Universidade de Lisboa

Faculdade de Medicina de Lisboa

CANNABINOID CB_1 RECEPTORS MODULATION OF HIPPOCAMPAL SYNAPTIC PLASTICITY AND THE CROSS TALK WITH ADENOSINE A_1 RECEPTORS

Armando Dulcídio da Silva Cruz

Orientadora: Professora Doutora Ana Maria Sebastião

Tese especialmente elaborada para obtenção do grau de

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Para a minha mãe,

Uma parte crucial da minha vida e desta odisseia que foi doutoramento.

“…We know that man is not whole as long as he is single, that he is essentially a possible member of society. Especially, ***one man´s experience is nothing if it stands alone****. If he sees what others cannot, we call it hallucination.”*

Charles S.Pierce

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TABLE 1 - DRUGS USED IN THE EXPERIMENTAL WORK 37

List of abbreviations

LTP – long-term potentiation

GABA – γ-aminobutyric acid

CNS – central nerve system

DAG – diacylglycerol

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

NMDA –N-methyl-D-aspartate receptor

AMPAR – AMPA receptor

NMDAR – NMDA receptors

PKC – protein kinase C

CaMKII – α-calcium calmodulin-dependent protein kinase II

eCB – endocannabinoid

eCBs – endocannabinoids

THC – tetrahydrocannabinol

2-AG – 2-Arachidonoylglycerol

NAPE – N-acyl-phosphatidylethanolamine

NAPE-PLD – NAPE-specific phospholipase D

FAAH – fatty acid amide hydrolase

DAGL – diacylglycerol lipase

GPCR – G-protein-coupled receptors

CB_1R – cannabinoid receptor 1

CB_2R – cannabinoid receptor 2

CB_3R – cannabinoid receptor 3TrpV1R – The transient receptor potential cation channel subfamily V member 1

A_1R – adenosine receptor 1

$A_{2A}R$ – adenosine receptor 2A

$A_{2B}R$ – adenosine receptor 2B

A_3R – adenosine receptor 3

CA – Cornu Ammonis

CCK – cholecystokinin

PV – parvalbumin

DSI – Depolarization-induced Suppression of Inhibition

DSE – Depolarization-induced Suppression of Excitation

ATP – adenosine triphosphate

VDCCs – voltage-dependent Ca^{2+} channels

DG – dentate gyrus

EC – entorhinal cortex

STDP – spike-time dependent plasticity

NMDARs – NMDA receptors

COX-2 – cyclooxygenase-2

PKA – protein kinase A

cAMP – cyclic adenosine monophosphate

CREB – cAMP response element-binding protein

PCR – polymerase chain reaction

aCSF – artificial cerebrospinal fluid

wTBS – weak theta-burst stimulation

sTBS – strong theta-burst stimulation

Resumo

Os efeitos independentes da cannabis e da cafeína sobre a memória são conhecidos, mas pouco se sabe acerca da interacção entre estas duas susbtâncias moduladoras da memória. A cafeína, um conhecido estimulante do sistema nervoso central, actua através do bloqueio dos receptores de adenosina, que faz parte do sistema purinérgico. A cannabis activa os receptores do sistema endocanabinóide. A adenosina e os endocanabinóides são dois importantes reguladores de actividade neuronal no sistema nervoso central, sendo denominados por neuromoduladores. Os seus receptores específicos são expressos por todo o cérebro, apresentando uma densidade particularmente elevada no córtex, cerebelo e hipocampo.

A adenosina activa quatro tipos de receptores acoplados a proteína G, dois de alta afinidade (A_1R e $A_{2A}R$) e dois de baixa afinidade ($A_{2B}R$ e A_3R). Os receptores A_1R e A_3R são acoplados a G_i enquanto que os receptores $A_{2A}R$ e $A_{2B}R$ são acoplados a G_s. Os receptores A_3R podem também estar acoplados a proteínas $G_{q/11}$. No hipocampo, o receptor da adenosina com maior expressão é o A_1R, estando presente em todos tipos de neurónios, excitatórios e inibitórios, e também em células da glia tais como os astrócitos. A adenosina extracelular tem origem em dois principais processos: 1) hidrólise de nucleótidos de adenina, como por exemplo do ATP e 2) transporte de adenosina intracelular para o meio extracelular através de transportadores.

Os endocanabinódes actuam em dois tipos de receptores acoplados a proteínas $G_{i/o}$, CB_1R e CB_2R. Os CB_1R são os receptores mais abundantes no sistema nervoso central, particularmente no cortéx e hipocampo. Os endocanabinóides, substâncias lipídicas, são sintetizados maioritariamente no neurónio pós-sináptico, sendo depois libertados para a fenda sináptica, onde activam receptores presinápticos específicos. Existem dois endocanabinóides principais, 2-araquidonilglicerol (2-AG) e a anandamida, que têm vias de síntese distintas. A síntese do 2-AG é exclusivamente pós-sináptica enquanto que a síntese de anandamida pode ocorrer também ao nível do terminal pré-sináptico.

O hipocampo é uma estrutura cerebral associada a aquisição de memória espacial e declarativa. Foi demonstrado que administração crónica de cafeína exacerba o défice de memória pela injecção de tetra-hidrocanabinol (THC), principal substância psicoactiva da

cannabis. Este efeito é dependente de CB_1R e poderá ser explicado pela sobreexpresão dos A_1R em consequência de consumo crónico de cafeína. Há também descrições de que o A_1R modula negativamente a capacidade inibitória dos CB_1R na transmissão excitatória e inibitória.

Considerando o desconhecimento sobre a interacção entre o sistema endocanabinóide e da adenosina em processos de plasticidade neuronal, o presente estudo teve como objectivo inicial a análise do efeito dos receptores hipocampais A_1R e CB_1R a nível da transmissão sináptica e da plasticidade sináptica.

Para responder a estas questões foi planeado e executado um trabalho experimental que envolveu o registo da actividade eléctrica neuronal no hipocampo de ratinhos através de técnicas eletrofisiológicas *ex vivo* (nomeadamente registos extracelulares e registos de *patch-clamp*). Foram utilizadas duas populações de ratinhos, alelo selvagem (wild-type, em inglês) e com deleção total dos A_1R. Para o estudo da plasticidade sináptica foi utilizado o modelo de potenciação de longa duração (LTP, em inglês). É um modelo celular de aprendizagem associativa e tem como expressão a manutenção, por um tempo prolongado, da potenciação da transmissão sináptica excitatória devido a uma estimulação de alta frequência nos seus aferentes.

Relativamente ao mecanismo de interacção A_1R e CB_1R em termos de plasticidade sináptica, foi demonstrado que os A_1R não interferem, endogenamente, na neuromodulação exercida pelos CB_1R na LTP. Adicionalmente, ao analisar a acção inibitória dos CB_1R na transmissão sináptica inibitória que projecta sobre neurónios piramidais, observou-se que o bloqueio dos A_1R previne essa acção dos CB_1R. Sugere-se assim uma possível interligação A_1R-CB_1R ao nível da comunicação entre interneurónios e células piramidais da região de CA1.

Para uma caracterização detalhada da acção dos endocanabinoides sobre a plasticidade sináptica, e dado que a libertação dos dois neuromoduladores, adenosina e endocanabinóides, depende da potência e da duração do estímulo de alta frequência, foram utilizados dois protocolos distintos para indução da LTP. Surprendentemente, observou-se a existência de um efeito bifásico dos endocanabinóides na LTP. Na LTP induzida pelo protocolo mais fraco, os resultados obtidos são compatíveis com a conclusão de que os endocanabinóides inibem a LTP enquanto que no protocolo com indução mais

forte, que induz maior libertação de endocanabinóides, verificou-se exactamente o oposto, permitindo concluir que os endocanabinoides facilitam a LTP. Os resultados deste estudo sugerem que os endocanabinóides funcionam como filtro passa-alto de maneira a reduzir o ruído com a inibição de sinais mais fracos e potenciando sinais mais fortes. Os resultados sugerem que o endocanabinóide responsável por este efeito bifásico é o 2-AG através da activação endógena dos receptores CB_1 localizados nos neurónios interneurónios inibitórios, GABAergicos. Os astrócitos não parecem participar nesta modelação.

Neste trabalho, foi ainda avaliado o efeito da activação exógena dos CB_1R utilizando o agonista WIN 55,212-2. A perfusão do WIN 55,212-2 levou a uma diminuição significativa da potenciação para valores quase nulos, utilizando o protocolo mais forte de indução de LTP. O mesmo efeito foi observado na população de murganhos com deleção do A_1R. O mesmo resultado foi obtido aquando do aumento de concentração endógena de anandamida produzida, sugerindo assim um efeito inibitório sobre a LTP. Quando testados fármacos que induzem aumento de 2-AG endógeno observou-se um aumento da potenciação de longa duração. O conjunto dos resultados sugerem uma proeminência do sistema inibitório via sistema endocanabinóide na potenciação de longa duração. Aliado a este facto, três quartos dos neurónios que expressam CB_1R são interneurónios sendo por isso revelante investigar no futuro que tipo de interneurónios estão associados a este efeito de filtragem.

Para avaliar os efeitos dos receptores hipocampais A_1R e CB_1R a nível da transmissão sináptica e da supressão da inibição induzida pela despolarização., do ingês "*depolarisation-induced suppression of inhibition",* (DSI), foi utilizada a técnica de *whole cell patch-clamp* para registo de correntes sinápticas inibitórias em células piramidais localizadas no *Stratum Pyramidale* na área de CA1 do hipocampo. Foram utilizados murganhos *wild type*.

Relativamente à modulação da transmissão sináptica, verificou-se que a activação dos CB_1R pelo WIN 55,212-2, inibe a transmissão sináptica em cerca de metade da população dos neurónios avaliados. Este resultado está de acordo com os dados obtidos na literatura, dado que nem todos os neurónios expressam o receptor CB_1. Também foi verificado que a perfusão de um antagonista selectivo de A_1R, previne o efeito inibitório de activação exógena dos receptores CB_1R.

Em suma, este projecto permitiu aprofundar o conhecimento sobre a neuromodulação da transmissão e plasticidade sináptica ao nível da região da CA1 do hipocampo. Foi identificado pela primeira vez o efeito bifásico da activação dos CB_1R por endocanabinóides, especificamente pelo 2-AG, indicando assim um importante contributo para o controlo homeostático da plasticidade sináptica. Foi também observada uma interligação dos A_1R e CB_1R ao nível da transmissão sináptica inibitória, sendo inexistente ao nível da plasticidade sináptica.

Abstract

Caffeine, an adenosine receptors antagonist, and marijuana, a cannabinoid receptor agonist, both affect human cognition. Their effects mostly result from their interference with the endogenous purinergic system and endocannabinoids (eCBs) system in the brain. Neuromodulators such as adenosine and endocannabinoids are important regulators of central nervous system neuronal activity. They are also able to modulate synaptic transmission individually. In the hippocampus, adenosine receptor type 1 (A_1R) and cannabinoid receptor type 1 (CB_1R) are the main receptors for adenosine and endocannabinoids, respectively. Both receptors are G-protein-coupled receptors that activate $G_{i/o}$. They are expressed in both inhibitory and excitatory neurons, and also in glial cells such as astrocytes. Chronic caffeine administration exacerbates spatial memory impairment caused by acute tetrahydrocannabinol (THC), which is the main psychoactive molecule of cannabis. In the hippocampus, A_1R and CB_1R are involved in memory impairment caused by CB_1R activation. It has also been shown that CB_1R activation decreases GABA and glutamate release in the hippocampus. These effects are partially reduced by co-activation of A_1R, suggesting an interaction between these modulatory pathways at the level of G-protein activation. The main sources of extracellular adenosine are 1) the extracellular production from the hydrolysis of adenine nucleotides and 2) transport from intracellular adenosine sources. eCB synthesis mostly results from the cleavage of postsynaptic membrane lipids. The activation of postsynaptic G-coupled glutamate metabotropic receptors induces the cleavage of postsynaptic membrane lipids, which is predominantly activated due to high neuronal firing. Thus, eCBs travel in a retrograde manner to activate astrocytic and nerve-terminal CB_1R, which inhibit neurotransmitter release and lead to several forms of short-term synaptic plasticity. CB_1Rs are endogenously activated by eCBs, mainly the fatty acids 2-arachidonoyl-sn-glycerol (2-AG) and anandamide. CB_1R agonists impair cognition and prevent long-term potentiation (LTP), synaptic plasticity induced by brief high-frequency neuronal firing, and synaptic transmission. Still, the influence of endogenously formed cannabinoids on hippocampal LTP remains ambiguous.

In this study, it was possible to evaluate the influence of endocannabinoids on synaptic plasticity. I also wanted to determine if adenosine, through A_1R, could affect eCB signalling.

The deletion or blockade of A_1R did not significantly change the modulatory effect of eCBs on LTP. I did not find evidence of a cross-talk mechanism at the synaptic plasticity level involving the two neuromodulators (A_1R and CB_1R).

LTP induced by a weak θ-burst stimulation (wTBS) protocol was facilitated when blocking the action of eCB on CB_1R. In contrast, LTP induced by a strong θ-burst stimulation (sTBS) protocol was inhibited when the endogenous activation of CB_1R was blocked. This suggests that eCB inhibit weak LTP and facilitate strong LTP. The dual effect is mediated by 2-AG, suggesting that it acts as a high-pass filter that likely reduces the signal-to-noise-ratio of synaptic strengthening. The facilitatory effect of eCBs upon strong LTP depends on the activity of GABAergic interneurons.

It was also described a modulatory role for A_1R on the CB_1R activation effect on inhibitory postsynaptic potential currents (IPSCs). When A_1R was blocked, the inhibitory effect of CB_1R activation on pyramidal cells was abolished, suggesting that an interaction between A_1R and CB_1R is at play.

Overall, this work provides a better understanding of the neuromodulation of synaptic transmission and plasticity in the hippocampal CA1 region. It was able to show that CB_1R plays an important homeostatic role in synaptic plasticity phenomena through an A_1R-independent process. However, A_1R seems to play a modulatory role in the action of eCB on inhibitory synaptic transmission.

1 Introduction

Understanding memory and its associated mechanisms have always been a major goal in neuroscience. An important property of synapses is the ability to modulate synaptic efficacy in response to specific patterns of activation, a process known as synaptic plasticity. Synaptic plasticity can be observed for a couple of seconds, or even for several hours or days (Abraham, 2003). Synaptic plasticity was first proposed as a mechanism for learning and memory, based on theoretical analysis (Attneave et al., 1950). A few years later, Hebb's postulate proposed that when one neuron drives the activity of another neuron, the connection between the two is potentiated. This postulate was first demonstrated in 1966 when Terje Lømo (Andersen and Lømo, 1967) found experimental evidence of a long-lasting potentiation of synaptic activity later renamed long-term potentiation (LTP) by Graham Goddard in 1975. However, in 1973 Tim Bliss and Terje Lømo finally published the first piece of the puzzle linking synaptic plasticity, memory, and Hebb's postulate. Long-term potentiation has been observed in a variety of neural structures, including the hippocampus, the cerebellum (Jörntell and Hansel, 2006), the amygdala, the corpus striatum (Chapman et al., 2003), and the cortex (Herry and Garcia, 2002). LTP has several properties related to the memory mechanism such as rapid induction, persistence, synaptic activity dependency, synapse specificity, and the fact that blockade of several LTP-related molecular pathways leads to memory deficits. These factors suggest that LTP may be more than just a potentiation mechanism for memory formation, but rather a model of synaptic and cellular events that underlie memory formation and may enhance our understanding of learning and memory. Several changes occur in the synaptic plasticity process involving, among others, the amount of neurotransmitter released in the synaptic cleft; the neurotransmitter receptors located at a synapse; the release of neuromodulators and activated intracellular pathways; and the physical strengthening or weakening at the synapses. These events happen both at excitatory and inhibitory synapses.

Synaptic transmission

Before detailing about synaptic plasticity proprieties, it is crucial to talk about the communication between two neurons, i.e. synaptic transmission, as well as the specialized site wherein two neural cells communicate with each other, the synapse. The term synapse was used for the first time at the end of the 19th century by Charles Sherrington and histologically described microscopically by Ramón y Cajal by about the same time (Tansey, 1997). The synapse is a fundamental unit of information transmission in the nervous system and allows neurons to transmit information via electrical or chemical signals to another neuron. Electrical synapses are less common than chemical synapses in the brain and are characterized by a faster and bidirectional signal but less powerful in comparison to chemical transmission. Electrical transmission occurs employing current flow through gap junctions that connect the cytoplasm of the primary neuron and next neuron. On the other hand, chemical synaptic transmission depends on the diffusion of a neurotransmitter on the synapse from the starting signal neuron (presynaptic) to communicate to the next neuron (postsynaptic) (For a review see Kandel et al., 2014). In this thesis, all the studies were focused directly or indirectly on chemical synapses.

Most neurotransmitters are low-molecular-weight molecules that bind to receptors in the postsynaptic membrane of the target cell, mainly at the synapse. In most chemical synapses, the neurotransmitter is released by exocytosis from specialized swellings in the axon called presynaptic terminals (afferent terminal of the presynaptic neuron which contains hundreds of synaptic vesicles, each of which with thousands of neurotransmitters), which allows the chemical transmitter to increase the signal gain in comparison to electrical synaptic transmission (Ceccarelli et al., 1973, 1979; Heuser and Reese, 1973; Katz, 1969; Raastad et al., 1992). The term chemical synaptic transmission will henceforth be referred to as synaptic transmission that can be illustrated as an incoming action potential into the presynaptic terminal that triggers calcium influx *via* depolarization through voltage-dependent calcium channels (N- and P/Q-type)(Brose et al., 1992). Next, the rise of intracellular calcium activates exocytosis of synaptic vesicles, leading to the release of the neurotransmitter into the synaptic cleft (a gap between the pre- and postsynaptic cells), activating specific receptors of the postsynaptic terminal. Through

diffusion in the synaptic cleft, neurotransmitters can also bind to presynaptic autoreceptors, which modulate the rate of transmitter release, or to receptors on the membrane of the postsynaptic cell (Kwon and Castillo, 2008; Schacht and Backer, 1979) (Figure 1.2.1). On the postsynaptic side, neurotransmitters then bind to specific receptors, leading to the opening of ion channels and an influx of ions such as Na^{+} and/or Ca^{2+} carrying on the signal to the next cell, which can induce a hyper- or depolarization of the postsynaptic neuron(Kauer et al., 1988; Lev-Tov et al., 1988; Llinás et al., 1981). Thus, neurotransmitters can exert either excitatory or inhibitory effects in the postsynaptic cells. On the one hand, excitatory neurotransmitters such as glutamate or acetylcholine trigger sodium and calcium channels, leading to an influx of positive charge and depolarisation of the postsynaptic terminal. On the other hand, inhibitory transmitters γ-aminobutyric acid (GABA) and glycine induce the opening of chloride channels to an influx of anions and hyperpolarisation of the postsynaptic neuron. In the early development stage, GABA receptors have depolarising effects (Ben-Ari et al., 1989). Glutamate and GABA are the main neurotransmitters in the central nervous system (CNS). There are two types of neurotransmitter receptors called ionotropic and metabotropic receptors. Ionotropic receptors are directly bound to ion channels and responsible for fast chemical synaptic transmission, whereby the neurotransmitter binding to the receptor leads to almost immediate ion channel opening. Metabotropic receptors act indirectly on ion channels by activating second-messenger cascades such as adenosine 3’,5’-cyclic adenosine monophosphate (Sutherland et al., 1968), Ca^{2+}, and diacylglycerol (DAG) (Kishimoto et al., 1980; Takai et al., 1979b, 1979a), through intermediary G-proteins within the cell. Ionotropic and metabotropic receptors can be found both pre- and postsynaptically, and the neurotransmitter released may influence both the pre- and postsynaptic cell (Petralia et al., 1996; Schwartz and Alford, 2000; Watabe et al., 2002) (Kandel et al., 2014).

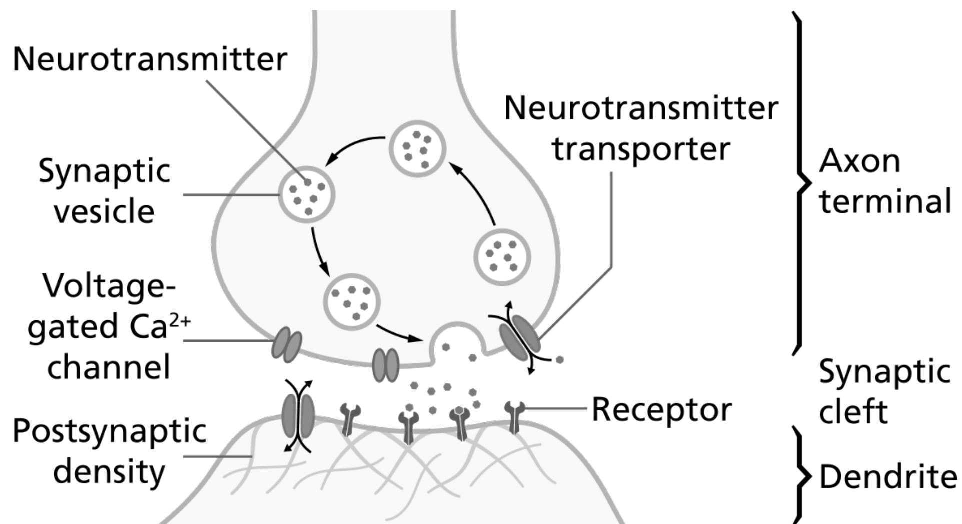

Figure 1.2.1 – Chemical neurotransmission. The presynaptic neuron axon releases neurotransmitters, which activate receptors on the postsynaptic dendritic cell—adapted from Thomas Splettstoesser (*www.scistyle.com*).

Glutamate can activate three classes of tetrameric ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors. The family of metabotropic glutamate receptors is composed of distinct receptor subunits, which are currently subdivided into eight groups (mGluR1–8) (Niswender and Conn, 2010). GABA has two types of receptors: $GABA_A$ receptor, a fast-acting pentameric receptor, and the slower metabotropic $GABA_B$. $GABA_A$ is the predominant inhibitory receptor in the brain and is permeable to monovalent anions such as Cl^-(Krnjević and Schwartz, 1967; Schofield et al., 1988). $GABA_B$ is coupled by an intracellular signal transduction cascade that triggers potassium and calcium channels opening (Dunlap and Fischbach, 1981; Hill, 1985; Newberry and Nicoll, 1985)(Figure 1.2.2).

Some mechanisms are preventing prolonged depolarisation or hyperpolarisation. One is the degradation by enzymes, or the reabsorption of the neurotransmitter by a neurotransmitter transporter from the synaptic cleft back to the neurons or astrocytes in a process called reuptake (Borden and Caplan, 1996). Another mechanism is the desensitization, whereby upon prolonged exposure to its neurotransmitter, a receptor can become unresponsive to the neurotransmitter (Sun et al., 2002).

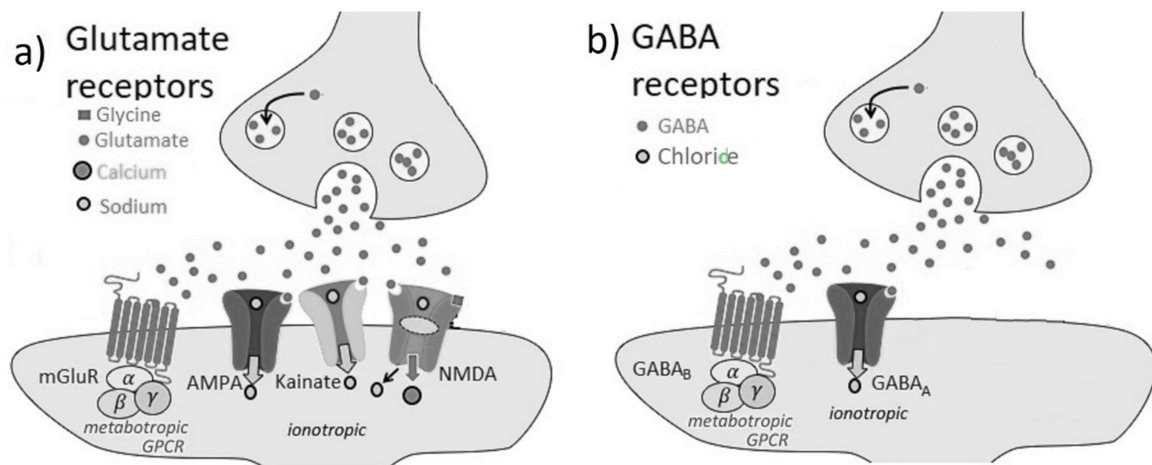

Figure 1.2.2 – Glutamate and GABA transmission and receptor. A) Following release from the presynaptic nerve terminal, glutamate binds AMPA, kainate, and NMDA (with glycine co-activation) ionotropic receptor channels leading to an influx of sodium through all receptors and calcium from the NMDA receptor on the postsynaptic site; mGluR (metabotropic glutamate receptor) activation by glutamate on the postsynaptic site. B) Following release from the presynaptic nerve terminal, GABA binds to the $GABA_A$ ionotropic receptor channel leading to chloride influx and $GABA_B$ (metabotropic receptor) on the postsynaptic site.

Long-term potentiation in the hippocampus (Schaffer collaterals)

There are at least three types of LTP in the hippocampus depending on the input and output of the circuit. These are the Schaffer collateral, mossy fibers, and direct perforant pathways. In all three, synaptic transmission is persistently enhanced in response to tetanic stimulation, but the mechanisms underlying LTP induction differ among them.

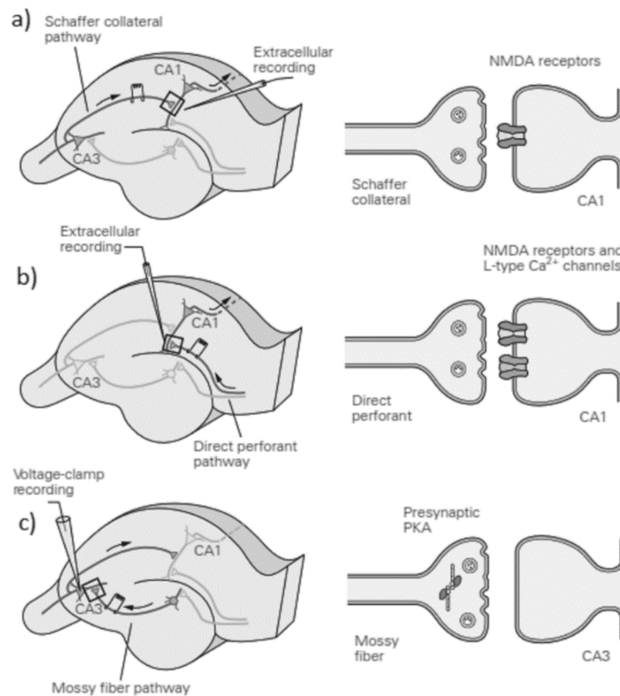

Figure 1.2.3 – Different neural mechanisms underlie long-term potentiation at each of the three synapses in the trisynaptic pathway in the hippocampus. A) Schaffer collateral pathway. At these synapses, LTP requires activation of NMDA receptors in the CA1 neurons. B) Direct perforant pathway. At these synapses, LTP requires activation of NMDA receptors and L-type voltage-gated Ca^{2+} channels simultaneously in the CA1 neurons. C) Mossy fiber pathway. At these synapses, LTP requires activation of protein kinase A but not NMDA receptors. Adapted from Kandel et al. (2014).

For example, the contribution of NDMA receptors to LTP induction depends on the microcircuit and the hippocampus area. In the direct perforant pathway, LTP depends partially on the activation of NMDA receptors and somewhat on the activation of L-type voltage-gated Ca^2+ channels (Figure 1.2.3; Remondes and Schuman, 2003). The mossy fiber pathway does not require the activation of NMDA receptors and is protein kinase A-dependent (Figure 1.2.3; Zalutsky and Nicoll, 1990). In the Schaffer collateral pathway, LTP depends on NMDA receptor activation in the CA1 neurons (Figure 1.2.3; Morgan and Teyler, 2001). This work focuses on the Schaffer collateral pathway. More detailed information about hippocampus morphology, cell types, and circuits is provided in section 1.3.

1.1.1 LTP properties

Hebbian LTP is a type of LTP that occurs in excitatory synapses. A non-Hebbian LTP typically found in integrative areas of the central nervous system that usually involves the release of GABA, as a way to inhibit its targets, was described in interneurons (Lamsa et al., 2007). Hebbian LTP has four typical features (Figure 1.2.4) (Bi and Poo, 2001; Hebb, 1949) LTP is specific (input specificity) because LTP at one synapse does not spread to other synapses unless it takes place over short distances. The second LTP property is associativity, which refers to the ability that, when a weak input of a single pathway is insufficient for the induction of LTP, simultaneous strong stimulation of another pathway will induce LTP at both paths. The third LTP propriety is cooperativity, the ability of several low-frequency stimuli to generate LTP if applied to several pathways that converge on a single patch of the postsynaptic membrane. The fourth feature of LTP is persistence, which is the ability to last from a couple of seconds to several months.

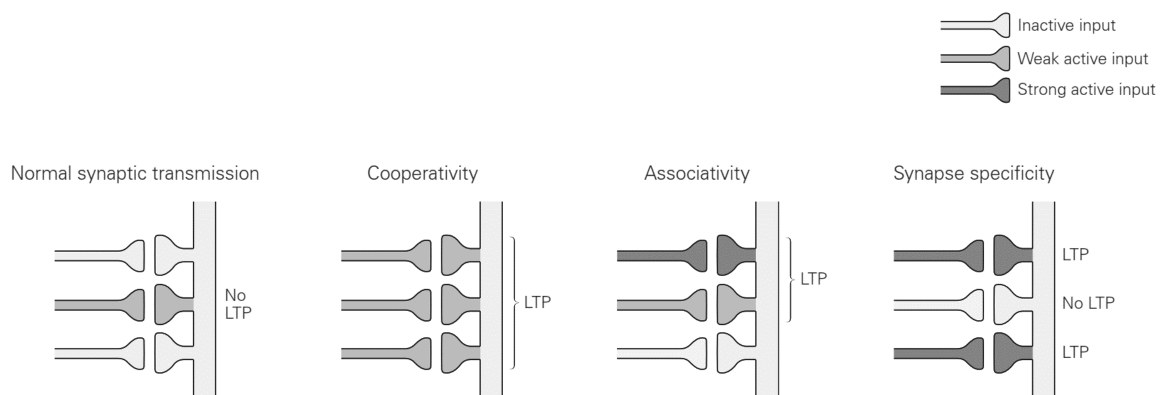

Figure 1.2.4 – Scheme showing three of the four properties of Schaffer collateral LTP: specificity, associativity, and cooperativity. Adapted from Kandel et al. (2014).

1.1.2 LTP induction

Experimentally, LTP induction is normally achieved by applying high-frequency tetanic stimulation to the synapses, or by using a 'pairing protocol' during which the postsynaptic cell is directly depolarised while low-frequency synaptic activation is sustained. An

additional method for induction of LTP (as well as long-term depression, LTD) involves protocols that generate so-called ‘spike-time dependent plasticity’ (STDP; Dan and Poo, 2006; Markram et al., 1997). In studies of STDP, LTP is induced if afferent stimulation generates a synaptic response within a discrete-time window prior to firing of the postsynaptic cell. The mechanisms for the induction of LTP at the CA3–CA1 synapse are postsynaptic (Kumar, 2011; Yeckel et al., 1999).

LTP induction can be structured into the following steps: high-frequency stimulation induces depolarization of neurons with an inward current of Na^+ from the AMPA receptor (AMPAR), permeable to monovalent charge. NMDA receptors (NMDARs) exhibit strong voltage dependence because of the block of their channels at negative membrane potentials by extracellular magnesium. As a result, NMDARs little contribute to the postsynaptic response during basal synaptic activity. Depolarisation unblocks the NMDA receptors by releasing the magnesium and allowing the influx of Ca^{2+} and Na^+ through the NMDAR channel for at least 2–3 seconds. This leads to an increase in postsynaptic calcium concentration, which likely has to reach a critical level to trigger an LTP (Fig. 1.2.1; Lynch et al., 1983; Malenka, 1991; Malenka and Nicoll, 1993; Mayer et al., 1984; Nowak et al., 1984).

1.1.3 LTP Maintenance

It has been demonstrated that Ca^{2+} rise is crucial to LTP induction. When the calcium concentration threshold for LTP is reached, the protein kinase C (PKC) is activated. PKC activation leads to the insertion of clusters of AMPA receptors in the postsynaptic membrane from a pool of intracellular receptors stored in recycling endosomal vesicles (Bredt and Nicoll, 2003; Lu et al., 2001; Shi et al., 1999). Another protein kinase activated is α-calcium-calmodulin-dependent proteinkinase II (CaMKII). CAMKII has autophosphorylating properties. The phosphorylation of subunit GluR1 of AMPAR increases the conductance, efficacy of the signal (Barria et al., 1997). CaMKII has been shown to influence the sub-synaptic localization of AMPA receptors, such that more AMPA receptors are delivered to the postsynaptic membrane. By increasing the efficiency and number of AMPA receptors at the synapse, future excitatory stimuli still generate larger postsynaptic responses. A high-frequency stimulation triggers glutamate silent and non-silent synapses. Silent synapses are excitatory glutamatergic synapses whose postsynaptic

membrane contains NMDAR but no AMPAR. The activation of CAMKII leads to the insertion of clusters of AMPA receptors in the postsynaptic membrane from a pool of intracellular receptors stored in recycling endosomal vesicles in silent synapses, making them active (Isaac et al., 1995; Kerchner and Nicoll, 2008; Liao et al., 1995; Malinow and Malenka, 2002).

All above mechanisms are part of the stage named LTP expression when the cell enhances the synaptic transmission. The expression is part of the first stage of LTP.

1.2 Neuromodulators

The term neuromodulation encompasses a broad and diverse array of phenomena. It describes the regulation of neuronal communication from the synaptic transmission to large areas of the brain involved with behavior. In this work, the definition of neuromodulation is limited to the cellular level and describes an alteration of the electrical or chemical properties of neural cells and their communication as a consequence of synaptic changes and/or exogenous drug action (Kaczmarek and Levitan, 1987). Neuromodulators are chemical compounds that potentiate or inhibit synaptic transmission and/or plasticity, regulating neuronal activity, but not acting as neurotransmitters. Endocannabinoids and adenosine are two major neuromodulators of the central nervous system (Di Marzo et al., 1998; Dunwiddie and Masino, 2001).

1.2.1 Endocannabinoid system

The history of the discovery of the endocannabinoid (eCB) system started with the use of the *Cannabis* plant by indigenous populations, followed by the discovery of the main cannabis compound — tetrahydrocannabinol (THC) —, and the identification of specific receptors until the isolation of their endogenous ligands, named endocannabinoids. The lipophilic nature of THC suggested that an endogenous cannabinoid would have a similar chemical profile (Katona and Freund, 2014). In 1992, Devane and colleagues isolated *N*-arachidonoylethanolamine, the first endocannabinoid identified, which was named

anandamide (Devane et al., 1992). eCBs are fatty acid derivatives and result from cleavage of postsynaptic membrane lipids. The name anandamide comes from the Sanskrit word *Ananda*, which means inner happiness, plus amide. Anandamide belongs to the large family of N-acylethanolamines. Three years later, the second major endocannabinoid, 2-arachidonoylglycerol (2-AG), was identified from canine intestines by Mechoulam and co-workers (Mechoulam et al., 1995). 2-AG is more abundant in the brain than anandamide (Stella et al., 1997) and is part of the 2-monoacylglycerols family.

Other fatty acid derivatives (endocannabinoids) include arachidonoyldopamine, 2-arachidonoylglycerylether (noladin ether) and O-arachidonoyl-ethanolamine (virodhamine) (Fezza et al., 2014), and N-docosahexaenoylethanolamine (Brown et al., 2010). Most studies on endocannabinoids have been published on 2-AG and anandamide.

1.2.1.1 Anandamide

1.2.1.1.1 Synthesis

The biogenesis of endocannabinoids is highly complex. Anandamide and related N-acylethanolamines are synthesized with apparent redundancy by at least five synthesis pathways of which *N*-acyl-phosphatidylethanolamine-phospholipase D is the main one. Anandamide is produced from phospholipid precursors when intracellular Ca^{2+} is elevated, following either neuron depolarisation or activation of metabotropic $G_{q/11}$-coupled receptors and cleavage by Ca^{2+}-dependent N-acyltransferase into *N*-acyl-phosphatidylethanolamine (NAPE) (Cadas et al., 1997; Di Marzo et al., 1994; Ueda et al., 2013). This process can be Ca^{2+}-independent through a Ca^{2+}-independent enzyme (Jin et al., 2007).

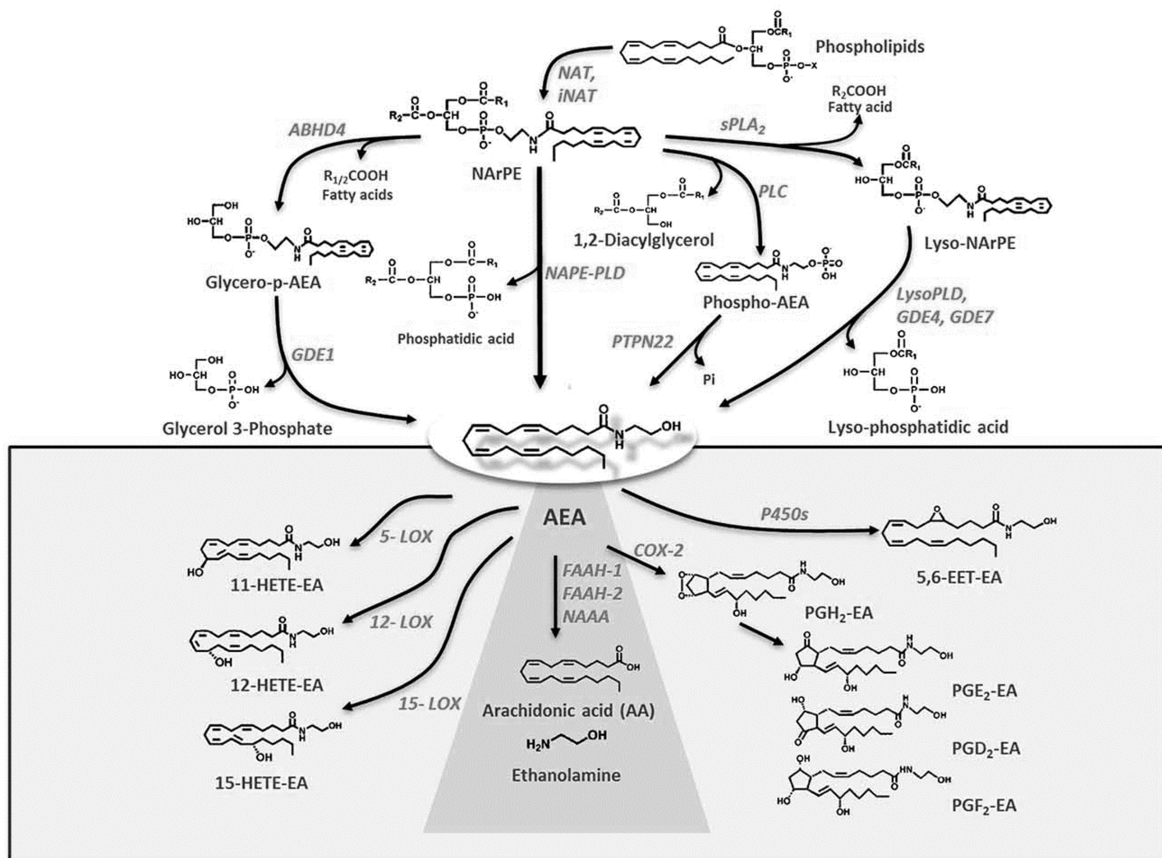

Figure 1.2.1 – Schematic illustration of parallel Anandamide synthesis, degradation and oxidation pathways. AEA: N-arachidonoylethanolamine (anandamide); NAT: N-acyltransferase; NAPE: N-acyl-phosphatidylethanolamine; NAPE-PLD: NAPE-specific phospholipase D; NArPE: N-arachidonoyl-phosphatidylethanolamine; ABHD4: α/β-hydrolase domain 4; GDE: glycerophosphodiesterase; PLC: phospholipase C; PTPN22: non-receptor protein tyrosine phosphatase 22; sPLA2: soluble phospholipase A2; FAAH: fatty acid amide hydrolase; NAAA: N-acylethanolamine-hydrolysing acid amidase; COX-2: cyclooxygenase-2; LOX: lipoxygenase; P450s: cytochrome P450 monooxygenases; PG-EA: prostaglandin-ethanolamide; HETE-EA: hydroxyeicosatetraenoyl-ethanolamide (hydroxy-AEA); EET-EA: epoxyeicosatrienoyl-ethanolamide. Adapted from Maccarrone (2017).

Anandamide is normally synthesized from NAPE-specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004), a pathway that seems to be the most relevant biosynthetic pathway of anandamide. NAPE-PLD is mainly expressed at presynaptic excitatory terminals in the hippocampus, with the highest density in the mossy fiber terminals lacking cannabinoid receptor 1 expression (Nyilas et al., 2008), suggesting a presynaptic synthesis of anandamide. Besides NAPE, other pathways such as phospholipases A_2 (Sun et al., 2004) and C (Liu et al., 2006) and *alpha*/*beta* domain serine hydrolase 4 (Simon and Cravatt, 2006, 2008) have been implicated in the synthesis of anandamide. Besides, anandamide can be formed by conjugation of arachidonic acid and ethanolamine, a process detected in brain

synaptosomes and which in turn occurs in the presynaptic component (Fig. 1.3.1; Devane and Axelrod, 1994).

1.2.1.1.2 Degradation and oxidation

Arachidonic acid and ethanolamine are final compounds of the anandamide degradation chain. Even though several enzymes can metabolize anandamide, fatty acid amide hydrolase (FAAH) is the main enzyme responsible for anandamide cleavage (Cravatt et al., 1996). Two isoforms of FAAH (FAAH-1 and FAAH-2) have been identified in humans, but only FAAH-1 is expressed in rodents (Giang and Cravatt, 1997). FAAH is also found in intracellular membranes of principal neurons (Egertova et al., 1998; Gulyas et al., 2004; Figure 1.2.1). N-acylethanolamine-hydrolysing acid amidase (NAAA), a lysosomal cysteine hydrolase, is another degradation enzyme (Tsuboi et al., 2007).

In addition to hydrolising pathways, anandamide is oxygenated by several enzymes such as cyclooxygenase-2 (COX-2; Kozak et al., 2001), several lipoxygenases (5, 12, and 15) (Hampson et al., 1995), as well as several cytochrome P450 monooxygenases (Snider et al., 2010) giving rise to prostaglandin-ethanolamides, hydroxy-anandamides, and epoxyeicosatrienoyl-ethanolamides, respectively (Figure 1.2.1).

1.2.1.1.3 Storage and transportation

For endocannabinoids anandamide and 2-AG to be widely produced and released postsynaptically, a trigger is necessary. However, some researchers have hypothesized that endocannabinoids are pre-synthesized and stored until a stimulus triggers their release (Alger and Kim, 2011; Hashimotodani et al., 2013; Maccarrone et al., 2010; Min et al., 2010). Evidence for this hypothesis is the presence of anandamide in adiposomes (lipid droplets), which represent a dynamic reservoir for the sequestration of eCBs (Oddi et al., 2008), opening a new chapter for specific (intracellular and extracellular) transporters for endocannabinoids (Maccarrone, 2017; Solymosi and Kofalvi, 2017;). Further research is

needed to elucidate all the steps involved in the storage and transportation of 2-arachidonoylglycerol.

1.2.1.2 2-Arachidonoylglycerol

1.2.1.2.1 Synthesis

2-arachidonoylglycerol (2-AG) is produced predominantly from phospholipase C *beta* and diacylglycerol lipase (DAGL). Ca^{2+} influx triggers phospholipase C *beta* activity (β1 and β4) (Hashimotodani et al., 2005; Maejima et al., 2005), which then transforms membrane phosphoinositide into a diacylglycerol. DAGL then cleaves diacylglycerol into 2-AG (Bisogno et al., 2003). Alternatively, 2-AG can also be synthesized by dephosphorylation of arachidonoyl-lysophosphatidic acid (Nakane et al., 2002) via phospholipase A1 and a lysophospholipase C (Higgs and Glomset, 1994; Sugiura et al., 1995; Tsutsumi et al., 1994). Two DAGL isoforms (DAGL-α and DAGL-β) have been identified (Bisogno et al., 2003), and there are some dissimilarities between them. The DAGL-α isoform is highly expressed in hippocampal pyramidal cells and dentate granule cells, whereas DAGL-β is expressed in lower levels in the hippocampal pyramidal cell layer. Additionally, some spatial differences exist between DAGL-α and β isoforms, suggesting that DAGL-α has a role in intercellular 2-AG signalling while DAGL-β plays a role in the intracellular membrane segments, including perinuclear lipid droplets (Bisogno et al., 2003; Jung et al., 2011; Katona et al., 2006; Yoshida et al., 2006, Figure 1.2.2).

1.2.1.2.2 Degradation and oxidation

Several enzymes may be involved in the degradation of 2-AG, depending on its localization (postsynaptic vs presynaptic). Postsynaptically, 2-AG can be cleaved into glycerol and arachidonic acid by the membrane serine hydrolase enzyme α-β-hydrolase

domain 6 (ABHD6, Blankman et al., 2007). Presynaptically, monoacylglycerol lipase metabolizes 2-AG, and this is the main degradation pathway.

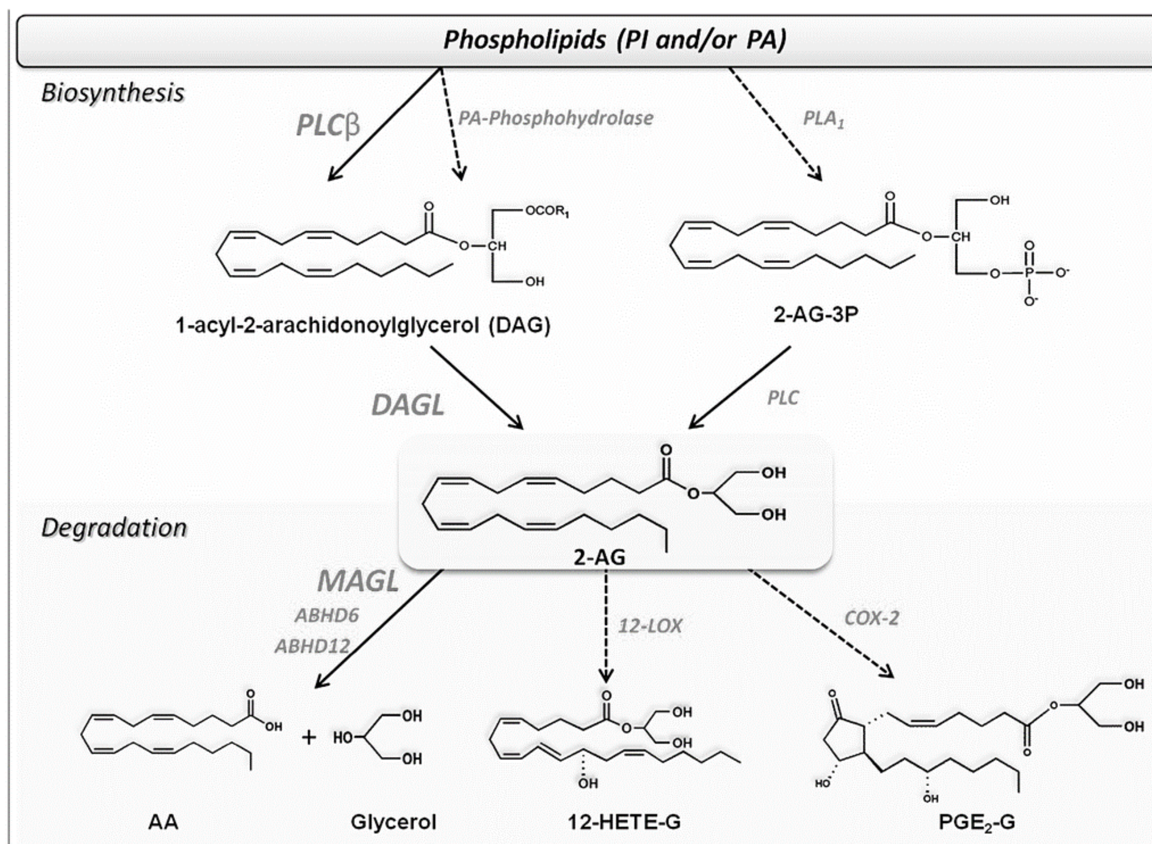

Figure 1.2.2 – Schematic illustration of parallel 2-AG synthesis, degradation, and oxidation pathways. 2-AG: 2-arachidonoylglycerol; PLC: phospholipase C; PLA: phospholipase A; DAGL: diacylglycerol lipase; 2-AG-3P: 2-arachidonoylglycerol-3-phosphate; MAGL: monoacylglycerol lipase; ABHD4: α/β-hydrolase domain 6; ABHD12: α/β-hydrolase domain 12; COX-2: cyclooxygenase-2; LOX: lipoxygenase; AA: arachidonic acid; 12-HETE-G: 12-hydroxyeicosatetraenoic acid; PGE_2-G: prostaglandin E2 glycerol ester. Adapted from Fezza et al. (2014).

Another serine hydrolase, α-β-hydrolase domain 12 (ABHD12), metabolizes 2-AG in the Golgi complex (Blankman et al., 2007). The final products of the three enzymatic pathways are arachidonic acid (AA) and glycerol (Freund et al., 2003). Additionally, FAAH can metabolize 2-AG *in vitro* (Blankman et al., 2007). Similar to what happens to anandamide, 2-AG can also be oxidized. Cyclooxygenase-2 (Hu et al., 2008; Sang et al., 2007) and lipoxygenases (Kozak and Marnett, 2002) can oxidize 2-AG, producing prostaglandin glycerol esters and hydroperoxy derivatives of 2-AG, respectively (Figure 1.2.2). Another

degradation pathway involves acylglycerol kinase, which can phosphorylate 2-AG into lysophosphatidic acid (Bektas et al., 2005).

1.2.1.3 Cannabinoid receptors

In the late 1980s, Devane, Howlett, and colleagues discovered the first cannabinoid receptor, known as cannabinoid receptor 1 (CB_1R), using the specific radio-labeled cannabinoid analog [3H]CP55940 (Bidaut-Russell et al., 1990; Devane et al., 1988). CB_1R belongs to the rhodopsin-like guanine-nucleotide-binding protein-coupled receptor (GPCR) superfamily and contains seven α-helical transmembrane domains bound to G_i protein subunits (Bidaut-Russell et al., 1990; Hua et al., 2016; Shao et al., 2016) that can inhibit adenylyl cyclase, lowering protein kinase A (PKA) activity and cyclic adenosine monophosphate (cAMP) availability. CB_1R is widely expressed in the central nervous system (e.g., hippocampus), in principal cells and their terminals (Herkenham et al., 1990, 1991).

Not long after the discovery of CB_1R, a second cannabinoid receptor (CB_2R) was identified (Munro et al., 1993). Because this receptor is mainly expressed by the immune system (Galiègue et al., 1995), as opposed to CB_1R, it was initially believed that CB_2R played a minor or no role in the brain. However, more recent studies have shown that CB_2R can be expressed in the central nervous system by several neuronal cell types, where it has a modulatory function (Atwood and MacKie, 2010; Kim and Li, 2015; Marsicano and Kuner, 2008). The two receptors share 44% amino acid sequence homology only, and the transmembrane domain is the region with greater homology (approximately 68%) (Munro et al., 1993). Similarly to CB_1R, CB_2R couples predominantly with $G_{i/o}$ proteins to inhibit adenylyl cyclase activity (Howlett, 1995, 2002).

The complex pharmacology of the endocannabinoid system and the lack of selective ligands have jeopardized the identification of new cannabinoid receptors. There are a few CB_3R candidates, including transient receptor potential cation channel subfamily V member 1 (TrpV1R), also known as capsaicin receptor and vanilloid receptor 1, which is activated by anandamide (Zygmunt et al., 1999). TrpV1R is found in the digestive tract and is mostly known for mediating the burning sensation of 'hot' chili peppers elicited by the molecule capsaicin, and is linked to the transduction of a range of sensorial stimuli such as temperature, electrical charge, light, olfactive, and taste stimuli (Pertwee et al., 2010).

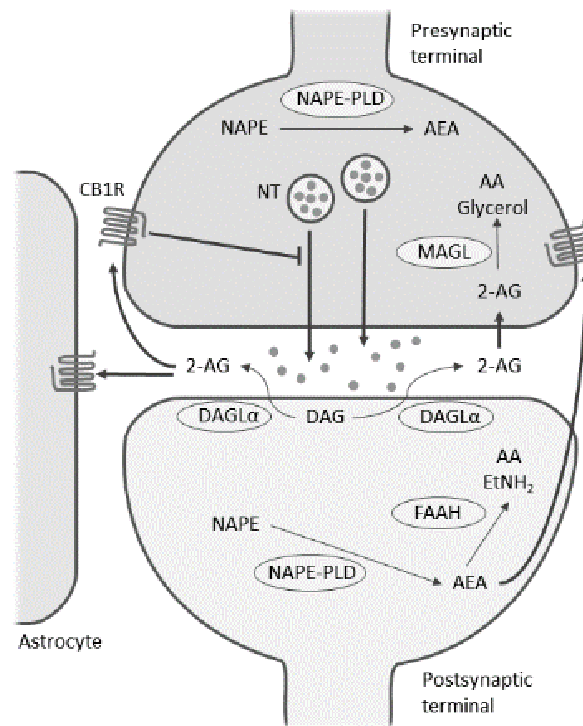

Figure 1.2.3 – Simplified scheme representing endocannabinoid retrograde signaling mediated trisynaptic transmission. Endocannabinoids are produced from postsynaptic terminals upon neuronal activation. As the two major endocannabinoids were shown in the scheme, 2-arachidonoylglycerol (2-AG) is biosynthesized from diacylglycerol (DAG) by diacylglycerol lipase-α ($DAGL_{\alpha}$), and anandamide (AEA) is synthesized from *N*-acyl-phosphatidylethanolamine (NAPE) by NAPE-specific phospholipase D (NAPE-PLD). As lipids, endocannabinoids, mainly 2-AG, readily cross the membrane and travel in a retrograde fashion to activate CB_1Rs located in the presynaptic terminals. Activated CB_1Rs will then inhibit neurotransmitter (NT) release through the suppression of calcium influx. 2-AG is also able to activate CB_1Rs located in astrocytes, leading to the release of glutamate. Extra 2-AG in the synaptic cleft is taken up into the presynaptic terminals, via a yet unclear mechanism, and degraded to arachidonic acid (AA) and glycerol by monoacylglycerol lipase (MAGL). On the other hand, AEA, synthesized in the postsynaptic terminal, activates intracellular CB_1R, and other non-CBR targets such as the transient receptor potential cation channel subfamily V member 1 (TRPV1). Although endocannabinoid retrograde signaling is mainly mediated by 2-AG, AEA can activate presynaptic CB_1Rs as well. Fatty acid amide hydrolase (FAAH) is primarily found in postsynaptic terminals and is responsible for degrading AEA to AA and ethanolamine ($EtNH_2$). Although NAPE-PLD is expressed in presynaptic terminals in several brain regions, it is not clear yet whether AEA is responsible for anterograde signalling in the endocannabinoid system. Note that alternative routes exist for the metabolism of endocannabinoids, depending on the brain region and physiological conditions. Thin arrows indicate enzymatic process; thick arrows indicate translocation; blunted arrow indicates inhibition. Adapted from Zou and Kumar (2018).

Marsch et al. (2007) described a modulatory effect on hippocampal synaptic plasticity mediated by TrpV1 via the endocannabinoid system (Marsch et al., 2007). Additionally, peroxisome proliferator-activated receptors (PPARs), which are nuclear hormone receptors of the nuclear receptor subfamily 1, group C, member 1 family are another type of receptor activated by anandamide with various roles, including to regulate lipid homeostasis, cellular differentiation, proliferation, and the immune response (Lu and MacKie, 2016; Pertwee et al., 2010). Class A G-protein-coupled receptors (GPCR) such as GPR18, GPR55,

and GPR119 have also emerged as possible additional members of the cannabinoid family. GPR55 was identified in the human striatum and is activated by 2-AG and anandamide (Sawzdargo et al., 1999; Sharir and Abood, 2010). However, their classification is still disputed due to the lack of pharmacological tools to validate them. To date, no GPCR has been classified by the International Union of Pharmacology as a novel CB_3R (Pertwee et al., 2010). Because this thesis focuses on CB_1R in the hippocampus, this receptor will be described in detail in the following sections.

1.2.1.4 CB_1R in the hippocampus

CB_1R is highly expressed in the hippocampus (Herkenham et al., 1991) and especially in the CA1 and CA3 regions (Marsicano and Lutz, 1999; Tsou et al., 1998) and has been detected in the following brain cell types: pyramidal cells, interneurons, astrocytes, oligodendrocytes, and microglia (Figure 1.2.3; Castillo et al., 2012; Katona et al., 2001; Kawamura, 2006; Navarrete and Araque, 2008; Stella, 2009; Wilson et al., 2001). In the dentate gyrus, glutamatergic mossy cells and cholecystokinin (CCK) interneurons express CB_1R. Interneurons can be classified into two groups based on the expression of the molecular markers CCK or parvalbumin (PV), which differ in CB_1R expression: CCK, but not PV interneurons, expresses CB_1R (Freund et al., 2003; Klausberger, 2005). Additionally, granule cells of the dentate gyrus do not express CB_1R (Johnston and Amaral, 2004; Kawamura, 2006; Monory et al., 2006). In the CA1 and CA3 regions, neuronal cells such as CCK interneurons contain CB_1R (Katona et al., 2006).

1.2.1.5 CB_1R subcellular level

Endocannabinoids are lipid-like compounds synthesized intracellularly, which suggest the existence of an intracellular target in combination with the extracellular cellular membrane target.

Besides the plasma membrane pool of CB_1R, a different intracellular pool of CB_1R in endosomes and lysosomes modulates calcium release from the endoplasmic reticulum and lysosomes (Figure 1.2.4; Brailoiu et al., 2011; Grimsey et al., 2010; Rozenfeld and Devi, 2008). Mitochondria is another cell organelle that expresses CB_1R. There is evidence of metabolic modulation of mitochondrial CB_1R, but the magnitude of the effect remains controversial (Figure 1.2.4; Bénard et al., 2012; Hebert-Chatelain et al., 2014a, 2014b).

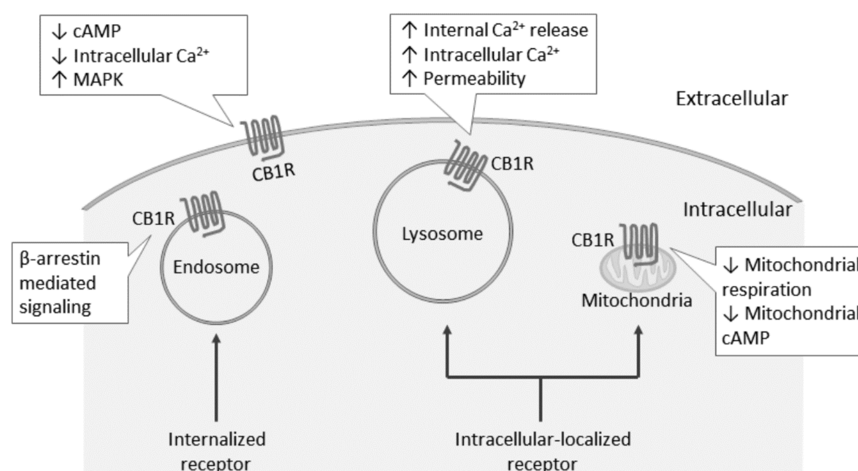

Figure 1.2.4 – Subcellular localization of the CB_1R. Typically, CB_1R is located at the cell surface and inhibits cyclic adenosine monophosphate (cAMP) formation and calcium influx upon activation. Constitutive and ligand-induced internalized CB_1Rs mediate signalling pathways through β-arrestin. Intracellular-localized CB_1R do not translocate to the plasma membrane. Instead, they form a subpopulation with pharmacological properties distinct from their plasma membrane-localized counterparts. CB_1R located on lysosomes can increase intracellular calcium concentrations through the release of internal calcium stores and increase the permeability of lysosomes. Mitochondrial CB_1R inhibits mitochondrial cellular respiration and cAMP production, hence regulating cellular energy metabolism. Adapted from Zou and Kumar (2018).

1.2.1.6 Role of (endo)cannabinoids on hippocampal LTP and inhibitory postsynaptic potential

It is well established that CB_1R activation inhibits GABAergic and glutamatergic transmission presynaptically (Castillo et al., 2012; Kano et al., 2009). Moreover, because it is predominantly expressed at inhibitory terminals, hippocampal CB_1R exerts a potent modulatory role on the inhibition of GABA release (Freund et al., 2003). As mentioned

above, CCK interneurons express CB_1R and control the information flow as well as encode fine-tuning network chronosynchrony (Freund et al., 2003; Keimpema et al., 2012). They are also essential for mediation of a form of short-term plasticity called depolarisation-induced suppression of inhibition (DSI). DSI is a regulatory mechanism of GABA release from presynaptic interneurons that involves an increase of calcium concentration in the postsynaptic neuron, which is mediated by the release of a retrograde messenger that reversibly inhibits afferent synapses via presynaptic mechanisms. DSI was first described in the early 1990s, but its regulation was then attributed to an unidentified substance (Llano et al., 1991; Pitler and Alger, 1992). Only a decade later would endocannabinoids be identified as the modulatory substances (Carlson et al., 2002; Ohno-Shosaku et al., 2012; Wilson et al., 2001). This was only achieved by assuming that endocannabinoid release was an "on-demand" mechanism. A similar mechanism, called depolarisation-induced suppression of excitation (DSE), occurs in excitatory neurons (Kreitzer and Regehr, 2001). Tanimura and co-workers identified 2-AG as the endocannabinoid that regulates DSI and DSE (Tanimura et al., 2010).

While inhibition by eCBs of neurotransmitter release and short-term plasticity are well-established mechanisms, eCBs action on synaptic plasticity induced by brief high-frequency neuronal firing (e.g., LTP) is highly controversial. Considering only the hippocampus, a brain area important for memory encoding and the one most used to study LTP phenomena, there are reports showing that eCBs restrict LTP (Bohme et al. 1999; Slanina et al. 2005) while others show that they facilitate it (Carlson et al. 2002; De Oliveira Alvares et al. 2006). This controversy is intriguing because LTP is a compelling cellular model for learning and memory (see Nicoll 2017), and exogenous cannabinoids, including the phytocannabinoids in marijuana and the synthetic agonists of CB_1R, harm learning and memory, both in humans and in laboratory animals (Lane et al., 2005; Miller et al., 1977; Sousa et al., 2011). Elegant studies aimed at determining the influence of eCBs upon LTP in different cell types and circuits in the hippocampus show that the action of eCBs may vary according to the cell type where CB_1Rs are expressed (Monory et al., 2015) and the hippocampal circuit where LTP is induced (Wang et al. 2016). Because eCBs formation results from the neuronal activity, the influence of eCBs on LTP also may varies as a function of the pattern of neuronal firing that induces plasticity. Evidence of that association would not only further

our understanding of the reasons for the conflicting data in the literature but also provide better insight into the subtleties of eCB use to control synaptic strengthening.

1.2.2 Adenosine

Neuromodulators vary significantly in nature, from lipids (endocannabinoids) to purines. Adenosine is a purine nucleoside composed of a molecule of adenine attached to a ribose molecule (Figure 1.2.5).

Adenosine is well-known as a physiological modulator since the 1920s. Drury and Szent-Györgyi discovered that adenosine lowers blood pressure, causing bradycardia, and dilates the coronary arteries (Drury and Szent-Györgyi, 1929). Since then, several modulatory roles have been described for adenosine in various physiological systems (e.g., cardiovascular, renal, respiratory, and central nervous system [Williams, 1990]).

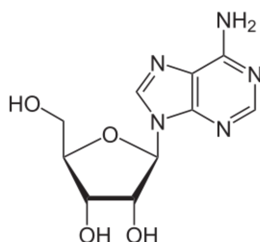

Figure 1.2.5 – Chemical structure of adenosine. Adenosine is a nucleoside, consisting of the purine adenine and a ribose ring.

In 1954, Holton and Holton suggested that adenosine triphosphate (ATP) and derivatives could be used as neurotransmitters of the central nervous system (Holton and Holton, 1954). However, because ATP is neither stored nor released as a classical neurotransmitter. It took 20 years to envisage a neuromodulation role for adenosine in neurotransmission, which was hypothesized based on the observation of its depressant effect on the responses of neurons (Phillis et al., 1974) or as cyclic adenosine monophosphate promoter (Sattin and Rall, 1970).

Currently, adenosine is considered a major neuromodulator, regulating neurotransmission indirectly in neuronal cells, including neurons (pre- and postsynaptically and outside the synapses) and astrocytes (Ribeiro and Sebastião, 2010; Sebastião and Ribeiro, 2009).

1.2.2.1 Synthesis, degradation, and transport

The two main sources of extracellular adenosine are the extracellular production from the hydrolysis of adenine nucleotides and the transport-mediated release of adenosine from intracellular adenosine sources. Adenosine can be generated by sequential ATP dephosphorylations by ecto-5'-nucleotidases (Meghji, 1993; Figure 1.2.6) and can also be released from neurons and astrocytes by nucleoside transporters (Pascual et al., 2005). Nucleoside transporters are involved in equilibrative adenosine transport from and into the cell (Latini and Pedata, 2001) depending on the adenosine concentration gradient across the cell membrane (Figure 1.2.6). Under basal conditions, the intracellular concentration of adenosine is lower than in the extracellular space (25 to 250 nM), so the influx of adenosine into the cell is prevails (Ballarin et al., 1991; Dunwiddie and Diao, 1994).

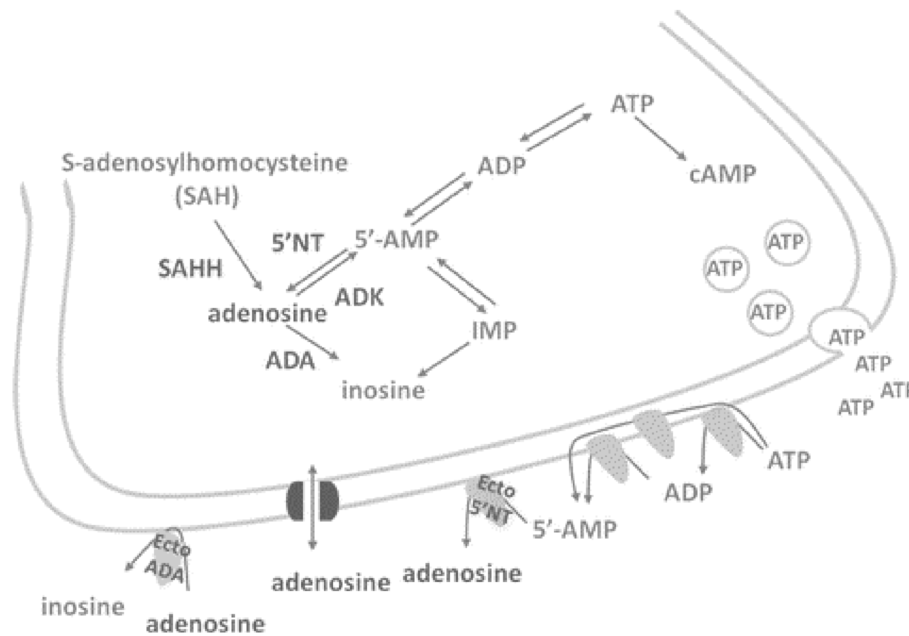

Figure 1.2.6 – Schematic representation of adenosine metabolism. Adenosine can be synthesized intra- and extracellularly. Inside the cell, adenosine is formed from AMP (adenosine monophosphate) metabolism through endo-5'-nucleotidase (5'NT) or by the transmethylation reaction catalyzed by S-adenosylhomocysteine hydrolase (SAHH), which converts S-adenosylhomocysteine (SAA) into adenosine and homocysteine. In the extracellular space, adenosine derives from the metabolism of ATP/ADP (adenosine diphosphate)/AMP, with the last reaction being catalyzed by ecto-5'-nucleotidase (Ecto 5'NT). The release of adenosine by equilibrative nucleoside transporters is an alternative source of adenosine. Regarding the clearance of extracellular adenosine, in some cases it can be converted into inosine by ecto-adenosine deaminase (ecto-ADA) in the extracellular space., However, in most cases, adenosine is taken up by the equilibrative nucleoside transporter into cells where adenosine can be phosphorylated to AMP by adenosine kinase (ADK) or deaminated to inosine by adenosine deaminase (ADA). Adapted from Sebastião et al. (2013).

However, under intense neuronal activity, this flux can be reversed, providing an additional source of extracellular adenosine (Cunha, 2001; Dunwiddie and Masino, 2001). Intracellularly, adenosine is produced by the dephosphorylation of adenosine 5' monophosphate (ATP) by endo-5'-nucleotidase (Meghji, 1993) and the hydrolysis of S-adenosyl-homocysteine by S-adenosyl-L-methionine hydrolase (Palmer and Abeles, 1979; Figure 1.2.6). Elimination of adenosine occurs primarily through phosphorylation to adenosine monophosphate by adenosine kinase, located intracellularly in neurons and astrocytes (Studer et al., 2006), and by adenosine metabolization by adenosine deaminase into inosine intracellularly and extracellularly (Dunwiddie and Masino, 2001; Figure 1.2.6).

1.2.2.2 Adenosine receptors

Two adenosine receptors were independently identified by two research groups and named A_1 and A_2 receptors by the van Calker group (Calker et al., 1979; Londos et al., 1980). Later, the A_2 receptor was subdivided into A_{2A} and A_{2B} receptors: the former is a high-affinity receptor in striatal membranes that induces synthesis of high levels of cAMP, and the latter is a low-affinity receptor ubiquitously distributed throughout the brain (Bruns et al., 1986). Simultaneously, a fourth adenosine receptor was proposed, but no pharmacological confirmation was provided (Phillis and Wu, 1981; Ribeiro and Sebastião, 1986; Stone, 1985).

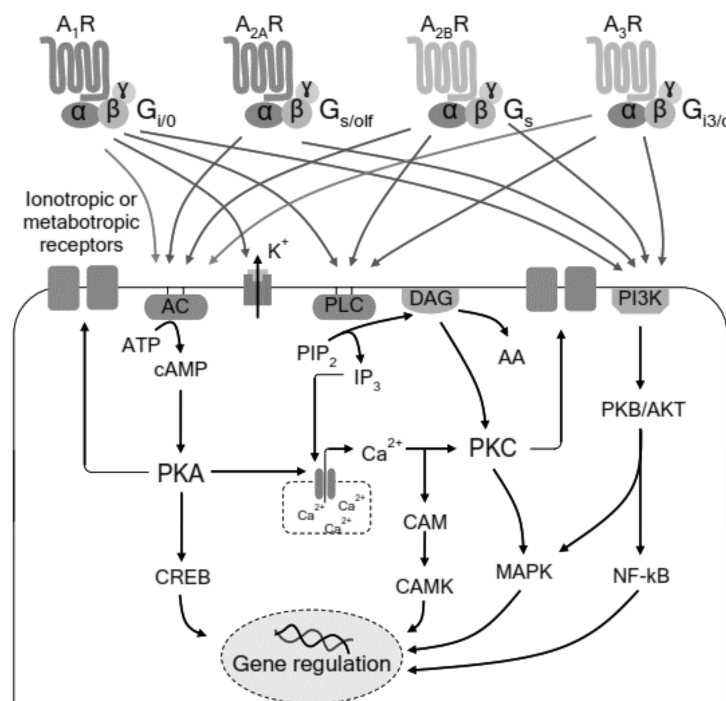

Figure 1.2.7 – Adenosine receptors and classical signaling pathways. Adenosine A_1R and A_3R are coupled to pertussis-sensitive $G_{i/o}$ proteins inhibiting the activity of AC (via G_α subunit) and increasing the activity of PLC (via $G_{\beta\gamma}$ subunits). A_1R also activates inwardly rectifying K^+ channels. Activation of $A_{2A}R$ and $A_{2B}R$ increases AC activity through activation of G_s proteins. $A_{2B}R$ is also positively coupled to PLC via $G_{\beta\gamma}$ subunits. All four subtypes of adenosine receptors induce the activation of PI3K, which may result in the activation of NF-κB and MAPK, and thus play a role in cell growth, survival, death, and differentiation. AA: arachidonic acid; AC: adenylate cyclase; ATP: adenosine 5'-triphosphate; Ca^{2+}: calcium ion; CAM: Ca^{2+}/calmodulin-dependent protein; CAMK: CAM kinase; cAMP: cyclic adenosine 5'-monophosphate; CREB: cAMP response element-binding protein; DAG: diacylglycerol; IP3: inositol 1,4,5-trisphosphate; K^+: potassium ion; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor-κB; PI3K: phosphatidylinositol 3-kinase; PIP2: phosphatidylinositol-4,5-bisphosphate; PKA: protein kinase A; PKB/AKT: protein kinase B; PKC: protein kinase C; PLC: phospholipase C; green arrow: activation; red arrow: inhibition. Adapted from Rombo (2015).

Only ten years after the discovery of the A_1 receptor (A_1R), four different G-protein-coupled receptors had already been cloned and characterized: A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_{2A} receptors have a high affinity for adenosine, whereas the A_{2B} and A_{3A} receptors show a relatively lower affinity for adenosine receptors (Figure 1.2.7; Fredholm et al., 2011). The receptors, which belong to the superfamily of G-protein-coupled receptors, have a seven-transmembrane α-helical structure and are linked to a variety of transduction mechanisms. The A_{2A} and A_{2B} receptors are coupled to $G_{s/olf}$ proteins. Their activation increases cAMP production, resulting in activation of protein kinase A and phosphorylation of the cAMP response element-binding protein (CREB). In contrast, A_1 and A_3 receptors are coupled to $G_{i/o}$ proteins, and their activation inhibits the above-mentioned signaling cascade. A_1R also activates inwardly rectifying K^+ channels (Calker et al., 1979; Haas and Greene, 1984; Segal, 1982; Trussell and Jackson, 1985), and A_3R can couple to G_q.

Adenosine receptors are expressed differently across the brain (for a review see Fredholm et al., 2001, 2011). A_1R is abundantly expressed in the neocortex, hippocampus, and cerebellum, whereas the A_{2A} receptor ($A_{2A}R$) is mainly expressed in basal ganglia and the olfactory bulb but is present in other brain structures at much lower concentrations. A_{2B} is widely expressed in the brain, whereas the A_3 receptor has a very low density in the brain. The physiological roles of $A_{2B}R$ and A_3R are poorly characterized. In humans, the A_3 receptor is expressed at intermediate levels in the cerebellum and hippocampus and at lower levels in other brain regions (Figure 1.2.8; Atkinson et al., 1997).

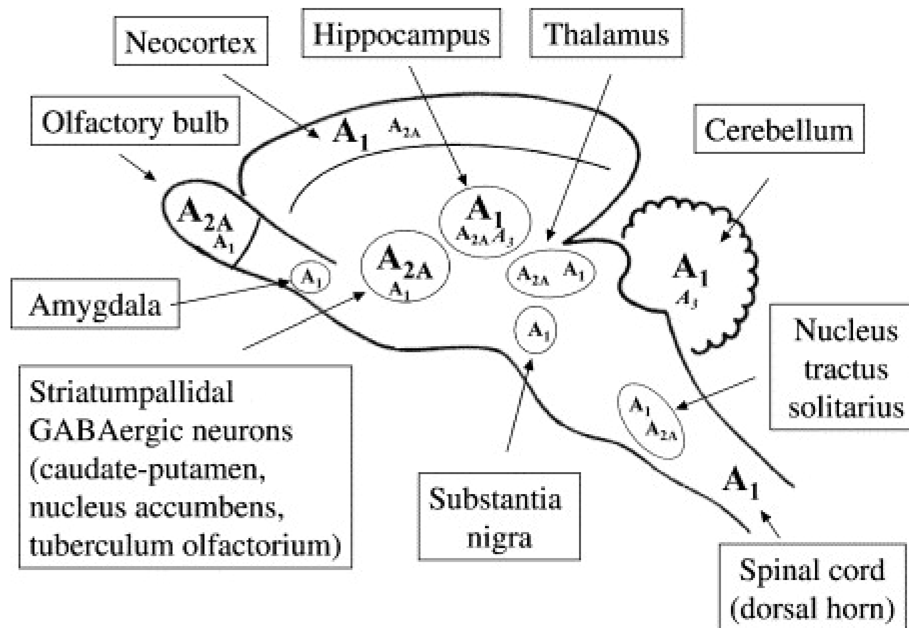

Figure 1.2.8 – Distribution of high-affinity adenosine receptors (A_1, A_{2A}, and human A_3) in the main regions of the central nervous system where adenosine has been proposed to interfere with brain dysfunctions and disease. High levels of expression are indicated by bigger alphabets. Adapted from (Ribeiro et al., 2002)

1.2.2.3 A_1R in the hippocampus and at the subcellular level

The A_1 receptor is well expressed in the hippocampus, in particular in the *stratum oriens* and *lacunosum-moleculare* layers, and less expressed in the *stratum piramidale* (Fastbom et al., 1987). Additionally, the A_1 receptor is expressed in all neuronal cell types. Excitatory neurons have A_1R at the pre- and postsynaptic densities (Rebola et al., 2003) as well as in interneurons from the *stratum oriens* and *stratum radiatum* (Ochiishi et al., 1999; Othman et al., 2003). Glial cells, including astrocytes, microglia, and oligodendrocytes, also express A_1R (Biber et al., 1997; Gebicke-Haerter et al., 1996; Othman et al., 2003).

1.2.2.4 Role of A_1R into hippocampal LTP and inhibitory postsynaptic potential

Endogenous adenosine is able to modulate mechanisms of synaptic plasticity (i.e., long-term potentiation) in the hippocampus (De Mendonça and Ribeiro, 1994).

A_1R plays a modulatory role in synaptic transmission both pre- and postsynaptically. Presynaptically, A_1R activation decreases glutamate release via voltage-dependent Ca^{2+} channels (VDCCs) (MacDonald et al., 1986; Schubert et al., 1986; Wu and Saggau, 1994) and decreases the spontaneous release of neurotransmitter (Scanziani et al., 1992). Postsynaptically, A_1R also activates inwardly, rectifying K^+ channels controlling neuronal depolarization and consequently burst-like activity in the central nervous system (Dragunow, 1988; Ehrengruber et al., 1997; Lesage et al., 1995; Ponce et al., 1996). A_1R exerts its modulatory role mostly upon excitatory transmission (Dunwiddie and Fredholm, 1989; Sebastião et al., 1990). A_1R is also able to modulate GABAergic transmission either directly or indirectly (Rombo et al., 2016). For example, vasoactive intestinal peptide enhances GABA release leading to increased excitatory synaptic transmission to CA1 pyramidal cells (Cunha-Reis et al., 2004, 2007).

1.2.2.5 Role of A_1R and CB_1R (cross-talk) in synaptic transmission and plasticity

CB_1R activation decreases GABA and glutamate release in the hippocampus, these effects being partially reduced by co-activation of A_1R, suggesting an interaction between these modulatory pathways at the level of G-protein activation in studies using rodents (Sousa et al., 2011). The endogenous activation of A_1R reduces or abrogates CB_1R inhibition of glutamate release (Hoffman et al., 2010). Moreover, A_1R modulates $GABA_AR$ currents, at the tonic level, CCK-positive basket cells that are CB_1R-positive (Rombo et al., 2016).

CB_1R in the hippocampus is involved in memory impairment by the administration of cannabinoids (Sousa et al., 2011), and chronic caffeine (adenosine receptors antagonist) administration exacerbates acute THC-induced memory impairment. Besides, while DSI was believed to result from CB_1R endogenous activation (Ohno-Shosaku et al., 2002; Wilson

et al., 2001), Gu and co-workers (Gu et al., 2005) showed that DSI depends on A_1R activation in the *nucleus accumbens* brain area.

Finally, A_1R–CB_1R can co-localize and interact with GPCRs (Straiker et al., 2002) in hippocampal Schaffer collateral axons and terminals.

1.3 Hippocampus

The hippocampus, named after the Greek word for seahorse due to its incurved structure, has held a central and fascinating role in brain research through time (Ramón y Cajal, 1909). Phylogenetically, the hippocampus is an ancient brain region that differs both anatomically and physiologically from the outer region of the cerebral cortex (neocortex), which is composed of several lobes. The hippocampus is part of the allocortex and differs from the cortex functionally, anatomically, and cytoarchitecturally; it receives its main inputs from the entorhinal cortex and sends outputs to the temporal cortex (neocortex) and other parts of the limbic system. The hippocampus belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory, and in spatial memory that enables navigation (Kandel et al., 2014).

The hippocampus contains two regions, the *Cornu ammonis* ('hippocampus proper') and the dentate gyrus. Some anatomists separate the hippocampus (hippocampal formation) into hippocampus proper (*Cornu ammonis*; CA), dentate gyrus (DG), subiculum, and entorhinal cortex (EC); and CA can be divided into subfields CA1, CA2, CA3, and CA4 from the EC to DG (Figure 1.3.1B). The main neuronal cell types of the DG and CA regions are the granule cells and pyramidal cells, respectively, which are glutamatergic excitatory (Amaral, 1978).

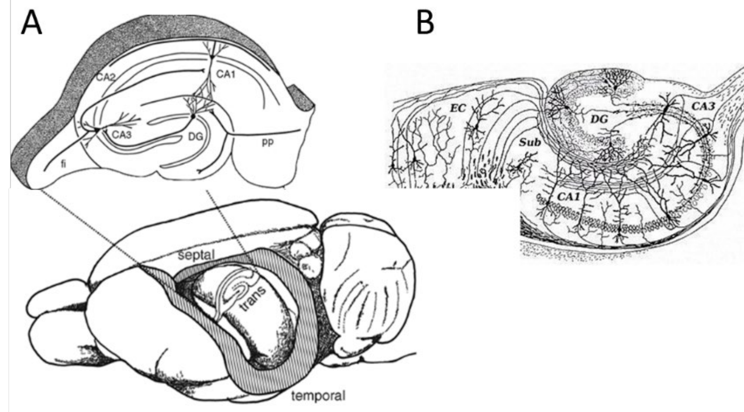

Figure 1.3.1 – Line drawing of the rat brain shows the septotemporal and transverse axes of the hippocampal formation. A) Schematic representation of the hippocampus when cutting transversally to the hippocampal septotemporal axis. fi: fimbria; CA1: Cornu ammonis 1; CA2: Cornu ammonis 2; CA3: Cornu ammonis 3: DG: dentate gyrus; PP: perforant path. Adapted from Andersen et al. (2009). B) Original drawing by Ramón y Cajal of the rodent hippocampus. EC: entorhinal cortex; sub: subiculum; CA1: Cornu ammonis 1; CA3: Cornu ammonis 3; DG: dentate gyrus. Adapted from Ramón y Cajal (1909).

One of the main characteristics of the anatomy of the hippocampus is its unique connectivity, forming a trisynaptic circuit, organized in a semi-closed, mono-directional pathway: perforant path-to-dentate gyrus-to-CA3-to-CA1. Per Andersen noted that thin slices could be cut out the hippocampus perpendicular to its axis, in a way that all of these connections were preserved (Andersen et al., 1971; Figure 1.3.1A).

The first link of the trisynaptic circuit corresponds to the main afference of the hippocampus, the pyramidal cells of the layer II of the entorhinal cortex, whose axons project to DG granule cells, named perforant path (Claiborne et al., 1986). The second link of the circuit consists of the granule cells projecting their axons, the mossy fibers, to the dendrites of the pyramidal cells of CA3. The pyramidal cells of CA3 send collateral projections, the Schaeffer collaterals, to the pyramidal cells of CA1 through the stratum radiatum forming the third link of the trisynaptic circuit. Later, several other pathways were discovered, including the direct projections from layers II and III of EC to CA3 and CA1, respectively (Figure 1.3.2; Amaral, 1993; Steward, 1976). For a review, see Andersen et al. (2009).

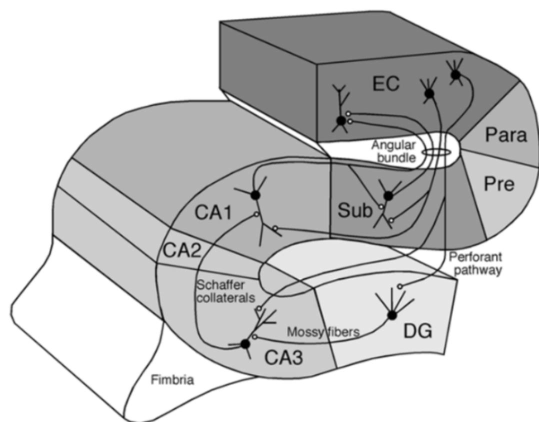

Figure 1.3.2 – The hippocampal trisynaptic circuit. Neurons in layer II of the entorhinal cortex project to the dentate gyrus and the CA3 field of the hippocampus proper via the perforant pathway. Neurons in layer III of the entorhinal cortex project to the CA1 field of the hippocampus and the subiculum via the perforant and alvear pathways (see text for a detailed description). The granule cells of the dentate gyrus project to the CA3 field of the hippocampus via mossy fiber projections. Pyramidal neurons in the CA3 field of the hippocampus project to CA1 via Schaffer collaterals. Pyramidal cells in CA1 project to the subiculum. Both CA1 and the subiculum project back to the deep layers of the entorhinal cortex. Adapted from Andersen et al. (2009).

The DG and CA can be divided into several layers or subfields. The DG is composed of the *stratum granulosum*, which contains the granule cells, and the *stratum moleculare*. The *stratum moleculare* has the proximal dendrites of the granule cells. The axons of the granule cells, called 'mossy fibers', establish synaptic contacts with the pyramidal cells of the CA3 region (Claiborne et al., 1986).

CA subfields are well characterized and are primarily composed of two regions, CA1 and CA3. However, there is some controversy regarding the CA2 and CA4 regions. For example, many consider the CA2 region as a small transitional zone between CA1 and CA3 without real identity, where the two classes of pyramidal neurons of CA1 and CA3 merge (Blaabjerg and Zimmer, 2007; Blackstad, 1956; Swanson et al., 1978; Tole et al., 1997; Zimmer and Haug, 1978). The CA4 region is also controversial; Lorente de Nó (1934) defined the CA4 region as located in the hilar region of the DG (Lorente de Nó, 1934).

CA1 and CA3 have seven layers, named from inside towards outside as *stratum moleculare*, *stratum lacunosum*, *stratum radiatum*, *stratum lucidum*, *stratum pyramidale*, *stratum oriens*, and the alveus. The *stratum pyramidale* is the principal layer of the CA region and contains the cell bodies of the pyramidal cells.

CCK-basket cells and CB_1R-positive basket cells are located in the *stratum lucidum*/*pyramidale* projecting to the pyramidal cells at CA1. A_1R and CB_1R expression have been described in the pyramidal cells and CCK- and CB_1R-positive basket cells at the same terminal, suggesting an interaction between CB_1R and A_1R at the level of G-coupled proteins (Sousa et al., 2011).

An important property of the hippocampus is its synaptic plasticity, the ability to respond to specific patterns of activation with long-lasting increases or decreases in synaptic efficacy. Synaptic plasticity is a critical component of the neural mechanisms underlying learning and memory. There is vast evidence that memory formation depends on a series of molecular steps in the CA1 region of the hippocampus that are similar to those of LTP, in the same region (Morris et al., 1986; Tsien et al., 1996). Thus, LTP has been identified as the main candidate for the cellular player of learning and memory in the CA1 region, as a good therapeutic target (for a review see Takeuchi et al., 2014).

2 Aim

The main goal of this work was to identify the role of endo and synthetic cannabinoids upon neuronal communication in the hippocampus and whether that role could be affected by another endogenous modulator, adenosine. I focused on CB_1R-mediated actions and on adenosine A_1R since these receptors are abundantly expressed in the hippocampus and share the transducing system.

To accomplish this goal, three specific objectives were pursued:

1. To evaluate the influence of the endogenous activation of CB_1R upon synaptic plasticity and identification of the endocannabinoids involved.

2. To identify and characterize a putative interaction between CB_1R and A_1R upon:

   2a) Hippocampal synaptic plasticity at the CA1 hippocampal area.

   2b) Inhibitory synaptic inputs to excitatory neurons at the CA1 hippocampal area

   2c) Short plasticity of inhibitory synaptic transmission to excitatory neurons at the CA1 hippocampal area.

3 Material and Methods:

3.1 Animals

All procedures were conducted following the United Kingdom Home Office Animals (Scientific Procedures) Act (1986), the Institutional Animal Care and Use Committee (IACUC) at Stockholm (Sweden) or Lisbon (Portugal), Portuguese and Swedish legislation on animal care, and the European Community guidelines (Directive 2010/63/EU).

Most experiments were performed using 8–18-week-old (most 9–13-week-old) male C57Bl6/J mice (Charles River Laboratories, Paris). In some cases, male and female mice were used to maximize the use of A_1R knockout mice; because of this, a few control experiments were performed using male and female mice, but no differences were detected between males and females (Figure 4.2.3). The adenosine A_1 receptor knockout ($A_1R^{-}/^{-}$) and wild type ($A_1R^{+}/^{+}$) mice were generated by inactivating the second protein-coding exon of the mouse A_1R gene from heterozygous breeding pairs of C57Bl6/J background strain mice (Johansson et al., 2001), obtained from a breeding colony derived from the original line housed at Karolinska Institute, Sweden, and genotyped as described by Yang et al. (2015). At Oxford, WT males were bought from the local animal facility. All animals were socially housed under standardized conditions of light (12-h light/12-h dark cycle), temperature (22–24 °C), humidity (55–65%), and environmental enrichment (cardboard tubes plus nest material) and had free access to food and tap water.

3.2 Animal Genotyping

Tissue was collected from mice ears and digested overnight in 50 µL of TDB solution (1 M KCl, 1 M Tris-HCl pH=9.0, 10% Triton X-100, 20 ng/mL proteinase K) at 56 °C. Proteinase K was inactivated at 95 °C for 15 min. The samples were stored at −20 °C until use. For polymerase chain reaction (PCR) assays, DreamTaq DNA Polymerase (5 U/µL; Thermo Scientific, Waltham, MA, USA) was used for a final solution consisting of 1 µL of each DNA

sample mixed with 1× Dream Taq Buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM Mix Primers (Reverse:; Forward:), 1.5 U DreamTaq DNA Polymerase (5 U/µL; Thermo Scientific), and water to a final volume of 25 µL. The amplification reaction was based on a first heating cycle at 94 °C for 3 min for enzyme activation, followed by 30 cycles of 30 s each at three discrete temperature steps: denaturation, 95 °C; annealing, 58 °C; extension/elongation, 72 °C; and a final extension at 72 °C for 5 min. PCR products were electrophoresed on 2% agarose gel with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA) at 100 V for approximately 1 h in 1× TAE buffer (40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA).

3.3 Hippocampal slice preparation

In vitro studies of central nervous system function offer some advantages over *in vivo* studies. One advantage is the possibility of studying in detail the cell physiology and pharmacology comparing to *in vivo* models, and reducing the complexity of the nervous system without losing the microcircuit integrity. Many preparations have been developed to study hippocampal function; in this study, we used transversal hippocampal slices. In the 1950s, McIlwain and colleagues demonstrated that it was possible to perform electrophysiological studies in living cortex slices (Li and McIlwain, 1957). Later, it was demonstrated that when 400-µm thick slices are cut perpendicularly to the long axis of the hippocampus, their physiological properties are preserved. The *in vivo* evoked responses were similar to *in vitro* records, indicating that synaptic activity was maintained (Richards and McIlwain, 1967; Skrede and Westgaard, 1971; Yamamoto and McIlwain, 1966). Since then, hippocampal slices have been used as an experimental model in numerous studies.

3.3.1 Field recordings

Mice were sacrificed by decapitation under deep isoflurane anaesthesia following Portuguese law on animal care and the European Community guidelines (86/609/EEC). The hippocampus was dissected free within ice-cold artificial cerebrospinal fluid (aCSF) solution composed of (mM) NaCl 124, KCl 3, $NaHCO_3$ 26, Na_2HPO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400-µm thick) were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for 1 h in a resting chamber filled with the same solution at room temperature and continuously gassed (Diogenes, 2004).

3.3.2 Patch-clamp

Mice were anaesthetised with an intraperitoneal injection of pentobarbitone sodium (20% w/v, dosage ± 0.2 mg/g; Pharmasol, Andover, UK). All animals were sacrificed by decapitation when the breathing had slowed down, and the response to stimulation of the limb withdrawal reflex had ceased. After decapitation, the brain was rapidly removed and placed in 4 °C oxygenated Krebs solution (in mM): sucrose 75, NaCl 87, KCl 2.5, $CaCl_2$ 0.5, $MgCl_2$ 7, NaH_2PO_4 1.0, $NaHCO_3$ 25, glucose 25 (pH 7.4). The frontal cortex and cerebellum were cut perpendicularly. The brain was mounted (cerebellum side) onto the vibratome specimen disc using superglue, orienting the sample such that the cortex faces the razor blade with a supporting piece of agar behind the brain to provide structural support during the slicing (Figure 3.3.1).

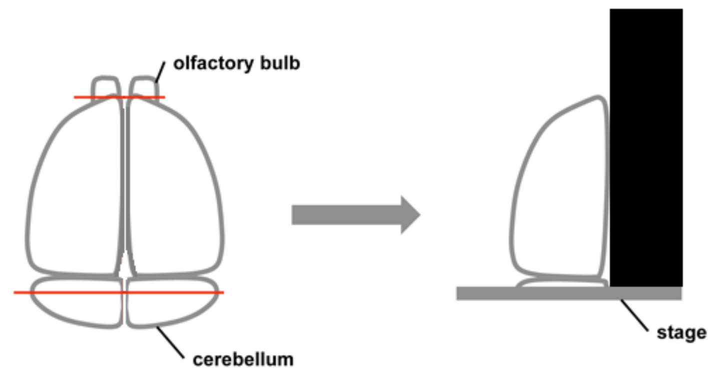

Figure 3.3.1 - Schematic diagrams of a mouse brain preparation. It was adapted from Japan Consortium for Glycobiology and Glycotechnology Database.

The two hemispheres were cut simultaneously. Transverse slices were obtained using a vibratome (Leica VT 1000S; Leica Microsystems, Germany or Microm HM650V, Carl Zeiss, UK) and the thickness was adjusted according to requirements: 250 to 300 µm-thick slices for experiments. Once cut, all slices were trimmed and immersed (Figure 3.3.2) in oxygenated cutting solution at 35 °C for 20–25 min to allow metabolic recovery. Following recovery, slices were transferred to an interface storage chamber containing oxygenated aCSF at room temperature (20–25 °C). Slices were stored for at least 60 min before starting experiments. The patch solution composition was (mM): 119 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 10 glucose pH 7.4.

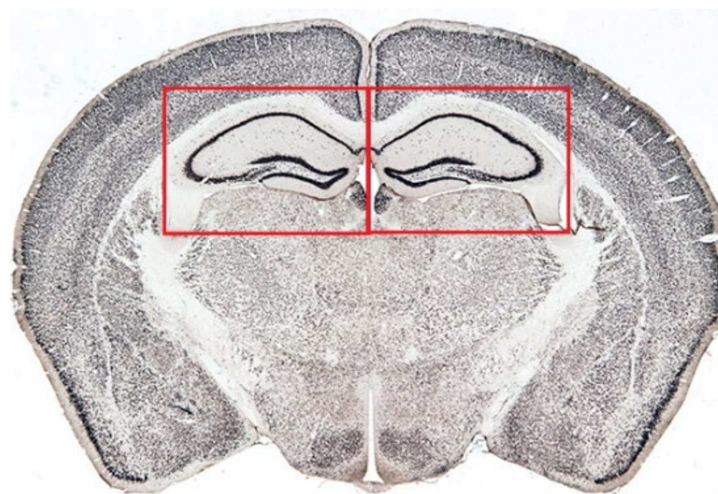

Figure 3.3.2 - Brain transversal cut and trimmed areas for patch-clamp. It was adapted from Melbourne Brain Centre.

3.4 Drugs

Table 1 - Drugs used in the experimental work

Drug	Chemical Name	Supplier	Biological activity	Stock solution [mM]	Final Concentration [µM]	K_i and/or IC_{50}
AM-251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide	Tocris Bioscience, Bristol, UK	CB_1R inverse agonist	10 mM in DMSO	1 µM	7.49 nM; 8 nM (Lan et al., 1999)
WIN 55,212-2 mesylate	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate	Tocris Bioscience, Bristol, UK	CB_1R agonist	10 mM in DMSO	0.5 and 1 µM	62.3 nM (Kuster JE, Stevenson JI, Ward SJ, D'Ambra TE, 1993)
SR 141716A	N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride	Tocris Bioscience, Bristol, UK	CB_1R antagonist	10 mM in DMSO	1 µM	1.98 nM (Rinaldi-Carmona et al., 1994);
Tetrahydr-olipstatin or Orlistat	N-Formyl-L-leucine (1S)-1-[[(2S,3S)-3-hexyl-4-oxo-2-oxetanyl]methyl]dodecyl ester	Tocris Bioscience, Bristol, UK	Diacylglyce--rol lipase inhibitor	10 mM in DMSO	10 µM	0.06 µM (Bisogno et al., 2006)
URB597	(3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate	Caymon Chemicals	inhibitor of fatty acid amide hydrolase (FAAH)	10 mM in DMSO	1 µM	4.6 nM (Kathuria et al., 2003)
JZL 184	4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester	Tocris	Monoacylglycerol Lipase Inhibitor (MAGL)	10 mM in DMSO	1 µM	8 nM (Long et al., 2009a)
JZL 195	4-[(3-Phenoxyphenyl)methyl]-1-piperazinecarboxylic acid 4-nitrophenyl ester	Tocris	Dual inhibitor of FAAH and MAGL	10 mM in DMSO	1 µM	2 and 4 nM respectively (Long et al., 2009b)
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine	Tocris Bioscience, Bristol, UK	A1 adenosine receptor antagonist	5 mM in DMSO	50 nM	3.9 nM (Bruns et al., 1987)
Picrotoxin		Tocris Bioscience, Bristol, UK	$GABA_A$ receptor antagonist	50 mM in DMSO	50 µM	0.37 µM (Mehta and Ticku, 2013)
NBQX disodium salt	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt	Tocris Bioscience, Bristol, UK	AMPA/ KA receptor antagonist	100 mM in H_2O	25 µM	0.15 µM, 4.8 µM (Sheardown et al., 1990)
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid	Tocris Bioscience, Bristol, UK	NMDA receptor antagonist	100 mM in H_2O	100 µM	1.4 µM (Evans et al., 1982)
Sodium fluorocitrate		Sigma-Aldrich	selective astrocyte metabolism reducer	2.33 mM in H_2O	200 µM	

THL, URB597, JZL 184, JZL 195 and NBQX disodium salt values are IC_{50}

Sodium fluorocitrate solution was prepared as described by Paulsen et al. (1987). Briefly, 8 mg of the barium salt of DL-fluorocitric acid (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1 M HCl, precipitated by the addition of 0.1 M Na_2SO_4, buffered with 0.1 mM Na_2HPO_4 and centrifuged at 1000 *g* for 5 min. The supernatant containing fluorocitrate was added to the aCSF solution at a final concentration of 200 µM (pH 7.4).

3.5 Electrophysiological recordings

In the late 18th century, Luigi Galvani described the first evidence that the nervous system is linked to electrical activity, creating the term electrophysiology, which is defined as the study of electrical properties of biological cells and tissues. Over the last 200 years, generations of investigators have invested immense effort into building instruments capable of measuring and controlling this electrical activity in different organs, like the heart and the brain. Researchers were then able to measure the voltage and electric current on a wide variety of scales from cell populations to a single ion channel in a single neuron, enhancing our understanding of nervous system function from the molecular to the behavioural level. In this thesis, two different electrophysiological techniques were used: field extracellular and patch-clamp recordings.

The extracellular recording is an electrophysiological technique that uses an electrode inserted into living tissue to measure electrical activity coming from adjacent cells, usually neurons. Nevertheless, several contributions were made by numerous researchers during the 20th century before the development of modern extracellular recording. Gasser and Newcomer (1921) were the first to apply valve amplifiers to nerve action potentials, and to display them on a cathode-ray oscilloscope (Gasser and Newcomer, 1921). In 1939, Hodgkin recorded the action potential of the giant nerve fiber of the squid from an electrode inserted into the fiber. Further research was interrupted by World War II, but in 1952 Hodgkin and A.F. Huxley used the intracellular recording from giant squid fibers to establish a thorough understanding of the electrical and ionic basis of the nerve impulse (Hodgkin and Huxley, 1952).

The patch-clamp technique was developed by Neher and Sakmann (Sakmann and Neher, 1976, 1984) between the 1970s and 1980s and enables the study of single or multiple ion channels in a variety of cell types such as neurons, muscle fibers, and cardiomyocytes. There are different approaches to the patch-clamp recording: inside-out, whole-cell, outside-out, perforated, and cell-attached patch (Figure 3.5.1). Conventional intracellular recording involves impaling a cell with a fine electrode; a patch-clamp microelectrode is a micropipette with a relatively large tip diameter. The microelectrode is placed next to the cell, and gentle suction is applied through the microelectrode to draw a piece of the cell membrane (the 'patch') into the microelectrode tip; the glass tip forms a high-resistance 'seal' with the cell membrane. This configuration is called 'cell-attached' mode, and it can be used for studying the activity of the ion channels in the membrane patch. If more suction is applied, the small patch of the membrane in the electrode tip can be displaced, leaving the electrode sealed to the rest of the cell. This 'whole-cell' mode allows a very stable intracellular recording. Compared to conventional intracellular recording with sharp electrodes, a downside is that the intracellular fluid of the cell mixes with the solution inside the recording electrode, which may cause some important components of the intracellular fluid to be diluted. A variant of this technique, the 'perforated patch' technique, tries to minimize these problems. Instead of applying suction to displace the membrane patch from the electrode tip, it is also possible to make small holes on the patch with pore-forming agents so that large molecules such as proteins can stay inside the cell and ions can pass through the holes freely. In addition, the membrane patch can be pulled away from the rest of the cell. This approach enables the membrane properties of the patch to be analysed pharmacologically.

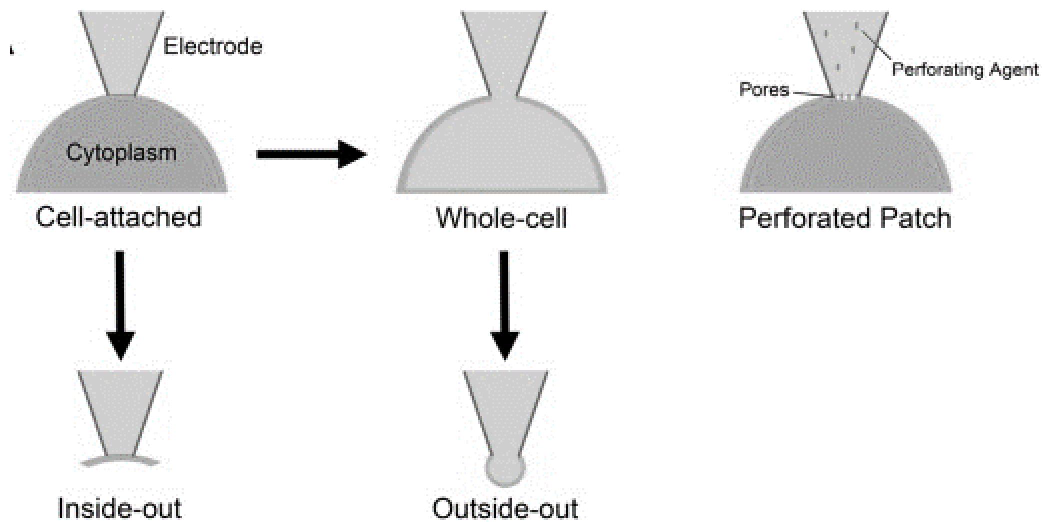

Figure 3.5.1 – Patch-clamp types. The pipette lumen and cytoplasm are represented by blue and orange, respectively, adapted from(Accardi et al., 2016).

3.5.1 Extracellular recordings

For electrophysiological recordings of field post-synaptic potentials (fEPSP), individual slices were transferred into a 1-mL recording chamber and continually superfused with gassed Krebs solution at a constant flow (3 mL/min) and temperature (32 °C). Stimulation (rectangular 0.1 ms pulses, once every 20 s) was delivered through a concentric electrode placed on Schaffer collateral-commissural fibers, in the stratum radiatum near the CA3-CA1 border. The intensity of the stimulus was set to the one eliciting near 50% of the maximal response and was maintained throughout the experiment. The recording was done through a microelectrode filled with 4 M NaCl (2–6 MΩ resistance), placed in the CA1 stratum radiatum, coupled to an Axoclamp 2B amplifier (Axon Instruments, Sunnyvale, CA, USA), and digitized using a BNC-2110 connector block (National Instruments, Austin, TX, USA). Individual responses were monitored, and averages of six (LTP protocols) or eight (input-output curves) consecutive responses were continuously stored on a personal computer using the WinLTP software (Anderson and Collingridge, 2001).

3.5.1.1 Basal synaptic transmission

fEPSPs were continuously recorded under basal stimulation frequencies, and drug-induced alteration in synaptic transmission was evaluated as the percentage change in the average slope of the fEPSP in the presence of the drug (at least 30 min after addition) in comparison to the average slope of the fEPSP measured during the 10 min before its addition.

3.5.1.2 Input/output curves

The input-output curves were performed after a stabilization period under the standard stimulation conditions; the stimulus intensity was increased by 20 µA every 6 min from 80 to 300 µA.

3.5.1.3 Long-term potentiation (LTP)

LTP control was induced only after obtaining stable fEPSP slope values for a minimum of 15 min. Test drugs were added to the perfusing aCSF at least 30 min before LTP induction, with LTP also being induced only after a stable baseline period of at least 15 min in the presence of the drugs. LTP was induced by theta(θ)-burst stimulation. Two different stimulation protocols were used in different experiments, namely **weak θ-burst** and **strong θ-burst** stimulation, which differed only in the number of trains delivered. The weak θ-burst protocol consisted of five trains, whereas the strong θ-burst was composed of 10 trains, and in both cases, the stimulation trains were separated by 200 ms. In both protocols, each train consisted of four stimuli delivered at 100 Hz. LTP magnitude was quantified as the % change in the average fEPSP slopes recorded from min 50 to 60 after LTP induction, with 0% magnitude defined as the averaged fEPSP slope recorded in the 10 min immediately before LTP induction.

3.5.2 Patch Clamp recordings

Slices in a submerged recording chamber (Luigs and Neumann) mounted on the stage of a BX51WI microscope (Olympus, Tokyo, Japan) were visualized using a 20× immersion objective in zoom 2 and 4 (i.e., up to 80× magnification) and DIC-IR in combination with a SensiCam CCD camera (PCO imaging, Kelheim, Germany). Slices were continuously superfused with aCSF patch solution at 5 mL/min and oxygenated with 95% O_2/5% CO_2 in a closed pump-driven circuit (Watson-Marlow, Falmouth, UK) at 32 °C.

Data were recorded with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA); recordings were low-pass filtered at 2 kHz using the built-in Bessel filter, digitized at 10 kHz with a Digidata 1400, and acquired with Clampex software version 10.2 (Molecular Devices). Patch pipettes (4–9 MΩ) were pulled from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter; Harvard Apparatus, Holliston, MA, USA) with a P-97 Puller (Sutter Instrument Co., Novato, CA, USA). Intracellular solution composition was (mM): KCl 135, HEPES 10, Na-ATP 2, Na-GTP 0.2, $MgCl_2$ 2, EGTA 0.1; with 285 mOsm, pH 7.4.

3.5.2.1 Inhibitory postsynaptic potential currents (IPSCs)

For the IPSCs experiments, 5 mM QX-314 (Tocris Bioscience, Bristol, UK) was added to block the firing of action potentials from the pyramidal cells. IPSCs were evoked as previously described by Rombo et al. (2016). Rectangular pulse stimuli (0.067 Hz, 1–15 µA) were delivered via bipolar concentric tungsten electrodes in or near the CA1 *stratum pyramidale*, 80–120 µm from the recorded cell. Recordings were performed in the continuous presence of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate (KA) receptor antagonists (50 µM DL-AP5 and 10 µM CNQX, respectively). Series resistance was not compensated for during voltage-clamp recordings but was regularly monitored throughout each experiment with a −5 mV, 50 ms pulse, and cells with >20% change in series resistance were excluded from the analysis. The amplitude of eight consecutive currents was averaged and the 5-min period

immediately before the application of the tested drug was considered as baseline. For statistical purposes, the drug effect was evaluated between min 8 to 10 after drug perfusion.

3.5.2.2 Depolarization-induced suppression of inhibition (DSI)

DSI tests were performed with two protocols. In protocol 1, DSI was performed every 90 seconds and consisted of 10 stimuli at 0.67 Hz with depolarisation from −60 mV to 0 mV after the 5th stimulus (Figure 3.5.2). DSI was calculated using the mean of the five IPSCs immediately before depolarisation (baseline) and the two IPSCs immediately after depolarisation (test): DSI (%) = 100 × (1 − (test /baseline)). DSI protocol 2 was designed to improve temporal resolution. DSI was performed every 120 s, consisted of 30 stimuli at 0.33 Hz with depolarisation from −60 mV to 0 mV for 5 s after the 13th stimulus (Figure 3.5.3). DSI was calculated using the mean of the 13 IPSCs immediately before depolarisation (baseline) and the three IPSCs immediately after depolarisation (test): DSI (%) = 100x (1 − (test /baseline)). It is thus possible to obtain small negative values for DSI because of statistical noise, especially when the measurement is based on a small number of trials in unitary connections that have high variability in IPSC amplitude.

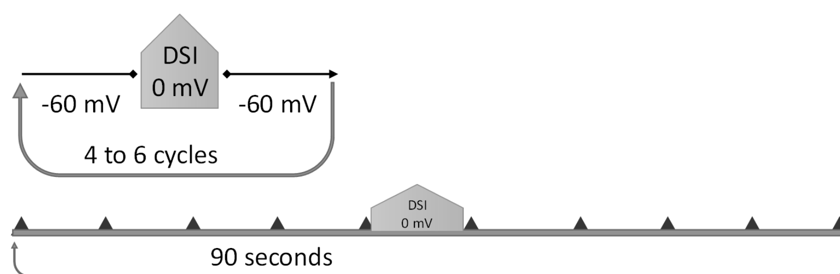

Figure 3.5.2 - Schematic diagrams of DSI protocol 1 The orange triangle represents the 5-s cell depolarization (from −60 mV to 0 mV) and occurs after the 5th stimulus, inducing the suppression of inhibitory postsynaptic potential current (IPSC). The stimuli are represented as red triangles along a 90-s cycle repeated at least four times.

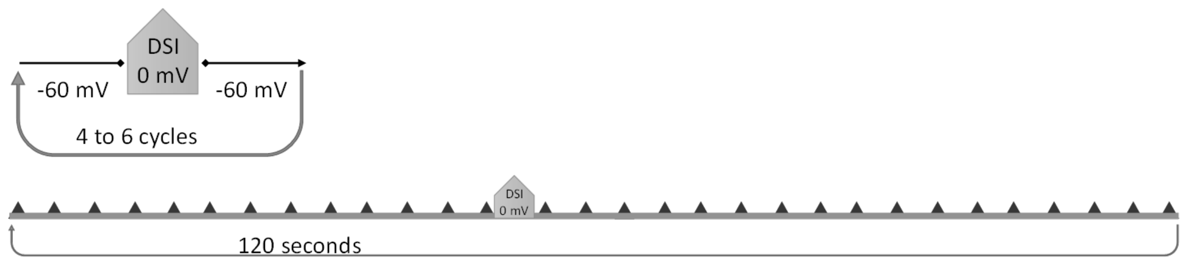

Figure 3.5.3 - Schematic diagrams of DSI protocol 2. The orange triangle represents the 5-s cell depolarization (from -60 mV to 0 mV) and occurs after the 13th stimulus, inducing the suppression of inhibitory postsynaptic potential current (IPSC). The stimuli are represented as red triangles along a 120-s cycle repeated at least four times.

3.6 Statistical analysis

3.6.1 Extracellular recordings

Data are expressed as the mean ± SEM; n corresponds to the number of experiments; in each experiment, only one slice was used per drug condition. At least one drug condition and the corresponding control were tested on each day of the experiment. Statistical significance was assessed by two-tailed Student's t-test when comparing two groups or by one-way analysis of variance (ANOVA) followed by Sidak's posthoc test when comparing multiple experimental groups. A p-value <0.05 was considered significant. All analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA).

3.6.2 Patch-clamp recordings

Data are expressed as the mean ± SEM of n cells from different slices. Statistical significance was assessed either by two-tailed Student's t-test when comparing two groups. A p-value <0.05 was considered significant. All analyses were performed using GraphPad Prism Software (GraphPad Software).

4 Results

4.1 Hippocampal synaptic plasticity modulated by cannabinoid CB_1 receptors

Part of the work presented in this Chapter was published in:

Silva-Cruz A, Carlström M, Ribeiro JA, Sebastiao AM (2017). Dual influence of endocannabinoids on Long-Term potentiation of synaptic transmission. Front. Pharmacol. 8. doi:10.3389/fphar.2017.00921.

4.1.1 Rationale

The impact of marijuana upon human cognition is due mainly because the weed disrupts the action of eCBs in the brain. eCBs are widely recognized as fine-tuning modulators of synaptic activity, their action mainly resulting from activation of G-protein-coupled CB_1R, which are widely distributed in the central nervous system, in particular in the hippocampus, cortex, basal ganglia, and cerebellum (Wilson et al., 2012). CB_1R are located both in excitatory and inhibitory neurons (Hoffman et al., 2010; Katona et al., 2001; Kawamura, 2006; Wilson et al., 2001) and also in astrocytes (Navarrete and Araque, 2008). CB_1R is endogenously activated by eCBs, mainly the fatty acid derived 2-AG and anandamide. eCB synthesis mostly results from cleavage of postsynaptic membrane lipids as a consequence of the activation of postsynaptic glutamate metabotropic receptors, which are predominantly activated as a result of high neuronal firing (Katona et al., 2006; Chevaleyre et al., 2006). eCBs thus travel in a retrograde manner to activate astrocytic and nerve-terminal-located CB_1R, resulting in inhibition of neurotransmitter release, and giving rise to several forms of short-term synaptic plasticity (Chevaleyre et al., 2006; Freund et al., 2003; Kano et al., 2009; Ohno-Shosaku et al., 2012). While the inhibitory action of eCBs upon neurotransmitter release is well established, their action upon synaptic plasticity induced by brief high-frequency neuronal firing, e.g., long-term potentiation, is highly

controversial. In fact, and considering only the hippocampus, a brain area important for memory encoding and the one most used to study LTP phenomena, there are reports showing that eCBs restrict LTP (Bohme et al., 1999; Slanina et al., 2005) while others show that they facilitate it (Carlson et al., 2002; De Oliveira Alvares et al., 2006). This controversy is intriguing since LTP is a compelling cellular model for learning and memory (see Nicoll, 2017), and exogenous cannabinoids, including the phytocannabinoids in marijuana and the synthetic agonists of CB_1R, harm learning and memory processes both in humans and in laboratory animals (Lane et al., 2005; Miller et al., 1977; Sousa et al., 2011). Elegant studies aimed at determining the influence of eCBs upon LTP in different cell types and circuits in the hippocampus show that the action of eCBs may vary according to the cell type where the CB_1Rs are located (Monory et al., 2015) and the hippocampal circuit where LTP is induced Wang et al. (2016). Because eCBs are formed as a result of neuronal activity, it was hypothesized that the influence of eCBs on LTP could also vary as a function of the pattern of neuronal firing that induces plasticity. Evidence of that association would not only further our understanding of the reasons for the conflicting data in the literature but also provide better insight into the subtleties of eCB use to control synaptic strengthening. This study aimed to determine the influence of eCBs on hippocampal LTP induced by a weak or a strong θ-burst train of stimulation in mice.

4.1.2 Endocannabinoids reduce LTP induced by a weak theta-burst stimulation (wTBS) protocol

The first series of experiments was designed to evaluate the influence of eCBs upon weakly induced LTP. The influence of eCBs was assessed by testing the effects of drugs that block CB_1R activation by eCBs or the synthesis of eCBs.

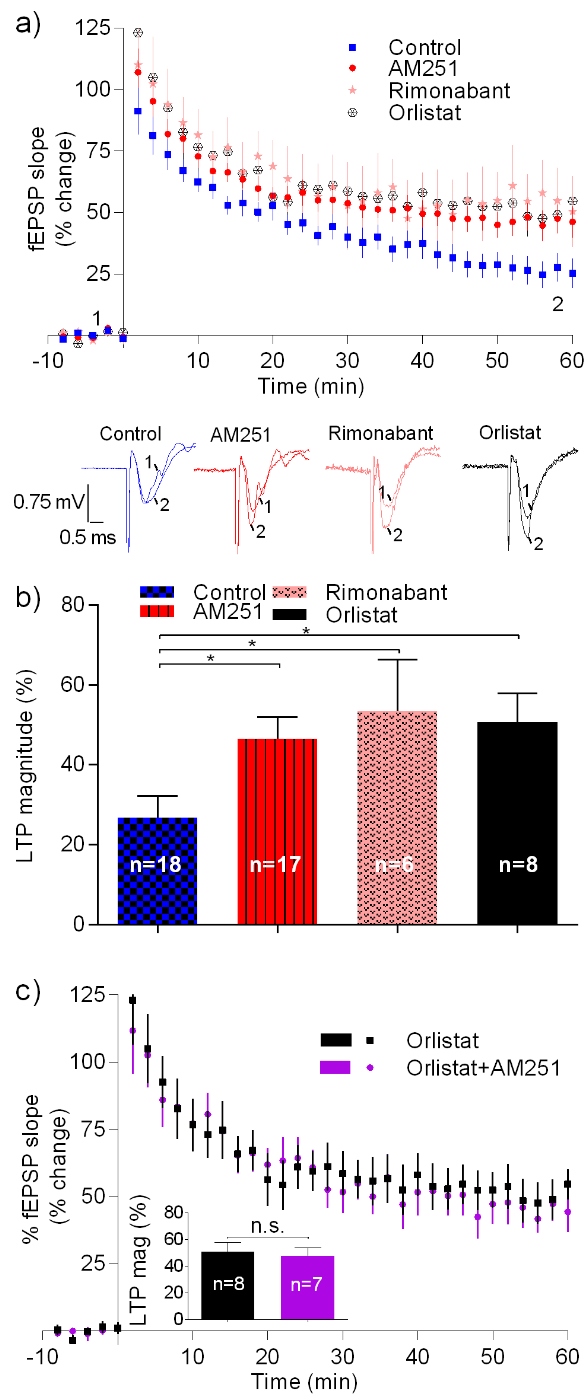

Figure 4.1.1 – Endocannabinoids inhibit weak-θ-burst-induced LTP stimulation protocol (five trains of 100 Hz, four stimuli separated by 200 ms). a) Time course of the averaged fEPSP slopes under control conditions (no drugs) or in the presence of 1 µM of AM251 (CB_1R inverse agonist), 1 µM rimonabant (CB_1R antagonist), or 10 µM orlistat (a fatty acid synthesis inhibitor). Data are expressed as % of the averaged fEPSP slope recorded for 10 min before LTP induction. Original traces taken from individual representative experiments and recorded at baseline (1) and 50–60 min after weak θ-burst induction (2) are shown below the time course panel. Each trace is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. **b)** Quantification of LTP magnitude under the drug conditions is indicated. LTP magnitude was quantified as the % increase in fEPSPs slope recorded at min 50–60 after LTP induction compared to the value recorded during the 10 min immediately before LTP induction. **c)** Non-additivity of the facilitating action of AM251 and orlistat when combined. Data are expressed as the time course of fEPSP slopes, and the inset shows average LTP magnitude (LTP mag) in the two conditions, the color of the bars corresponding to the color of the symbols in the time course. All values are mean ± standard error of the mean (SEM) from n experiments; n values are indicated inside the bars. *$P < 0.05$ (one-way ANOVA with Sidak's posthoc test correction); ns: $P>0.05$ (Student's t-test).

In the control slices, fEPSP slopes recorded 50–60 min after induction of LTP with a weak θ-burst stimulation were 26.7% ± 5.5% higher than before LTP induction (n=18; Figure 4.1.1b). In slices where the CB_1R inverse agonist AM251 (1 µM) was added to the perfusion at least 30 min before LTP induction, the magnitude of LTP was 46.5% ± 5.4% (n=17, $p < 0.05$ vs control, Figure 4.1.1b), which corresponds to a near 80% increase in LTP magnitude. A similar result was obtained in the presence of another CB_1R blocker, the selective CB_1R antagonist rimonabant (1 µM): LTP magnitude was 47.8% ± 12.2% (n=6, $p < 0.05$ vs control, Figure 4.1.1b). In the presence of orlistat (10 µM), an inhibitor of diacylglycerol (DAG) lipase, the enzyme responsible for the conversion of DAG into 2-AG, the magnitude of LTP was also enhanced by 50.7% ± 7.2% (n=8, $p < 0.05$; Figure 4.1.1b). Importantly, when both CB_1R activation and 2-AG synthesis were blocked together by the simultaneous presence of AM251 (1 µM) and orlistat (10 µM), the magnitude of LTP was enhanced at the same degree as with each drug alone. This lack of additive effect indicates that both drugs facilitate LTP due to their shared ability to block eCB signalling.

In summary, these findings show that either CB_1R blockade by endocannabinoids or inhibition of eCB synthesis results in marked facilitation of weak-θ-burst-induced LTP, thus suggesting that eCBs inhibit such form of LTP.

4.1.3 Endocannabinoids enhance LTP with a strong theta-burst stimulation (sTBS) protocol

Next, the influence of eCBs on LTP induced by a strong θ-burst stimulation (sTBS) protocol was evaluated. All other experimental conditions being similar to those used with the weak θ-burst.

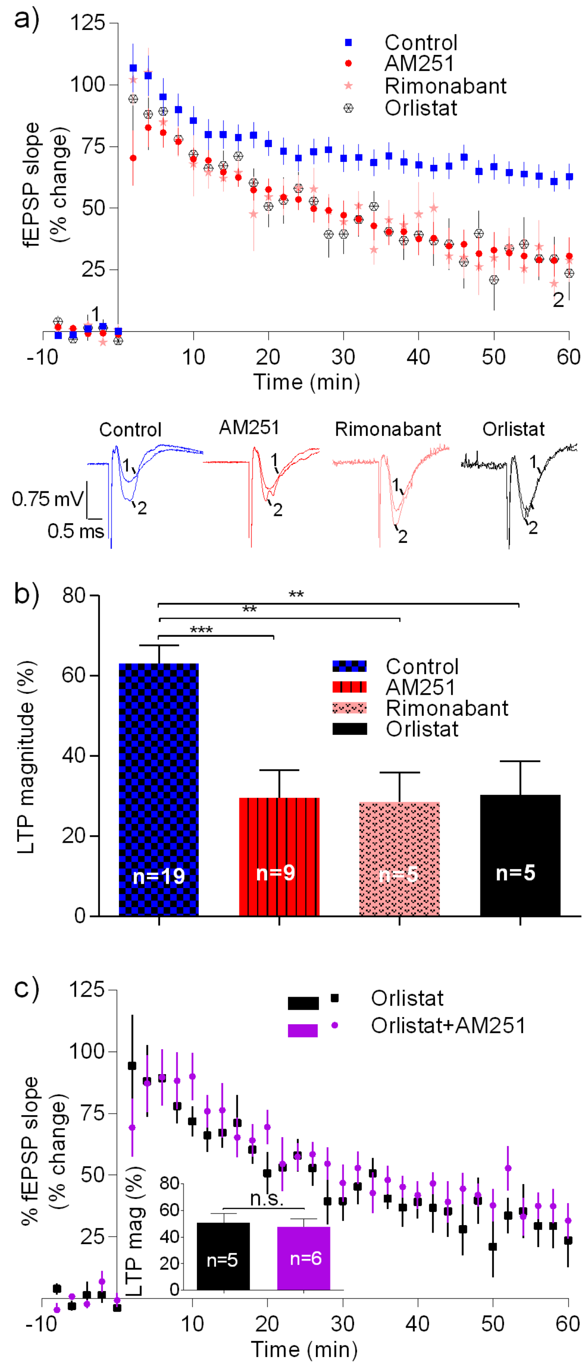

Figure 4.1.2 – Endocannabinoids enhance LTP induced by a strong θ-burst stimulation (sTBS) protocol (10 trains of 100 Hz, four stimuli separated by 200 ms). a) Time course of the averaged fEPSP slopes under control conditions (no drugs) or in the presence of 1 μM of AM251 (CB_1R inverse agonist), 1 μM rimonabant (CB_1R antagonist), or 10 μM orlistat (a fatty acid synthesis inhibitor). **b)** Quantification of LTP magnitude under the drug conditions indicated. **c)** Non-additive effect of the inhibitory effect of AM251 and orlistat when combined. **P < 0.01; ***P < 0.001 (one-way ANOVA with Sidak's correction). For further details, see legend to Figure 4.1.1.

Under control conditions, LTP magnitude was 63.1% ± 4.5% (n=19) higher 50–60 min after strong θ-burst stimulation than at pre-θ-burst stimulation. LTP magnitude decreased to roughly 30% in the presence of AM251 (29.6% ± 6.8%, n=9, $p < 0.001$; Figure 4.1.2b) or rimonabant (28.5% ± 7.4, n=5, $p < 0.01$; Figure 4.1.2b). In the presence of orlistat, the LTP magnitude also decreased to similar values (30.3% ± 8.4%, n=5, $p < 0.01$; Figure 4.1.2b). It is noteworthy that the effect of AM251 was not additive to that of orlistat regarding inhibition of LTP induced by a strong θ-burst.

When both drugs were combined, LTP magnitude was 38.5% ± 6.4% (n=6), a significantly lower value ($p < 0.05$) compared to that recorded under control conditions but of similar magnitude to that obtained in the presence of each drug separately (Figure 4.1.2bc). Likewise, the wTBS protocol, this result suggests that the ability of AM251 and orlistat to inhibit strong θ-burst-induced LTP results from their shared ability to block eCB signalling.

In summary, these results show that either CB_1R blockade by eCBs or inhibition of eCB synthesis inhibits strong θ-burst-induced LTP. Also, in contrast to weak θ-burst-induced LTP, LTP induced by a **strong θ-burst** is facilitated by eCBs.

4.1.4 LTP is diminished by CB_1R activation with exogenous cannabinoids

The approach described in the previous sections was directed towards the effects of blocking CB_1R activation by eCBs. Based on what is currently known about the inhibitory action of cannabinoids on neuronal activity, it was unexpectedly finding that strong θ-burst-induced LTP is reduced if CB_1R activation by eCBs is blocked. It was decided to assess how this form of LTP is affected by overactivation of CB_1R using two approaches: 1) by assessing the effect of a CB_1R agonist and 2) by exogenously activating CB_1R in a sustained manner.

To evaluate CB_1R-mediated effects, CB_1R agonist WIN (500 nm) was tested with AM251. As it is known that the effect of WIN on the synaptic transmission is delayed (Serpa et al. 2009), slices were preincubated with WIN for at least 60 min before transfer to the acquisition chamber. Slices were then stabilized for at least 20 min, with LTP being only induced when fEPSP slope values remained stable for at least 15 min. In these experiments, LTP was virtually abolished (LTP magnitude: 5.5% ± 10.5%, n=7, p>0.05 vs. pre-LTP induction; p < **** vs. LTP magnitude under control conditions; Figure 4.1.3b). However, the inhibitory effect of WIN was blocked when the slices had been preincubated with AM251. In fact, under such conditions, the inhibitory effects of both the agonist and the antagonists seem to annul each other since LTP magnitude recorded in slices incubated with both AM251 and WIN (55.2% ± 11.2%, n=7, Figure 4.1.3b) was similar (p>0.05) to that recorded under control conditions.

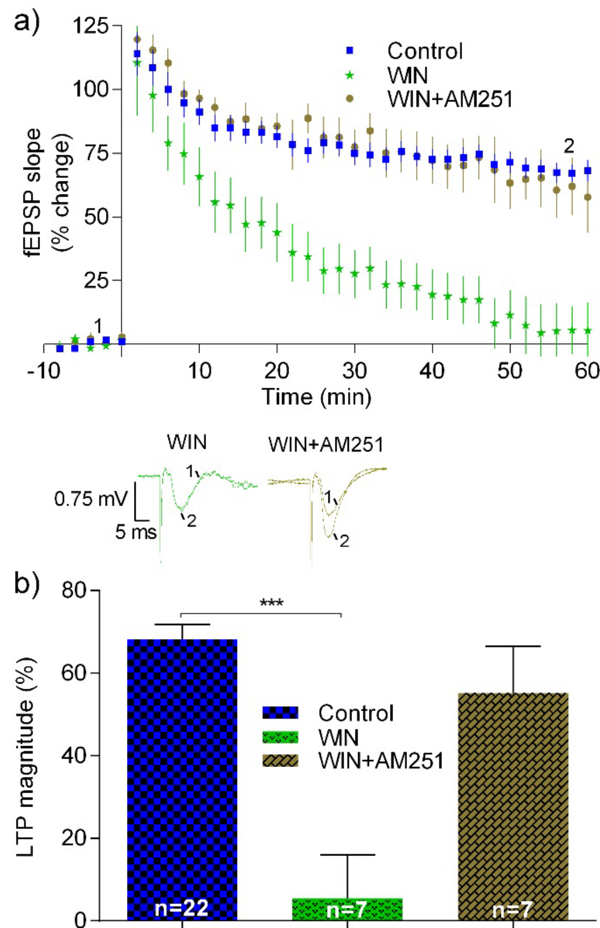

Figure 4.1.3 – Strong θ-burst-induced LTP is inhibited when CB₁R is overactivated by exogenous cannabinoids, and the effect is reverted when CB₁R is blocked. a) Time course of the averaged fEPSP slopes under control conditions (no drugs) or in the presence of 500 nM WIN55,212-2 (WIN, CB₁R agonist) or 1 µM AM251 together with 500 nM WIN. **b)** Quantification of LTP magnitude under the drug conditions indicated; control data in panels a) and b) are the same as those shown in Figure 4.1.2a and 2b, but are displayed here to allow comparison with the drug conditions. ***$p < 0.001$ (one-way ANOVA with Sidak's correction). For further details, see the legend of Figure 4.1.1.

In summary, these results suggest that sustained overactivation of CB₁R induced by an exogenous agonist leads to inhibition of the LTP induced by a **strong θ-burst**. This effect is reverted when CB₁R is blocked by AM251.

4.1.5 LTP is modulated differently by endogenous 2-AG and anandamide

The approach described in the previous sections was directed towards the effects of CB_1R activation by exogenous cannabinoids. Based on what is currently known about the inhibitory action of cannabinoids on neuronal activity, our finding that strong θ-burst-induced LTP is reduced by blocking CB_1R activation was unexpected. It was decided to assess how this form of LTP is affected by endogenous overactivation of CB_1Rs in animal populations. I tested the influence of inhibitors of eCB hydrolysis and thus replicated the effects of sustained enhanced levels of eCBs. Data shown in Figure 4.1.4 summarise the findings using inhibitors of enzymes that block the hydrolysis of eCBs in wild type mice. Besides, several pharmacological drugs were used to evaluate the endogenous increase of both endocannabinoids (2-AG and anandamide) blocking degradation via FAAH and MAGL, separately and together.

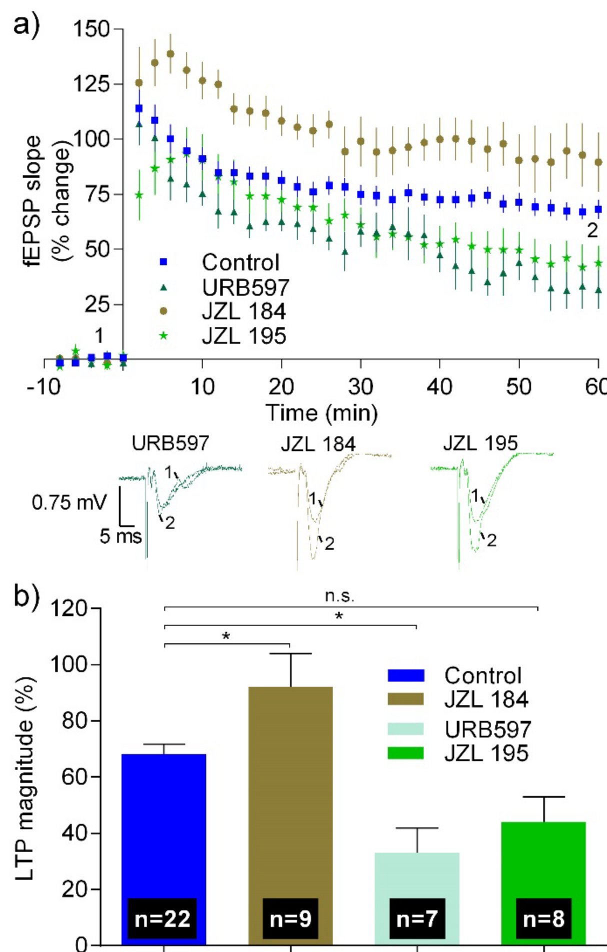

Figure 4.1.4 – Strong θ-burst-induced LTP is enhanced when endogenous 2-AG concentration increases and inhibited when endogenous anandamide concentration increases. a) Time course of the averaged fEPSP slopes under control conditions (no drugs) or in the presence of 1 μM URB 597 (FAAH inhibitor), 1 μM JZL 184 (MAGL inhibitor), or 1 μM JZL 195 (inhibitor of both FAAH and MAGL). **b)** Quantification of LTP magnitude under the drug conditions indicated; control data in panels a) and b) are the same as those shown in Figure 4.1.2a and 2b, but are displayed here to allow comparison with the drug conditions. *$p < 0.05$ (one-way ANOVA with Sidak's correction). For further details, see the legend of Figure 4.1.1.

When using JZL 184 (1 μM), a selective inhibitor of MAGL, the enzyme that hydrolyzes 2-AG, the magnitude of LTP was enhanced by 92.3 % ± 11.2% (n=9, t=2.6, p<0.05 vs control conditions, Figure 4.1.4b), corresponding roughly to a 40% increase in LTP magnitude compared to control conditions.

As a FAAH inhibitor, URB 597 (1 μM) was used. Interestingly, in the presence of URB 597, LTP magnitude increased by only 33.3% ± 8.6% (n=7, t=3.4, $p < 0.05$; Figure 4.1.4b), nearly half of the value recorded under control conditions.

In the presence of JZL 195 (1 μM), a powerful and selective inhibitor of both FAAH and MAGL, LTP magnitude reached an intermediate value (44.2% ± 8.8%, n=8, Figure 4.1.4b) between the values recorded with URB 597 and in the absence of any drug. This was not significantly different (t=2.4, p>0.05) in either condition.

The effect of CB_1R blocker AM251 was null for JZL 184 and JZL 195, corresponding to LTP magnitude of 85.4% ± 15.4% (n=5, Figure 4.1.5b) and 42.3%, for JZL 184 and JZL 195 in the presence of AM251. respectively. This is similar to the magnitude obtained without AM251. However, in the presence of URB597, LTP magnitude was slightly rescued from 33.3% ± 8.6% to 39.9% ± 3.6% (n=6, Figure 4.1.5b).

In summary, these data suggest that sustained overactivation of CB_1R induced by blockade of eCBs degradation leads to a dual effect on strong θ-burst-induced LTP. This finding suggests that enhancing the levels of the predominant eCB in the hippocampus, 2-AG (Piyanova et al., 2015) facilitates LTP induced by strong θ-burst stimulation. However, LTP was inhibited following an accumulation of anandamide, another endogenous cannabinoid.

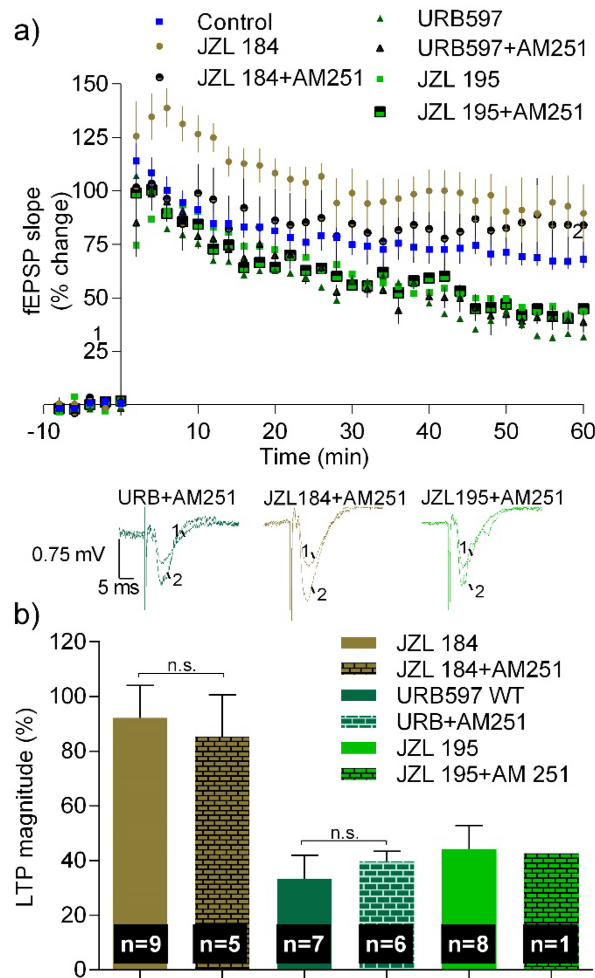

Figure 4.1.5 – AM251 does not prevent the increase in the effect of endocannabinoids on LTP. a) Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in the presence of 1 µM JZL 184 (inhibitor of MAGL, the enzyme that hydrolyzes 2-AG), 1 µM URB597 (inhibitor of FAAH, the enzyme that hydrolyzes anandamide), or 1 µM JZL 195 (inhibitor of both FAAH and MAGL) in combination with CB_1 receptor blocker. **b)** Quantification of LTP magnitude under the drug conditions indicated. n.s.: not significant; $F_{(3, 23)} = 8.3$. For further details see legend to Figure 4.1.1.

4.1.6 Astrocytes do not contribute to the enhancement of LTP caused by eCBs

Astrocytes are known to contribute to the facilitating action of eCBs on glutamatergic transmission (Navarrete and Araque, 2010). It was hypothesized that the apparent facilitating action of eCBs on strong θ-burst-induced LTP would involve the astrocytes. To address this possibility, slices were incubated with the metabolic poison gliotoxin

fluorocitrate (200 μM) for at least 20 min and allowed the fEPSP slopes to stabilize for at least 15 min before inducing LTP either in the presence or absence of AM251.

As expected from previous reports (Bonansco et al., 2011), LTP magnitude was reduced in slices incubated with fluorocitrate (cf. data in Figure 4.1.6 with Figure 4.1.2). Remarkably, however, under these conditions the CB_1R inverse agonist, AM251, was still able to significantly inhibit LTP (fluorocitrate: 43.0% ± 13.5%, n=7; fluorocitrate +AM251: 7.9% ± 15.8%, n=9, p<0.05 vs. fluorocitrate alone; Figure 4.1.6b).

These findings suggest that the apparent facilitating action of eCBs on strong θ-burst-induced LTP does not involve the astrocytes.

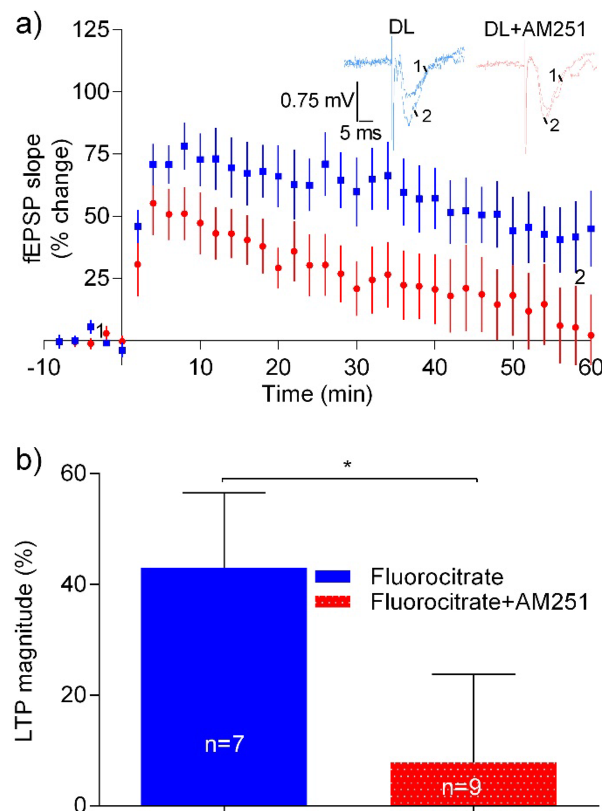

Figure 4.1.6 – Astrocytes do not contribute to the enhancement of LTP caused by eCBs. a) Data obtained in slices treated with 200 μM Fluorocitrate to inhibit astrocyte metabolism, either in the absence (control) or in the presence of 1 μM of AM251. **b)** Quantification of LTP magnitude under the indicated conditions. *p < 0.05 (Student t test). For further details see legend to Figure 4.1.1.

4.1.7 The eCB-mediated increase of LTP is GABAergic transmission-dependent

Next, it was tested if the apparent facilitating action of eCBs on LTP caused by a strong θ-burst stimulation could be due to the effective inhibition of GABA release controlling glutamate release. To this end, experiments were performed in the presence of the $GABA_AR$ antagonist picrotoxin (PTX, 50 µM). LTP magnitude was smaller in the presence than in the absence of picrotoxin (cf. Figure 4.1.7 with Figure 4.1.2), which may result from overactivity of glutamatergic transmission even before LTP induction. However, importantly, in slices with picrotoxin, the inhibitory action of AM251 on LTP was lost (PTX: 34.3% ± 9.7%, n=6; PTX+AM251: 31.8% ± 7.7%, n=5; $p>0.05$, Figure 4.1.7b). The ability of picrotoxin to block the inhibitory action of AM251 on LTP should not be attributed to its ability to inhibit LTP, because fluorocitrate also inhibited LTP by a similar degree (cf. data in Figure 4.1.6 and Figure 4.1.7) but did not prevent the inhibitory effect of AM251 on LTP.

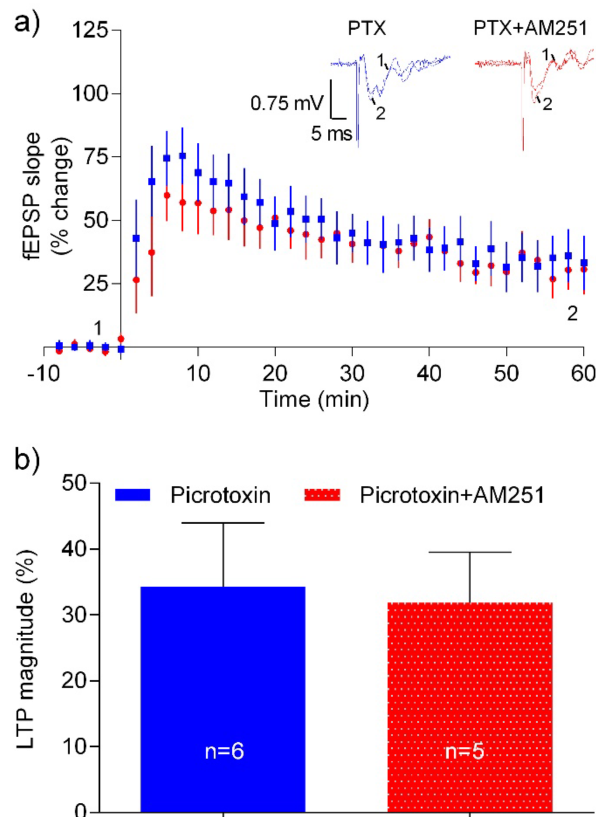

Figure 4.1.7 – The influence of eCBs on strong θ-burst-induced LTP is lost when blocking GABAergic transmission. a) Data for slices treated with the $GABA_A$ receptor antagonist picrotoxin (50 µM) in the absence (control) or in the presence

of 1 µM of AM251. **b)** Quantification of LTP magnitude under the conditions indicated. No significant difference was found between the two conditions ($p > 0.05$, Student's t-test). For further details, see the legend of Figure 4.1.1.

These findings suggest that the apparent facilitating action of eCBs on strong θ-burst-induced LTP involves $GABA_AR$-mediated GABAergic transmission, most likely resulting from eCB-induced inhibition of GABA release, with subsequent activation of glutamatergic neurons.

4.1.8 Blockade of CB_1R activation by eCBs did not affect basal excitability

LTP can be impacted by changes in basal synaptic transmission, and the manipulation of eCBs may alter the basal synaptic transmission. The influence upon the basal synaptic transmission was studied with input/output (I/O) curves were compared in the absence and presence of AM251, rimonabant, or orlistat. As shown in Figure 4.1.8, none of these drugs significantly modified I/O curves, compared to control conditions. Conversely, exogenous activation of CB_1R using the CB_1R agonist WIN significantly altered the I/O curve, which can be attributed to the ability of WIN to inhibit glutamatergic transmission. This result indicates that transiently released eCBs have a predominant influence over LTP, rather than over basal synaptic transmission.

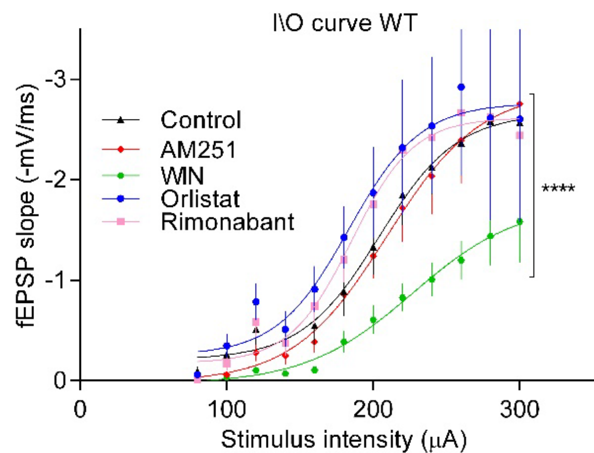

Figure 4.1.8 – WIN impairs synaptic transmission. Input-output curves showing fEPSP slope values plotted against the stimulation intensity (80–300 µA) in hippocampal slices under the drug conditions indicated. Data are expressed as mean ± SEM, n = (5–8). Statistical significance was assessed by two-way ANOVA followed by Sidak's post-hoc test comparing multiple experimental groups; no significant differences were detected among AM251, rimonabant, and orlistat, compared to control. ($F_{interaction}(_{44,360}) = 0.5$; F_{row} factor($_{11,360}$) = 38.1; F_{column} factor($_{4,360}$) = 14.4; t-values: control/AM251, 0.6; control/WIN, 5.2; control/rimonabant, 1.1; control/orlistat, 2.1).

4.1.9 Discussion

The main finding of this study is that blocking CB_1R activation may have opposing effects on CA1 LTP, in mice. The results show that blocking CB_1R activation facilitates weakly induced LTP, whereas a stronger LTP is inhibited when CB_1R cannot be activated. This finding suggests a dual action for eCBs on CA1 LTP, namely inhibiting weak LTP while facilitating more robust LTP.

To our knowledge, this is the first study to demonstrate a dual role for cannabinoids in hippocampal LTP, in mice. It has long been known that mice lacking CB_1Rs have enhanced high-frequency induced hippocampal LTP (Bohme et al., 1999; Jacob et al., 2012). Similarly, Slanina et al. (2005) showed that weak LTP induced by a small number of pulses delivered at the CA1 area of hippocampal slices is facilitated by CB_1R blockade, also indicating that endocannabinoids inhibit weakly induced LTP. However, LTP induced by strong high-frequency stimulation (100 pulses for 1 s, or twice that separated by 20 s) was unaffected by blocking of CB_1R (Slanina et al., 2005), in stark contrast to the results reported for robust LTP in the current study. Nevertheless, species differences (mice in our study vs rats in theirs) or the age of experimental animals (adults in our study vs adolescents in theirs) may account for these differences.

Facilitation of LTP by eCBs has been previously reported, but again those studies did not suggest a dual role for eCBs as a function of LTP magnitude. By reporting that eCBs enable LTP induction by trains of EPSPs that are ineffective if eCBs are inhibited, Carlson et al. (2002) provided the first evidence that eCBs facilitate CA1 hippocampal LTP. This action could be attributed to an eCB-mediated inhibition of GABAergic synapses (Carlson et al., 2002), and indeed it was later reported that by removing synaptic inhibition in a restricted

area of the dendritic tree, endocannabinoids selectively prime nearby excitatory synapses, facilitating induction of CA1 hippocampal LTP (Chevaleyre and Castillo, 2004). Those studies used single-cell recording, and thus enhanced our understanding of the action of eCBs at the local circuitry level, but the overall impact of eCBs on LTP of a population of pyramidal neurons was yet unknown. Alvares et al. (2006) reported, using adult rats, a marked inhibition of high-frequency-induced CA1 LTP of fEPSPs by AM251, indicating that eCBs are required for robust LTP phenomena. More recently, Wang et al. (2016) reported that eCB signalling is required for LTP of the lateral perforant path input to dentate gyrus neurons. In the study by Wang et al. (2016), robustly induced CA1 LTP was unaffected by the manipulation of eCB signalling. In contrast, our results point towards a facilitating role for eCBs on CA1 LTP induced by robust stimulation. The type of stimulation (θ-burst in both cases) or age (adult animals in both cases) alone cannot account for the differences observed. In fact, these differences may be due to the characteristics of the perfusion chamber, which may affect the accumulation of endogenous substances around the synapses. A slice submersion chamber was used, while Wang et al. (2016) used an interface chamber, and submerging chambers likely favor the accumulation of endogenous substances. Moreover, mice were used while Wang et al. (2016) used rats when testing the influence of eCBs on CA1 LTP, but species differences are unlikely to account for the conflicting results since no marked differences were detected by Wang et al. (2016) when comparing LPP-LTP data in mice and rat hippocampal slices.

Astrocytes release several neuromodulatory substances, including purines (Henneberger et al., 2010; Lalo et al., 2014), and have been shown to contribute to the facilitating action of eCBs on hippocampal glutamatergic transmission (Navarrete and Araque, 2010). However, the metabolic inhibition of astrocytes, a condition known to affect astrocytic signaling and release of gliotransmitters (Bonansco et al., 2011; Paulsen et al., 1987; Swanson and Graham, 1994), did not affect the influence of AM251 on LTP. This result suggests that astrocytes do not play a major role in the facilitating action of eCBs on LTP.

A common conclusion in all studies reporting facilitation of LTP by eCBs is that it can be accounted for by influence upon GABAergic neurons (Carlson et al., 2002; Chevaleyre and Castillo, 2004; Wang et al., 2016). Accordingly, it was observed that the ability of the CB_1R

blocker AM251 to inhibit LTP was lost in the presence of the $GABA_AR$ antagonist picrotoxin, thus supporting the idea that eCBs facilitate LTP by restraining the inhibition of LTP imposed by GABAergic inputs. It has been shown that the deletion of CB_1R in GABAergic neurons leads to a diminished hippocampal CA1 LTP, whereas the deletion of eCBs in glutamatergic neurons leads to enhanced LTP (Monory et al., 2015). It is therefore likely that the two stimulation conditions used in the current study lead to a differential influence of eCBs on GABAergic interneurons and glutamatergic neurons so that the influence of eCBs upon GABAergic neurons predominates under strong LTP induction conditions.

CB_1R is widely distributed in the central nervous system, and mainly in the hippocampus, cortex, basal ganglia, and cerebellum (Marsicano and Lutz, 1999; Wilson et al., 2012). The receptor is localized in excitatory and inhibitory neurons (Katona et al., 2001; Kawamura, 2006; Wilson et al., 2001) and also in astrocytes (Han et al., 2012; Hoffman et al., 2010). Considering the neuronal compartment only, it has been estimated that approximately ¾ of all CB_1R in the hippocampus are expressed in GABAergic neurons, whereas glutamatergic neurons contain approximately the other ¼ of all hippocampal CB_1Rs (Steindel et al., 2013). However, not all GABAergic hippocampal neurons express CB_1R, these receptors being localized in cholecystokinin (CCK)-positive neurons. CCK-positive neurons express higher levels of CB_1R than pyramidal cells (Marsicano et al., 2003; Marsicano and Lutz, 1999; Monory et al., 2006). It is thus not surprising that the apparent facilitating action of eCBs on strongly induced LTP results from their action on GABAergic neurons, most likely by suppressing the inhibitory control exerted by CCK-positive basket cells over the pyramidal neurons.

Another relevant finding of the current study is the similarity between the effect of drugs that block eCB-mediated CB_1R activation and the effect of drugs that lead to overactivation of CB_1R. It should also be noted that the action of orlistat and the action of URB597 were both counteracted by the CB_1R receptor blocker AM251, clearly indicating that their ability is due to alterations in the level of CB_1R activation. However, while inhibition of 2-AG synthesis is expected to reduce the levels of eCBs, inhibition of 2-AG degradation increases them (Basavarajappa et al., 2014). It has been long known that exogenous application of an eCB, direct activation of CB_1R, or increased eCB levels inhibit LTP (Basavarajappa et al., 2014; Collins et al., 1995; Nowicky et al., 1987; Paton et al., 1998;

Stella et al., 1997; Terranova et al., 1995), these inhibitory actions are usually interpreted in the light of the knowledge that exocannabinoids inhibit excitatory synaptic transmission. However, the novelty of the current study lies in the ability to show the action of drugs that strongly exacerbate CB_1R activation with those that reduce CB_1R activation by eCBs. Our findings contribute to the interpretation of apparently conflicting phenomena and strongly support the notion that an optimal level of eCBs signalling is required to sustain LTP.

4.2 Influence of adenosine on the modulation of synaptic plasticity by cannabinoids.

4.2.1 Rationale

Endocannabinoids and adenosine are widely recognized as fine-tuning neuromodulators of synaptic activity. Adenosine A_1 receptors are the major adenosine receptors in the hippocampus, where their activation suppresses glutamatergic synapses on pyramidal neurons (Dunwiddie and Diao, 1994; Hoffman et al., 2010). There are two major sources of extracellular adenosine: ATP catalysis in the synaptic cleft (Richardson et al., 1987; Zimmermann, 1996) and transporter-mediated release of adenosine (Fredholm et al., 1994). Interestingly, the effects of endocannabinoids are partially blocked by co-activation of A_1R, suggesting an interaction between these modulatory pathways (Hoffman et al., 2010; Sousa et al., 2011). Moreover, A_1 receptors and CB_1 receptors are co-localized and share G-protein-coupling (Hoffman et al., 2007; Sousa et al., 2011).

The results in section 4.1 indicating that transiently released eCBs can either inhibit or facilitate LTP depending on the strength of the LTP-inducing protocol led us to hypothesize that the strong θ-burst used could lead to the recruitment of other neuromodulators that would affect the neuromodulatory effect of eCBs. Purines are released during high-frequency neuronal firing (Cunha et al., 1996) and are abundantly expressed in the hippocampus, and adenosine is known to inhibit LTP through A_1R activation. Moreover, A_1R can affect CB_1R signalling (Hoffman et al. 2010; Sousa et al. 2011).

This study aimed to determine the role of A_1R in CB_1R modulation upon mice hippocampal LTP induced by two stimulation protocols. Two different approaches were used to block A_1 receptor activity: genetic ($A_1R^{-/-}$ mice) and pharmacological (selective A_1 receptor antagonist, DPCPX).

4.2.2 Characterization $A_1R^{-/-}$ mice versus WT

Before evaluating the possible role of A_1R in synaptic plasticity, differences in synaptic transmission between wild type (WT) and adenosine A_1 receptor knockout mice were evaluated. To examine these differences, input/output curves were obtained by recording fEPSP responses as a function of increasing stimulation intensities delivered to hippocampal slices from $A_1R^{-/-}$ mice and control littermates.

The maximum fEPSP slope and amplitude recorded for slices taken from $A_1R^{-/-}$ mice was similar to that from slices taken from WT mice (Figure 4.2.1), indicating that there is no change in basal synaptic transmission between adenosine receptor 1 knockout mice and control littermates ($Emax_{slope}WT = -2.67 \pm 0.25$, n=8 vs. $Emax_{slope}A_1RKO = -2.66 \pm 0.39$, n=6, p=0.21 (Student's t test); $Emax_{amplitude}WT = -2.04 \pm 0.16$, n=6 vs. $Emax_{amplitude}A_1RKO = -2.38 \pm 0.17$, n=6, p=0.51).

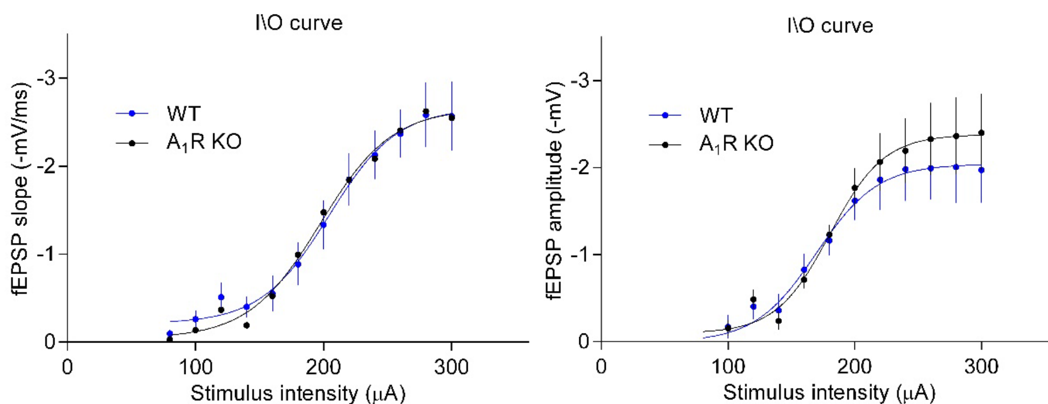

Figure 4.2.1 – Input/output curves showing fEPSP slopes and amplitude values plotted against stimulation intensities (80–300 μA) in hippocampal slices of WT and A_1R knockout mice. Data are expressed as mean ± SEM. Statistical significance was assessed by the Student's t test, n = (6-8); no significant differences were detected between slope and amplitude. Slope (p-value = 0.98, $F_{slope(4,160)} = 0.94$) and amplitude (p-value = 0.45, $F_{amplitude(4,122)} = 0.93$). Two-way ANOVA: slope – $F_{interactopm}$ $factor_{(11,144)} = 0.6$, F_{row} $factor_{(11,144)} = 18.6$, F_{column} $factor_{(1,144)} = 0.04$; amplitude – $F_{interactopm}$ $Factor_{(11,96)} = 0.2$, F_{row} $Factor_{(11,96)} = 19.0$, F_{column} $factor_{(1,96)} = 1.4$.

Another statistical approach to this question was testing whether the same curve adequately described the data (fEPSP slope and amplitude) distribution for both wild type littermates and A_1R knockout mice. The slope and amplitude of the fEPSP values (p>0.05)

were compared. Two-way ANOVA was used to compare each individual (average) slope value for each stimulus intensity step and found no significant differences.

After the differences in synaptic transmission between the animal models were determined, a series of experiments were designed to examine the differences in LTP induced by weak θ-burst and strong θ-burst stimulation between wild type and adenosine A_1 receptor knockout mice.

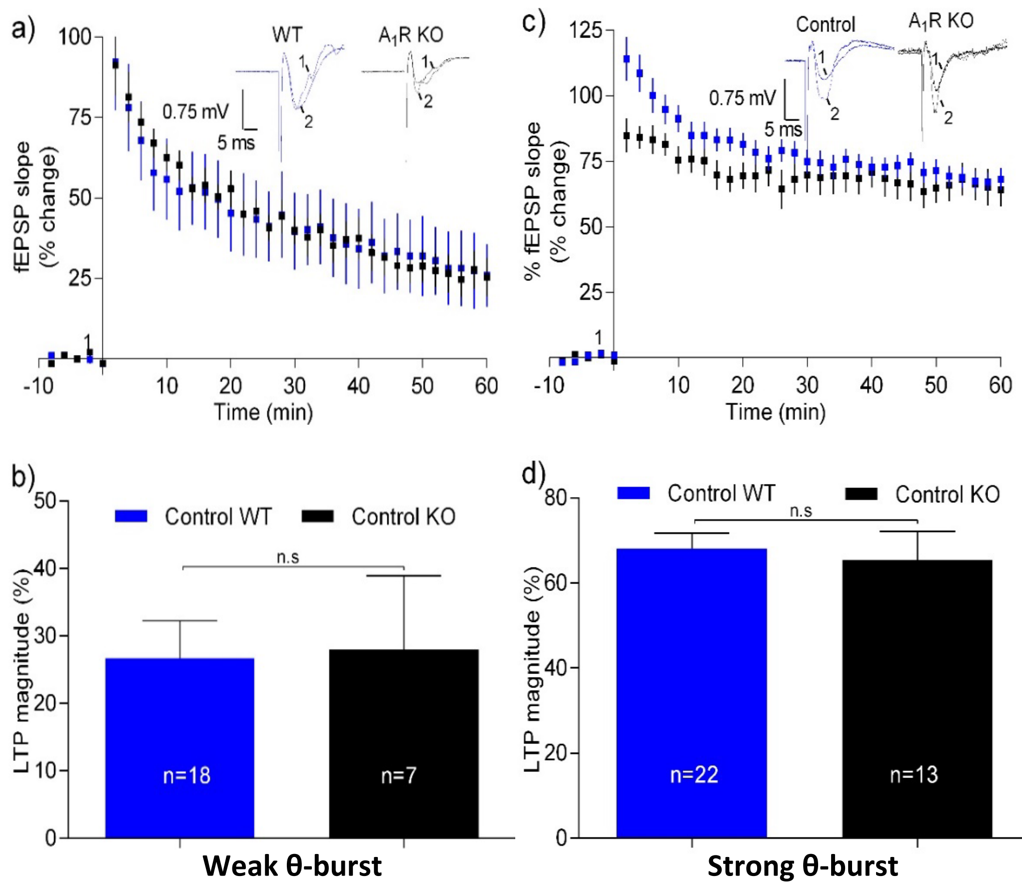

Figure 4.2.2 – Weak θ-burst-induced LTP (five trains of 100 Hz, four stimuli separated by 200 ms) and strong θ-burst (10 trains of 100 Hz, four stimuli separated by 200 ms) is not affected by adenosine receptor 1 deletion. a) Time course of the averaged fEPSP slopes under control conditions (wild type littermates) or in A_1R knockout mice induced by a weak θ-burst. **b)** Quantification of LTP magnitude induced by a weak θ-burst. **c)** Time course of the averaged fEPSP slopes under control conditions (wild type littermates) or in A_1R knockout mice induced by a strong θ-burst. **d)** Quantification of LTP magnitude induced by a strong θ-burst. For further details, see the legend of Figure 4.1.1.

LTP magnitude induced by a **weak θ-burst** was similar in A_1RKO and WT mice (28.0% ± 11.0% vs. 26.7% ± 5.5%, p>0.05; Figure 4.2.2b). In addition, LTP magnitude in control WT mice induced by a **strong θ-burst** was 68.1% ± 3.7% (n=22) higher than at pre-θ-burst stimulation and 65.5% ± 6.7% (n=13) in $A_1R^{-/-}$ mice, and there was no significant difference in LTP magnitude (p>0.05) between A_1R+/+ (WT) and A_1R−/− mice (Figure 4.2.2d).

In some cases, male and female mice were used to maximize the use of A_1R knockout mice; because of this, control experiments using male and female mice were performed. Nevertheless, no significant differences were detected between males and females (Figure 4.2.3).

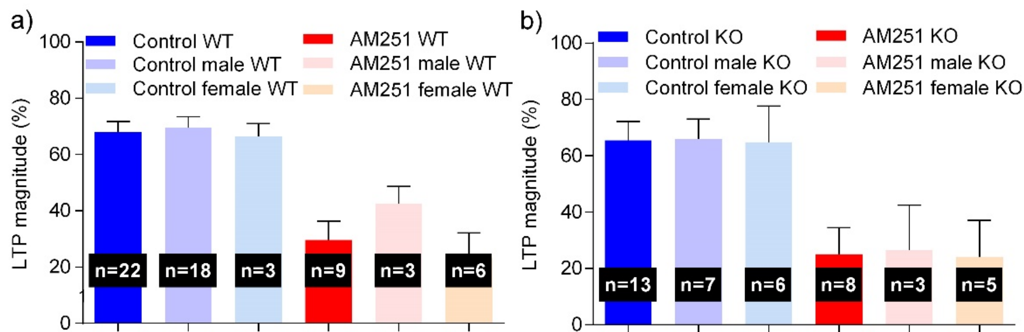

Figure 4.2.3 – CB_1R blockade inhibits strong θ-burst-induced LTP stimulation, both in male and female mice. Quantification of LTP magnitude under the conditions indicated. All values are mean ± standard error of the mean (SEM) from n experiments; n values are indicated inside the bars. No significant differences were found between the groups as determined by one-way ANOVA. For further details, see the legend of Figure 4.1.1.

In summary, these results show that the input/output curves and magnitude of LTP induced by weak and strong θ-burst protocols are not significantly different between wild type and adenosine A_1 receptor knockout mice.

4.2.3 Endocannabinoids reduce weak θ-burst stimulation (wTBS) induced LTP protocol

The role of eCBs was assessed by testing the effects of using antagonists to block CB_1R activation by eCBs. In A_1RKO slices treated with the CB_1R inverse agonist AM251 (1 µM), the magnitude of LTP increased by 42.4% ± 5.4% but was not significantly different from that of control A_1R knockout mice ($n=5$, $t=1.1$, $p=0.91$, Figure 4.2.4c). However, LTP magnitude was significantly higher in WT mice treated with AM251 than in control KO mice treated with AM251 ($n=17$, $t=2.5$, $p<0.05$ vs control, Figure 4.2.4c).

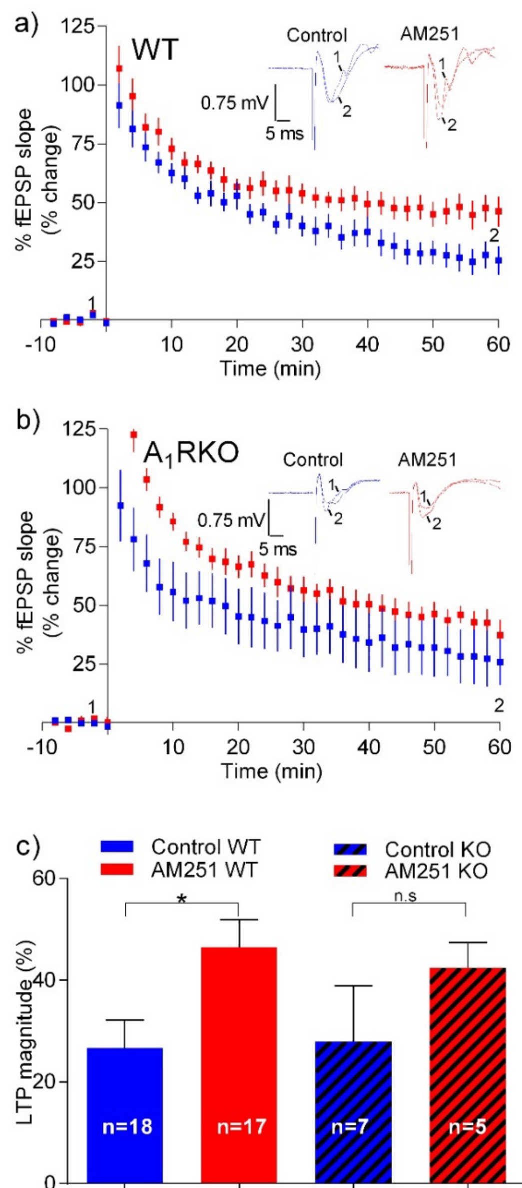

Figure 4.2.4 – CB_1R blockade enhances weak θ-burst-induced LTP stimulation. a) Time course of the averaged fEPSP slopes and original traces of fEPSP recordings in wild type mice under control conditions (no drugs, in blue) or in the presence of 1 µM AM251 (CB_1R inverse agonist, in red). **b)** Time course of the averaged fEPSP slopes under control conditions in A_1R knockout mice and in the presence of 1 µM AM251. **c)** Quantification of LTP magnitude. Data are expressed as mean ± standard error of the mean (SEM) from n experiments; n values are indicated inside the bars. $F(3, 43) = 0.73$, ns: non-significant. For further details, see the legend of Figure 4.1.1.

Collectively, these results suggest that A_1R plays no relevant role in the inhibitory effect of endogenous CB_1R on weak θ-burst-induced LTP.

4.2.4 Physiologically released endocannabinoids enhance strong θ-burst stimulation (sTBS) induced LTP protocol independently of A_1R

After investigating the role of A_1R on the inhibitory effect of CB_1R activation by eCBs on LTP induced by a weak θ-burst, the A_1R effect was also studied using a strong θ-burst protocol as described in section 4.1.3. The CB_1R inverse agonist AM251 inhibited LTP at the same level, both between genotypes (WT $A_1R^{+/+}$: 29.6% ± 6.8%, n=9; $A_1R^{-/-}$: 25.1% ± 9.3, n=8; $p>0.05$) and within the $A_1R^{-/-}$ genotype (control $A_1R^{-/-}$ taken vs $A_1R^{-/-}$+AM251; $p<0.05$) (Figure 4.2.5b).

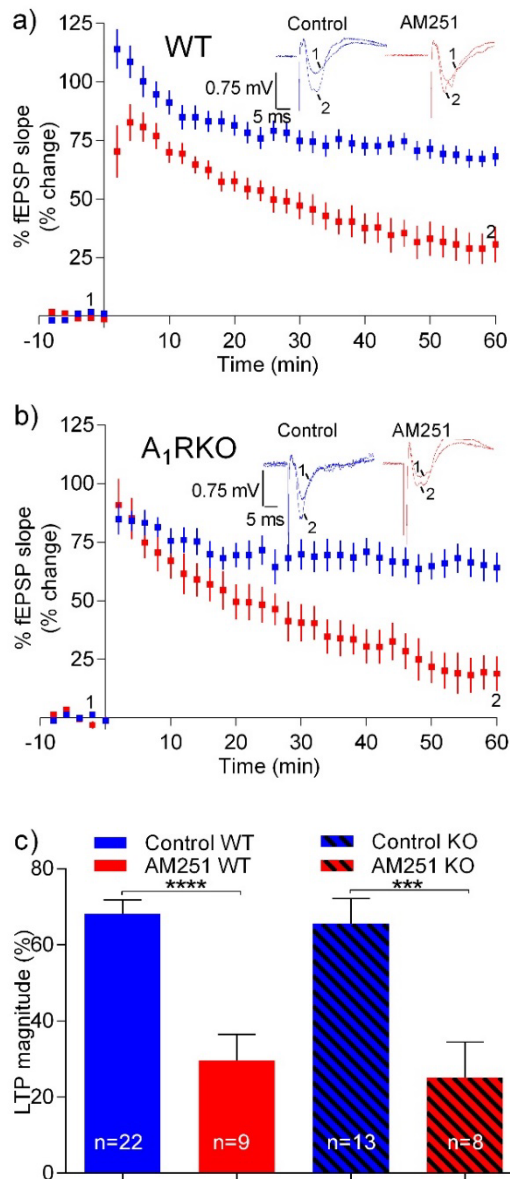

Figure 4.2.5 – CB_1R blockade inhibits strong θ-burst-induced LTP stimulation. a) Time course of the averaged fEPSP slopes and original traces of fEPSP recordings in wild type mice under control conditions (no drugs) or in the presence of 1 µM AM251 (CB_1R inverse agonist). **b)** Time course of the averaged fEPSP slopes in A_1R knockout mice under control conditions and in the presence of 1 µM AM251. **c)** Quantification of LTP magnitude. Data are expressed as mean ± standard error of the mean (SEM) from n experiments; n values are indicated inside the bars. $F_{(3, 48)}$ = 13.6, *** p<0.001, **** p<0.0001. For further details, see the legend of Figure 4.1.1.

These results suggest that inhibition of strong LTP by the CB_1R inverse agonist does not result from enhanced activation of A_1R by released adenosine.

To provide further confirmation of our findings and, to rule out any adaptation-like process caused by the genetic removal of A_1R, AM251 action was evaluated in $A_1R^+/^+$ mice,

after the A_1R blockade with DPCPX. Again, and despite the presence of DPCPX at a concentration (50 nM) around 100 times its Ki for A_1R (Bruns et al., 1987) expected to fully block A_1R signalling, AM251 significantly inhibited strong θ-burst-induced LTP (DPCPX: 68.0% ± 9.3%, n=9; DPCPX+AM251: 32.5% ± 5.2%, n=7, t=3.3, $p<0.05$; Figure 4.2.6b).

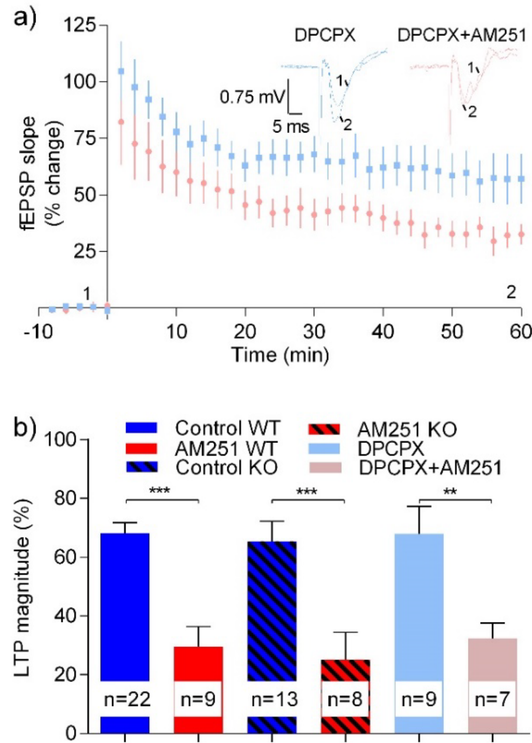

Figure 4.2.6 – CB_1R blockade inhibition of LTP induced by strong θ-burst stimulation does not result from endogenous A_1R activation. a) Data for slices from wild type mice treated with the A_1R antagonist DPCPX (50 nM) either in the absence (blue) or presence (pink) of AM251 (1 µM) **b)** Quantification of LTP magnitude. In all cases, LTP was induced by a strong θ-burst. Data for WT mice in the absence of DPCPX (control WT, AM251 WT) are the same as those shown in Figure 4.1.2 but are displayed here to allow comparisons with WT slices in the presence of DPCPX. **$p<0.01$; ***$p<0.001$; $F(5, 62) = 10.0$, one-way ANOVA with Sidak's correction. For further details, see the legend of Figure 4.1.1.

Together, these results indicate that inhibition of LTP caused by eCBs blocking of CB_1R is not due to enhanced activation of A_1R by endogenous adenosine.

Next, a CB_1R antagonist (rimonabant) and a 2-AG synthesis blocker (orlistat) were used. For $A_1R^{-/-}$ mice, a single experiment was performed with rimonabant and orlistat, and, as expected, LTP magnitude was inhibited (52.7% and 46.9%, respectively; Figure 4.2.7b).

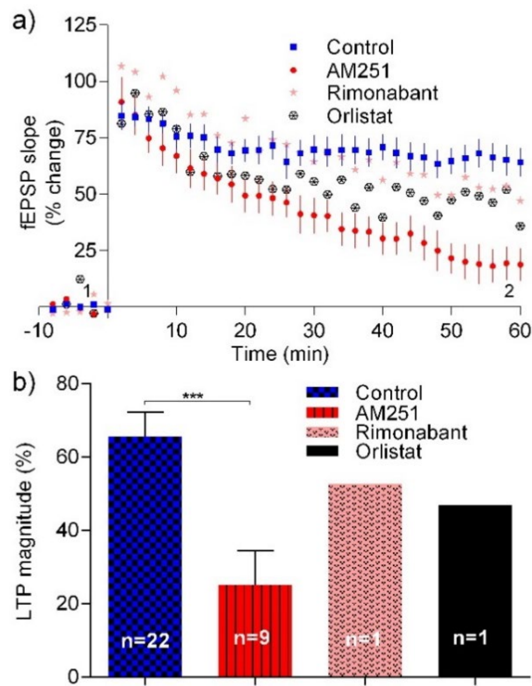

Figure 4.2.7 – Endocannabinoids enhance strong θ-burst-induced LTP stimulation in A_1R knockout mice. a) Time course of the averaged fEPSP slopes and original traces of fEPSP recordings under control conditions (no drugs) or in the presence of 1 µM AM251, 1 µM rimonabant, or 10 µM orlistat. **b)** Quantification of LTP magnitude. ***p<0.001, Student's t-test. For further details, see the legend of Figure 4.1.1.

4.2.5 Exogenous activation of CB_1R leads to LTP inhibition

To assess how LTP is affected by exogenous activation of CB_1R, a CB_1 receptor agonist (WIN 55,212-2; 500 nM) was used to exogenously activate CB_1Rs in a sustained manner (as described in chapter 4.1.4).

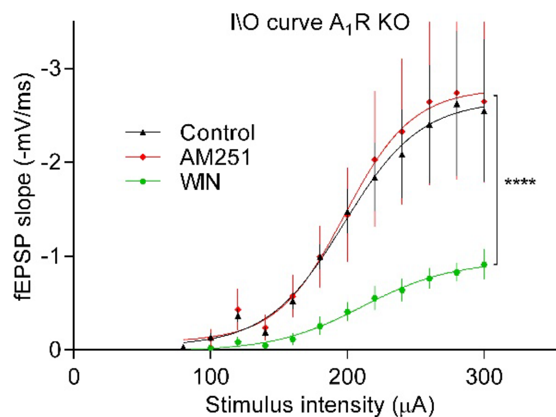

Figure 4.2.8 – WIN impairs synaptic transmission in A_1R knockout mice. Data are expressed as the mean ± SEM, n = (5–8). Statistical significance was assessed by two-way ANOVA followed by Sidak's post-hoc correction comparing multiple experimental groups; no significant differences were detected among drug treatments compared to the other drugs. A_1R knockout mice: $F_{interaction(22,144)}$ = 0.7, F_{row} factor$_{(11,144)}$ = 9.7, F_{column} factor$_{(2,144)}$ = 15.2, t-values: control/AM251, 0.5; control/WIN, 4.8). ****p<0.0001.

In $A_1R^{-/-}$ mice, LTP magnitude was significantly lower in the presence of 500 nM WIN (−0.57% ± 9.4%, n=5, t=4.1, p<0.0001) than in the absence of WIN and similar to the LTP magnitude in slices from WT mice treated with 500 nM WIN (t=0.5, p>0.05, Figure 4.2.8). This effect was not observed when AM251 was added (58.5% ± 15.0%, n=8, Figure 4.2.9).

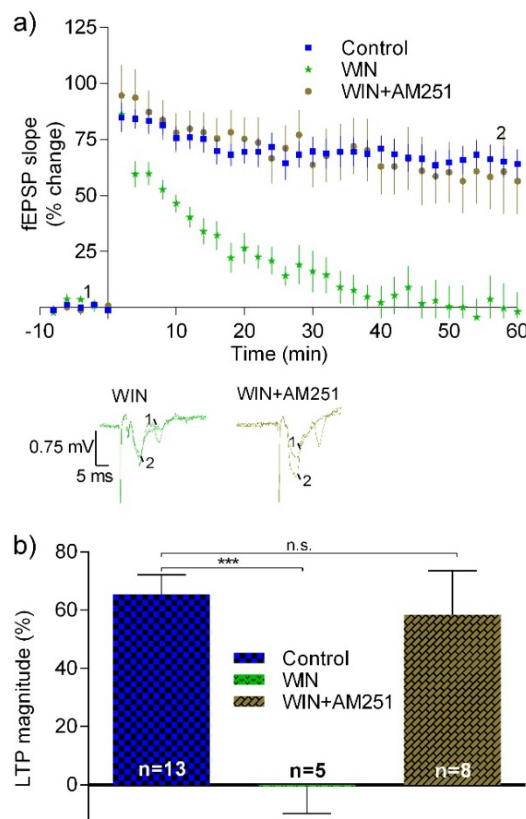

Figure 4.2.9 – Strong θ-burst-induced LTP is inhibited when CB_1Rs are continuously activated in A_1R knockout mice. a) Time course of the averaged fEPSP slopes and original traces of fEPSP recordings under control conditions (no drugs) or in the presence of 500 nM WIN55,212-2 (WIN, CB_1R agonist) or 500 nM WIN + 1 µM AM251. **b)** Quantification of LTP magnitude. ****p<0.0001; $F_{(2, 33)}$ = 8.8, one-way ANOVA with Sidak's correction. For further details, see legend to Figure 4.1.1.

In conclusion, the absence of the A_1 receptor did not affect the inhibition of LTP magnitude by the CB_1R agonist.

4.2.6 Overactivation of CB_1R by endogenous anandamide and 2-AG leads to a dual LTP effect in A_1RKO mice

The overactivation of CB_1R by endocannabinoids was also evaluated in A_1 receptor knockout mice, as described in chapter 4.1.5. In A_1 receptor knockout mice, the role of anandamide on LTP magnitude was evaluated using URB597. In this case, it was 24.8% ± 15.4% (n=4, Figure 4.2.10b), similar to the mean LTP magnitude recorded in WT mice.

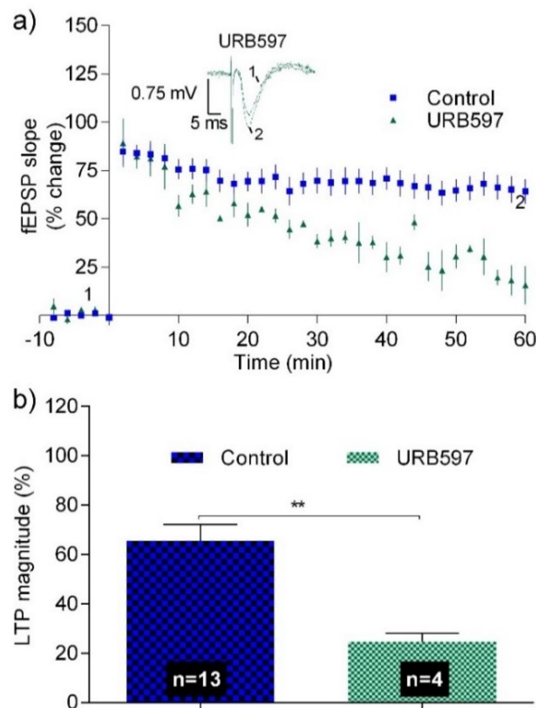

Figure 4.2.10 – Anandamide hydrolysis inhibitors differently affect strong θ-burst-induced LTP in A_1R knockout mice. a) Time course of the averaged fEPSP slopes and original traces of fEPSP recordings under control conditions (no drugs) or in the presence of 1 µM URB597. **b)** Quantification of LTP magnitude. **p<0.01; Student's t-test. For further details, see the legend of Figure 4.1.1.

4.2.7 Astrocytes do not contribute to enhancing LTP induced by eCBs in A_1RKO mice

The astrocytic role on the effect of A_1R on CB_1R-mediated LTP modulation was also examined as described in section 4.1.6, but this time using A_1RKO (A_1R knockout) mice. In A_1RKO slices treated with fluorocitrate (200 µM), LTP magnitude increased by 41.7% ± 11.5% (n=6, Figure 4.2.11b), a result similar to that obtained with WT littermates (Figure 4.1.6). In addition, LTP magnitude was significantly lower in A_1RKO slices treated with fluorocitrate plus the CB_1R blocker AM251 (6.1% ± 8.9%, n=6, t=2.4, $p<0.05$ vs. 41.7% ± 11.5%, Figure 4.2.11b).

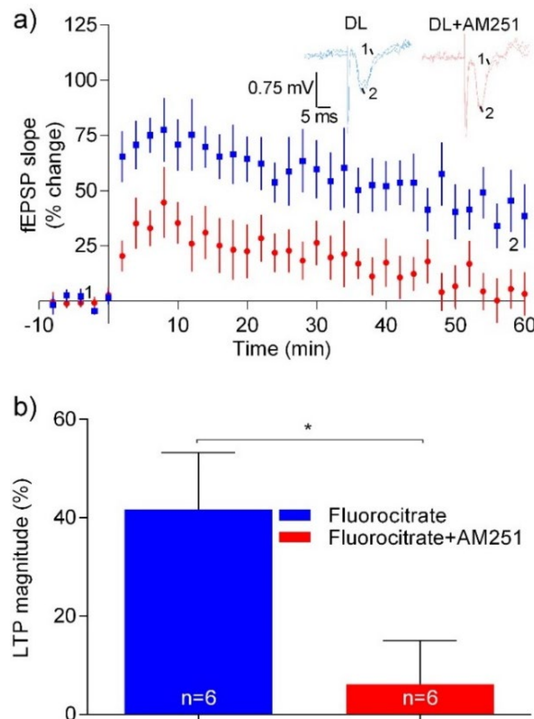

Figure 4.2.11 – Astrocytes do not contribute to enhancing LTP induced by physiologically released eCBs in A_1R knockout mice. a) Data for slices treated with 200 µM fluorocitrate to inhibit astrocyte metabolism, either in the absence (control) or presence of 1 µM AM251. **b)** Quantification of LTP magnitude. In all cases, LTP was induced by a strong θ-burst. *$p<0.05$ ($F_{(5, 5)}$ = 1.7, Student's t-test). For further details, see the legend of Figure 4.1.1.

These results suggest that the seemingly facilitating action of eCBs on strong θ-burst-induced LTP does not involve astrocytes in A_1R knockout mice as well as in WT mice.

4.2.8 In A_1RKO mice, eCB-mediated enhancement of LTP is GABAergic transmission-dependent

Analyzing the impact of interneurons on the modulatory effect of CB_1R on LTP in WT mice led to unexpected results: an opposite effect was observed when using A_1R knockout mice. In the presence of AM251 and picrotoxin, LTP magnitude was lower by approximately 67%, from 48.9% ± 11.3% to 14.1% ± 8.4% (n=5 and n=6 respectively, Figure 4.2.12b).

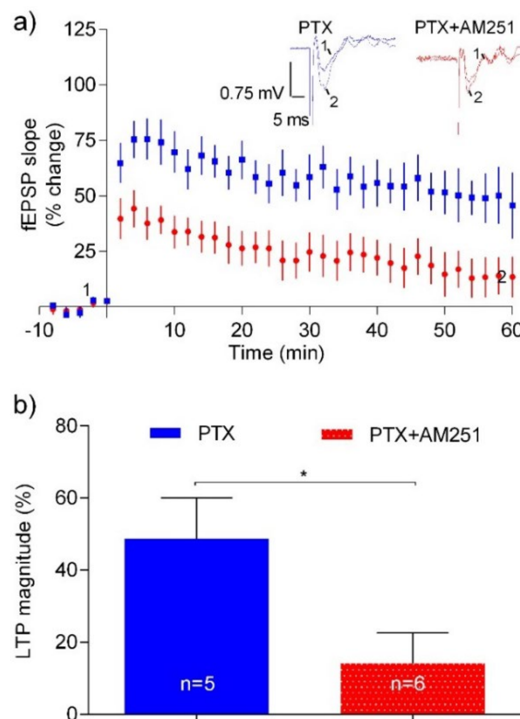

Figure 4.2.12 – The influence of physiologically released eCB on strong θ-burst-induced LTP remains when GABAergic transmission in A_1R knockout mice is blocked a) Data for slices treated with the $GABA_A$ receptor antagonist picrotoxin (PTX, 50 µM) in the absence (control) or presence of 1 µM AM251. **b)** Quantification of LTP magnitude. The LTP magnitude difference was detected between PTX and PTX+AM251 ($F_{(4, 5)} = 1.5$, $p>0.05$, Student's t-test). Astrocytes do not contribute to enhancing LTP induced by physiologically released eCBs in wild type littermates. For further details, see the legend of Figure 4.1.1.

These results suggest that in the case of A_1R deletion, the modulatory effect of CB_1R on LTP occurs through different mechanisms than those observed in WT mice.

4.2.9 Discussion

The main findings of this subchapter are that blocking of CB_1R activation by eCBs leads to a dual effect on CA1 LTP and that endogenous activation of A_1R plays no role in this dual effect, with one exception.

Pyramidal hippocampal neurons are under the inhibitory control of GABAergic synapses, but also under the control of several modulatory substances. Adenosine and endocannabinoids, both molecules released by neurons and glia, are able to modulate synaptic transmission and plasticity by operating high-affinity G-protein-coupled receptors (Sebastião and Ribeiro, 2015). Their receptors co-localize in the hippocampal CA1 region and a modulatory effect of A_1R over CB_1R has been demonstrated at the synaptic transmission level. The possibility that A_1R mediated the eCBs effect was rejected when examining A_1R deletion and A_1R blockade. The procedure did not significantly affect the action of eCB on either LTP stimulation protocol (weak vs strong θ-burst).

The results were largely the same for WT and A_1R knockout mice, indicating no role for endogenous activation of A_1R on the modulatory effect of CB_1R on LTP. The exception was when the inhibitory transmission was impaired by the $GABA_A$ receptor antagonist picrotoxin, resulting in an opposite effect in the case of $A_1R^{-/-}$ mice as compared to the WT mice. In A_1R knockout mice, the facilitating effect of endogenous CB_1R activation remained, even though GABAergic transmission was blocked. Rombo and co-workers (Rombo et al., 2016) found that activation of adenosine A_1R suppresses tonic, but not phasic $GABA_A$ currents in hippocampal pyramidal cells via CB_1R-positive interneurons, which makes it rather more difficult to envisage a possible mechanism for A_1–CB_1 receptor cross-talk. This unexpected result may be explained by a modification of G-protein-coupled receptors CB_1R (presynaptically or postsynaptically or both) with the deletion of A_1R through either direct or indirect mechanisms. Directly, it would involve direct interaction between A_1R and CB_1R via G-protein-coupled receptors, as previously suggested by Sousa et al. (2011). In this case, possible interactions could include a cross-talk between signaling pathways, an intracellular cross-talk mechanism, or a non-heterodimerization between A_1R–CB_1R modulation of the CB_1R effect on LTP at the pyramidal cells. Firstly, both receptors co-localize in hippocampal CA3 pyramidal neuron axon terminals that project to CA1 pyramidal cells and in these cells

(Dunwiddie and Hoffer, 1980; Shen et al., 1996). A_1R and CB_1R activation inhibits adenylyl cyclase activity and consequently reduces cyclic adenosine monophosphate (cAMP) production (Calker et al., 1979; Howlett et al., 1986; Howlett and Fleming, 1984). Serpa et al. (2015) found an additive effect of A_1R and CB_1R on cAMP production in the rat hippocampus. Thus, it is unlikely that the influence of A_1R deletion results from the cAMP signal transduction pathway, and other pathways may be involved such as the cyclic guanosine monophosphate (cGMP) pathway (Makara et al., 2007; Pinto et al., 2016). Secondly, adenosine receptors have a high propensity to be organized in a dimeric form (homo- and heterodimer) (Ciruela, 2006; Ciruela et al., 1995; Cristovão-Ferreira et al., 2011). A_1R–CB_1R dimerization in the hippocampus has not yet been described, in contrast to what occurs with $A_{2A}R$ in other brain areas (Borroto-Escuela et al., 2014; Vallabhajosyula et al., 2009). To understand this possible mechanism, it is important to define the terms heteromer and interaction hub. A heteromer is a macromolecular complex consisting of two or more functional receptors with different biochemical characteristics of individual receptors that alter the binding properties of receptors in the complex. The term interaction hub is a mathematical/physics term for a protein or assembled protein complex that can interact with numerous distinct partner proteins or lipids, either one at a time or simultaneously following several criteria (Vallabhajosyula et al., 2009). However, the deletion of A_1R may move the A_1R–CB_1R heterodimer pool toward CB_1R acting alone, which would probably act through the cAMP signal pathway, ruling out this hypothesis. Thus, an indirect mechanism could explain the enhancement effect of endogenous CB_1R activation with impaired inhibitory transmission and deletion of A_1R via $A_{2A}R$–CB_1R or A_1R–A_2A–CB_1R heteromers. $A_{2A}R$–CB_1R heterodimers have been well characterized in presynaptic terminals in other brain areas such as the striatum (Carriba et al., 2007; Ferreira et al., 2015). Moreover, A_1R, A_2R, and CB_1R have also been identified in presynaptic pyramidal cell terminals (Castillo et al., 2012; Kano et al., 2009; Rebola et al., 2005). The deletion of A_1R erases the tonic inhibition from presynaptic sites, shifting the balance predominantly to $A_{2A}R$ activation. $A_{2A}R$ activation was shown to facilitate synaptic transmission in hippocampal pyramidal cells (Lopes et al., 2002) and may possibly increase the release of endocannabinoids at the synaptic cleft, leading to LTP. A secondary effect on the synaptic transmission is the reduction of inhibitory synaptic transmission by CB_1R (Ferreira et al.,

2015; Martire et al., 2011). Postsynaptically, the three receptors (A_1R, A_2R, and CB_1R) are expressed in postsynaptic terminals, and the presynaptic interaction is a valid possible explanation for our findings, especially bearing in mind that CB_1R can negatively modulate NMDA receptors, and then LTP.

4.3 Hippocampal inhibitory synaptic transmission modulated by adenosine A_1 and cannabinoid CB_1 receptors

4.3.1 Rationale

CB_1R is highly expressed in the hippocampus, especially in the CA1 region (Freund et al., 2003; Herkenham et al., 1990). CB_1 receptors are located on several cell types, including pyramidal cells, interneurons, and astrocytes (Navarrete and Araque, 2010; Rivera et al., 2014). The hippocampus shows high A_1R expression (Goodman and Synder, 1982), and A_1 receptors are found in pre- and postsynaptic neurons (Rebola et al., 2003; Rombo et al., 2016), interneurons (Rivkees et al., 1995), and astrocytes (Biber et al., 1997). A_1 receptors and CB_1 receptors co-localize and share G-protein-coupling (Hoffman et al., 2007; Rombo et al., 2016; Sousa et al., 2011).

CB_1R activation exerts an inhibitory control of GABA and glutamate release from presynaptic terminals, enabling CB_1R to modulate neurotransmission (Gerdeman and Lovinger, 2001; Katona et al., 1999). Endogenous CB_1R activation plays an important role in short-term plasticity, including depolarisation-induced suppression of inhibition (DSI) (Carlson et al., 2002; Wilson and Nicoll, 2002). A_1R modulates excitatory synaptic transmission (Sebastião et al., 1990), having no direct presynaptic role on GABAergic transmission in mature hippocampal neurons (Yoon and Rothman, 1991) or on GABA release from isolated nerve terminals (Cunha and Ribeiro, 2000). However, A_1R activation attenuates the CB_1R-mediated inhibition of GABA and glutamate release, and this interaction is found at the G-protein activation level (Sousa et al., 2011). However, the effect of A_1R on glutamate release was absent in A_1R knockout mice (Hoffman et al., 2010), indicating that CB_1R function can be directly controlled by brain interstitial adenosine levels.

eCBs are released from hippocampal neurons in an activity-dependent manner to initiate short- and long-term changes in synaptic efficacy following activation of CB_1R (Carlson et al., 2002; Freund et al., 2003; Wilson and Nicoll, 2001). eCB mediates several forms of synaptic plasticity such as DSI and blocking of A_1R reduces the decrease in GABA release in the rat *nucleus accumbens* (Gu et al., 2005).

This chapter aimed to determine the influence of CB_1R on inhibitory synaptic transmission in hippocampal pyramidal cells and its interaction with A_1R. We also examined the effect of the A_1R–CB_1R interaction on DSI using young male wild type mice.

4.3.2 A_1R blockade prevents the decrease IPSCs by CB_1R activation

First, it was explored how CB_1R activation modulates $GABA_AR$-mediated inhibitory postsynaptic potential currents (IPSCs) evoked by electrical afferent fiber stimulation at the stratum. Monosynaptic IPSCs in pyramidal cells were recorded in the presence of NMDA (50 µM DL-AP5) and AMPA/kainate (10 µM CNQX) receptor antagonists to block glutamatergic inputs to pyramidal cells. Pyramidal cells showed a nonparametric distribution in response to WIN (84.9 ± 5.6, 1 µM; Student's t-test, n=11, Figure 4.3.1), and two different cell subpopulations were identified. WIN showed a significant and robust effect in a subset of the pyramidal cell population (mean ± SEM: 71.1% ± 5.5% of the baseline value, n=6). However, in the remaining pyramidal cells, IPSC amplitude was unchanged by WIN (amplitude: 101.5% ± 0.9% of the baseline value, n = 5, Figure 4.3.1).

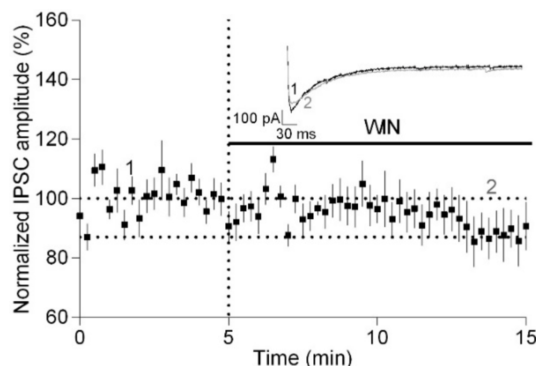

Figure 4.3.1 – CB_1 receptor activation inhibits $GABA_A$ inhibitory postsynaptic potential currents (IPSCs) in pyramidal cells. Time course plot showing that synaptic IPSCs evoked by electrical stimulation were diminished by 1 µM WIN55,212-2 (n=11). Representative IPSC recorded from one pyramidal cell corresponds to the average of 10 consecutive responses at baseline (1) and after 12 min in the presence of WIN. The IPSCs were recorded in the presence of NMDA (50 µM DL-AP5) and AMPA/kainate (10 µM CNQX) receptor antagonists. The intracellular solution contained 5 mM QX-314 (blocker of voltage-activated Na^+ channels) to prevent firing of action potential from the pyramidal cells.

Next, the role of A_1R was evaluated by testing if the A_1R receptor antagonist DPCPX (50 nM) could affect the CB_1R effect. The A_1R antagonist suppressed or inverted the inhibitory effect of the CB_1R agonist (109.0% ± 8.8%, n=8, 1 µM; p=0.027, Figure 4.3.2). These results show that CB_1R activation inhibits the hippocampal inhibitory transmission, and this effect is suppressed or even inverted by blocking A_1R.

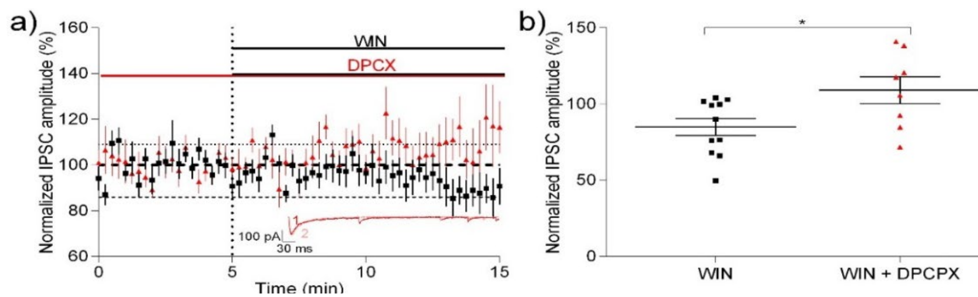

Figure 4.3.2 – A_1R blockade suppresses or inverts the inhibition caused by CB_1 receptor activation on inhibitory postsynaptic potential currents (IPSCs) in pyramidal cells. a) Time course plot showing that synaptic IPSCs evoked by electrical stimulation were diminished by 1 µM WIN55,212-2 (n=11, black squares), but the effect was inverted or suppressed after preincubation with the A_1R antagonist DPCPX (50 nM) (n=8, red triangles). Representative IPSC recorded from one pyramidal cell corresponds to the average of 10 consecutive responses at baseline with DPCPX (1) and after 12 min in the presence of the A_1R antagonist WIN. The IPSCs were recorded in the presence of DL-AP5 and CNQX. The intracellular solution contained 5 mM QX-314 (blocker of voltage-activated Na^+ channels) to prevent action potential firing from the pyramidal cells. Representative IPSC recorded from one pyramidal cell at baseline with DPCPX (1) and in the presence of WIN plus DPCPX (2). **b)** Baseline-normalized IPSCs recorded in the presence of WIN (n=11) and WIN plus DPCPX (n=8) from all individual cells sampled. All values are mean ± standard error of the mean (SEM) from n experiments. *p<0.05 (two-tailed Student's t-test).

4.3.3 DSI magnitude does not depend on stimulation protocol interval

After, it was investigated how endogenous CB_1R activation is modulated by A_1R in inhibitory currents in hippocampal CA1 pyramidal cells. For this set of experiments, a blocker of voltage-activated Na^+ channels (QX 314) was used to prevent action potential firing. I started by using 5-s depolarisation protocols to maximize DSI magnitude (Ohno-Shosaku et al., 2002).

First, it was examined if depolarization of postsynaptic neurons can induce DSI in mice hippocampal slices. When pyramidal neurons were depolarised from −70 to 0 mV for 5 s in voltage-clamp mode, the subsequent IPSCs from the depolarised neurons were momentarily suppressed. IPSC amplitude decreased by approximately 30% compared to

baseline after depolarisation (68%), calculated by comparing the two IPSCs immediately after depolarisation and a 40-s baseline before depolarisation (chapter 3.5.2.2, Figure 3.5.2). DSI magnitude in protocol 1 was 26.2% ± 2.6% (n=12, Figure 4.3.3). The 10-s interval between IPSCs gives a low temporal resolution to the DSI. Thus, a second stimulation protocol (DSI protocol 2) was designed, consisting of 30 stimuli at 0.33 Hz (3-s interval between evoked IPSCs) with depolarisation from −70 mV to 0 mV for 5 s after the 13th stimulus (chapter 3.5.2.2, Figure 3.5.3). IPSC amplitude decreased by 63% after depolarisation, and DSI magnitude was 22.1% ± 2.9% (n=11, Figure 4.3.3), suggesting that there is no difference in DSI magnitude between DSI protocols 1 and 2.

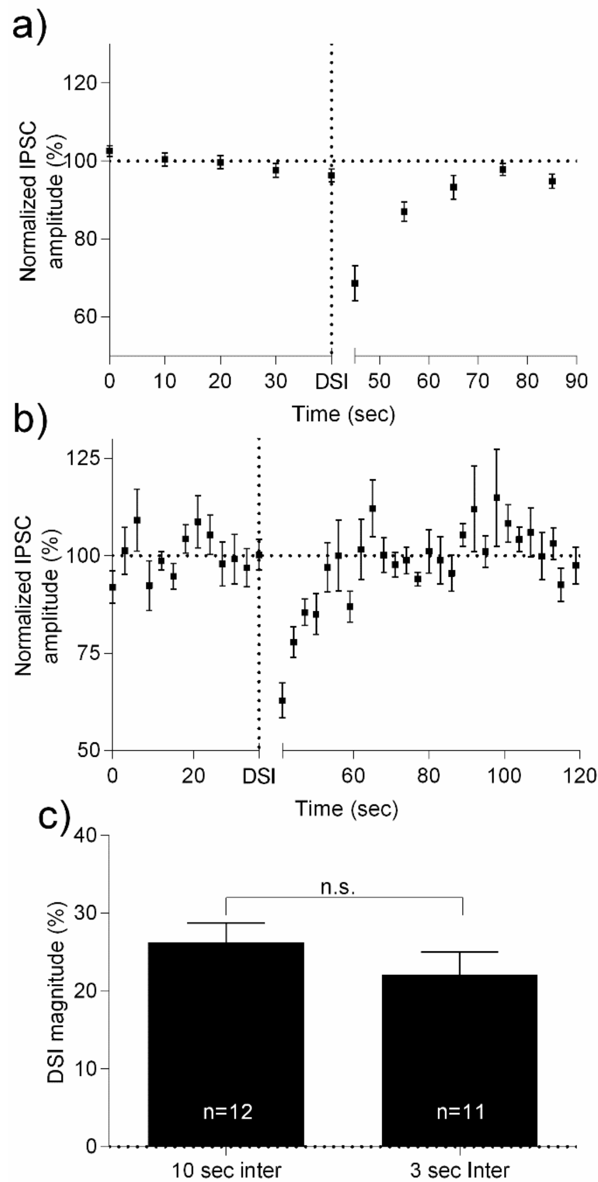

Figure 4.3.3 – Transient suppression of IPSCs induced by depolarization of a CA1 pyramidal neuron in the hippocampal slice is not stimulation interval-dependent. The IPSCs were recorded in the presence of receptor antagonists L-AP5 (50 µM) and CNQX (10 µM). **a)** Time course of the change in IPSC amplitudes with DSI protocol 1. IPSCs were evoked at 0.67 Hz. The pyramidal neuron was depolarised from −70 mV to 0 mV for 5 s at the times indicated by the arrows. **b)** Time course of the change in IPSC amplitudes with DSI protocol 2. IPSCs were evoked at 0.33 Hz. **c)** DSI magnitude is expressed as percentage depression in IPSC amplitude during the first 10 s after depolarisation (two sweeps for protocol 1 and four sweeps for protocol 2), averaged across three to five trials per sampled cell. No significant differences were detected between the two protocols ($p>0.05$, $F_{(11, 10)} = 1.1$, Student's t-test).

4.3.4 A_1R blockade reduces DSI magnitude with protocol 1

Next, the modulatory effect of A_1R on DSI was evaluated. DPCPX (50 nM) was perfused to the slice for at least 10 min before starting the DSI protocol. Using DSI induction protocol 1, IPSC amplitude depression in DPCPX slices was lower than in control slices (≈80%) with a DSI magnitude in DPCPX slices of only 10.1 % ± 4.9% (n=12, p<0.01, Figure 4.3.4).

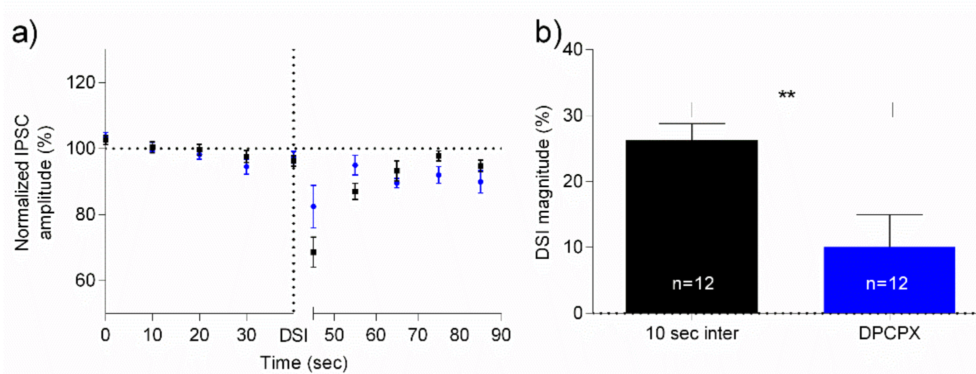

Figure 4.3.4 – DSI magnitude induced by protocol 1 is diminished in the presence of an A_1R antagonist. a) Time course of the change in IPSC amplitude in DSI protocol 1 under control conditions (black squares) and in the presence of 50 nM DPCPX (blue circles). IPSCs were evoked at 0.67 Hz. The pyramidal neuron was depolarised from −70 mV to 0 mV for 5 s at the times indicated by the arrows. **b)** DSI magnitude under control conditions and in the presence of DPCPX. Quantification of DSI magnitude. **p<0.01, ($F_{(11, 11)}$ = 3.7, Student's t-test).

4.3.5 A_1R blockade reduces DSI magnitude with protocol 2

The results using DSI induction protocol 2 were similar to those of DSI protocol 1, and showed a reduction of the suppressive effect of DSI when A_1R was blocked (DSI magnitude: control, 22.1% ± 2.9%, n=12; DPCPX, 14.1% ± 2.0% n=5; p<0.05, Figure 4.3.5).

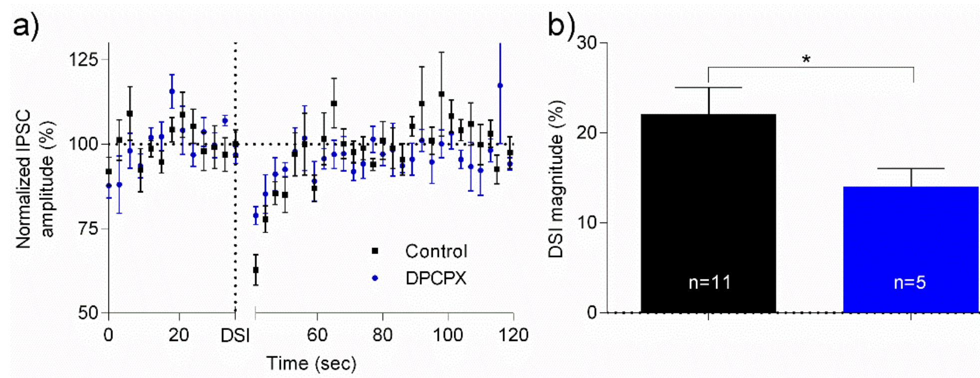

Figure 4.3.5 – DSI magnitude induced by protocol 2 is diminished in the presence of the A_1R antagonist, DPCPX. a) Time course of the change in IPSC amplitude in DSI protocol 2 under control conditions (black squares) and in the presence of 50 nM DPCPX (blue circles). IPSCs were evoked at 0.33 Hz. The pyramidal neuron was depolarised from −70 mV to 0 mV for 5 s at the times indicated by the arrows. **b)** DSI magnitude under control conditions and in the presence of DPCPX. Quantification of DSI magnitude. *p<0.05, ($F_{(10, 4)}$ = 4.5, Student's t-test).

4.3.6 Depolarization-induced suppression of inhibition is CB_1R independent

To evaluate if the suppression of inhibition is CB_1R-dependent, DSI was induced in the presence of the CB_1R inverse agonist AM251 (2 µM), and the result was surprising. DSI magnitude was similar following the blockade of CB_1R (AM251: 22.3% ± 3.7%, n=4) and under control conditions (control: 22.1% ± 2.9%, n=12). Thus, suppression of inhibition does not depend on CB_1R (Figure 4.3.6).

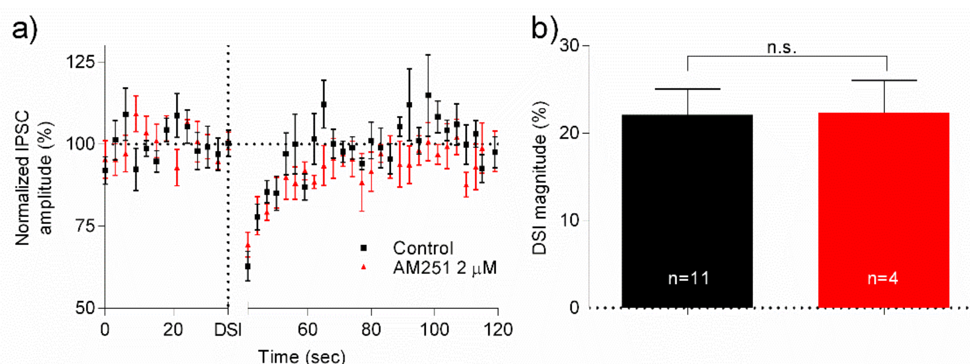

Figure 4.3.6 – DSI is CB_1R independent. a) Time course of the change in IPSC amplitude in DSI protocol 1 under control conditions (black squares) and in the presence of 2 µM AM251 (red triangles). IPSCs were evoked at 0.33 Hz. **b)** DSI magnitude under control conditions and in the presence of AM251. No significant differences were found between control and AM251 slices (p>0.05, $F_{(10, 3)}$ = 1.6, Student's t test).

4.3.7 Discussion

Over the last two decades, the consensus was that adenosine contributes to synaptic inhibition in the hippocampus, albeit with no direct effect on GABAergic synapses on pyramidal neurons (Cunha-Reis et al., 2007; Lambert and Teyler, 1991; Thompson et al., 1992; Yoon and Rothman, 1991). It is currently known that the adenosine A_1R receptor modulates tonic GABAergic transmission (Rombo et al., 2016), and thus it is clear that A_1R can only modulate phasic inhibitory transmission via other neuromodulators (Rombo et al., 2016). The inhibitory effect of WIN, a CB_1R agonist, has been well established for almost two decades (Wilson et al., 2001). Here, it was demonstrated that the A_1R blockade prevents the reduction of IPSCs by CB_1R activation in CA1 pyramidal cells. The excitatory transmission was completely inhibited by CNQX (AMPA/kainite receptor blocker) and DL-AP5 (NMDA receptor blocker) to allow isolation of inhibitory postsynaptic potential (IPSP) inputs. The A_1R blockade via CB_1R activation supports previous findings that A_1R activation attenuates the CB_1R-mediated inhibition of GABA release and the A_1R–CB_1R interaction occurs at the G-protein activation level (Sousa et al., 2011). However, in that study, the authors used different techniques and approaches.

The second part of this subchapter focused on a short-term plasticity mechanism, DSI, and the hypothesis of a possible interaction between A_1R and CB_1R modulating this phenomenon. DSI consists of a transient depression of IPSCs induced by brief depolarization of pyramidal neurons. When the inhibitory GABA-mediated neurotransmission is reduced, the IPSC is depressed, because the postsynaptic release of endocannabinoids activates CB_1R presynaptically. This short-term plasticity has been well characterized and is endocannabinoid-, 2-AG- (Min et al., 2010; Yoshino et al., 2011), and synthesis-dependent (Carlson et al., 2002; Ohno-Shosaku et al., 2012; Wilson et al., 2001). However, no studies have demonstrated an interaction between CB_1R and A_1R mediating the IPSC depression at pyramidal cells. Here, it was showed that the A_1R blockade reduces DSI magnitude, indicating a possible interaction between A_1 and CB_1 receptors.

Surprisingly, the CB_1R antagonist failed to prevent DSI under control conditions. This unexpected finding was recently verified in our laboratory using a different protocol.

Importantly, our finding that A_1R blockade prevents DSI in the hippocampus is consistent with previous findings such as those of Gu and co-workers (Gu et al., 2005).

5 General Discussion and Conclusions

The initial aim of this study was to elucidate the connection between the main hippocampal receptors for adenosine (A_1R) and endocannabinoids (CB_1R) in synaptic transmission and synaptic plasticity in the hippocampal CA1 region. Previous research has suggested a possible cross-talk mechanism between A_1R and CB_1R in hippocampal synaptic transmission and plasticity, but this mechanism has never been fully demonstrated for synaptic plasticity. In fact, when considering only long-term potentiation, a cellular plasticity model, there are no reports on an interaction between A_1R and CB_1R. Sousa et al. (2011) reported on a possible interaction between adenosine receptors and CB_1R upon chronic administration of caffeine, as an adenosine receptor antagonist, and THC, a CB_1R agonist and the main psychoactive constituent of cannabis. That study also found that caffeine exacerbated spatial memory deficit caused by THC and increased the expression of A_1R. Moreover, the authors showed that A_1R exerted a negative modulatory effect on CB_1-mediated inhibition of GABA and glutamate release.

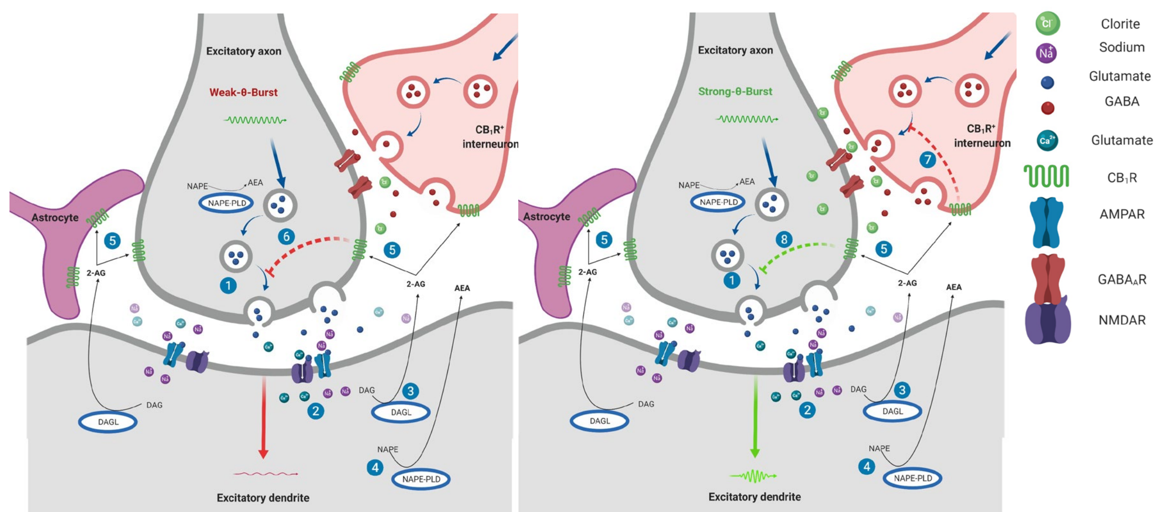

Figure 5.1– Schematic representation of the dual 2-AG effect on LTP. 1) Presynaptic release of glutamatergic transmitters into the synaptic cleft; 2) intracellular influx of sodium and calcium via glutamate ionotropic receptors NMDA and AMPA; 3) and 4) the increase of intracellular calcium triggers the synthesis of endocannabinoids such as 2-AG and anandamide (AEA), which are released into the synaptic cleft; 5) 2-AG activates CB_1R in astrocytes, interneurons, and pyramidal cells; 6) inhibition of glutamate release in pyramidal cells when the postsynaptic input is weak, inhibiting the postsynaptic signal; 7) inhibition of GABA release interneurons projecting to pyramidal cells when the postsynaptic input is strong; 8) enhancement of glutamate release in the pyramidal cells at the synaptic cleft, potentiating the postsynaptic signal.

In this work, I found no significant cross-talk between A_1R and CB_1R modulating synaptic plasticity (LTP), either when using an A_1R antagonist or A_1R knockout mice.

A crucial finding of the current study was that blocking of CB_1R activation might have a dual effect on LTP. In chapter 4.1, I demonstrated that eCB inhibits weak LTP while facilitates robust LTP, acting as a high-pass filter and likely reducing the signal-to-noise-ratio of synaptic strengthening. Moreover, I was able to show that the enhancement of LTP is dependent on the activity of interneurons, of which CCK-positive basket interneurons are the main candidates because they can express CB_1R and are localised at the *stratum lucidum*/*pyramidale* projecting to the pyramidal cells at the CA1 region.

I was also able to discriminate the modulatory effect of the two major endocannabinoids, 2-AG, and anandamide (AEA), on strong LTP. 2-AG and anandamide have opposite effects. On the one hand, the increase of endogenous anandamide by inhibition of its degradation diminished LTP. On the other hand, the increase of 2-AG enlarged LTP. These findings suggest that 2-AG plays a major role both in the facilitating effect of eCB on strong LTP and the inhibitory effect on weak LTP. When the levels of both eCBs (2-AG and AEA) increased, the prevailing effect was LTP inhibition via anandamide (Figure 4.1.4).

I also demonstrated a modulatory role for A_1R in the inhibitory effect caused by CB_1R activation at the synaptic transmission. When the A_1R was blocked, the inhibitory effect of CB_1R activation upon inhibitory inputs to excitatory neurons was abolished, suggesting an interaction between A_1R and CB_1R. Moreover, I also describe an interaction at the level of short term plasticity of inhibitory transmission, since DSI was attenuated by blocking A_1R, suggesting another modulation variable of inhibitory synaptic transmission and short-term plasticity mediated by endocannabinoids.

Overall, this work provides a better understanding of the neuromodulation of synaptic transmission and plasticity in the hippocampal CA1 region. Our findings strongly suggest that eCB (via CB_1R) play an important role in the homeostatic control of synaptic plasticity phenomena. By using A_1R knockout mice and selective A_1 antagonists, I was also able to show that most actions of endocannabinoids on synaptic plasticity are independent of endogenous A_1R activity.

This may seem somehow contradictory with the detected interaction between A_1R and CB_1R while evaluating short-term plasticity if inhibitory synapses. Still, it is important to keep in mind that synaptic transmission, short-term plasticity, and long-term plasticity phenomena result from different, though related mechanisms. Furthermore, the plasticity of inhibitory and excitatory synapses likely involves quite distinct processes.

6 Future perspectives

This thesis answered several questions, but many pieces of the A_1R–CB_1R cross-talk puzzle remain to be unveiled, especially at the synaptic transmission and plasticity levels. One unanswered question is the role of A_1R in DSI, a form of short-term plasticity. Also, a valid model to test the association of A_1R and DSI is required. It would be interesting to determine the role of A_1R in depolarisation-induced suppression of excitation (DSE) to appraise better the role of A_1R in cannabinoid-mediated short-term plasticity forms. Also, it would be important to investigate DSE and DSI using A_1R knockout mice to determine if the role of CB_1R on inhibitory transmission impairment is affected. Similarly, it would be crucial to understand the effect of endogenous 2-AG overactivation and the role of A_1R upon inhibitory synaptic transmission.

Similarly, several pieces of the synaptic plasticity puzzle remain unknown and need to be elucidated to enhance our understanding of the role of endocannabinoids 2-AG and AEA in LTP not only in young adult but also in old mice. The knockout of CB_1R in specific interneurons would make it possible to determine which interneuron type is responsible for the facilitating effect on LTP. In this thesis, I used pharmacological tools to block the synthesis and degradation of 2-AG. Due to pharmacological limitations, it was only possible to block the degradation of anandamide. The development of novel pharmacological tools could improve our understanding of the effect of endogenous anandamide on LTP.

Several approaches can be used to investigate the transduction and molecular mechanism responsible for LTP enhancement by eCBs. For instance, it would be important to determine the expression levels of several second messengers before and after LTP induction, focusing on the main candidates such as cyclic guanosine monophosphate. It would also be interesting to use the same technical approach as in Serpa et al. (2015) but focusing on cyclic guanosine monophosphate. In addition, a possible role for $A_{2A}R$ must be investigated under the same conditions. Finally, an important issue is to distinguish between the presynaptic and postsynaptic effect of 2-AG and AEA.

In general, a major challenge in the study of neuroscience is to understand how the endocannabinoid system is involved in pathological conditions such as neurodegenerative diseases or dementia. The endocannabinoid system may be an important piece in the

development of new targets to treat diseases related to memory impairment. Analogues of 2-AG may mitigate the memory deficit caused by the diseases with little or no harmful effects.

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9 Annexe 1

frontiers in Pharmacology

ORIGINAL RESEARCH
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Dual Influence of Endocannabinoids on Long-Term Potentiation of Synaptic Transmission

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Cannabinoid receptor 1 (CB_1R) is widely distributed in the central nervous system, in excitatory and inhibitory neurons, and in astrocytes. CB_1R agonists impair cognition and prevent long-term potentiation (LTP) of synaptic transmission, but the influence of endogenously formed cannabinoids (eCBs) on hippocampal LTP remains ambiguous. Based on the knowledge that eCBs are released upon high frequency neuronal firing, we hypothesized that the influence of eCBs upon LTP could change according to the paradigm of LTP induction. We thus tested the influence of eCBs on hippocampal LTP using two θ-burst protocols that induce either a weak or a strong LTP. LTP induced by a weak-θ-burst protocol is facilitated while preventing the endogenous activation of CB_1Rs. In contrast, the same procedures lead to inhibition of LTP induced by the strong-θ-burst protocol, suggestive of a facilitatory action of eCBs upon strong LTP. Accordingly, an inhibitor of the metabolism of the predominant eCB in the hippocampus, 2-arachidonoyl-glycerol (2-AG), facilitates strong LTP. The facilitatory action of endogenous CB_1R activation does not require the activity of inhibitory A1 adenosine receptors, is not affected by inhibition of astrocytic metabolism, but involves inhibitory GABAergic transmission. The continuous activation of CB_1Rs via exogenous cannabinoids, or by drugs known to prevent metabolism of the non-prevalent hippocampal eCB, anandamide, inhibited LTP. We conclude that endogenous activation of CB_1Rs by physiologically formed eCBs exerts a fine-tune homeostatic control of LTP in the hippocampus, acting as a high-pass filter, therefore likely reducing the signal-to-noise ratio of synaptic strengthening.

Keywords: endocannabinoids, cannabinoid CB_1 receptor, long-term potentiation, adenosine A_1 receptor, hippocampus

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INTRODUCTION

The influence of marijuana upon human cognition mostly results from its ability to interfere with the action of endocannabinoids (eCBs) in the brain. eCBs are widely recognized as fine-tune modulators of synaptic activity, their action mainly resulting from activation of G protein-coupled cannabinoid receptor type 1 receptors (CB_1R), which are widely distributed in the central nervous system, in particular in the hippocampus, cortex, basal ganglia, and cerebellum

(Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Marsicano and Lutz, 1999; Wilson and Nicoll, 2002). CB_1Rs are localized in neurons, both excitatory and inhibitory (Katona et al., 2001; Wilson et al., 2001; Kawamura, 2006; Hoffman et al., 2010), and also in astrocytes (Navarrete and Araque, 2008). CB_1Rs are endogenously activated by eCBs, mainly the fatty acid derivatives 2-arachidonoyl-sn-glycerol (2-AG) and anandamide. eCB synthesis mostly results from cleavage of postsynaptic membrane lipids as a consequence of the activation of postsynaptic G-coupled glutamate metabotropic receptors, which are predominantly activated as a consequence of high rate of neuronal firing (Chevaleyre et al., 2006; Katona et al., 2006). eCBs thus travel in a retrograde manner to activate astrocytic and nerve-terminal located CB_1R, resulting in inhibition of neurotransmitter release, and giving rise to several forms of short-term synaptic plasticity (Freund et al., 2003; Chevaleyre et al., 2006; Kano et al., 2009; Ohno-Shosaku et al., 2012). While the inhibitory action of eCBs upon neurotransmitter release is quite consistent, their action upon synaptic plasticity induced by brief high frequency neuronal firing, as long-term potentiation (LTP), is much more controversial. Indeed, and considering only the hippocampus, a brain area important for memory encoding and the mostly used to study synaptic plasticity phenomena, there are reports showing that eCBs restrict LTP (Bohme et al., 1999; Slanina et al., 2005) while others show that they facilitate LTP (Carlson et al., 2002; De Oliveira Alvares et al., 2006). This is intriguing since LTP is a compelling cellular model for learning and memory (see Nicoll, 2017), and exogenous cannabinoids, including the phytocannabinoids present in marijuana and the synthetic CB_1Rs agonists, have a negative impact upon learning and memory in humans and in laboratory animals (Miller et al., 1977; Lane et al., 2005; Sousa et al., 2011; Mouro et al., 2017). Elegant studies aiming at understanding the influence of eCBs upon LTP in different cell types or circuits in the hippocampus show that the action of eCBs may vary according to the cell type where the CB_1Rs sit (Monory et al., 2015) as well as the hippocampal circuit where LTP is induced (Wang et al., 2016). Knowing that eCBs are formed as a function of neuronal activity, we hypothesized that the influence of eCBs upon LTP could also vary as a function of the pattern of neuronal firing that induces plasticity. Evidence for that would not only contribute to further clarify reasons for discrepant data in the literature but also to better insight on the subtleties eCBs use to control synaptic strengthening. The present work was thus designed to evaluate the influence of eCBs upon hippocampal LTP induced by two types of stimulation, while keeping a θ-burst stimulation pattern, known to be related to hippocampal-dependent memory function (Buzsáki, 2002). We used a weak or a strong-θ-burst train of stimulation since previous evidence lead us to hypothesize that modulation of strong or weak forms of LTP may differ. Data obtained allow to suggest that eCBs act as a high pass filter, inhibiting LTP of low magnitude while facilitating robust LTP. Thus, eCBs likely reduce the signal-to-noise ratio of activity-dependent synaptic strengthening at the CA1 area of the hippocampus.

MATERIALS AND METHODS

Animals

The experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) from Stockholm (Sweden) or Lisbon (Portugal), and conducted in accordance with Portuguese and Swedish legislation on animal care and the European Community guidelines (Directive 2010/63/EU).

Most of the experiments were performed using male C57Bl6/J mice, aged between 8 and 18 weeks (most frequently 9–13 weeks) (Charles River Laboratories, Paris). In some cases, male and female mice were used to maximize the use of A1R knockout mice; because of this, control experiments using male and female mice have been performed. No appreciable differences between data obtained in males or females were detected (**Supplementary Figure S1**). The adenosine A_1 receptor knockout ($A_1R^{-/-}$) and wild-type ($A_1R^{+/+}$) mice were generated by inactivating the second protein coding exon of the mouse A_1R gene, from heterozygous breeding pairs with C57Bl6/J background strain (Johansson et al., 2001), obtained from a breeding colony derived from this original line that is housed at Karolinska Institutet, Sweden, and genotyped as described previously (Yang et al., 2015). All animals were social housed under standardized conditions of light (12-h light/12-h dark cycle), temperature (22–24°C), humidity (55–65%), and environmental enrichment (cardboard tubes plus nest material) and had free access to food and tap water.

Hippocampal Slices

Hippocampal slices were prepared as previously (e.g., Diógenes et al., 2004). The animals were sacrificed by decapitation under deep isoflurane anesthesia. The hippocampus was dissected free within ice-cold artificial cerebrospinal fluid (aCSF) solution composed of (millimeter): NaCl 124, KCl 3, $NaHCO_3$ 26, Na_2HPO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400-μm thick) were cut perpendicularly to the long axis of hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for 1 h in a resting chamber filled with the same solution, at room temperature and continuously gassed.

Extracellular Recordings

For electrophysiological recordings of field excitatory postsynaptic potentials (fEPSP), individual slices were transferred into a submerged recording chamber (dual submerged chamber) over the nylon mesh and continually superfused with gassed aCSF solution at a constant flow (3 ml/min) and temperature (32°C). This allows oxygenation in both slice surfaces while permitting a relatively fast flow rate to facilitate drug replacement. Stimulation (rectangular 0.1 ms pulses, once every 20 s) was delivered through a concentric electrode placed on Schaffer collateral-commissural fibers, in the stratum radiatum near the CA3–CA1 border. The intensity of the stimulus was set to the one eliciting near 50% of the maximal response, and was maintained throughout the experiment except in those experiments designed to perform input–output curves. In such experiments, after a

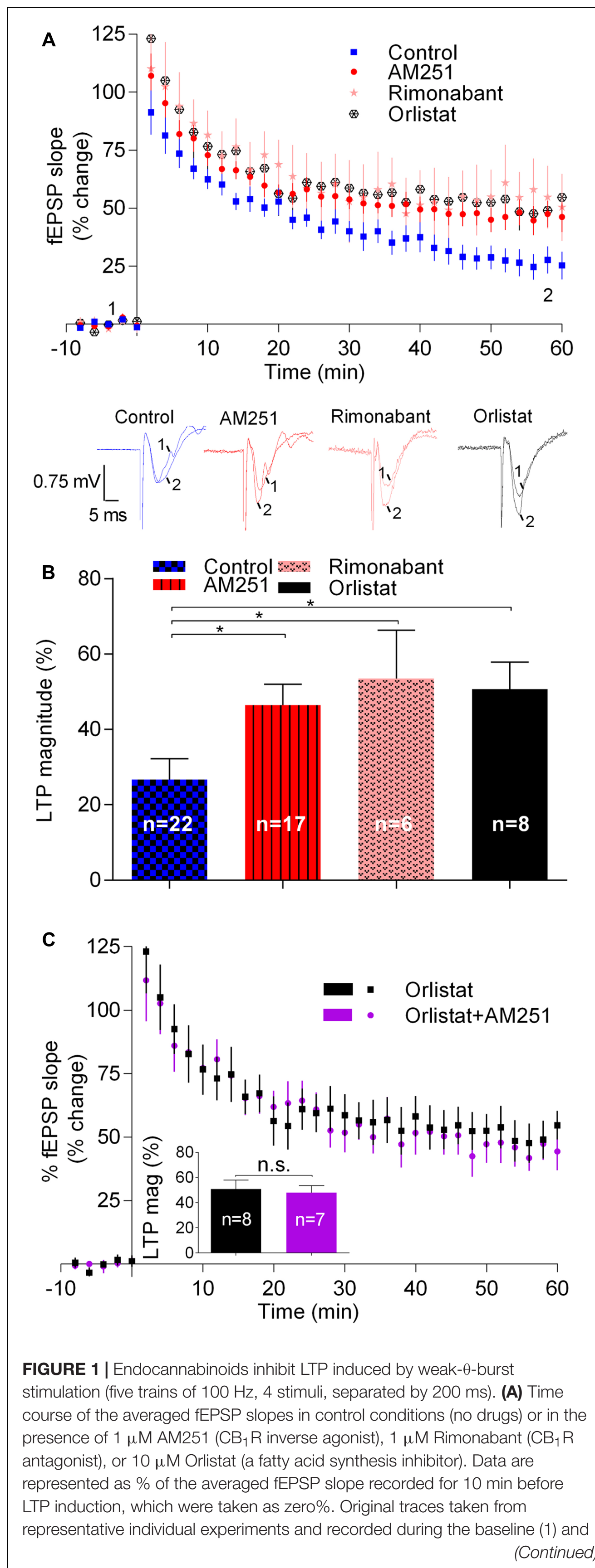

FIGURE 1 | Endocannabinoids inhibit LTP induced by weak-θ-burst stimulation (five trains of 100 Hz, 4 stimuli, separated by 200 ms). **(A)** Time course of the averaged fEPSP slopes in control conditions (no drugs) or in the presence of 1 μM AM251 (CB_1R inverse agonist), 1 μM Rimonabant (CB_1R antagonist), or 10 μM Orlistat (a fatty acid synthesis inhibitor). Data are represented as % of the averaged fEPSP slope recorded for 10 min before LTP induction, which were taken as zero%. Original traces taken from representative individual experiments and recorded during the baseline (1) and 50–60 min after weak-θ-burst induction (2) are shown below the time course panel. Each trace is composed by the stimulus artifact, followed by the presynaptic volley and the fEPSP. **(B)** Quantification of LTP magnitude under the indicated drug conditions. LTP magnitude was quantified as the % increase in fEPSP slope recorded at the 50–60 min after LTP induction, compared to the value recorded during the 10 min immediately before LTP induction; zero% represents no LTP and 100% would correspond to fEPSP slopes (at 50–60 min after LTP induction) twice the value recorded before LTP induction. *$p < 0.05$ ($F_{(4,51)} = 2.986$, one-way ANOVA with Sidak's correction). **(C)** Non-additivity of the facilitatory action of AM251 and Orlistat, when added together. Data are represented as time course of fEPSP slopes and inset shows average LTP magnitude (LTP mag, defined as in **B**) in the two conditions, the color of the bars corresponding to the color of the symbols in the time course. Data for Orlistat in **(A)** and **(C)** (time course) and in **(B)** and **(C)** (LTP magnitude) are repeated to allow comparison between the action of Orlistat in the absence or presence of AM251. All values are mean ± standard error of mean (SEM) from *n* experiments; *n* values are indicated on the bars. $F_{(7,6)} = 1.8$, ns: $p > 0.05$ (Student's *t*-test).

stabilization period under the standard stimulation conditions, the stimulus intensity was increased by 20 μA every 6 min, within a range of 80–300 μA. fEPSP recording was through a microelectrode filled with NaCl 4 M (2–6 MΩ resistance), placed in CA1 stratum radiatum, coupled to an Axoclamp 2B Amplifier (Axon Instruments) and digitized BNC-2110 (National Instruments). Individual responses were monitored, and averages of six consecutive responses were continuously stored on a personal computer with the WinLTP Software (Anderson and Collingridge, 2001). fEPSPs were continuously recorded under basal stimulation frequencies and LTP was induced only after obtaining stable fEPSP slope values for at least a 15 min. Test drugs were added to the perfusing aCSF at least 30 min before LTP induction, or initiation of the input–output curves. Changes in stimulus frequency (LTP induction) or intensity (input–output curves) were only initiated after at least a 15 min stable baseline in the presence of the drugs.

Long-term potentiation was induced by θ-burst stimulation. Two different stimulation paradigms were used in different experiments, weak-θ-burst and strong-θ-burst protocols, which differed only in the number of trains delivered. The weak-θ-burst consisted of five trains whereas the strong-θ-burst was composed of 10 trains, in both cases the stimulation trains were separated by 200 ms. In both paradigms each train was composed of four stimuli delivered at 100 Hz. LTP magnitude was quantified as the % change in the average fEPSP slopes recorded from 50 to 60 min after LTP induction, taking as 0% the averaged fEPSP slope recorded for 10 min immediately before LTP induction. Throughout the text, while referring to weak LTP or to strong LTP we mean LTP induced by a weak-θ-burst or by a strong-θ-burst, respectively.

Drugs

The following drugs were used: WIN55,212-2 (WIN) mesylate, AM251, 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), picrotoxin (PTX), JZL 184, and JZL 195 from Tocris. SR141716A (Rimonabant), tetrahydrolipstatin (Orlistat) from Biogen,

URB597 from Cayman Chemicals, barium salt of DL-fluorocitric acid from Sigma-Aldrich.

WIN55,212-2 was used as CB_1R agonist at a concentration (500 nM) 250 times higher than its K_i value for these receptors (Kuster et al., 1993). AM251 was used as a CB_1 receptor inverse agonist at a concentration (1 μM) 100 times higher than its K_i value for these receptors (Lan et al., 1999). Rimonabant was used as a CB_1 receptor antagonist at a concentration (1 μM) 500 times higher than its K_i for this receptor (Rinaldi-Carmona et al., 1994). DPCPX was used as an adenosine A_1 receptor antagonist at a concentration (50 nM) 100 times higher than its K_i value for this receptor (Bruns et al., 1987). PTX was used as a $GABA_A$ receptor antagonist at a concentration (50 μM) 100 times higher than its K_i value this receptor (Mehta and Ticku, 2013). JZL 184 was used as potent and selective monoacylglycerol lipase (MAGL) inhibitor at concentration (1 μM) 125 times higher than its IC_{50} for this enzyme (Long et al., 2009a). JZL 195 was used as potent inhibitor of both fatty acid amide hydrolase (FAAH) and of MAGL at a concentration (1 μM), respectively, 500 and 250 times higher than its IC_{50} for these enzymes (Long et al., 2009b). Orlistat was used as a diacylglycerol (DAG) lipase inhibitor at a concentration (10 μM) 100 times higher than the IC_{50} to inhibit DAG lipases α (Bisogno et al., 2006). URB597 was used as a selective FAAH inhibitor at a concentration (1 μM) 200 times the IC_{50} to inhibit this enzyme (Kathuria et al., 2003). Care was taken to use drug concentrations within selectivity ranges and according to previously published work using the same drugs for similar purposes. Solutions of all these drugs were prepared as stock solutions in 100% dimethylsulfoxide (DMSO). Aliquots of these stock solutions were diluted in aCSF in the day of the experiment. The concentration of the stock solution was chosen so that the final concentration of DMSO in the perfusion solutions was ≤0.1% (v/v).

Sodium fluorocitrate, an astrocyte metabolism inhibitor (Bonansco et al., 2011), was prepared as described by Paulsen et al. (1987): 8 mg of the barium salt of DL-fluorocitric acid was dissolved in 0.1 M HCl, precipitated by the addition of 0.1 M Na_2SO_4, buffered with 0.1 mM Na_2HPO_4 and centrifuged at 1000 × g for 5 min; the supernatant containing fluorocitrate was added to aCSF at a final concentration of 200 μM (pH 7.4).

Statistical Analysis

Data are expressed as the mean ± SEM; n corresponds to the number of experiments; in each experiment, only one slice was used per drug condition. At least one drug condition and the corresponding control was tested in each experimental day. Statistical significance was assessed by two-tailed Student's t-test when comparing two groups, or by one-way ANOVA with treatment as the between-subject factor, followed by Sidak's *post hoc* test when comparing multiple experimental groups. For the input–output curves, statistical significance was assessed by two-way ANOVA with treatment as the between-subject factor, followed by Sidak's *post hoc* test when comparing multiple experimental groups. A p-value of <0.05 was considered to account for significant difference. Analyses were performed with the GraphPad Prism 6 Software.

RESULTS

Physiologically Released Endocannabinoids Reduce LTP Induced by a Weak-θ-Burst

The first series of experiments was designed to evaluate the influence of eCBs upon weakly induced LTP. The influence of eCBs was assessed by testing the consequences of drugs that prevent CB_1R activation by eCBs or the synthesis of eCBs. We focused upon the synthesis of a predominant eCB at the hippocampus, 2-AG (Piyanova et al., 2015).

In control slices, fEPSP slopes recorded 50–60 min after inducing LTP with a weak-θ-burst, were 26.7 ± 5.5% higher than before LTP induction (n = 18; **Figure 1**). In slices where the CB_1R inverse agonist, AM251 (1 μM) was added to the perfusion at least 30 min before LTP induction, the magnitude of LTP was 46.5 ± 5.4% (n = 17, t = 2.6, $p < 0.05$ vs. control, **Figure 1**), which corresponds to near 80% increase in LTP magnitude. A similar result was obtained in the presence of another CB_1R blocker, the selective CB_1R antagonist, rimonabant (1 μM) (LTP magnitude: 53.5 ± 12.8%, n = 6, t = 2.5, $p < 0.05$ vs. control, **Figure 1**). In the presence of Orlistat (10 μM), an inhibitor of DAG lipase, the enzyme responsible for the conversion of DAG into 2-AG, the magnitude of LTP was also enhanced toward 50.7 ± 7.2% (n = 8, t = 2.5, $p < 0.05$; **Figure 1**). Importantly, when both CB_1R activation and 2-AG synthesis were prevented together, by the simultaneous presence of AM251 (1 μM) and Orlistat (10 μM) the magnitude of LTP was enhanced at the same degree as obtained with each of the drugs alone (t = 0.3, $p > 0.05$, **Figure 1C**). This lack of additivity indicates that both drugs facilitate LTP due to their common ability to prevent eCB signaling.

Summarizing, the above results show that drugs known to prevent the activation of CB_1R by eCBs or drugs known to inhibit the synthesis of 2-AG, the predominant eCB in the hippocampus (Piyanova et al., 2015), cause a marked facilitation of LTP induced by a weak-θ-burst, thus suggesting that eCBs inhibit such form of LTP.

Physiologically Released Endocannabinoids Enhance LTP with a Strong-θ-Burst

We then assessed the influence of eCBs on LTP induced by a strong-θ-burst protocol, all other experimental conditions being similar to those used before. Fifty–sixty minutes after the strong-θ-burst stimulation, LTP magnitude in control conditions was 68.1 ± 3.7% (n = 22) of pre-θ-burst stimulation. LTP dropped off by around 40% in the presence of AM251 (29.6 ± 6.8%, n = 9, t = 5.1, $p < 0.001$; **Figure 2**) or of rimonabant (28.5 ± 7.4%, n = 5, t = 4.2, $p < 0.01$; **Figure 2**). In the presence of Orlistat, the magnitude of LTP also decreased toward similar values (30.3 ± 8.4%, n = 5, t = 4.0, $p < 0.01$; **Figure 2**). It is worthwhile to note that in what concerns to the inhibition of LTP induced by a strong-θ-burst, the effect of AM251 was also not additive with that of Orlistat. Indeed, when both drugs were present, the LTP

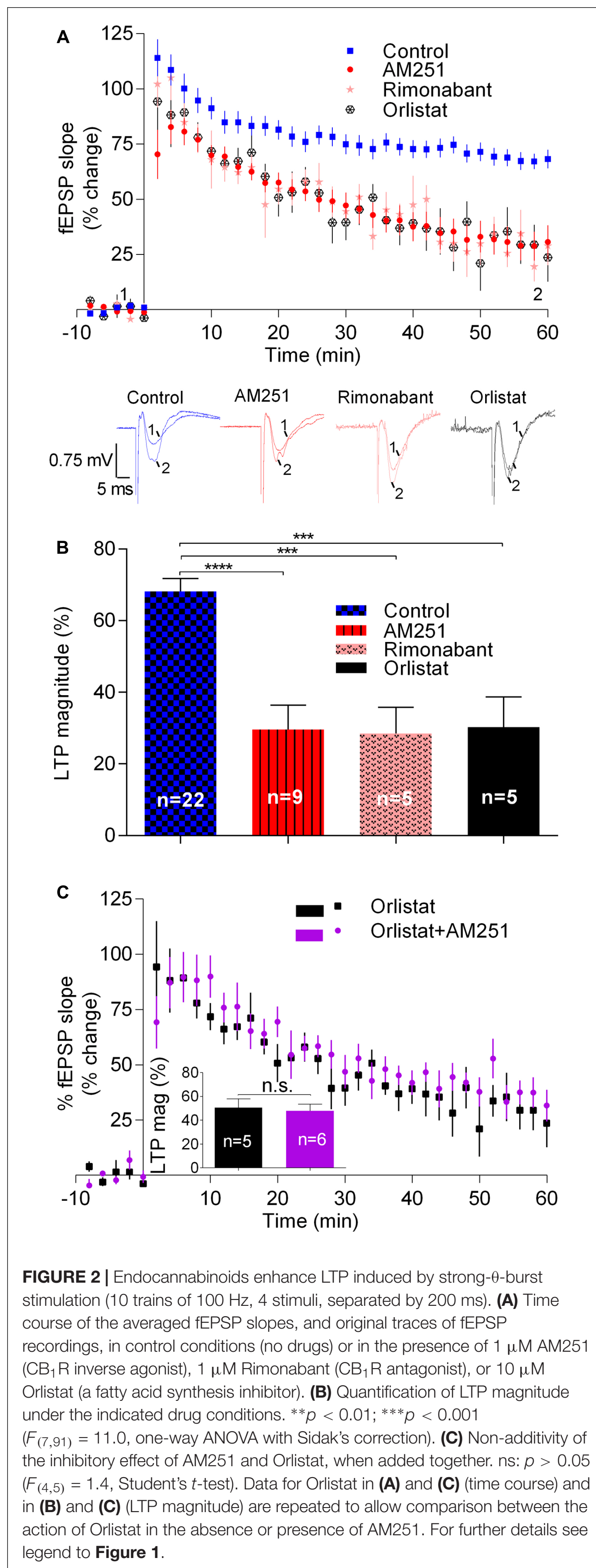

FIGURE 2 | Endocannabinoids enhance LTP induced by strong-θ-burst stimulation (10 trains of 100 Hz, 4 stimuli, separated by 200 ms). **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 1 μM AM251 (CB_1R inverse agonist), 1 μM Rimonabant (CB_1R antagonist), or 10 μM Orlistat (a fatty acid synthesis inhibitor). **(B)** Quantification of LTP magnitude under the indicated drug conditions. $^{**}p < 0.01$; $^{***}p < 0.001$ ($F_{(7,91)} = 11.0$, one-way ANOVA with Sidak's correction). **(C)** Non-additivity of the inhibitory effect of AM251 and Orlistat, when added together. ns: $p > 0.05$ ($F_{(4,5)} = 1.4$, Student's t-test). Data for Orlistat in **(A)** and **(C)** (time course) and in **(B)** and **(C)** (LTP magnitude) are repeated to allow comparison between the action of Orlistat in the absence or presence of AM251. For further details see legend to **Figure 1**.

magnitude was 38.5 ± 6.4% ($n = 6$), a value significantly different ($t = 0.8$, $p < 0.05$) from that obtained in control conditions, but of similar magnitude as that obtained in the presence of each of the drugs separately (**Figure 2**). Again, this suggests that the ability of these drugs to inhibit strongly induced LTP results from their common ability to prevent eCB signaling.

Summarizing, the results reported in this section show that drugs known to prevent CB_1R activation by eCBs or to inhibit 2-AG synthesis lead to an inhibition of LTP induced by strong-θ-burst. These data are in clear contrast with what was observed when inducing LTP with a weak-θ-burst, and suggest that LTP induced by a strong-θ-burst is facilitated by eCBs.

Continuous Stimulation of CB_1R or the Non-prevalent eCB Leads to LTP Inhibition

The approach described in the previous sections was always directed toward the consequences of preventing CB_1R activation by eCBs. On the light of what is known about the inhibitory action of cannabinoids on neuronal activity, our finding that LTP induced by a strong-θ-burst is reduced by preventing CB_1R activation was unexpected. We thus decided to assess how this form of LTP is affected by continuous activation of CB_1Rs. To do so we used two approaches: (1) test the influence of inhibitors of eCB hydrolysis and in such way create conditions for sustained enhanced levels of eCBs, or to (2) use a CB_1 receptor agonist to exogenously activate CB_1Rs in a sustained way.

Data shown in **Figure 3** summarize the findings while using inhibitors of enzymes that prevent hydrolysis of eCBs. When using JZL 184 (1 μM), a selective inhibitor of MAGL, the enzyme that hydrolyses 2-AG, the magnitude of LTP was enhanced toward 92.3 ± 11.2% ($n = 9$, $t = 2.6$, $p < 0.05$ as compared with absence of drugs, **Figure 3**), corresponding to a value about 40% higher than that obtained in the absence of any drug. This finding suggests that enhancement of the levels of the predominant eCB in the hippocampus, 2-AG (Piyanova et al., 2015), facilitates strong LTP, thus in line with previous results showing that blockade of CB_1R or inhibition of synthesis of 2-AG inhibit strong LTP.

Remarkably, in the presence of URB 597, which at the concentration used (1 μM) inhibits FAAH, but not MAGL, the magnitude of LTP decreased toward 33.3 ± 8.6% ($n = 7$, $t = 3.4$, $p < 0.05$ as compared with absence of drugs, **Figure 3**), thus toward near half of the value obtained in control conditions. Since FAAH hydrolyses anandamide, this data suggest that accumulation of the non-predominant eCB in the hippocampus, anandamide (Piyanova et al., 2015), inhibits LTP in clear contrast with what occurs with the influence of the most abundant eCB in the hippocampus, 2-AG. This conclusion is further supported by the experiments where a non-selective inhibitor of both enzymes, FAAH and MAGL, was used. Thus, in the presence of JZL 195 (1 μM), the LTP magnitude was decreased toward a value (44.2 ± 8.8%, $n = 8$, **Figure 3**) between that obtained with URB 597 and that obtained in the absence of any drug, being not significant different ($t = 2.4$, $p > 0.05$) from any of these conditions. Altogether, the data with JZL 184, URB 597, and

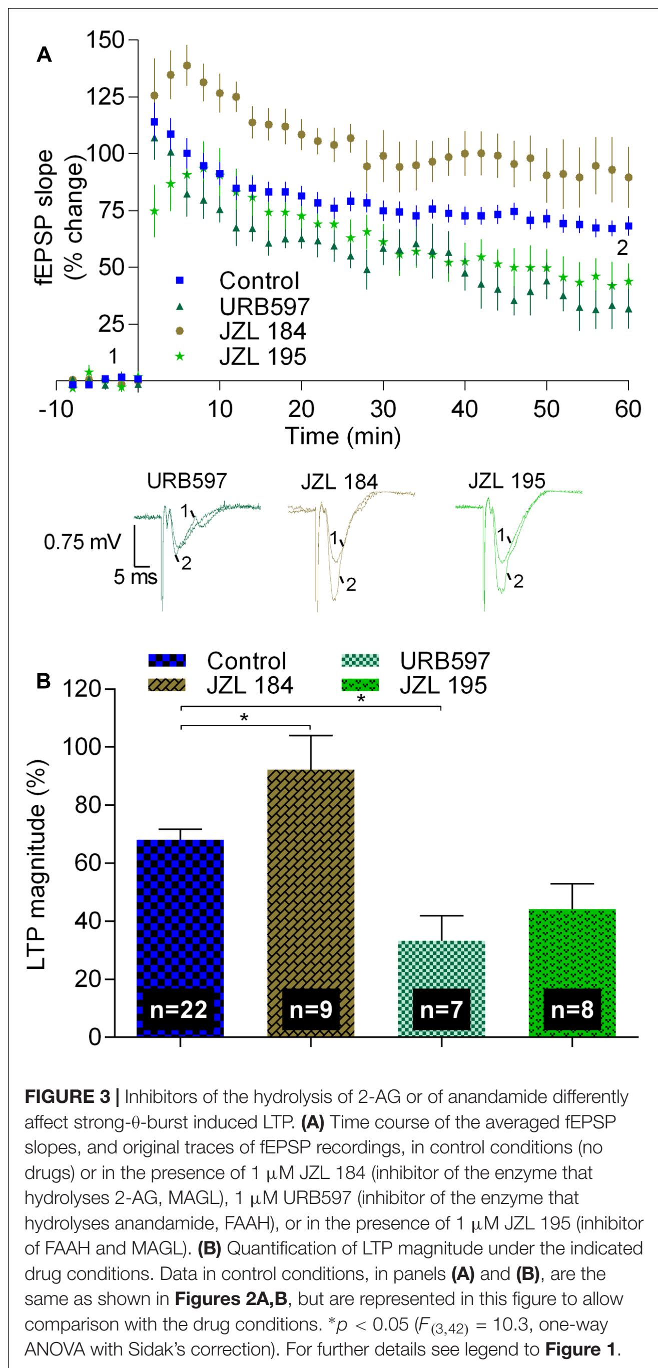

FIGURE 3 | Inhibitors of the hydrolysis of 2-AG or of anandamide differently affect strong-θ-burst induced LTP. **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 1 μM JZL 184 (inhibitor of the enzyme that hydrolyses 2-AG, MAGL), 1 μM URB597 (inhibitor of the enzyme that hydrolyses anandamide, FAAH), or in the presence of 1 μM JZL 195 (inhibitor of FAAH and MAGL). **(B)** Quantification of LTP magnitude under the indicated drug conditions. Data in control conditions, in panels **(A)** and **(B)**, are the same as shown in **Figures 2A,B**, but are represented in this figure to allow comparison with the drug conditions. $^{*}p < 0.05$ ($F_{(3,42)} = 10.3$, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

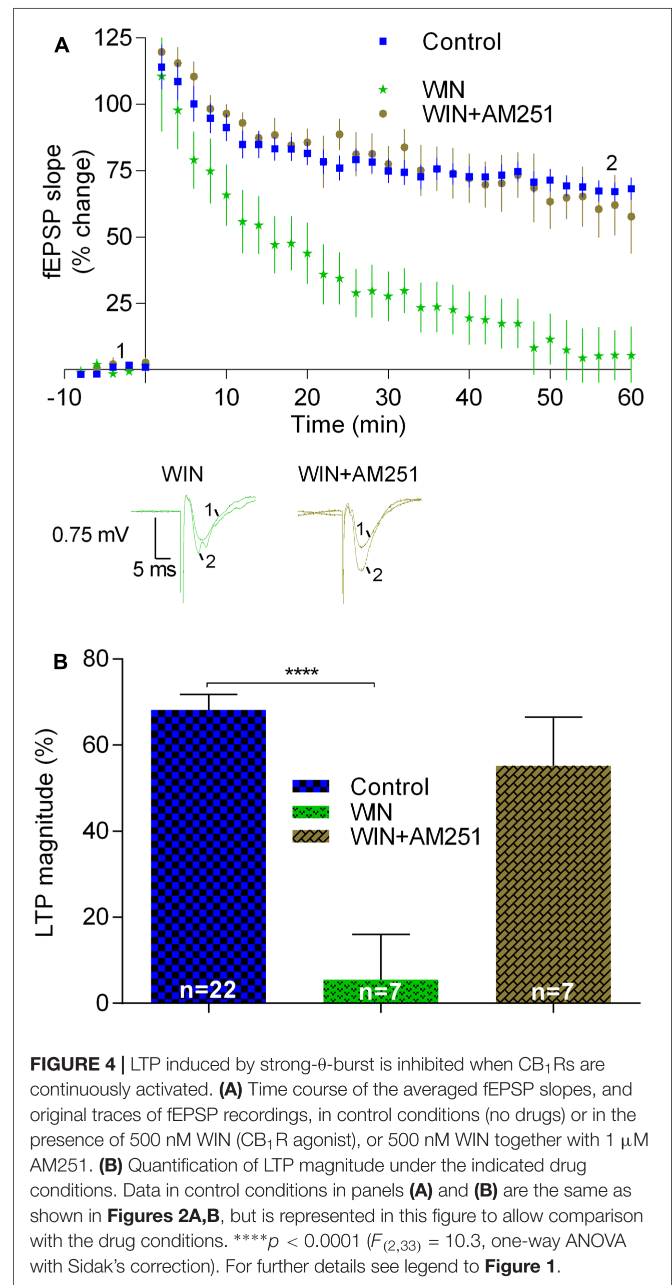

FIGURE 4 | LTP induced by strong-θ-burst is inhibited when CB_1Rs are continuously activated. **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 500 nM WIN (CB_1R agonist), or 500 nM WIN together with 1 μM AM251. **(B)** Quantification of LTP magnitude under the indicated drug conditions. Data in control conditions in panels **(A)** and **(B)** are the same as shown in **Figures 2A,B**, but is represented in this figure to allow comparison with the drug conditions. $^{****}p < 0.0001$ ($F_{(2,33)} = 10.3$, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

JZL 195 allow to suggest that enhanced production of 2-AG and enhanced production of anandamide affect strong LTP in an opposed way. However, the possibility that the inhibitory action of URB 597 results from non-CB1-related mechanisms (Kathuria et al., 2003; Ratano et al., 2017) cannot be fully excluded.

Secondly, we tested the effect of WIN (500 nM), a compound known to activate CB_1R. Since the effect of WIN upon synaptic transmission is known to be rather slow (Serpa et al., 2009), the slices were pre-incubated with WIN for at least 60 min before transfer to the acquisition chamber. Then, the slices were stabilized for at least 20 min, LTP being only induced when fEPSP slope values remained stable for at least 15 min. In such experiments LTP was virtually abolished (LTP magnitude: $5.5 \pm 10.5\%$, $n = 7$, $t = 6.5$, $p < 0.05$ vs. pre-LTP induction; $p < 0.0001$ vs. LTP magnitude in control conditions; **Figure 4**). This inhibitory effect of WIN was prevented when the slices had been pre-incubated with the CB_1R inverse agonist, AM251, before addition of WIN. Indeed, under such conditions the inhibitory effects of both the agonist and the antagonists seem to be reciprocally canceled since LTP magnitude obtained in slices in the presence of AM251 and WIN ($55.2 \pm 11.2\%$, $n = 7$, **Figure 4**) was similar ($t = 1.3$, $p > 0.05$) to that obtained in the absence of any drug.

Summarizing, the data reported in this section suggest that sustained activation of CB_1Rs induced by adding an exogenous agonist as well as prevention of degradation of the non-prevalent eCB in the hippocampus leads to inhibition of LTP induced by the strong-θ-burst. This is in clear contrast with the conclusions that could be drawn while assessing the action of a drug known to prevent the hydrolysis or prevent the formation of the predominant eCB in the hippocampus as well as when accessing the action of transiently released eCBs by using CB_1R blockers. Altogether, the data indicate that while physiologically released eCBs are required to facilitate LTP induced by a strong θ-burst, non-physiological activation of CB_1R leads to inhibition of this form of LTP.

Inhibition of LTP during CB_1R Blockade Is Not a Result of Enhanced A_1R Activation

The above results indicating that physiologically released eCBs can facilitate LTP lead us to hypothesize that the strong-θ-burst could lead to the recruitment of other neuromodulators that would affect the neuromodulatory influence of eCBs. Purines are released during high-frequency neuronal firing (Cunha et al., 1996) and adenosine is known to inhibit LTP through activation of A_1R (De Mendonça and Ribeiro, 2000; Wang et al., 2016), which are abundantly expressed in the hippocampus. Furthermore, A_1R can affect CB_1R signaling (Hoffman et al., 2010; Sousa et al., 2011). To test if the apparent facilitatory action of eCBs upon strong-θ-burst-induced LTP could be due to any interference with endogenous adenosine acting on A_1R, we used two different approaches: genetic ($A_1R^{-/-}$ mice) or pharmacological (selective A_1R antagonist, DPCPX) prevention of A_1R activity.

The magnitude of LTP induced by the strong-θ-burst in slices from $A_1R^{-/-}$ mice (65.5 ± 6.7%, n = 13) was not significantly different (t = 0.35, p > 0.05) from that obtained in $A_1R^{+/+}$ (68.1 ± 3.7%, n = 22, **Figure 5**). Remarkably, the CB_1R inverse agonist AM251 inhibited LTP toward a similar value in both genotypes [$A_1R^{+/+}$: 29.6 ± 6.8%, n = 9; $A_1R^{-/-}$: 25.1 ± 9.3%, n = 8, t = 0.4, p > 0.05 when comparing genotypes; t = 4.1, p < 0.05 when assessing the effect of AM251 in $A_1R^{-/-}$ (control $A_1R^{-/-}$ vs. $A_1R^{-/-}$: AM251), **Figure 5**]. These data suggest that the inhibition of strong LTP caused by the inverse agonist of CB_1R does not result from an enhanced A_1R activation by released adenosine. To further confirm this, and to preclude any adaptation-like process due to genetic removal of A_1R, we tested action of AM251 in $A_1R^{+/+}$ mice following inhibition of the A_1R with DPCPX. Again, and in spite the presence of DPCPX at a concentration (50 nM) near 100 times its K_i for A_1R (Bruns et al., 1987), thus expected to fully block A_1R signaling, AM251 caused a marked inhibition of LTP induced by the strong-θ-burst (DPCPX: 68.0 ± 9.3%, n = 9; DPCPX+AM251: 32.5 ± 5.2%, n = 7, t = 3.3, p < 0.05, **Figure 5**).

Altogether the above data seem to indicate that the inhibition of LTP caused by preventing CB_1R activation by eCBs is not due to enhanced activation of A_1R by endogenous adenosine.

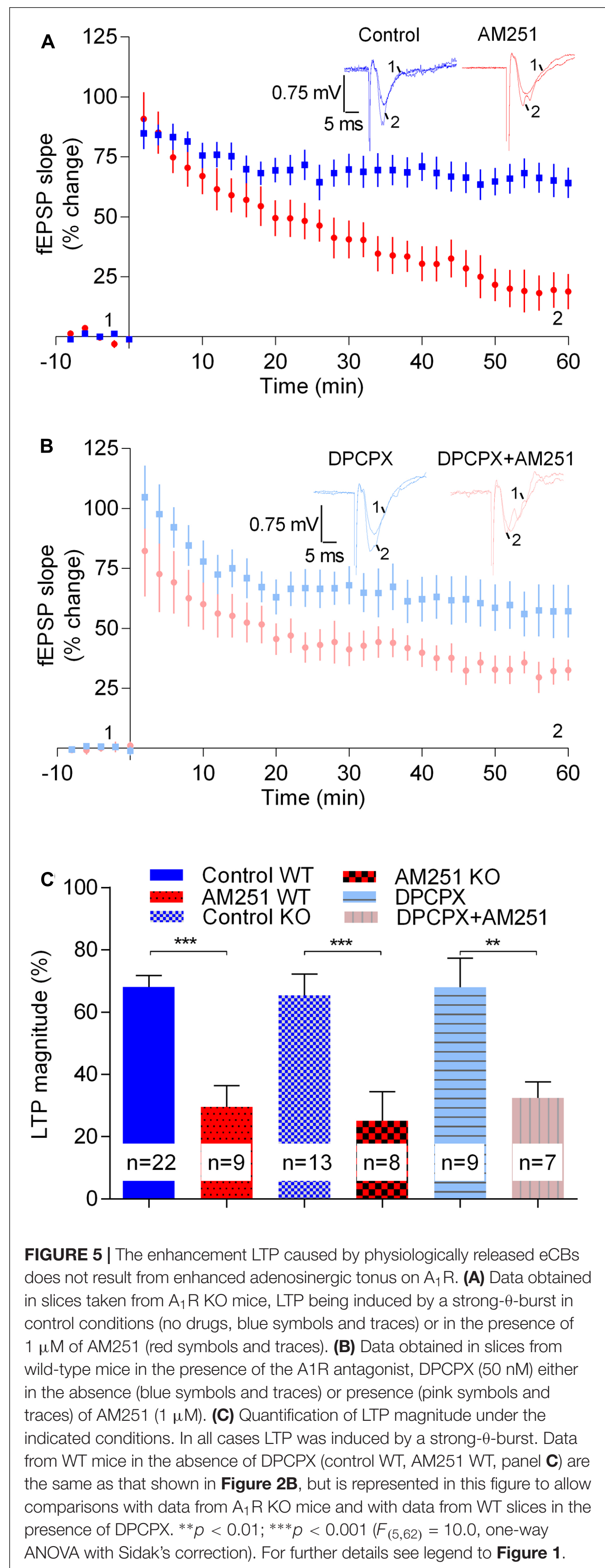

FIGURE 5 | The enhancement LTP caused by physiologically released eCBs does not result from enhanced adenosinergic tonus on A_1R. **(A)** Data obtained in slices taken from A_1R KO mice, LTP being induced by a strong-θ-burst in control conditions (no drugs, blue symbols and traces) or in the presence of 1 μM of AM251 (red symbols and traces). **(B)** Data obtained in slices from wild-type mice in the presence of the A1R antagonist, DPCPX (50 nM) either in the absence (blue symbols and traces) or presence (pink symbols and traces) of AM251 (1 μM). **(C)** Quantification of LTP magnitude under the indicated conditions. In all cases LTP was induced by a strong-θ-burst. Data from WT mice in the absence of DPCPX (control WT, AM251 WT, panel **C**) are the same as that shown in **Figure 2B**, but is represented in this figure to allow comparisons with data from A_1R KO mice and with data from WT slices in the presence of DPCPX. $^{**}p$ < 0.01; $^{***}p$ < 0.001 ($F_{(5,62)}$ = 10.0, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

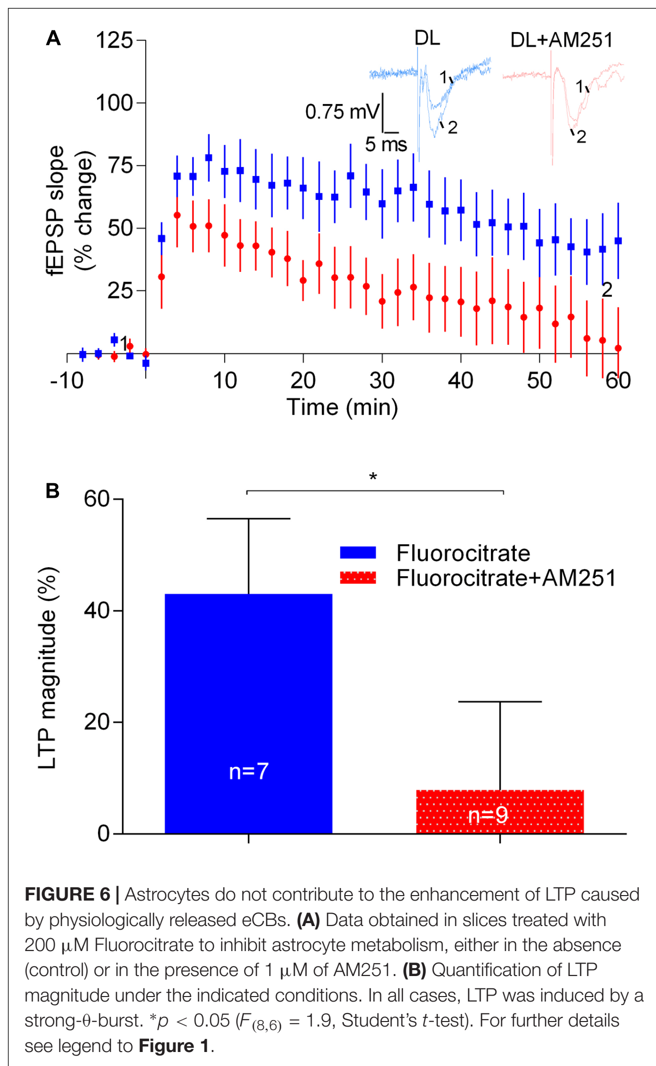

FIGURE 6 | Astrocytes do not contribute to the enhancement of LTP caused by physiologically released eCBs. **(A)** Data obtained in slices treated with 200 µM Fluorocitrate to inhibit astrocyte metabolism, either in the absence (control) or in the presence of 1 µM of AM251. **(B)** Quantification of LTP magnitude under the indicated conditions. In all cases, LTP was induced by a strong-θ-burst. *$p < 0.05$ ($F_{(8,6)} = 1.9$, Student's *t*-test). For further details see legend to **Figure 1**.

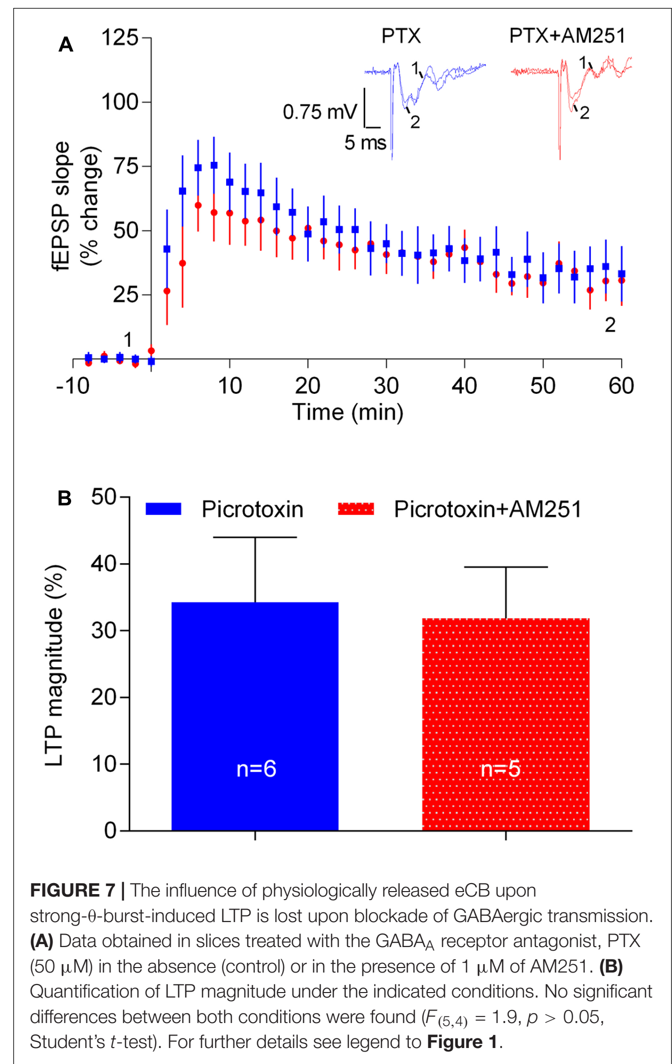

FIGURE 7 | The influence of physiologically released eCB upon strong-θ-burst-induced LTP is lost upon blockade of GABAergic transmission. **(A)** Data obtained in slices treated with the $GABA_A$ receptor antagonist, PTX (50 µM) in the absence (control) or in the presence of 1 µM of AM251. **(B)** Quantification of LTP magnitude under the indicated conditions. No significant differences between both conditions were found ($F_{(5,4)} = 1.9$, $p > 0.05$, Student's *t*-test). For further details see legend to **Figure 1**.

The absence of A_1Rs also did not affect the inhibitory action of the CB_1R agonist (500 nM WIN) upon LTP magnitude. Thus, in $A_1R^{(-/-)}$ mice the magnitude of LTP in the presence of 500 nM WIN (-0.57 ± 9.4, $n = 5$) was significantly lower ($t = 4.1$, $p < 0.0001$) than in the absence of WIN and not different ($t = 0.5$, $p > 0.05$) from LTP magnitude in slices from WT mice in the presence of 500 nM WIN.

Astrocytes Do Not Contribute to the Enhancement of LTP Caused by eCBs

Astrocytes are known to contribute to the facilitatory action of eCBs upon glutamatergic transmission (Navarrete and Araque, 2010). In addition, it is known that astrocytes, by releasing gliotransmitters, which then act in pre- and post-synaptic receptors, affect neuronal signaling and plasticity (Pascual et al., 2005; Henneberger et al., 2010). We thus hypothesized that the apparent facilitatory action of eCBs upon LTP induced by the strong-θ-burst would involve the astrocytes. To address that possibility we incubated the slices with the metabolic gliotoxin fluorocitrate (200 µM) for at least 20 min and allowed the fEPSP slopes to stabilize for at least 15 min before inducing LTP either in the presence or absence of AM251. As expected from previous reports (Bonansco et al., 2011) LTP magnitude was reduced in slices incubated with fluorocitrate (cf. data in **Figure 6** with **Figure 2**). Remarkably, however, under such conditions the CB_1R inverse agonist, AM251, was still able to markedly inhibit LTP (fluorocitrate: $43.0 \pm 13.5\%$, $n = 7$; fluorocitrate +AM251: $7.9 \pm 15.8\%$, $n = 9$, $t = 2.3$, $p < 0.05$ vs. fluorocitrate alone; **Figure 7**).

These data suggest that the apparent facilitatory action of eCBs upon strong-θ-burst-induced LTP does not involve the astrocytes. This data also show that, at least under some experimental conditions, the strength of LTP induction may be even more determinant of the direction of the influence of eCBs upon LTP than the magnitude of LTP itself.

The eCB-Mediated Enhancement of LTP Is GABAergic Transmission Dependent

Next we hypothesized that the apparent facilitatory action of eCBs upon LTP caused by the strong-θ-burst stimulation could

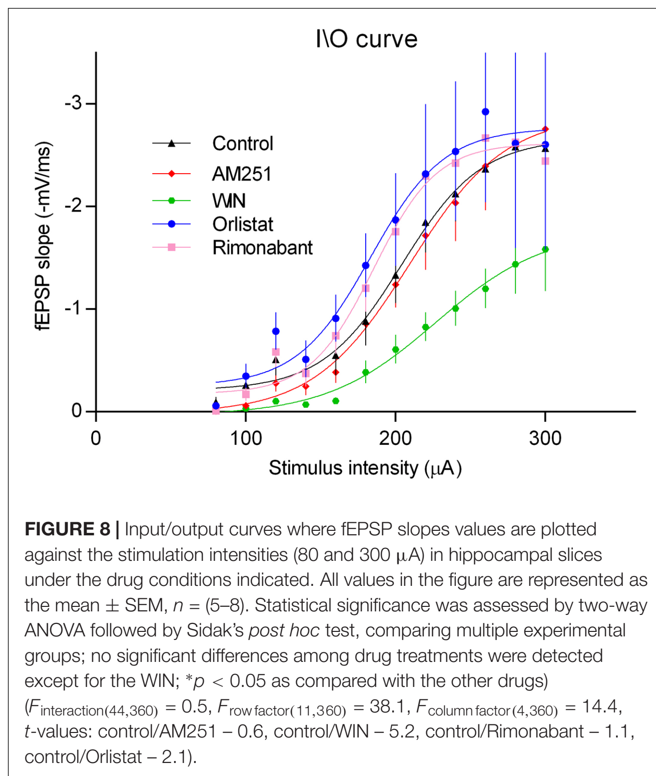

FIGURE 8 | Input/output curves where fEPSP slopes values are plotted against the stimulation intensities (80 and 300 μA) in hippocampal slices under the drug conditions indicated. All values in the figure are represented as the mean ± SEM, n = (5–8). Statistical significance was assessed by two-way ANOVA followed by Sidak's *post hoc* test, comparing multiple experimental groups; no significant differences among drug treatments were detected except for the WIN; *$p < 0.05$ as compared with the other drugs) ($F_{\text{interaction}(44,360)} = 0.5$, $F_{\text{row factor}(11,360)} = 38.1$, $F_{\text{column factor}(4,360)} = 14.4$, t-values: control/AM251 – 0.6, control/WIN – 5.2, control/Rimonabant – 1.1, control/Orlistat – 2.1).

be due to a preponderant inhibition of GABA release over glutamate release. To test this possibility, experiments were performed in the presence of the $GABA_AR$ antagonist, PTX (50 μM). LTP magnitude was smaller in the presence of PTX, as compared with its absence (cf. **Figures 2**, **7**), which may result from overactivity of glutamatergic transmission even before LTP induction. Remarkably, in slices in the presence of PTX, the inhibitory action of AM251 upon LTP was lost (PTX: 34.3 ± 9.7%, n = 6; PTX+AM251: 31.8 ± 7.7%, n = 5; 0.1, $p > 0.05$, **Figure 7**). The ability of PTX to prevent the inhibitory action of AM251 upon LTP should not be attributed to its ability to diminish LTP, since fluorocitrate also inhibited LTP and did not prevent the action of AM251 (cf. **Figures 6**, **7**).

The above data suggest that the apparent facilitatory action of eCBs upon LTP induced by the strong-θ-burst involves $GABA_AR$-mediated GABAergic transmission, most probably resulting from eCB-induced inhibition of GABA release with consequent disinhibition of glutamatergic neurons.

Prevention of CB_1R Activation by eCBs Does Not Affect Basal Excitability

Long-term potentiation can be influenced by changes in basal synaptic transmission. To evaluate if manipulation of 2-AG signaling could have a global influence upon excitability, input/output (I/O) curves were compared in the absence and presence of AM251, Rimonabant, or Orlistat. As illustrated in **Figure 8**, none of these drugs appreciably modified I/O curves compared with the control. Exogenous activation of CB_1R using the CB_1R agonist WIN, did however clearly altered the I/O curve, an action that can be attributed to its well-known ability to inhibit synaptic transmission at the CA1 area of the hippocampus (Serpa et al., 2009). The absence of influence of AM251, Rimonabant, or Orlistat in I/O curves but their influence upon LTP, indicates that transiently released 2-AG have a predominant influence over LTP rather than over basal synaptic transmission.

DISCUSSION

A main finding in the present work is that prevention of CB_1R activation may affect CA1 LTP in an opposing way, depending on the strength of LTP induction and the magnitude of LTP itself. Thus, we show that a CB_1R inverse agonist, a CB_1R antagonist as well as an inhibitor of the formation of 2-AG, the main eCB in the hippocampus (Piyanova et al., 2015), leads to a facilitation of weakly induced LTP but to an inhibition of strongly induced LTP. This suggests that eCBs inhibit weak LTP while facilitating a more robust LTP. In accordance to the idea that physiologically released eCBs favor robust LTP is also the finding that an inhibitor of the degradation of 2-AG facilitates LTP induced by the strong-θ-burst. However, continuous activation of CB_1R with an exogenous agonist or overproduction of a non-prevalent eCB, anandamide, leads to inhibition of strongly induced LTP. Overall, these findings are suggestive of dual actions of eCBs upon CA1 LTP depending both on the strength of LTP induction as well as on the nature of CB_1R activators.

To our knowledge, this is the first time that dual action of cannabinoids upon hippocampal LTP is clearly shown. It is known for a long time that mice lacking CB_1Rs have enhanced hippocampal LTP (Bohme et al., 1999; Jacob et al., 2012), compatible with the general idea of inhibitory actions of cannabinoids in the brain. Similarly, the work by Slanina et al. (2005) clearly showed that weak LTP, induced by a small number of pulses delivered at the CA1 area of hippocampal slices, is facilitated by CB_1R blockade, also allowing the suggestion that eCBs inhibit weakly induced LTP. However, LTP induced by strong non-θ-burst high frequency stimulation (100 pulses for 1 s, or twice this paradigm separated by 20 s) was unaffected by CB_1R blockade (Slanina et al., 2005), in clear contrast with the results herein reported for robust θ-burst-induced LTP. Species differences (mice in our study vs. rats in the study by Slanina et al., 2005) or age of the animals (adults in our study vs. adolescents in the study by Slanina et al., 2005) may account for these differences.

Facilitation of LTP by eCBs has been also reported, but again, those studies do not highlight a dual action of eCBs as a function of LTP magnitude. First evidence that eCBs facilitate CA1 hippocampal LTP was provided by the report that eCBs enable LTP induction by trains of EPSPs that are ineffective if eCBs are not allowed to act (Carlson et al., 2002). This action could be attributed to a eCB-mediated inhibition of GABAergic synapses (Carlson et al., 2002), and indeed it was later reported that upon removal of synaptic inhibition in a restricted area of the dendritic tree, there is a selective priming of nearby excitatory synapses by eCBs, which facilitate induction of CA1 hippocampal LTP (Chevaleyre and Castillo,

2004). Those studies allowed to understand the action of eCBs at the local circuitry and at the single neuron level, but do not inform on the global impact of eCBs upon LTP of pyramidal neurons. Using adult rats, De Oliveira Alvares et al. (2006) reported a marked inhibition of non-θ-burst high-frequency-induced CA1 LTP of fEPSPs by AM251, thus pointing that eCBs are required for robust LTP phenomena. More recently, Wang et al. (2016) reported that 2-AG and CB_1R signaling is required for LTP of the lateral perforant path input to dentate gyrus neurons. In the study by Wang et al. (2016), however, robustly induced CA1 LTP was unaffected by preventing CB_1R activation. In contrast, our data clearly point toward a facilitatory action of 2-AG and CB_1R signaling on CA1 LTP induced by robust stimulation. Pattern of stimulation (θ-burst in both cases), or age (adult animals in both cases), cannot account for the differences. The difference may reside in the characteristics of the perfusion chamber, which may impact upon the accumulation of endogenous substances around the synapses. We used a slice submersion chamber, while Wang et al. (2016) used an interface chamber; submerging chambers likely favor the accumulation of endogenous substances. Lower level of oxygenation in submerged chambers may, in some studies, account for differences between data obtained in submerged or interface chambers (Hájos and Mody, 2009). However, this might not be the case since our chambers are provided with nylon mesh thus allowing oxygenation in both surfaces of the slice. Under our experimental conditions the oxygen pressure in the perfusion solution inside the chamber is 500–600 mmHg (Sebastiao et al., 2001). We used mice while Wang et al. (2016) used rats when testing the influence upon CA1 LTP, but species differences are unlikely to account for the dissimilarities since no marked differences were detected by Wang et al. (2016) while comparing LPP–LTP data in mice and rat hippocampal slices.

Pyramidal hippocampal neurons are under inhibitory control of GABAergic synapses, but also under control of several modulatory substances. Adenosine, an ubiquitous molecule released by neurons and glia, is able to modulate synaptic transmission and plasticity by operating high affinity G-protein-coupled receptors (Sebastião and Ribeiro, 2015). The adenosine A_1R is highly expressed in the hippocampus and with a clear inhibitory action upon synaptic transmission and LTP (Dunwiddie and Masino, 2001; Boison, 2005; Sebastião and Ribeiro, 2015). The inhibitory action of CB_1R upon GABA and glutamate release, as well as on synaptic transmission in the hippocampus are partially reduced by co-activation of A_1R (Hoffman et al., 2010; Sousa et al., 2011), suggesting an interaction between these two modulatory pathways at the hippocampus (but see Serpa et al., 2009, 2015). The possibility that A_1R-mediated attenuation of an inhibitory effect of eCBs could justify the apparent excitatory action of eCBs upon strongly induced LTP led us to test if the action of a CB_1R blocker was affected by A_1R deletion or A_1R blockade. However, none of these significantly influenced the inhibitory action of AM251 upon strong LTP.

Astrocytes release several neuromodulatory substances, including purines (Henneberger et al., 2010; Lalo et al., 2014), and have been shown to contribute to the facilitatory action of eCBs upon hippocampal glutamatergic transmission (Navarrete and Araque, 2010). Metabolic inhibition of the astrocytes, a condition known to affect astrocytic signaling and release of gliotransmitters (Paulsen et al., 1987; Swanson and Graham, 1994; Bonansco et al., 2011) did, however, not affect the influence of AM251 upon LTP. This suggests that astrocytes do not play a major role in the facilitatory action of eCBs upon LTP.

A common conclusion in all studies reporting facilitation of LTP by eCBs is that it can be accounted by an influence upon GABAergic neurons (Carlson et al., 2002; Chevaleyre and Castillo, 2004; Wang et al., 2016). Accordingly, we also observed that the ability of the CB_1R blocker, AM251, to inhibit LTP was lost in the presence of the $GABA_AR$ antagonist, PTX, thus reinforcing the conclusion that physiologically released eCBs facilitate LTP by restraining the inhibition of LTP imposed by GABAergic inputs. It has previously been shown that deletion of CB_1R in GABAergic neurons leads to a decreased hippocampal CA1 LTP, whereas deletion of CB_1R in glutamatergic neurons leads to enhanced LTP (Monory et al., 2015). It is therefore likely that the two stimulation conditions used in the present work lead to a differential influence of eCBs in GABAergic interneurons and glutamatergic neurons, so that under strong LTP induction conditions the influence of eCBs upon GABAergic neurons predominates.

CB_1R are widely distributed in the central nervous system, mainly in the hippocampus, cortex, basal ganglia, and cerebellum (Marsicano and Lutz, 1999; Wilson and Nicoll, 2002). This receptor is localized in excitatory and inhibitory neurons (Katona et al., 2001; Wilson et al., 2001; Kawamura, 2006) and also in astrocytes (Hoffman et al., 2010; Han et al., 2012). Considering the neuronal compartment only, it has been estimated that about three quarters of all CB_1R present in hippocampi are on GABAergic neurons while glutamatergic neurons contain about one quarter of all hippocampal CB_1R (Steindel et al., 2013). Not all GABAergic hippocampal neurons express CB_1R, these receptors being localized in cholecystokinin (CCK) positive neurons. CCK-positive neurons express higher levels of CB_1R than the pyramidal cells (Marsicano and Lutz, 1999; Marsicano et al., 2003; Monory et al., 2006). It is thus not surprising that the apparent facilitatory action of eCBs upon strongly induced LTP results from an action upon GABAergic neurons, most probably by suppressing the inhibitory control exerted by CCK-positive basket cells over the pyramidal neurons.

Another relevant finding in the present work is the similarity between the effect of drugs that block CB_1Rs or inhibit formation of 2-AG, the predominant eCB in the hippocampus (Piyanova et al., 2015), and the effect of sustained activation of CB_1R by an exogenous agonist. It is worthwhile to note that the inhibitory action of Orlistat (2-AG synthesis inhibitor) and the inhibitory action of WIN (CB_1R agonist) were both counteracted by the CB_1R receptor blocker, AM251, clearly indicating that both are due to alterations in the level of CB_1R activation. It is known for a long time that the CB_1R activation inhibits LTP

(Nowicky et al., 1987; Collins et al., 1995; Terranova et al., 1995; Stella et al., 1997; Paton et al., 1998; Basavarajappa et al., 2014), these inhibitory actions being usually interpreted on the light of the knowledge that exocannabinoids inhibit excitatory synaptic transmission. However, the novelty of the present work is the possibility to contrast, under the same experimental conditions, the action of drugs that continuously activate CB_1R with those that reduce CB_1R activation, allowing to suggest that CB_1R can either facilitate or inhibit LTP as a function of several conditions, including the characteristics of CB_1R activation, the strength of LTP induction, as well as the magnitude of LTP itself. Also worthwhile to note is the contrast, under the same experimental conditions, between the influence of a drug known to inhibit hydrolysis the predominant eCB at the hippocampus, 2-AG, which facilitates strong LTP in line with the idea of a facilitatory action of endogenous activation of eCBs, with that of drugs that unselectively prevent eCB metabolism or that prevent metabolism of anandamide only, both of which inhibit strong LTP. These findings highlight differences in the modulatory actions of the eCBs, which may be relevant to interpret some age-dependent differences in the neuromodulatory actions of cannabinoids. Indeed, the relative abundance of anandamide over 2-AG increases throughout age (Piyanova et al., 2015). Our data thus contribute to interpret apparently discrepant data, and strongly support the idea of a dual action of eCB signaling to sustain LTP.

CONCLUSION

The data herein reported clearly show that manipulating eCB signaling may have opposing effects upon LTP, depending on the strength of LTP induction, inhibiting weak LTP and facilitating stronger LTP. This suggests that eCBs act as a high-pass filter, therefore likely reducing the signal-to-noise ratio of synaptic strengthening. Importantly, we also show that under the same LTP inducing conditions, prolonged activation of CB_1R with exocannabinoids or blockade of CB_1R may both impair LTP. Our data with drugs known to increase the accumulation of anandamide or 2-AG suggest that these two eCBs may differently affect LTP. Altogether, the data herein reported highlight a clear homeostatic control of eCBs and CB_1Rs upon LTP. Disruption of this finely tuned homeostatic role of eCBs upon synaptic plasticity phenomena likely underlies the known deleterious influence of cannabinoid-based drugs upon memory.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Directive 2010/63/EU." The protocol was approved by the "iMM's Institutional Animal Welfare Body – ORBEA-iMM and the National competent authority – DGAV (Direcção Geral de Alimentação e Veterinária)."

AUTHOR CONTRIBUTIONS

AS-C performed the experiments and quantified the data. AS and AS-C designed the experiments, analyzed, and discussed the data. MC provided breeding pairs for A1R knockout and WT mice. AS-C, MC, JR, and AS contributed to manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2017.00921/full#supplementary-material

FIGURE S1 | No appreciable differences between data obtained in males or females were detected. Values are represented as the mean ± SEM. The number of experiments in each condition is indicated in the bars. No significant differences between males and females were found, $F_{(11,102)} = 7.2$, *t*-values – Control WT:Control WT male = 0.9, Control WT:Control WT female = 0.5, AM251 WT:AM251 WT male = 0.9, AM251 WT:AM251 WT male = 0.6, Control KO:Control KO male = 0.06, Control KO:Control KO female = 0.05, AM251 KO:AM251 KO male = 0.1, AM251 KO:AM251 KO female = 0.07. For further details on the way to calculate LTP magnitude see the section "Materials and Methods" and legend to **Figure 1**.

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

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