistently increase fEPSCs neither in tamoxifen-treated GFAP.CB1.WT (111.6 ± 22.75, as compared to 100, n=7), nor in GFAP.CB1.KO (264.4 ± 96.54, as compared to 100,

n=9; $F_{1,28} = 1.79$, $p > 0.05$). As this protocol could not induce synaptic potentiation in the WT littermates, we decide to increase the intensity of stimulation thereby trying a stronger protocol.

In the second preliminary experiment, we decide to apply a HFS protocol of 3 trains of 100Hz (1s) separated apart by 20 seconds to induce LTP (Blitzer et al., 1995). With this protocol, the tamoxifen-treated GFAP.CB1.WT mice are able to induce an increase over baseline although they cannot sustain it during the 40 minutes period (106.9 ± 23.63, as compared to 100, n=9) ($F_{1,15}=0.22$, $p>0.05$) (Figure 29C and D). GFAP.CB1.KO mice were not able to induce changes over baseline (96.54 ± 15.93, as compared to 100, n=8). Because this protocol was not able to induce a potentiation in the GFAP.CB1.WT we decided to overcome the technical limitations described above and to change the stimulation/recording areas to the ipsilateral CA3-CA1 pathway (Clarke et al., 2010).

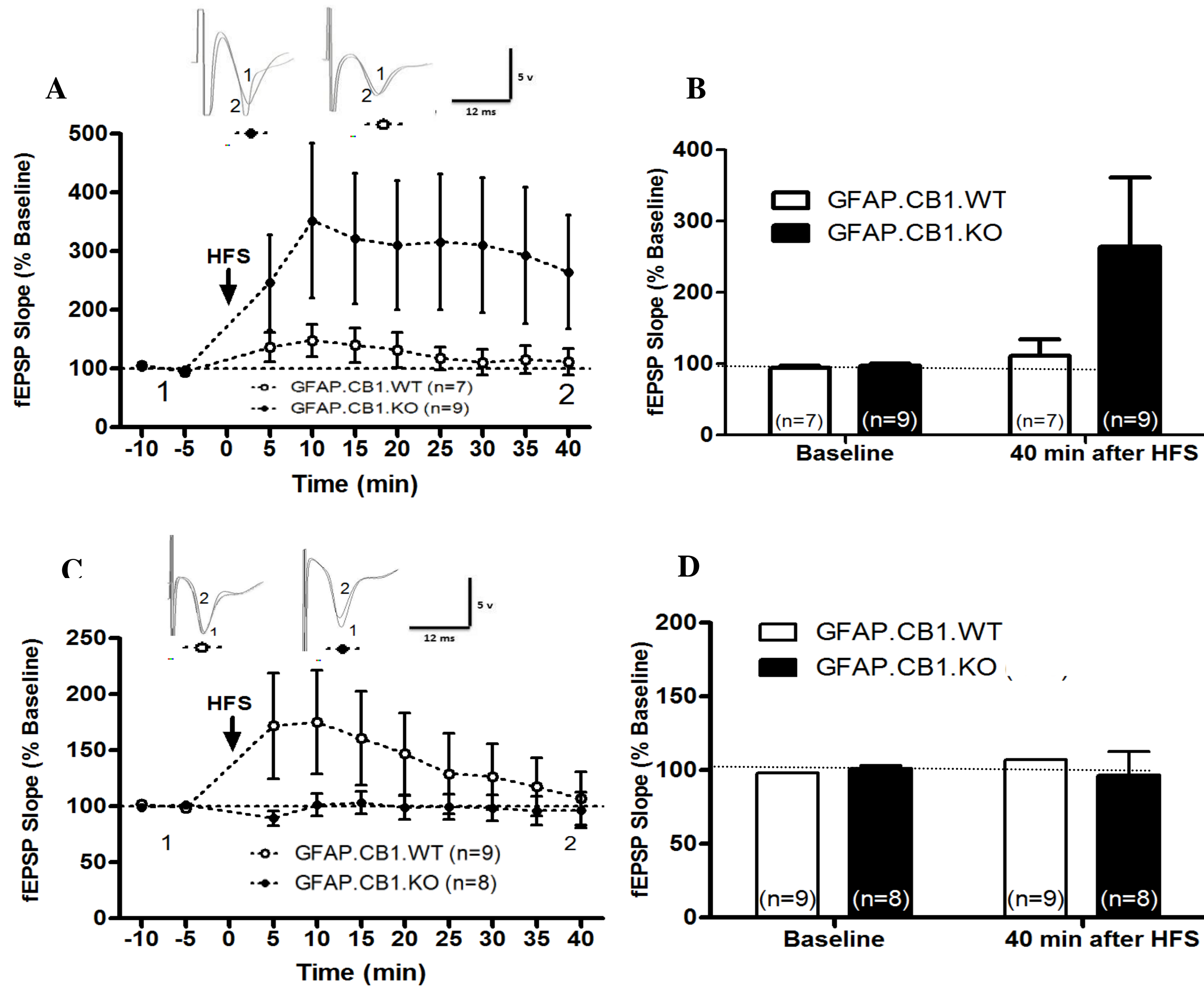

FIGURE 29 – PRELIMINARY DATA. (A) Plots of normalized fEPSP slopes in anesthetized mice show that 1 train of 100Hz (1s) is not able to induce long term potentiation in VHC-CA1 synapses from GFAP.CB1.WT littermates although inducing long term potentiation GFAP.CB1.KO mice. (**C**) Plots of normalized fEPSP slopes in anesthetized mice show that 3 train of 100Hz (1s) is also not able to induce long term potentiation in VHC-CA1 synapses from neither GFAP.CB1.WT littermates or from GFAP.CB1.KO. Representative fEPSP traces before (1) and 40 min after (2) high frequency stimulation (HFS) are shown above the plot. (**B** and **D**) Histograms summarizing the average percent changes of fEPSP slope in baseline (1) and 40 min after (2) HFS of **A** and **D**, respectively.
All summary graphs show means ± S; n = numbers of animals recorded (A, B) in each group. Two-way ANOVA (**B:** $F_{1,28} = 1.79$, $p > 0.05$; **D:** $F_{1,15} = 0.22$, $p > 0.05$).

Astroglial CB1 receptors are necessary for *in vivo* long term potentiation in CA3-CA1 synapse

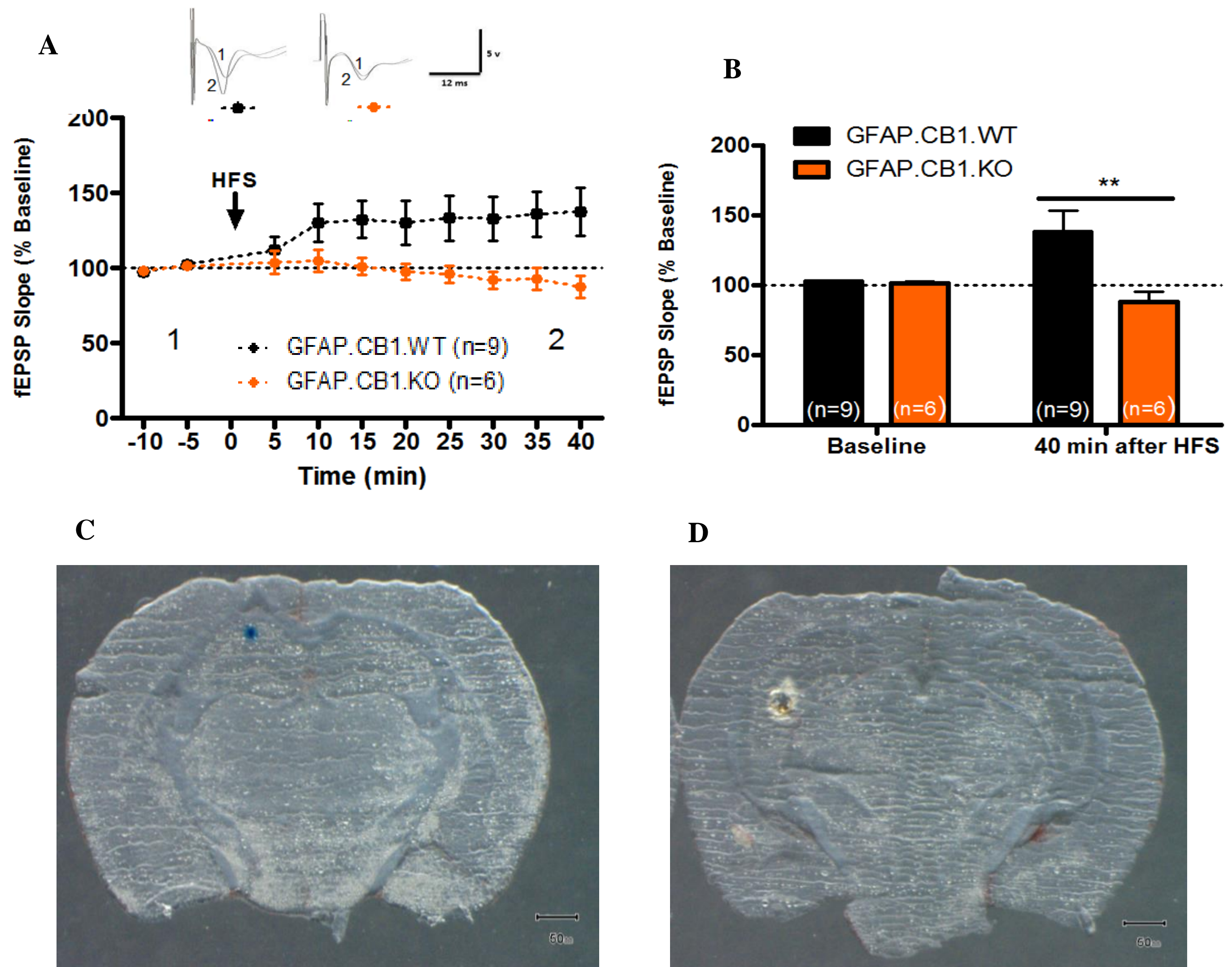

FIGURE 30 - ASTROGLIAL CB1 RECEPTORS ARE NECESSARY FOR IN VIVO LONG TERM POTENTIATION IN CA3-CA1 SYNAPSES. (A) Plots of normalized. fEPSP slopes in anesthetized mice show that 3 trains of 100Hz (1s) are able to elicit long term potentiation in wild-type (GFAP-CB1-WT) but not in GFAP-CB1-KO mice. Representative fEPSP traces before (1) and 40 min after (2) high frequency stimulation (HFS) are shown above the plot. (B) Histogram summarize the average percent changes of fEPSP slope in baseline (1) and 40 min after (2) HFS. (C and D) Histological control for the recording electrode (C) and for the stimulation electrode (D). The scale bar represents 50 mm. All summary graphs show means ± SEM; n = numbers of animals recorded (A, B) in each group. **$p < 0.01$ versus control, Bonferronni post-hoc test after two-way ANOVA (B: $F_{1,13} = 6.24$, $p < 0.05$).

In vivo recordings of field excitatory postsynaptic potentials (fEPSP) from CA3-CA1 synapses in anesthetized mice shows that tamoxifen-treated wild-type mice littermates were able to induce and maintain a constant potentiation finishing after 40 min with an increase over baseline close to 40% (137,7 ± 15.97, as compared to 100, n=9) (Figure 30 A, B). fEPSP from GFAP.CB1.KO mice do not show changes over baseline during the all 40 minutes after HFS, even showing a small decrease (87.76 ± 7.36, as compared to 100, n=6) (Figure 30 A and 2B). This fEPSP recorded from the GFAP.CB1.KO is significantly different from the GFAP.CB1.WT littermate mice ($F_{1,13} = 6.24$, $^{**}p<0.01$). Therefore, astroglial CB1 receptors deletion in the GFAP-CB1-KO prevent LTP induction in CA3-CA1 hippocampal synapses in vivo. Mice in which the recording electrode didn’t target the stratum radiatum of CA1 and mice in which the stimulation electrode didn’t target the CA3 axons were excluded from analysis (figure 30 C, D). As this LTP induction procedure proved to be effective in WT littermates in our experimental conditions, further analyses were conducted using the same areas of stimulation/recording.

Astroglial CB1 dependent long term potentiation is mediated by NMDAR transmission

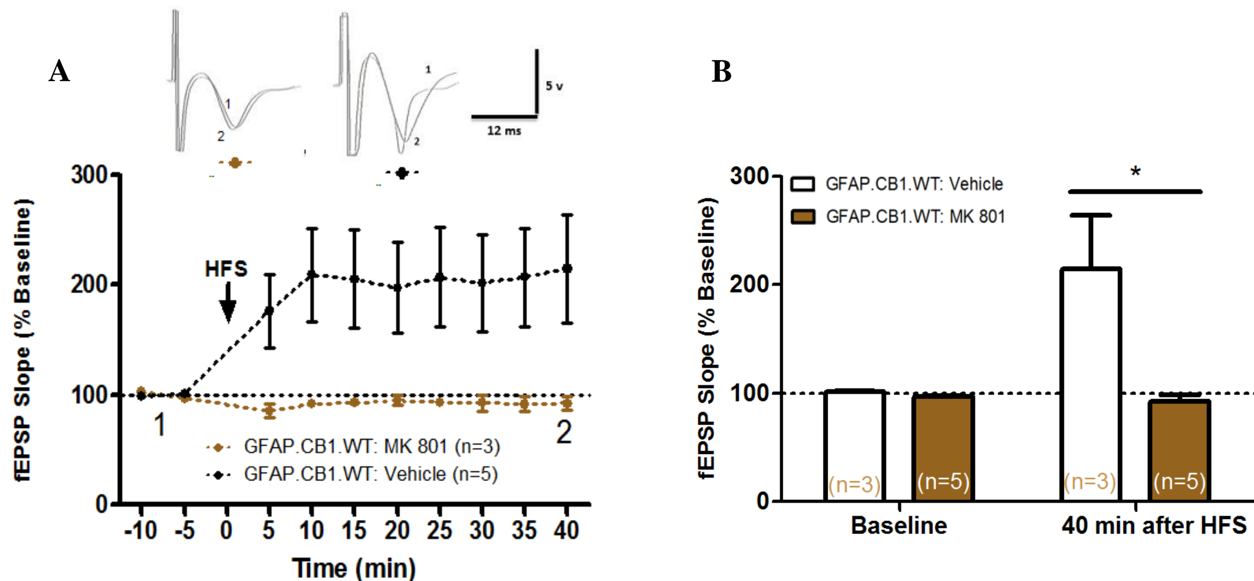

FIGURE 31 - ASTROGLIAL CB1 DEPENDENT LONG TERM POTENTIATION IS MEDIATED BY NMDAR TRANSMISSION. (A) PLOTS OF NORMALIZED fEPSP SLOPES IN ANESTHETIZED MICE SHOW THAT THE I.P. INJECTION OF 3.0 MG/KG MK 801 ONE HOUR BEFORE HFS IS ABLE TO ABOLISH LONG TERM POTENTIATION IN GFAP.CB1.WT MICE. REPRESENTATIVE fEPSP TRACES BEFORE (1) AND 40 MIN AFTER (2) HIGH FREQUENCY STIMULATION (HFS) ARE SHOWN ABOVE THE PLOT. (B) HISTOGRAM SUMMARIZES THE AVERAGE PERCENT CHANGES OF fEPSP SLOPE IN BASELINE (1) AND 40 MIN AFTER (2) HFS. All summary graphs show means ± SEM; n = numbers of animals recorded (**A, B**) in each group. *p < 0.05 versus control, Bonferronni post-hoc test after two-way ANOVA (**B:** $F_{1,6} = 3.36$, $p < 0.05$).

Previous data from the host laboratory indicates that the blockade of hippocampal NMDAR by AP-5, an antagonist acting on the glutamate binding-site (Paoletti & Neyton, 2007), is able to impair object recognition memory in wild-type mice, showing that this form off memory is both dependent on astroglial CB1 receptors and on NMDAR transmission in the hippocampus (Robin et al., unpublished results). Since the LTP phenomenon in the hippocampus is thought to participate in object recognition memory (Ennaceur, 2010), we propose to examine whether the astroglial CB1-dependent hippocampal LTP in vivo described before share the same mechanisms as the astroglial CB1-dependent object recognition memory process. We thus hypothesized that the LTP observed in tamoxifen-treated wild-type mice littermates from the previous experiment is also NMDAR dependent. In order to test our

hypothesis, we injected systemically MK 801 (3 mg/kg; i.p.), a specific non-competitive antagonist of the NMDA receptor wildly used systemically in in vivo experiments (Zhang et al., 1992), before the HFS procedure.

As it is illustrated in figure 31A, the systemic treatment with MK 801 (3 mg/kg), one hour before HFS, is able to fully impair LTP in the tamoxifen-treated GFAP.CB1.WT (92.54 ± 6.16, as compared to 100, n=3) as compared with vehicle treatment (214.9 ± 49.03, as compared to 100, n=5) ($F_{1,6} = 3.36$, $p < 0.05$) (Figure 31A and B). The results support our hypothesis indicating that long term potentiation observed in GFAP.CB1.WT is dependent of NMDAR transmission.

Exogenous D-Serine rescues in vivo long term potentiation impairment induced by Astroglial CB1 deletion

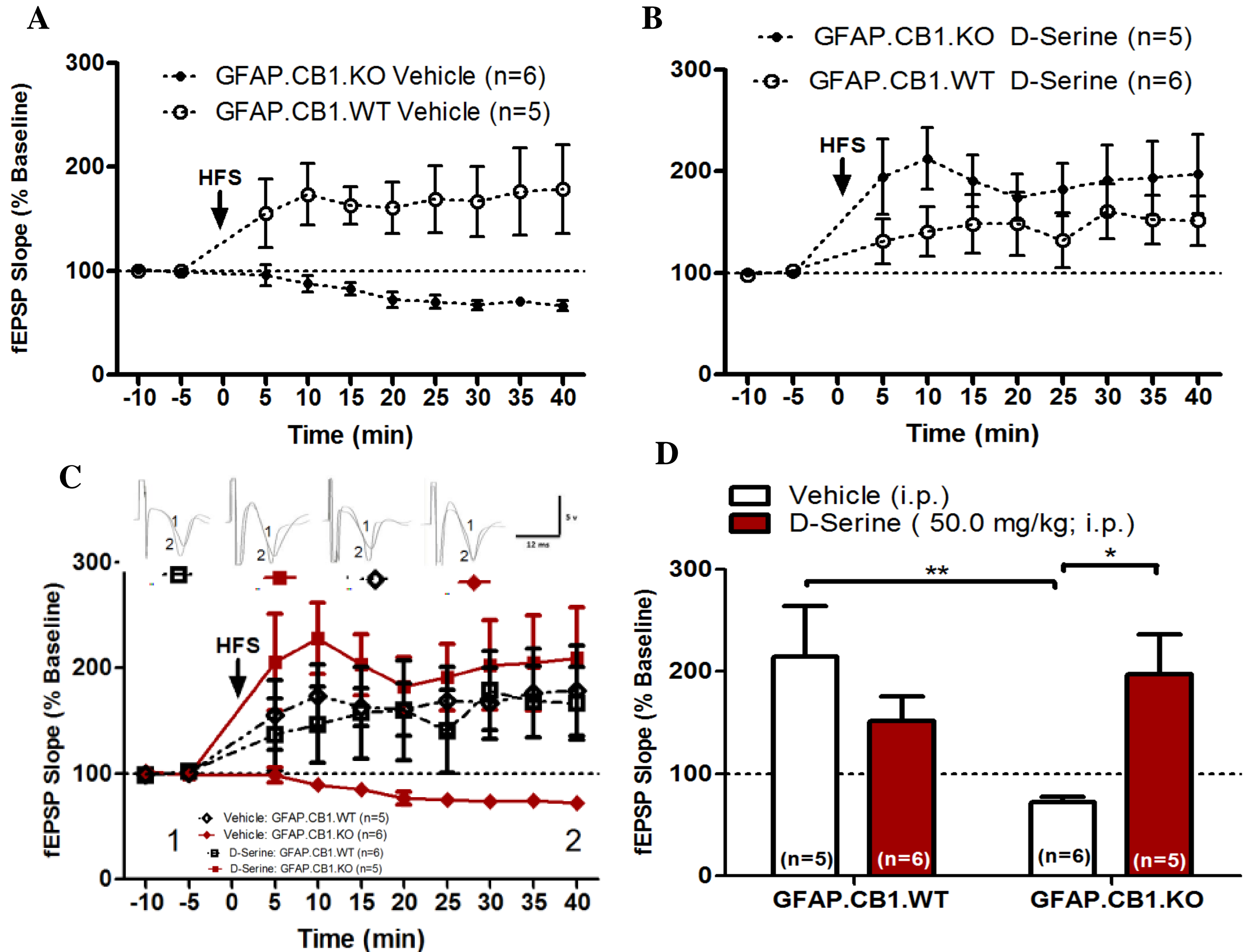

Figure 32 - Exogenous D-Serine rescues in vivo long term potentiation impairment induced by astroglial CB1 deletion. (A-C) Plots of normalized fEPSP slopes in anesthetized mice show that the i.p. injection of D-Serine (50 mg/kg) rescues the long term potentiation impairment observed in the GFAP.CB1.KO mice. Representative fEPSP traces before (1) and 40 min after (2) high frequency stimulation (HFS) are shown above the plot. (**D**) Histogram summarizes the average percent changes of fEPSP slope 40 min after HFS. All summary graphs show means ± SEM; n = numbers of animals recorded (A, B) in each group. $*p < 0.05$, $**p < 0.01$ Bonferroni post-hoc test after two-way ANOVA (**D:** $F_{1,19} = 9.75$, $p < 0.05$).

Following unpublished results from Robin et al., we hypothesize that the same D-Serine dose that is able to restore a normal long-term OR memory in GFAP.CB1.KO is also capable of rescuing the NMDAR dependent long term potentiation impairment observed in GFAP.CB1.KO mice. To test this hypothesis we injected 50 mg/kg of D-Serine between two and three hours before HFS in GFAP.CB1.KO mice and WT littermates. The time window chosen took in consideration the time necessary for exogenous D-Serine to pass the blood brain barrier thereby achieving the highest levels in the brain (Pernot et al., 2012).

In vivo recordings of field excitatory postsynaptic potentials from GFAP.CB1.KO mice and WT littermates after vehicle treatment confirmed the results obtained in the first set of experiments: GFAP.CB1.WT mice are able to induce and maintain a potentiation 40 minutes after HFS (214.9 ± 49.03, as compared to 100, n=5) and significantly different ($p<0.01$) from GFAP.CB1.KO mice which showed no LTP induction (72.15 ± 5.2, as compared to 100, n=7) (Figure 32 A). Strikingly, GFAP.CB1.KO mice treated with D-Serine are able to clearly rescue the LTP impairment (197.3 ± 39.10, as compared to 100, n=5) shown in GFAP.CB1.KO mice treated with vehicle (Figure 32 B and C). The long term potentiation exhibited is not significantly different neither from WT D-Serine treated littermates (151.4 ± 24.42, as compared to 100, n=6) ($p>0.05$) or from vehicle treated WT littermates ($p>0.05$) (Figure 32 D). These results indicate that the exogenous i.p. injection of 50 mg/kg of D-Serine can rescues in vivo long term potentiation impairment induced by astroglial CB1 deletion potentially through a NMDAR dependent mechanism.

Chapter IV – Discussion

Our results indicate that the cannabinoid receptor 1 on astrocytes is essential for the regulation of hippocampal synaptic plasticity. We found that the astroglial CB1 is necessary for the induction of a NMDAR-dependent long term potentiation in CA3-CA1 synapses. Furthermore we show that this impairment can be rescued through the systemic injection of D-Serine, a NMDAR co-agonist. Altogether these results support the growing idea that endocannabinoid-mediated neuron-astrocyte communication through the astroglial CB1 plays a key role in the modulation of hippocampal synaptic plasticity that ultimately may underlie learning and memory.

The hippocampus being crucial for object recognition memory, electrophysiological studies in this area may provide important insights to study elicited changes in synaptic plasticity (Ho et al., 2011). To investigate the potential role of astroglial CB1 in hippocampal synaptic plasticity, Valentin Langlais in Dr Oliet's laboratory used in vivo electrophysiology on brain slices to examine changes in long term synaptic plasticity in the CA3-CA1 Schaffer Collateral Pathway. Interestingly, there were no differences between genotypes concerning input/output curves or in the ratio between NMDARs and AMPAR currents in the mutants. Both mutant and wild type littermates did not elicited changes neither in long term potentiation or long term depression. These results suggest that astroglial CB1 does not alter basic properties of glutamatergic transmission in CA1 synapse from Schaffer Collaterals pathway in brain slices.

Because astrocytic properties in vivo may differ from in vitro or cultures properties, we decided to check the same pathway in vivo. Astroglial CB1 receptors signaling did not alter NMDAR-dependent long term potentiation in a protocol requiring endogenous D-serine release from astrocytes on brain slices (Henneberger et al.), However, in our study, mice lacking astroglial CB1 cannot induce long term potentiation in vivo using a similar induction protocol. These striking results show that astroglial CB1 is necessary for in vivo long term synaptic plasticity. These apparent contrasting results in in vivo and in vitro electrophysiological approaches can be explained by several technical distinctions between both procedures.

First of all, the slice preparations (e.g. cutting the brain) may disrupt neuronal and astroglial networks that may support synaptic functions during the high frequency stimulation. Similarly, the

cannabinoids-induced, astroglial CB1 receptor -dependent long term depression can be observed in vivo (Han et al., 2012) but not on brain slices (Chevaleyre and Castillo, 2006). Indeed, the impact of astroglial CB1 receptor on synaptic plasticity might be masked on brain slices if this control involves an extended network processing. It is know that astrocytes communicate among themselves and display huge calcium waves that can travel over several microns (Kuga et al., 2011). In this context, we do not know to which extend the slice preparation may disrupt these connections and thereby mask a possible astrocytic global and not local modulation over the glutamatergic synapses through, for example, the trafficking of molecules and ions (Giaume et al, 2010).

Another important issue is to take in consideration that the slices suffer from a huge insult when they are cut. In this period they must recover before recording and they are kept in an artificial environment with not physiological temperatures (room temperature) that may alter intrinsically properties of astrocytes that are still poorly understood and many times not taken in consideration (Aston-jones and Siggins, 2000).

Another important fact is that in slices, there is inhibition of GABAergic transmission through bath application of Picrotoxin which is a noncompetitive antagonist for GABA A receptors (Olson and DeLorey, 1999). This objective of this inhibition is to record more specifically glutamatergic excitatory currents without being contaminated with GABAergic inputs (Verdier et al., 2004). In in vivo electrophysiology it is not possible to fully inhibit the GABAergic transmission and this issue must be taken in consideration. It would be interesting to redo electrophysiology in slices but this time without Picrotoxin to check if there is a different phenotype. Another interesting experiment still more technically difficult would be to inhibit in vivo GABAergic transmission through a local injection of Picrotoxin and check whether there are changes in the phenotypes obtained. The technical difficulty relies on the fact that there is no established evidence of this inhibitor in in vivo electrophysiology. Problems with drug diffusion and lack of specificity may blur possible results. Still, it would be interesting to check possible outcomes within in scenario. Independently, we must take in consideration a possible of role GABAergic transmission in the whole process of astroglial CB1-dependent synaptic transmission.

Robin et al., (unpublished data) demonstrated that hippocampal NMDAR transmission is a necessary component for object recognition memory. Our results show that after the injection of an NMDAR antagonist, MK 801, the long term potentiation elicited in wild type littermates is completely

abolished, thus allowing the conclusion that NMDAR transmission is directly involved in the astroglial CB1-dependent long term potentiation.

Calcium increase in astrocytes is believed to underlie gliotransmission mechanisms (Agulhon et al., 2012). The astroglial CB1 receptor has been proposed to act through a G_q protein that is coupled to a PLC that can mobilize calcium stores inside astrocytes (Navarrete and Araque 2008, 2010). D-Serine is believed to be docked in the membrane of astrocytes and readily available to be released (Martineau et al., 2013). Nevertheless, it has also been shown that abolishing calcium mobilization in astrocytes can impair LTP through the reduction of the NMDAR co-agonist occupancy levels (Henneberger et al., 2010). In our study, we show that D-Serine administration can rescue the NMDAR-dependent long term potentiation impairment in mice lacking Astroglial CB1. Within this scenario where exogenous D-Serine rescues the phenotype observed in the conditional KO, and following the functional studies from Navarrete and Araque (2008, 2010), it is possible to theorize that astroglial CB1 receptor signaling leads to a calcium rise in astrocytes that possibly underlies the release of D-Serine. This D-Serine would then participate in the NMDAR-dependent long term potentiation by increasing the occupancy levels of the NMDAR co-agonist binding sites, leading to a more efficient and stronger activation of NMDAR.

Recently, it has been proposed that endocannabinoids can bind to astroglial CB1 receptors in CA1 astrocytes from brain slices, thus increasing calcium concentrations inside astrocytes. This increase stimulates the release of glutamate into the presynaptic terminal that will act on metabotropic glutamate receptor type 1 leading to the release of glutamate (Navarrete and Araque, 2008, 2010). In 2012, Han et al., described a mechanism in which the exogenous administration of cannabinoids can induce an NMDAR –dependent LTD in CA3-CA1 synapses in vivo. These apparently contradictory results must take in consideration the fact the exogenous cannabinoids used by Han and colleagues may mask the locally and spatially –controlled “on demand” (Piomelli, 2003; Marsicano et al., 2003) release of endocannabinoid, a tightly regulated endogenous mechanism that can lead to different physiological phenomena.

Taking in consideration an endogenous point of view, it is possible to conjecture that the release of D-Serine acting in NMDA receptors may potentiate the excitatory glutamatergic synapses during high frequency stimulation. Nevertheless, more studies are needed to further investigate the possible mechanism underlying this long term potentiation.

One key experiment to confirm this hypothesis of astrocytic modulation of synaptic plasticity through the release of D-Serine would be the direct measurement of Glycine and D-Serine levels either in vitro (in primary cultures of hippocampal astrocytes WT and lacking CB1 receptors) or in vivo through intrahippocampal microdialysis in freely moving mice performing the object recognition task, which would be the ideal experimental condition, albeit technically more difficult. If our hypothesis is correct, the stimulation of WT primary astrocytes by, for instance, increasing bath calcium concentration, would lead to a specific increase of the extracellular concentrations of D-Serine as compared to as compared to other amino acid, a phenomenon which would be blocked in CB1-KO astrocytes. In in vivo experiments, it would be expectable a similar conclusion. In WT it would be predictable that D-Serine levels would increase after ORT as compared to naive mice and this increase would not be seen in littermate mice lacking astroglial CB1 receptor.

Recently it has been proposed that synaptic and extrasynaptic NMDAR are gated by different endogenous co-agonists (Papouin et al., 2012). In this context, extrasynaptic GluN2B-NMDAR heteromers have higher affinity to Glycine where synaptic GluN2A-NMDARs heteromers have stronger affinity for D-serine (Papouin et al., 2012). These conclusions merged together with our study may raise the following question: is D-Serine the required NMDAR co-agonist or the high levels of D-Serine mask a putative effect featured by glycine? Glycine being a primary inhibitory neurotransmitter in the spinal cord and brainstem, the in vivo administration of this molecule produces bradycardia and may not be compatible with anesthetize organisms during the long period of recording (Olsson and Hahn, 1999). One of the strategies to overcome these issues concerning exogenous administration of glycine would be the regulation of endogenous levels by inhibition glycine uptake through glycine transporter 1 inhibitors (Paoletti et al., 2013). This scenario should be tested both in in vivo electrophysiology and object recognition task. Nevertheless studies with co-administration of D-Serine and a GluN2A-NMDAR/GluN2B-NMDAR specific antagonist would provide less background and more answers regarding this issue. In this context it would be interesting to test, during object recognition task, the effect of co-administration of D-Serine with either a specific inhibitor of GluN2B-NMDAR heteromers (such as Ro 25-6981 (Fischer et al., 1997)) or specific inhibitor of GluN2A-NMDAR heteromers (such as NVP-AAM077 (Neyton and Paoletti, 2006). This would give a more clear picture whether D-Serine rescues the object recognition impairment in mice lacking astroglial CB1through specific NMDAR heteromers. Furthermore, if D-Serine rescues the phenotype specifically through GluN2B-NMDAR heteromers, and following recent evidence showing that this

subunit has higher affinity for glycine as compared with D-Serine (Papouin et al., 2012), one could hypothesize that D-Serine may rescue the phenotype by overcoming defective glycine levels. This would give rise to more complex questions regarding the role of astrocytes in the control of glycine levels either spatially (synaptic vs. extrasynaptic) and temporally (basal levels or increase after induction of long term synaptic plasticity). Nevertheless is extremely important to keep in mind a broader view concerning co-agonist's availability and NMDAR location, composition and function (Paoletti et al., 2013).

Another interesting question concerns the role of neuronal CB1 in synaptic plasticity events. It is known that mice bearing a full deletion of CB1 receptors (full knockout) or mice lacking CB1 receptor in GABAergic or Glutamatergic neurons do not display impairments in object recognition memory (Metna-Laurent, unpublished data). The production of endocannabinoid that previously acted on astroglial CB1 may redirect their targets after the induction of astroglial CB1 deletion. Although it has been shown that astroglial CB1 is necessary for both object recognition memory and the induction of a NMDAR-dependent long term potentiation, we don't know in what extent neuronal CB1 may participate in the exhibited phenotypes. Equally it would be interesting to check in both in vivo electrophysiology and object recognition task if there are still the described phenotypes after administration of CB1 specific antagonist such as Rimonabant. Important insights concerning the selective role of neuronal and astroglial CB1, both in memory and synaptic plasticity, could be gathered from this experiment.

Here we showed that astroglial CB1 receptor signaling control a form of in vivo hippocampal NMDAR–dependent long term potentiation that can be mimicked by exogenous D-serine administration when CB1 receptor on astrocytes are suppressed. Strikingly, these mechanisms are shared by the astroglial CB1 receptor –dependent object recognition memory (Robin et al., unpublished results) because normal (i.e. wild-type –like) behavioral performances in this task need an intact hippocampal NMDAR transmission and can be also restored by intra-hippocampal D-serine administration. Together, these results point to a scenario where astroglial CB1 receptor signaling controls object recognition memory by regulating NMDAR-dependent long term potentiation through the occupancy of their co-agonist-binding site. Although it is not possible to establish a direct correlation between an object recognition memory impairment and impaired long term potentiation, it is possible to assume that activity dependent synaptic changes are important to neuronal tasks (Neves

et al., 2008). In this context it would be interesting to check in mice lacking astroglial CB1 receptor through in vivo electrophysiology in awake mice performing ORT, if there are changes in synaptic strength between before training and after test 2 (Figure in 27). In a hypothetical scenario of a different phenotype, what would be the putative effect of D-Serine administration?

It would be also important to check whether astroglial CB1 receptors modulate other synaptic plasticity processes. It has been reported that learning a new environment underlies a facilitation of hippocampal LTD, even without its induction (Manahan-Vaughan et al., 1999). In this context it would be interesting to investigate whether this form of long term synaptic plasticity is also impaired in mice lacking astroglial CB1. Another interesting experiment would be to check if other known hippocampal dependent memory functions are also impaired. Recently it has been reported that astrocytic signaling controls endocannabinoid mediated a spike timing-dependent long term depression (t-LTD) at neocortical synapses (Min and Nevian, 2012). It would be also interesting to check in a conditional KO whether astroglial CB1 is important in the induction of the astrocytic intracellular calcium concentrations that the authors described as essential for the induction of t-LTD.

Another interesting point is the general network effect through the activation of CB1 on astrocytes. This effect could underlie possible impairments on glial-neuronal communication leading to lack of support of synapses that are under intense neuronal activity. Recently, through a functional study in rat hippocampal organotypic slices, it has been proposed that astrocytes contribute to a positive feedback loop that induces excessive neuronal discharge through an astroglial CB1 related mechanism (Coiret et al., 2012). Moreover, the authors propose that calcium increases spreading through the astrocytic networks are of the most importance in the modulation of epileptiform activity. D-Serine has been implied in epilepsy (Radzishevsky et al., 2012). Harai and colleagues reported that in Serine Racemase KO mice (Serine Racemase is responsible for D-Serine synthesis through the direct conversion of L-Serine) there is a partially resistant to seizures induced by a GABA antagonist, pentylenetetrazol (Harai et al., 2012). Since the over activation of the NMDA receptor has been implicated in the development of epileptic activity (Harai et al., 2012) and following the idea that astroglial CB1 may control D-Serine availability in glutamatergic synapses, it would be interesting to check whether astroglial CB1 conditional KO mice are resistant to induced epileptic seizures.

In general terms, all new evidence boosts the novel concept of astrocytes involvement in synaptic plasticity and also the concept of astroglial CB1 function in the modulation of synaptic plasticity.

Chapter V – Conclusion

The results obtained during this study indicate that astroglial CB1 is an important component in the regulation of long term synaptic plasticity. Astroglial CB1 receptor is crucial for induction and maintenance of hippocampal NMDAR-dependent long term potentiation in the CA3-CA1 Shaffer Collateral pathway in vivo. Moreover, the systemic injection of the NMDAR co-agonist D-Serine is able to fully rescue the impaired LTP possibly due to the increase in the occupancy levels of the co-agonist binding site in the NMDAR. Although it is not possible to imply directly LTP in memory performance, being long term synaptic changes believed to underlie learning and memory, it is possible to correlate the failure in the induction on long term potentiation with the object recognition memory impairment displayed by mice lacking CB1 receptors on astrocytes. Altogether these results boost the growing concept that endocannabinoid system is crucial for neuronal-glial bidirectional communication, participating in the astrocytic-dependent regulation of synaptic plasticity in hippocampal glutamatergic synapses that is accepted as a component of the molecular basis of memory processes.

Chapter VI – References

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