

THE EFFECT OF CANNABIDIOL
IN THE IMMATURE BRAIN

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Ana Caulino. The effect of cannabidiol in the immature brain.



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The effect of cannabidiol in the immature brain

Ana Veiga Caulino Rocha



ANA VEIGA CAULINO ROCHA

THE EFFECT OF CANNABIDIOL IN THE IMMATURE BRAIN

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Orientador

Doutora Maria da Graça Borges Lobo
Professora Auxiliar Convidada
Instituto de Ciências Biomédicas Abel Salazar
(ICBAS)
Universidade do Porto

Coorientador

Doutor Paulo Correia de Sá
Professor Catedrático
Instituto de Ciências Biomédicas Abel Salazar
(ICBAS)
Universidade do Porto

Laboratório de Farmacologia e Neurobiologia /
MedInUP
Departamento de Imuno-Fisiologia e Farmacologia
(ICBAS-UP)



Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

A planta *Cannabis Sativa* e os seus diferentes constituintes têm sido usados para fins medicinais desde a antiguidade. Atualmente, sabemos que esta planta é constituída por mais de 100 químicos biologicamente ativos, dos quais os mais conhecidos são o tetrahydrocannabinol (Δ^9 -THC) e o canabidiol (CBD). Com a descoberta desta classe de substâncias e dos seus efeitos no sistema nervoso, descobriram-se várias moléculas endógenas, denominadas endocanabinóides, seguindo-se a revelação de um sistema responsável pelos seus efeitos, até aí desconhecido, o Sistema Endocanabinóide (ECS).

O sistema endocanabinóide é constituído por dois recetores de membrana - o recetor canabinóide tipo 1 (CB₁R) e o recetor canabinóide tipo 2 (CB₂R), os endocanabinóides e todas as enzimas responsáveis pela sua síntese e metabolismo.

Apesar da canábis ter diversos efeitos terapêuticos, o seu uso está mais associado a efeitos psicoativos. Estes efeitos psicoativos induzidos pelos canabinóides advêm da ligação a recetores canabinóides tipo 1, o que leva a que o Δ^9 -THC, um grande constituinte da canábis, seja a principal razão pela qual a canábis não é recomendável para uso terapêutico. Porém, o CBD, outro constituinte importante da planta canábis, não parece partilhar esta característica e não provoca efeitos psicotrópicos, uma vez que não ativa recetores canabinóides.

O CBD atua em diferentes alvos farmacológicos e os seus efeitos estão a ser explorados para uso em diversas terapias, como por exemplo a epilepsia. Os mecanismos responsáveis pela ação do CBD como antiepilético não estão completamente elucidados e muita da sua eficácia clínica foi recolhida a partir de ensaios clínicos. Apesar disso, o CBD foi aprovado para o tratamento de dois tipos de epilepsias infantis refratárias aos antiepiléticos, as síndromes de Lennox-Gastaut (LGS) e de Dravet (DS). A problemática na base da epilepsia infantil sugere uma possível dependência da idade, sendo o cérebro imaturo mais suscetível a ataques epiléticos. Para além disto, durante o desenvolvimento, a função do sistema endocanabinóide modifica-se: na fase embrionária exerce funções na neurogénese, durante o período perinatal e adolescência é importante para a gliogénese, mielinização e aprimoramento dos circuitos neuronais, e no adulto influencia a homeostase neuronal. Tendo estes dados em consideração, decidimos estudar os mecanismos subjacentes à libertação de [³H]GABA e [¹⁴C]glutamato em sinaptossomas de ratazana obtidos de animais imaturos e adultos, na presença e na ausência de CBD.

Resultados preliminares sugerem que a libertação de [^3H]GABA e [^{14}C]glutamato, a partir do hipocampo de animais imaturos é semelhante aos animais adultos. Os resultados sugerem também que a exocitose dependente de cálcio desempenha um papel menor na libertação evocada de [^3H]GABA e [^{14}C]glutamato. Em relação aos iões sódio, os resultados destacam a sua importância na manutenção da libertação normal de neurotransmissores e sugerem o envolvimento de reversão de transportadores de GABA e glutamato como meio principal para a sua libertação, pelo menos em sinaptossomas hipocámpais de ratas adultas, enquanto que no caso do glutamato em animais imaturos pode estar envolvido um outro mecanismo na sua libertação. No entanto, ainda são necessários mais estudos para consolidar estas conclusões.

O CBD (3, 10 e 30 μM) causou uma diminuição da libertação evocada de [^3H]GABA e [^{14}C]glutamato, efeito inibitório este consistente com outros estudos feitos com outros canabinóides. A inibição do CBD em sinaptossomas de hipocampo foi mais evidente na libertação evocada de [^3H]GABA quando comparada com [^{14}C]glutamato, não se tendo observado diferenças significativas entre os dois grupos de idades. Contrariamente, em condições de repouso, o CBD (10 e 30 μM) aumentou transitoriamente a libertação de [^3H]GABA e [^{14}C]glutamato, e este efeito foi mais evidente em animais imaturos em comparação com animais adultos.

Estudos anteriores realizados com células em cultura revelaram que o CBD, quando usado em concentrações na ordem do micromolar, pode causar danos na membrana plasmática. Para descartar a possibilidade de que o aumento da libertação basal de [^3H]GABA e [^{14}C]glutamato, observado na presença de CBD 10 e 30 μM em sinaptossomas do hipocampo não estimulados, fosse causado por danos na membrana, pesquisámos a libertação de LDH no meio de perfusão; os resultados revelaram que nenhuma das concentrações de CBD estudadas causou rutura da membrana.

Várias linhas de evidência sugerem que o efeito do CBD pode derivar da modulação do cálcio. Os nossos resultados mostram que o cálcio extracelular parece modificar a libertação de [^3H]GABA e [^{14}C]glutamato em sinaptossomas não despolarizados do hipocampo de ratas imaturos, sem afetar significativamente a libertação evocada tanto de [^3H]GABA e como de [^{14}C]glutamato.

A adenosina (Ado) é uma molécula essencial para a vida de qualquer célula e pode ter efeitos anticonvulsivantes endógenos no cérebro; estudos prévios sugerem que o efeito terapêutico do CBD na epilepsia pode resultar da sua capacidade de aumentar os níveis de Ado em roedores adultos, por redução da recaptação de Ado. No entanto, com os

resultados obtidos no nosso trabalho, não foi possível comprovar que o efeito inibitório do CBD na libertação de $[^3\text{H}]\text{GABA}$ e $[^{14}\text{C}]\text{glutamato}$ a partir dos sinaptossomas hipocampais, despolarizados com KCl, é mediado pela ativação dos recetores da adenosina A_1 .

Estas conclusões são preliminares e deve destacar-se que o número de experiências é reduzido. No entanto, até onde sabemos, este é o primeiro estudo realizado com CBD, comparando os seus efeitos em ratos na idade de desmame (imaturos) e ratos adultos para estudo da libertação síncrona de $[^3\text{H}]\text{GABA}$ e $[^{14}\text{C}]\text{glutamato}$.

Abstract

Cannabis sativa and its most idiosyncratic chemical components have been used for medical purposes throughout history, since ancient China. Currently, it is known that *cannabis* is constituted by over 100 active substances from which the more ubiquitous are tetrahydrocannabinol (Δ^9 -THC) and cannabidiol (CBD). Thanks to the discovery of this class of chemicals and its effects on the nervous system, endogenous molecules now known as endocannabinoids (eCBs) were revealed, followed by the detection of a whole new system activated by eCBs, the Endocannabinoid System (ECS).

The ECS is composed by two membrane receptors, the cannabinoid receptor 1 (CB₁R) and the cannabinoid receptor 2 (CB₂R), endogenous cannabinoids (eCBs) and all the biochemical machinery necessary to its synthesis and metabolism.

Despite having therapeutic effects in various diseases, the use of *Cannabis sativa* is commonly associated to its psychotropic effects; these effects are consequent to activation of CB₁R. Δ^9 -THC is the central psychotropic ingredient of *Cannabis*, and the main reason for its inadvisable therapeutic use. Notwithstanding CBD being a cannabinoid, it appears to be devoid of these undesirable effects because it does not seem to bind to cannabinoid receptors (CbRs).

CBD holds a plethora of pharmacological activities that are being explored for different therapeutic applications, including epilepsy. CBD antiepileptic mechanisms are not yet fully elucidated, and much of the evidence for its clinical effectiveness has been collected from case reports or smaller surveys. Even so, its use was approved for the treatment of two kinds of drug resistant epilepsy affecting children, the Lennox-Gastaut (LGS) and the Dravet (DS) syndromes. The problematic aspects behind paediatric epilepsy suggest a possible age-specific susceptibility to seizures, being immature brain more susceptible to seizures. Moreover, in the course of brain development, the function of the ECS changes: in embryonic stages exert a role in neurogenesis, in the perinatal and adolescent brain is important when gliogenesis, myelination and circuit refinement occurs, and in the adult influence neuronal homeostasis. Therefore, we set up to study in parallel and under the same experimental conditions, the mechanisms underlying [³H]GABA and [¹⁴C]glutamate release from rat hippocampal synaptosomes obtained from immature and adult rats, in the presence and absence of CBD.

Preliminary results suggest that [^3H]GABA and [^{14}C]glutamate release from the hippocampus of immature animals is similar to adult animals. Data also indicate that Ca^{2+} -dependent vesicle exocytosis plays a minor role on depolarization-evoked release of [^3H]GABA and [^{14}C]Glutamate. Regarding Na^+ , results show that these ions are important in the maintenance of normal neurotransmitter release and support the involvement of high-affinity amino acid transporter's reversal as the main path to release [^3H]GABA and [^{14}C]Glutamate from depolarized hippocampal synaptosomes at least in adult rats, whereas the release of [^{14}C]Glutamate may involve other mechanism in immature rats. This however remains to be better explored.

CBD (3, 10 and 30 μM) inhibited depolarization-evoked release in [^3H]GABA and [^{14}C]glutamate release, consistent with previous studies with other cannabinoids. CBD was more potent at inhibiting the release of [^3H]GABA compared to [^{14}C]Glutamate from depolarized hippocampal synaptosomes, but with no significant differences between the two groups of ages. In a different way, under resting conditions CBD (10-30 μM) transiently increased the outflow of [^3H]GABA and [^{14}C]Glutamate, and was more evident in immature compared to adult animals.

Previous studies demonstrated that when used in micromolar concentrations, CBD may cause damage to the plasma membrane of cultured cells. To rule out the possibility of release of [^3H]GABA and [^{14}C]Glutamate from resting hippocampal synaptosomes being caused by membrane damage, we measured LDH release into the medium; our results disclosed that neither concentration of CBD studied caused membrane leakage.

Several lines of evidence suggest that the CBD effects may be derived by modulation of calcium (Ca^{2+}). Our results indicate that extracellular Ca^{2+} appears to modify the resting release of [^3H]GABA and [^{14}C]Glutamate from hippocampal synaptosomes of immature rats, without significantly affecting the evoked release of the two amino acids.

Adenosine (Ado) is a pivotal molecule in the life of any cell that can act as an endogenous anticonvulsant in the brain; reports suggest that CBD therapeutic effect in epilepsy is originated by its ability to increase Ado levels in adult rodents by reducing Ado reuptake. Nevertheless, we failed to prove that adenosine A_1 receptors activation mediates the inhibitory effect of CBD on [^3H]GABA and [^{14}C]Glutamate release from hippocampal synaptosomes depolarized by high KCl.

It is extremely important to mention that this study is still preliminary and that the number of experiments is low. However, to our knowledge, this is the first study comparing

the effects of rats in weanling age (immature) with adult rats reporting the effects of CBD in $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{Glutamate}$ release simultaneously.

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List of Abbreviations

2-AG, 2-arachidonoyl glycerol
AA, Arachidonic acid
ABC, ATP-binding cassette proteins
ABHD12, alpha/beta hydrolase domain 12
ABHD6, alpha/beta hydrolase domain 6
AC, Adenylate cyclase
ADA, Adenosine deaminase
ADK, Adenosine kinase
Ado, Adenosine
ADP, Adenosine diphosphate
AEA, anandamide
AK, Adenosine kinase
AMP, Adenosine monophosphate
AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA, Analysis of variance
AOAA, Aminooxyacetic acid
AR, Adenosine receptor
ATP, Adenosine triphosphate
BCA, Bicinchoninic acid
BGT1, Betaine-GABA transporter
Ca²⁺, Calcium
CaM, Calmodulin
CaMKII, Calmodulin kinase II
cAMP, Cyclic AMP
CB₁R, type 1- cannabinoid receptor
CB₂R, type 2- cannabinoid receptor
CBD, Cannabidiol
CBs, cannabinoids
CBsR, cannabinoid receptor
CCK, cholecystokinin
CD73, Ecto-5'-nucleotidase
Cl⁻, Chloride

CNS, Central nervous system
CNT, concentrative nucleoside transporters
COX, cyclooxygenase
CPMs, ScCintilations per minute
DAG, diacylglycerol
DAGL, diacylglycerol lipase
DGL, diacylglycerol lipase
DNA, deoxyribonucleic acid
DPMs, Disintegrations per minute
DS, Dravet syndrome
DSE, depolarization-induced suppression excitation
DSI, depolarization-induced suppression of inhibition
EAATs, Excitatory amino acid transporters
eCB, endocannabinoid
ECS, Endocannabinoid System
EGTA, Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
EMA, European Medicines Agency
EMT, endocannabinoid membrane transporter
E-NPPs, Ectonucleotide pyrophosphatase and/or phosphodiesterases
E-NTPDases, Ectonucleoside triphosphate diphosphohydrolases
EPSC, Excitatory postsynaptic current
ER, endoplasmic reticulum
ERK, extracellular signal-regulated kinases
FAAH, fatty acid amide hydrolase
FDA, Food and drug administration
GABA, γ -aminobutyric acid
GABAR, GABA receptor
GABAR, GABA receptor
GABAT, GABA transaminase
GAD, Glutamate decarboxylase
GATs, GABA transporters
Gln, glutamine
GPCR, G-protein coupled receptors
GPR55, G protein-coupled receptor 55
HCO³⁻, Bicarbonate

HEA, homo-linolenylethanolamide
HEPES, 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
iGlu_t, ionotropic glutamate receptor
IP₃, Inositol 1,4,5-trisphosphate
IPSPs, Inhibitory postsynaptic currents
K⁺, Potassium
KA, Kainate
KCC2, K⁺-Cl⁻ cotransporter
KO, Knock-out
LOX, Lipoxygenase
LTD, Long-term depression
LTP, Long term potentiation
LysoPLC, lysoPI-specific PLC
MAGL, monoacylglycerol lipase
MAPK, Mitogen Activated Protein Kinases
Mg²⁺, Magnesium
MGL, Monoacylglycerol lipase
mGluR, Metabotropic glutamate receptor
min, Minutes
Na⁺, Sodium
NAD⁺, Nicotinamide adenine dinucleotide
NADA, *N*-arachidonoyldopamine
NADP⁺, Nicotinamide adenine dinucleotide phosphate
NAPE-PDL, *N*-acyl phosphatidylethanolamine phospholipase D
NAT, *N*-acetyltransferase
NMDA, *N*-methyl-D-aspartate
NMDG, *N*-methyl-D-glucamine
PAG, glutaminase
PBS, Phosphate-buffered saline
PCR, polymerase chain reaction
PEA, palmitoylethanolamide
PKA, Protein kinase A
PKC, Protein kinase C
PLA1, Phospholipase A1
PLC, Phospholipase C

PNS, Peripheral nervous system

RNA, ribonucleic acid

slc1, Solute carrier family

TCA, Tricarboxylic acid

TRPV1, transient receptor potential vanilloid-1

UDP, Uridine diphosphate

UTP, Uridine triphosphate

VGATs, Vesicular GABA transporters

VGCCs, voltage-gated Ca^{2+} channels

VGLUTs, Vesicular glutamate transporters

VNUT, Vesicular nucleotide transporters

xCT, glutamate-cysteine antiporter

Zn^{2+} , Zinc

Δ^9 -THC, delta-9-tetrahydrocannabinol

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Table 1: Targets and actions reported for CBD, adapted from (Franco *et al.*, 2019).13

Chapter 1

Introduction

1.1. Cannabinoids

The plant *Cannabis sativa*, popularly known as marijuana, has long been used for medical purpose throughout human history. The first record can be traced back to ancient China *circa* 5000 years ago, where extracts of the plant were used for the relief of cramps and pain (Ligresti *et al.*, 2016; Zou *et al.*, 2018).

Over nearly a century, numerous attempts were made to isolate the active marijuana constituents and to elucidate their structures, but these attempts were unsuccessful, and research on cannabis advanced slowly (Mechoulam *et al.*, 2013). Now we can understand the reason for this lack of success. Up to this point, more than 100 chemically and biosynthetically related cannabinoids have been identified in cannabis flowers, *per se* or after desiccation, in different amounts depending on the plant variety studied or even not detected in another cannabis plant (Izzo *et al.*, 2009; Ligresti *et al.*, 2016).

Currently, besides the distinction between the origin of the cannabinoids (CBs)-phytocannabinoids, endocannabinoids (eCs) and synthetic cannabinoids - there are five distinct classes based on their molecular pharmacology: (1) the classical cannabinoids (e.g., tetrahydrocannabinol (Δ^9 -THC), HU210); non-classical cannabinoids (e.g., CP-55,940); indoles (e.g., WIN 55,212), eicosanoids (e.g., eCs) and antagonist/inverse agonists (e.g., SR141716A and AM251 for CB₁R, SR145528 and AM630 for CB₂R) (Console-Bram *et al.*, 2012).

Although less known, cannabidiol (CBD) was one of the first phytocannabinoids discovered and isolated in 1940 by Adams and co-workers (Adams *et al.*, 1939). Still, the chemical structure of this compound was not elucidated until 1963 by Mechoulam and Shvo about 33 years after its discovery (Gaoni *et al.*, 1964). Following this breakthrough, Δ^9 -THC, the chief psychotropic ingredient of *Cannabis*, was isolated in 1964 by Gaoni and Mechoulam (Gaoni *et al.*, 1964). Shortly after that, Δ^9 -THC was synthesized in laboratory (Gaoni *et al.*, 1964; Ligresti *et al.*, 2016).

Even though CBD and Δ^9 -THC were identified at nearly the same time, most pharmacological efforts were dedicated to understand the mechanism of action of Δ^9 -THC, and, CBD, by contrast, remained a more puzzling and poorly studied target. This can be explained considering that Δ^9 -THC is responsible for the psychoactive effects of marijuana, by contrast with CBD that does not compete with conventional orthosteric radioligands to binding at the cannabinoid receptor 1 (CB₁R,) neither elicit distinctive psychoactive effects (Di Marzo, 2018; Straiker *et al.*, 2018 a). This scenario has slowly changed; studies with

different strains of cannabis sativa showed that varying the $\Delta 9$ -THC/CBD ratio yields different effects (Zuardi, 2008).

Thankfully to the discovery of CBs, research focused on understanding how these compounds, especially $\Delta 9$ -THC, exerted their effects. This curiosity led to the discovery of binding sites in the brain which distribution was found to be consistent with the pharmacological properties of psychotropic phytocannabinoids; this was the start of a remarkable revolution, the discovery of a whole system based on CBs (Mechoulam *et al.*, 2013; Ligresti *et al.*, 2016; Di Marzo, 2018).

1.1.1. The Endocannabinoid System

Cannabis sativa and its most idiosyncratic chemical components have the merit to led humanity to discover one of the most captivating and pleiotropic endogenous signaling system, the endocannabinoid system (ECS) (Ligresti *et al.*, 2016).

Even though the psychoactive properties of $\Delta 9$ -THC were recognized immediately, its mechanism of action was not. Initially, due to its hydrophobic nature, it was thought that CBs could act by influencing membrane fluidity, rather than by combining with a specific receptor (Di Marzo *et al.*, 1998; Piomelli, 2003). This viewpoint changed after the development of a new class of potent and selective $\Delta 9$ -THC analogues (HU-210 and CP-55,245) which led to the pharmacological identification of CBs-sensitive binding sites in the brain (Di Marzo *et al.*, 1998). This was the first step to the discovery of the ECS (Di Marzo *et al.*, 2004).

Presently, it is known that the ECS is represented by two membrane receptors, the cannabinoid receptor 1 (CB₁R) and the cannabinoid receptor 2 (CB₂R), endogenous ligands (eCBs) that activate them and all the biochemical machinery to produce and metabolize these lipids (Izzo *et al.*, 2009; Lu *et al.*, 2016).

1.1.2. Cannabinoid receptors

The first cannabinoid receptor (CBR) designated CB₁, was discovered (Devane *et al.*, 1988) and subsequently cloned (Matsuda *et al.*, 1990) on the basis of its responsiveness to $\Delta 9$ -THC and related synthetic analogues (Busquets-Garcia *et al.*, 2018).

The CB₁R is likely the most numerous metabotropic receptor in the mammalian brain and its activation is directly related to the typical psychoactivity of certain plant-derived CBs (Aizpurua-olaizola *et al.*, 2017). They are present in many different cell types, but their expression levels are astonishingly variable amongst different areas in the central nervous system (CNS) (Busquets-Garcia *et al.*, 2018). CB₁R is one of the most abundant G protein-coupled receptors (GPCRs) found so far in the CNS, with the highest densities of CB₁R present in the basal ganglia, *substantia nigra*, *globus pallidus*, *cerebellum* and *hippocampus* but it is also present in the peripheral nervous system (PNS) and several peripheral organs (Di Marzo *et al.*, 2004). The high CB₁R levels in the sensory and motor regions are consistent with the important role of this receptor in motivation and cognition, and the observed effects of CBs, including impairments in cognition, memory, learning and motor coordination (Demuth *et al.*, 2006).

CB₁Rs appears to be involved in γ -aminobutyric acid (GABA) and glutamate neurotransmission, and are found on GABAergic and glutamatergic neurons (Mechoulam *et al.*, 2013), however with different levels of expression, e.g. cortical GABAergic interneurons contain high levels of CB₁R protein, but cortical glutamatergic neurons have much lower levels of these receptors (Busquets-Garcia *et al.*, 2018).

After the discovery of CB₁R, a second cannabinoid receptor was isolated by a PCR-based strategy designed to isolate GPCRs, named CB₂R (Munro *et al.*, 1993). It was originally assumed that CB₂R was solely present in tissues and cells of the immune system (e.g. spleen macrophages, tonsils, B cells and natural killer cells, monocytes, neutrophils, and T cells) being absent from the CNS, in notable contrast with the well-known distribution of CB₁R. However, they have now been identified throughout the CNS in glial cells, including microglia and astrocytes, neural and oligodendroglial progenitors, and certain neuronal subpopulations in different brain structures of a variety of species (Fernández-Ruiz *et al.*, 2008; Mechoulam *et al.*, 2013). An important aspect of activation of CB₂R is that unlike the CB₁R, CB₂R is completely devoid of psychoactive effects.

Both CB₁R and CB₂R are members of class A GPCRs (Fernández-Ruiz *et al.*, 2008; Busquets-Garcia *et al.*, 2018), which are characterized by being structurally similar to rhodopsin, for having an extracellular N-terminal domain, a seven α -helical transmembrane domain, and a C-terminal domain of 73 (for CB₁R) or of 59 (for CB₂R) amino acids (Navarro *et al.*, 2016). These receptors primarily couple to G proteins of the G_i and G_o classes and can initiate signalling events typical of this class of transducing proteins. These include inhibition of adenylyl cyclases (AC) (with the consequent decrease in cytosolic cAMP concentrations) and promotion of mitogen-activated protein kinase (MAPK),

a mechanism by which CBs affect synaptic plasticity, cell migration, and possibly neuronal growth (Di Marzo *et al.*, 2004; Console-Bram *et al.*, 2012; Lu *et al.*, 2016; Aizpurua-Olaizola *et al.*, 2017; Busquets-Garcia *et al.*, 2018). However, CB₁R depending on the availability of the subunits of G proteins, dimerization with other proteins in particular cellular environments (cell type, subcellular location, cellular functional state, and so on) can result in binding to different G proteins, such as G_s or G_{q/11}, which could have implications for the signal-regulating mechanism (Aizpurua-Olaizola *et al.*, 2017; Busquets-Garcia *et al.*, 2018); and, in the case of CB₁R can inhibit certain voltage-dependent calcium (Ca²⁺) channels (N- and P/Q-type voltage-activated calcium (Ca²⁺) channels), as well as the stimulation of inwardly rectifying potassium (K⁺) currents (Figure 1) (Piomelli, 2003; Di Marzo *et al.*, 2004; Demuth *et al.*, 2006; Ohno-Shosaku *et al.*, 2014).

Activation of CB₁R or CB₂R receptors exerts diverse consequences on cellular physiology, including synaptic function, gene transcription, and cell motility (Lu *et al.*, 2016).

Despite only CB₁R and CB₂R are widely-acknowledged as CBRs, the actions of eCBs are not restricted to these receptors, several others, ranging from other GPCRs to ion channel and nuclear receptors, have been reported to interact with CBs (Zou *et al.*, 2018).

GPR55, an orphan GPCR is a supposed new CBR -“CB₃R”, has been linked to intracellular Ca²⁺ increase and extracellular signal-regulated kinases (ERK) phosphorylation. This receptor shares structural similarities in transmembrane domains 1, 2, and 3 when compared with the CBRs, which may explain why it is targeted by various CBs (Ibeas Bih *et al.*, 2015), but its pharmacological property is somewhat different from those of CB₁R and CB₂R: it is activated by Δ⁹-THC, CP55,940, and eCBs including anandamide (AEA), 2-arachidonoylglycerol (2-AG), noladin ether, and virodhamine but not by WIN55,212-2 and can be activated by palmitoylethanolamide and oleoylethanolamide, which are not the ligands for the common CBRs (Ohno-Shosaku *et al.*, 2014).

Another receptor involved in cannabinoid signaling is the transient receptor potential cation channel subfamily V member 1 (TRPV1). This receptor is a Ca²⁺ permeable, nonselective cation channel, is the best-documented for its significant role in synaptic transmission and pain regulation. So far, several endogenous substances (endovanilloids) have been found to activate TRPV1 like AEA, *N*-arachidonoyldopamine, and several lipoxygenase (LOX) products of arachidonic acid (AA) (Ohno-Shosaku *et al.*, 2014).

Overall, it has been shown that the ECS has a homeostatic role in a variety of physiological and pathological conditions. Indeed, its characteristic on-demand

biosynthesis and release make it a system capable of adapting quickly to changing conditions (Aizpurua-Olaizola *et al.*, 2017).

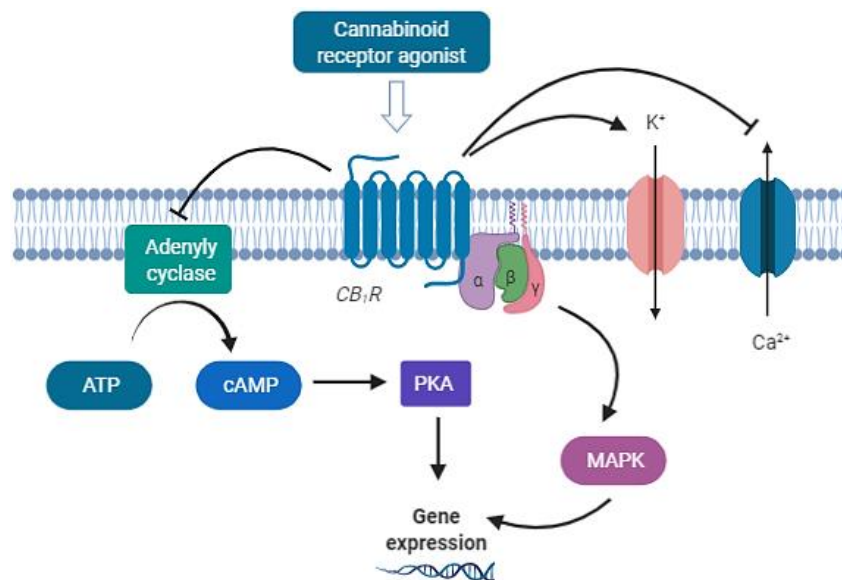


Figure 1: Signalling pathways associated with CBRs activation

1.1.3. Endocannabinoids

After the discovery of CBRs, researchers questioned why specific receptors existed solely to be activated by exogenous molecules such as CBs. This assessment gave rise to the search for endogenous molecules, which could stimulate or inhibit these receptors in the mammalian body; and since all plant-derived or synthetic cannabinoids are lipid-soluble compounds it was assumed that endogenous cannabinoids would also be lipids (Di Marzo *et al.*, 1998; Mechoulam *et al.*, 2013). This assumption was ultimately proved to be right (Di Marzo *et al.*, 1998; Mechoulam *et al.*, 1998). eCBs are lipid mediators present in brain and peripheral tissues that include amides, esters, and ethers of long-chain polyunsaturated fatty acids. So far, the most bioactive eCBs are AEA and 2-AG, yet the eCBs family includes also virodhamine, noladinether, and *N*-arachidonoyldopamine (NADA), besides homo-linolenylethanolamide (HEA), docosate- traenylethanolamide (DEA), and other related compounds such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Battista *et al.*, 2012; Ohno-Shosaku *et al.*, 2014).

The first endogenous ligand of CB₁R was identified in 1992, in porcine brain (Battista *et al.*, 2012). This substance is an amide of AA with ethanolamine, and was named anandamide (AEA) from the Sanskrit word '*ananda*' meaning 'bliss' (Di Marzo *et al.*, 1998; Piomelli, 2003). AEA acts as a high-affinity, partial agonist of CB₁R and is almost inactive at CB₂R (Zou *et al.*, 2018) which agrees with the fact that AEA is mostly present in the brain (Console-Bram *et al.*, 2012; Mechoulam *et al.*, 2013; Lu *et al.*, 2016). As AEA cannot fully reproduce the effects induced by Δ^9 -THC, the search lead to the discovery of 2-AG.

Another major endocannabinoid, 2-AG was originally isolated from canine gut and rat brain (Mechoulam *et al.*, 1995). Contrary to AEA, 2-AG appears to be localized primarily in the peripheral tissues and acts as a full agonist at both CBRs with moderate-to-low affinity (Console-Bram *et al.*, 2012; Mechoulam *et al.*, 2013; Lu *et al.*, 2016; Zou *et al.*, 2018) suggesting that 2-AG is a true natural ligand for the CBRs (Ohno-Shosaku *et al.*, 2014). 2-AG is present at substantially higher concentrations (nmol g⁻¹) than AEA (pmol g⁻¹) and functions as the key retrograde signalling molecule in most forms of eCB-mediated synaptic regulation in the brain (Soltesz *et al.*, 2015).

eCBs have crucial roles in a wide range of neuronal phenomena including brain development, cognition, learning and memory, motor behaviour, regulation of appetite, temperature regulation, pain perception and several psychiatric and neurological disorders (Soltesz *et al.*, 2015).

1.1.4. Endocannabinoid metabolism

eCBs ligands are not stored as classical neurotransmitters, instead both eCBs are quickly synthesized by neurons on-demand, in response to increased intracellular Ca²⁺ concentration (Wilson *et al.*, 2002; Zou *et al.*, 2018). However, AEA and 2-AG are synthesized, transported and inactivated in respective target tissues by distinct pathways.

AEA synthesis might be due to several metabolic routes, however *N*-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is currently considered the key enzyme responsible for AEA synthesis (Figure 2) (Battista *et al.*, 2012). AEA formation in neurons is a two-step process; the first step is the stimulus-dependent cleavage of the phospholipid precursor *N*-arachidonoylphosphatidylethanolamine (NAPE). This reaction is mediated by NAPE-PLD (or via other routes not involving NAPE-PLD) and produces AEA and phosphatidic acid, a metabolic intermediate that is used by cells in the synthesis of other glycerol-derived phospholipids (Piomelli, 2003; Zou *et al.*, 2018).

The brain contains small quantities of NAPE (20–40 pmol g⁻¹) thereby the cellular stores of this precursor are replenished by the enzyme *N*-acyltransferase (NAT), which catalyses the intermolecular passage of an AA group from the *SN*-1 position of phosphatidylcholine to the head group of phosphatidylethanolamine (PE). NAT activity is controlled by two intracellular second messengers: Ca²⁺ and cAMP. NAT is inactive in the absence of Ca²⁺, consequently Ca²⁺ is required for its activation while cAMP works through protein kinase A (PKA)-dependent phosphorylation to enhance NAT activity. Even though catalysed by separate enzymes, AEA and NAPE synthesis proceed in parallel because Ca²⁺-stimulated AEA production is generally accompanied by *de novo* formation of NAPE (Piomelli, 2003; Zou *et al.*, 2018).

Being a monoacylglycerol, 2-AG is at the crossroads of multiple routes of lipid metabolism, where it can serve conversely as an end-product for one pathway and precursor for another. These diverse metabolic roles explain why 2-AG concentration in the brain is about 200-fold greater than AEA and suggest that a significant fraction of 2-AG in the brain is engaged in housekeeping functions rather than merely in signalling (Piomelli, 2003; Zou *et al.*, 2018).

In a different manner, the synthetic pathways for 2-AG are simpler than the pathways for AEA (Figure 2) (Lu *et al.*, 2016). 2-AG is produced from 1,2-diacylglycerol (DAG) by either DAG lipase (DAGL) α or β , in the adult CNS, DAGL α appears to be the isoform responsible for most 2-AG production that contributes to synaptic plasticity (Lu *et al.*, 2016). This product regulates protein kinase C (PKC) activity — an important second messenger function — and is a substrate for two enzymes: DAG kinase, which attenuates DAG signalling by catalysing its phosphorylation to phosphatidic acid; and DAG lipase (DGL), which hydrolyses DAG to monoacylglycerol. The rate-limiting and Ca²⁺-sensitive step in 2-AG production, like AEA, is the formation of DAG, which is converted from phosphoinositides (membrane lipids) by phospholipase C (PLC) following activation of G_{q/11}-coupled receptors (Soltesz *et al.*, 2015). Other pathways for 2-AG generation so far proposed include the sequential reactions by phospholipase A1 (PLA1) and lysoPI-specific PLC (lysoPLC), the conversion from 2-arachidonoyl lysophosphatidic acid (2-arachidonoyl PLA) to 2-AG by phosphatase, and the formation from 2-arachidonoyl phosphatidic acid through 1-acyl-2- arachidonoylglycerol (Ohno-Shosaku *et al.*, 2014).

After eCBs synthesis they are released into the intracellular space unlike classical transmitters and neuropeptides, which can diffuse through the water-filled space that surrounds neurons, eCBs are hydrophobic compounds and tend to remain associated with lipid membranes (Piomelli, 2003). The release and uptake of eCBs is ascribed to a

supposed “endocannabinoid membrane transporter (EMT)” but irrefutable evidence of its molecular identity is still lacking and the mechanism is not yet well understood (Di Marzo *et al.*, 2004; Battista *et al.*, 2012; Zou *et al.*, 2018).

Once eCBs are taken up by the cells, they can be degraded through hydrolysis and/or oxidation. There are two pathways for the breakdown of AEA in the CNS. The first and main route is through the enzyme fatty acid amide hydrolase (FAAH), which cut down AEA into free AA and ethanolamine (Lu *et al.*, 2016; Zou *et al.*, 2018). Mice deficient in FAAH show significantly increased levels of brain AEA, implying that FAAH helps regulate eCBs tone (Wilson *et al.*, 2002).

The metabolism of 2-AG is primarily due to three hydrolytic enzymes, monoacylglycerol lipase (MGL), alpha/beta domain-containing hydrolase 6 (ABHD6), and alpha/beta domain-containing hydrolase 12 (ABHD12) (Lu *et al.*, 2016; Araque *et al.*, 2017). The three enzymes have different subcellular localizations, which likely define metabolism of 2-AG in different cellular compartments. MGL is widespread in the adult nervous system, mostly localized in synaptic terminal and astrocytes, and it is the primary and best-studied route (Lu *et al.*, 2016). MGL hydrolyses 2-AG into AA and glycerol (Zou *et al.*, 2018).

Oxidation of both AEA and 2-AG could involve cyclooxygenase-2 (COX-2) and several LOXs (Lu *et al.*, 2016). COX enzymes in mammalian tissues include three forms: COX-1 to 3. Among them, COX-1 and COX-2 preferably recognize AA as a substrate. COX-2 acts on AEA and produces prostaglandin-ethanolamides (Ohno-Shosaku *et al.*, 2014).

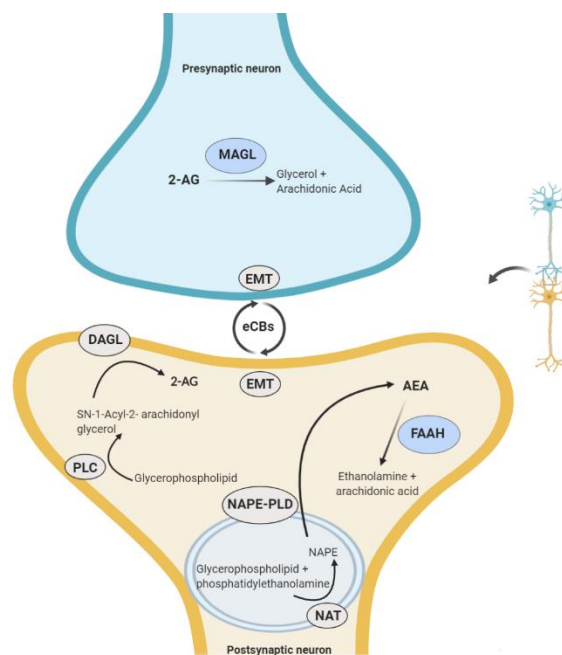


Figure 2: Anabolic and catabolic pathways of eCBs

1.1.5. Cannabinoid signalling

Both enzymes responsible for eCBs synthesis (NAPE-PLD and the DAGLs) are substantially stimulated by high Ca^{2+} concentration supporting the evidence of Ca^{2+} influx or its mobilization from intracellular stores triggers AEA and 2-AG biosynthesis. This on-demand nature of endocannabinoid production fits very well with the local modulatory role proposed for the ECS under pathological conditions, which are normally accompanied by rises in intracellular Ca^{2+} to high (mM) concentrations. This is perhaps the most important role of eCBs in the adult brain (Ligresti *et al.*, 2016). eCBs are released from postsynaptic neurons, particularly 2-AG, and travel “backwards” to activate CB_1R , very often located on presynaptic nerve terminals, and suppress transmitter release either transiently of either glutamate or GABA by decreasing the local release probability of synaptic vesicles (Wilson *et al.*, 2002; Trezza *et al.*, 2008), thus participating in both short- and long-term synaptic plasticity, including depolarization-induced inhibition of excitatory (DSE) or inhibitory (DSI) currents, long-term depression (LTD) of both excitatory and inhibitory signalling (Ligresti *et al.*, 2016). eCBs regulate synaptic function (Wilson *et al.*, 2002) by moving backward across synapses, violating the traffic rules of the bulb (Freund *et al.*, 2003). This is due to diverse mechanisms, including presynaptic inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), activation of presynaptic K^+ channels and cAMP/PKA signalling. Termination of synaptic eCBs signalling is initiated by re-uptake followed by intracellular breakdown (Araque *et al.*, 2017). Furthermore, eCBs released by neurons can modulate presynaptic and postsynaptic circuit elements through a bidirectional signalling communication between astrocytes and neurons, via activation of CB_1R expressed on astrocytes (Araque *et al.*, 2017). Synaptic neurotransmitters activate astroglial GPCRs with production of inositol 1,4,5-trisphosphate (IP3) and Ca^{2+} release from the endoplasmic reticulum (ER). Evidence show that eCBs and astroglial CB_1R play a part in the tripartite synapse (Figure 3). CB_1Rs in hippocampal astrocytes are activated by eCBs released by neurons, with elevation of intracellular Ca^{2+} through GPCRs signalling, stimulation of astrocytic glutamate release and participation in synaptic plasticity (Oliveira da Cruz *et al.*, 2016). Astrocytes can synthesize eCBs and participate in the endocannabinoid turnover in brain. Biosynthetic enzymes as well as FAAH and MAGL are expressed in astrocytes; neurons and astrocytes co-ordinately regulate 2-AG levels and CB_1 -mediated synaptic plasticity and behaviour (Ligresti *et al.*, 2016).

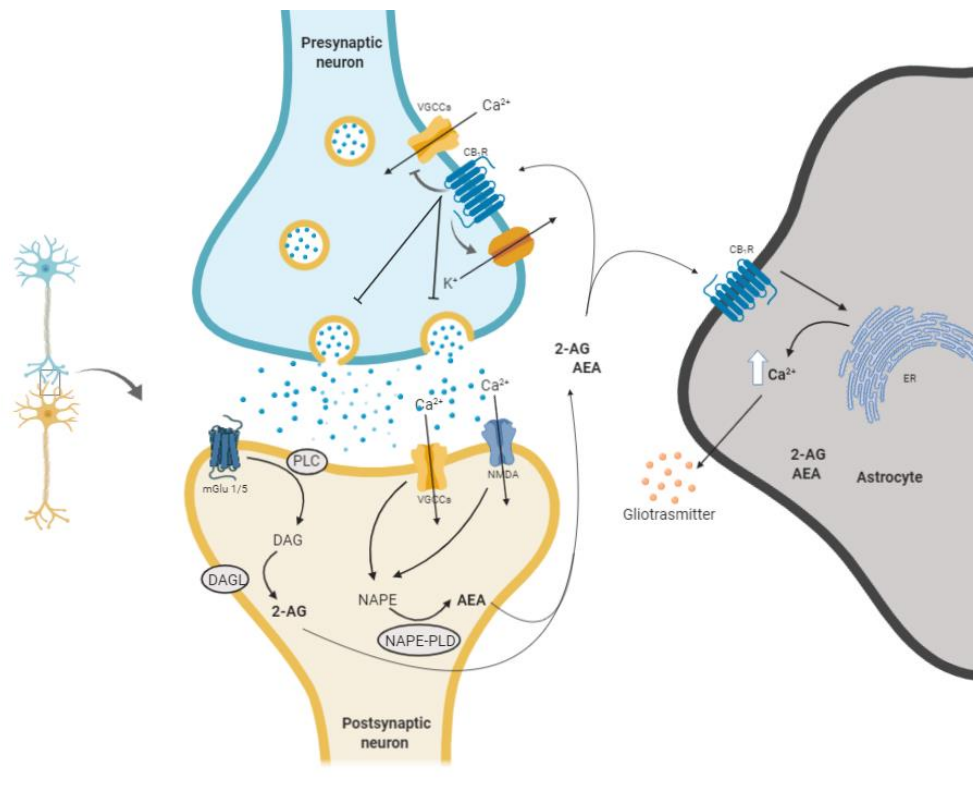


Figure 3: Schematic of eCBs signalling at the tripartite synapse

1.2. Cannabidiol

The road to the clinical development of ECS-based drugs has been paved with great hopes and bitter disappointments. In the meantime, CBD, either alone or in combination with Δ^9 -THC, has come back into the limelight as efficacious and moderately safe therapeutic drugs (Di Marzo, 2018).

CBD is the *major* nonpsychotropic constituent present on *cannabis sativa*; it has been investigated in a number of studies to evaluate its activity at CBRs and does not seem to exert its effects by binding to these receptors (Vilela *et al.*, 2015; Ligresti *et al.*, 2016; Reddy *et al.*, 2016; Saroya, 2017; Straiker *et al.*, 2018 b). Despite this, it holds a plethora of pharmacological activities that are being explored for different therapeutic applications many of them associated with both central and peripheral actions (Reichlin, 2005; Peters *et al.*, 2007). Several findings suggest that this compound act as an anxiolytic, antidepressant, antipsychotic, anticonvulsant, anti-nausea, antioxidant, anti-inflammatory, antiarthritic, and antineoplastic (Ligresti *et al.*, 2016; Reddy *et al.*, 2016), and has a therapeutic potential in neuropsychiatric disorders including anxiety, schizophrenia, addiction and epilepsy (Straiker *et al.*, 2018 b).

Even though the mechanism of action of CBD is not yet elucidated and much of the evidence for its clinical efficacy as an antiepileptic drug has been collected from case reports or smaller surveys (Ghovanloo *et al.*, 2018), its use was approved by the US Food and Drug Administration (FDA) in June 2018 and by European Medicines Agency's (EMA) in July 2019 (brand name Epidiolex) for two types of epilepsy affecting children, the Lennox-Gastaut (LGS) and the Dravet (DS) syndromes (U.S. Food and Drug Administration, 2018; European Medicines Agency, 2019). The anticonvulsant mechanisms of CBD's are still unclear, but most likely involve non-CBRs pathways (Ghovanloo *et al.*, 2018).

1.2.1. Mechanisms of action of Cannabidiol

Non-psychotropic phytocannabinoids exert multiple pharmacological effects via different mechanisms, thus CBD has several proposed mechanisms associated with its actions. The most recently investigated are modulation of: ECS, TRP channels, the peroxisome proliferator-activated receptor γ (PPAR γ), GPR55, glycine $\alpha 1$ and $\alpha 1\beta$ receptors, Ado receptors, LOX and COX-2 enzymes, and Ca^{2+} homeostasis (Izzo *et al.*, 2009; Ibeas Bih *et al.*, 2015; Reddy *et al.*, 2016); a list of targets and actions reported for CBD based on results from studies with different experimental models and systems are present below (Table 1).

The broad spectrum of CBD effects can be explained by the several mechanisms through which CBD acts, albeit further research is needed to illuminate the precise mechanisms that underlie some of the potentially beneficial effects of CBD (Zuardi, 2008).

Table 1: Targets and actions reported for CBD, adapted from (Franco *et al.*, 2019)

Receptor/target	Action of CBD at the indicated receptor/target
•CB ₁	•Inverse agonist/negative allosteric modulator
•CB ₂	•Inverse agonist/negative allosteric modulator
•TRPV1-3	•Agonist
•TRPV4	•Agonist
•TRPM8	•Antagonist
•TRPA1	•Agonist
•α1, α3 glycine	•Agonist
•5-HT1A	•Activation through direct, allosteric or indirect effects
•GPR55	•Antagonist
•GPR3, GPR6 and GPR12	•Inverse agonist
•PPAR-γ	•Agonist
•TNFα	•Antagonist
•Voltage-gated T-type Ca ²⁺ channels	•Antagonist
•Voltage-gated sodium currents	•Inhibition
•Ado reuptake	•Inhibitor
•A ₁ and A ₂ receptors	•Modulator
•AEA reuptake	•Inhibitor
•Fatty acid amide hydrolase	•Inhibitor

1.2.2. Cannabidiol and Epilepsy: State of the art

The use of cannabis whether for recreational or therapeutic purposes is a polarizing concept shielded in myth and misconceptions. There is now a fundamental reconsideration of the medical uses of cannabis and epilepsy finds itself in the heart of this argument (Friedman *et al.*, 2017).

Cannabis has gained visibility in the treatment of pediatric epilepsy through the “Charlotte’s Web” medical marijuana. It has very little Δ9-THC, with greater than a minimum of 30:1, CBD: Δ9-THC ratio. Charlotte had intractable DS epilepsy and her parents moved to Colorado (where cannabis is legal) and used this treatment to their child which attenuated her seizures; that led to the popularity of medical marijuana (Maa *et al.*, 2014; Interaction, 2015; Franco *et al.*, 2019). After the huge attention that “Charlotte’s Web” form of marijuana obtained it is often perceived for some parents of children with refractory epilepsy that there

is 'no other option' and against medical specialist and the law, administer cannabis to their child without the knowledge of their treating physician, potentially causing adverse effects or dangerous drug interactions (Yap *et al.*, 2015).

The problem of pediatric epilepsy suggests a possible age-specific susceptibility to seizures. It is believed that the immature brain is more excitable, in comparison with the mature brain, boosting its susceptibility to seizures (Cavalheiro *et al.*, 1987; Haut *et al.*, 2004). In fact, some glutamate receptors are transiently overexpressed in the immature brain, encouraging excitability (Cottrell *et al.*, 2000; Chapman *et al.*, 2012). Additionally, GABA exerts a net excitatory effect in immature neurons due to development differences in the chloride (Cl⁻) gradient (Cherubini *et al.*, 1991). Although ontogenically necessary for activity-dependent developmental processes (like neuronal proliferation and synaptogenesis), this excitatory predisposition is also thought to create a susceptibility to pathological and excessive neuronal activity that is characteristic of seizures (Chapman *et al.*, 2012). Thus, CBD emerged as a possible treatment option.

CBD has been investigated and evidenced an important anticonvulsant effect in various animal models, such as the acute model of seizures induced by pilocarpine and the penicillin model of partial seizure (Jones *et al.*, 2012), together with anecdotal and often ambiguous reports of unrefined cannabis extracts containing high-level of CBD exerting beneficial effects in treatment-resistant paediatric epilepsies, has led to formal human clinical trials of CBD (Devinsky *et al.*, 2014; Koo *et al.*, 2017; O'Connell *et al.*, 2017).

The DS is a catastrophic early-onset encephalopathic childhood epilepsy that is associated with drug-resistant seizures and a high mortality rate; it is a rare genetic form of epileptic encephalopathy predominantly due to loss-of-function mutations in the *SCN1A* gene encoding Na⁺ channels (namely Na_v1.1) (Patel *et al.*, 2016), where seizures are truly difficult to control and associated with the risk of sudden unexpected death. The safety and effectiveness of a standardized oral solution of CBD was tested in an open-label trial involving 214 children and young adults with drug-resistant epilepsy. This trial showed that CBD reduced the frequency of convulsive seizures among children and young adults with the DS over a 14-week period, but it was associated with adverse events including somnolence, loss of appetite, diarrhoea and increases in hepatic enzymes (Mellis, 2018).

Another clinical trial with CBD was performed in children with LGS. This syndrome is a rarer yet severe form of epileptic encephalopathy with early childhood onset, which typically prolongs throughout life with phenotypic and origin of seizures often varying with age. The syndrome typically manifests during the first 8 years of age with a peak incidence between age 3 and 5 years. It is characterized by the occurrence of multiple seizure types,

including the so-called drop attacks (i.e., sudden falls due to seizures), slow spike-and-wave activity on electroencephalograms, and cognitive impairment. CBD add-on therapy to the existing antiepileptic drugs significantly reduced the frequency of seizures in severe treatment-resistant patients with LGS. CBD was generally well tolerated in this population, although CBD has been associated with adverse events i.e. somnolence and transient elevations of the liver enzymes (Thiele *et al.*, 2018).

Despite an extensive preclinical evidence base suggesting a therapeutic value for CBD in several neurological disorders, a proven lack of CB₁R-mediated psychoactivity, and good tolerability, it remains unidentified the specific molecular target(s) through which CBD exerts its reported antiepileptic effects (Ibeas Bih *et al.*, 2015).

1.3. Neurodevelopment

In the course of brain development, the ECS is early active during neurogenesis in embryonic stages, as well as in the perinatal and adolescent brain when gliogenesis, myelination and circuit refinement occurs. The ECS also contributes to neuronal homeostasis in the adult brain, in addition to its relevance to cellular plasticity actions that are able to affect neuronal remodelling in the immature brain (Martínez-Orgado *et al.*, 2018). Bioactive signalling lipids are widely recognized as crucial for neuronal and glial differentiation, besides their influence in synaptic plasticity (MacCarrone *et al.*, 2014). CB₁R and CB₂R proceed via different signalling pathways to the control of neural cell generation and maturation during developmental stages (Galve-Roperh *et al.*, 2013; Martínez-Orgado *et al.*, 2018).

CBRs present in neural precursors (NPs) at neurogenic niches are engaged by eCBs (2-AG and AEA) synthesized by surrounding neurons and, in a paracrine/autocrine manner, by NPs themselves and ependymal cells. Neurodevelopmental eCBs production is unlike that occurring in the adult nervous system where eCBs are produced mainly on-demand by cellular activity (Galve-Roperh *et al.*, 2013). NPs produce and release eCBs in response to increased intracellular Ca²⁺ concentration and the eCB tone contributes to basal and stimulus-induced NP proliferation via CB₁R, which levels are associated to increased expression of markers of differentiation in neuronal lineages (Galve-Roperh *et al.*, 2013). Additionally, it has been demonstrated that 2-AG is present in neurogenic niches and plays an active role in NP cell regulation (Galve-Roperh *et al.*, 2013).

In neuronal cells, CBRs show opposite patterns of expression, with CB₁R increasing and CB₂R decreasing along neuronal differentiation (Galve-Roperh *et al.*, 2013; Martínez-Orgado *et al.*, 2018). CB₁R_s are expressed at low levels in NP cells with levels increasing until reach mature differentiated neurons; in NPs CB₁R regulates cell proliferation and promotes the transition from radial glial towards intermediate progenitors. The ability of CB₁R receptor to regulate NP proliferation is conserved later on in adult neurogenic areas, thus influencing neurogenesis as well as oligodendrogenesis (Martínez-Orgado *et al.*, 2018). CB₂R activity in NPs regulates cell proliferation and neurogenesis in a cell-autonomous manner and exerts complementary, sometimes overlapping, neural cell survival actions which may justify some of the beneficial effects of CBs in brain insults to the immature brain (Martínez-Orgado *et al.*, 2018).

1.4. Purinergic Signalling

Adenosine 5'-triphosphate (ATP) was proposed as the neurotransmitter responsible for non-adrenergic, non-cholinergic transmission in the gut and bladder. Even though the idea was initially received with scepticism, the term 'purinergic' was coined by Burnstock in 1972 (Kumaria *et al.*, 2008; Khakh *et al.*, 2009; Burnstock, 2018). Presently it is known that purine nucleotides and nucleosides constitute decisive extracellular signalling molecules acting as neurotransmitters and neuromodulators in CNS and PNS (Burnstock, 2006; Ciruela *et al.*, 2019).

In the brain, both neurons and glial cells act as cellular sources of extracellular ATP. ATP is taken up into synaptic vesicles by a vesicular nucleotide transporter vNuT and the nucleotide is released as a principal neurotransmitter and/or co-transmitter with classical neurotransmitters from nerve terminals, astrocytes and microglia (Abbracchio *et al.*, 2009). Moreover, ATP can be released through ABC transporters, connexin or pannexin hemichannels, maxi-ion channels, CALMH1 (calcium homeostasis modulator 1), P2X₇ receptors and, ultimately, by dying cells (Bodin *et al.*, 2001; Abbracchio *et al.*, 2009; Burnstock, 2009; Fredholm *et al.*, 2011; Blum *et al.*, 2017).

Extracellular ATP is rapidly broken down to Ado by the sequential action of ectonucleotidases, the enzymes responsible for the extracellular hydrolysis of adenine and uracil nucleotides (Gessi *et al.*, 2011; Cardoso *et al.*, 2015). Ectonucleotidases include: (1) the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), (2) ecto-5'-nucleotidase (CD73), (3) ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), and (4) alkaline and acid phosphatases (Burnstock, 2009, 2018; Blum *et al.*, 2017). E-

NTPDases and CD73 are known to be important in supporting purinergic signalling (Figure 4); of the eight known E-NTPDases, three are expressed extracellularly in brain, i.e., E-NTPDase-1 (also called CD39 and ATPDase), E-NTPDase-2 (also called CD39L1 and ecto-ATPase), and E-NTPDase-3 (also called CD39L3 and HB6). E-NTPDase-1 hydrolyzes ATP, ADP, UTP, and UDP and NTPDase-2 and -3 cleave ATP and UTP, forming ADP and UDP, respectively (Cardoso *et al.*, 2015; Blum *et al.*, 2017). ATP has a dual role since it may act directly on its own P2 receptors and indirectly via its breakdown into Ado by ectonucleotidases, which then activates the P1 receptor family (Gessi *et al.*, 2011; Cardoso *et al.*, 2015).

While ATP may function as a neurotransmitter in some brain areas, Ado is neither stored nor released as a classical neurotransmitter (Gessi *et al.*, 2011). Ado is arguably the most ubiquitous neuromodulator involved in regulation of synaptic transmission in virtually every brain region. In addition, Ado is a powerful neuroprotective agent, and it acts on many peripheral systems, being involved in regulation of blood pressure, immune response, pain pathways, angiogenesis and many other trophic functions (Burnstock *et al.*, 2012). Ado is present in a higher concentration inside cells, but it can translocate through the plasma membrane via facilitated diffusion by nucleoside transporters (Burnstock *et al.*, 2012), which may be equilibrative (ENT1-4) or concentrative (CNT1-3) (Baldwin *et al.*, 2004; E. Parkinson *et al.*, 2011). ENT is the predominant transporter type in the CNS; it can transport bi-directionally both purine and pyrimidine nucleosides following their chemical gradients. ENTs are particularly abundant in the hippocampus, cerebellum, cortex and striatum. These transporters are further classified due to their sensitivity (es or ENT1) or insensitivity (is or ENT2) to the selective inhibitor nitrobenzylthioinosine (NBMPR). Conversely, CNTs are unidirectional and mediate the influx of nucleosides driven by the transmembrane electrochemical sodium (Na^+) gradient (E. Parkinson *et al.*, 2011). Upon reversal of the transmembrane Na^+ gradient make these transporters also important contributors to increase extracellular Ado accumulation (E. Parkinson *et al.*, 2011).

Being a neurotransmitter/cotransmitter in the brain, ATP is released in a “stop and go” manner, with subsequent generation of Ado in the extracellular space. Normally, the extracellular Ado accumulation is kept at low levels either by deamination into inosine by adenosine deaminase (ADA) or by cellular uptake via ENTs. Interestingly, the driving force for Ado uptake into the cells is the rapid phosphorylation of the nucleoside by high affinity intracellular adenosine kinase (ADK) (Jacobson *et al.*, 2006; Gessi *et al.*, 2011; Burnstock, 2018).

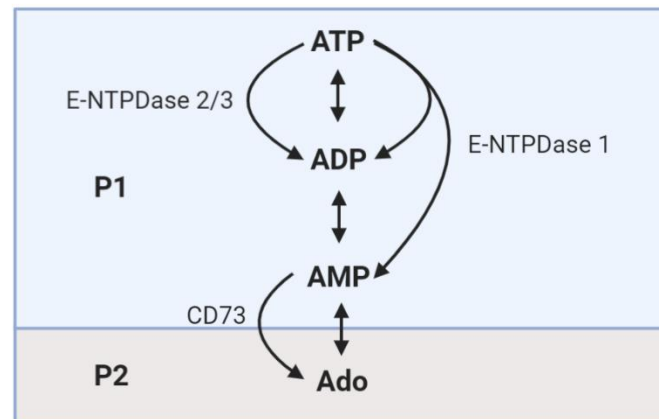


Figure 4: Extracellular catabolism of ATP by the ecto-nucleotidases.

The concept of purinergic neurotransmission and the potent actions of extracellular ATP on many different cell types implicated that there were purinergic membrane receptors (Burnstock, 2007, 2018). These receptors, which emerged early in evolution, are among the most abundant in living organisms controlling many physiological actions (Abbracchio *et al.*, 2009; Burnstock, 2009; Ciruela *et al.*, 2019). Purinoceptors (i.e., receptors for ATP/UTP, ADP/UDP and Ado) modulate both short-term signalling in neurotransmission, neuromodulation and secretion and long-term signalling in cell proliferation, differentiation and death in development and regeneration (Burnstock, 2017).

In 1978, Burnstock established the existence of two families of purinoceptors, named P1 and P2 receptors (Burnstock, 2017). While P1 receptors are activated by Ado, P2 receptors are activated by purine and pyrimidine nucleotides (Ciruela *et al.*, 2019). Based on the mechanism of action, pharmacology and molecular cloning, P2 receptors were subdivided into ionotropic P2X and metabotropic P2Y subtypes (Abbracchio *et al.*, 2009; Burnstock, 2017).

Ado is a pivotal molecule in the life of any cell: it is an intrinsic part of genetics (DNA and RNA), epigenetics (the *major* methyl donor is S-adenosylmethionine), redox balance (the “A” in NADH) and bioenergetic (the “A” in ATP). Predictably, for such an ubiquitous molecule, it has also evolved to become a paracrine signal to inform cells of the metabolic activity (Cunha, 2019).

Ado functions as a signalling molecule that modulates synaptic transmission and passive membrane properties through activation of high-affinity (A_1 and A_{2A}) and low-affinity (A_{2B}) receptor subtypes; A_3R affinity for Ado is species-dependent and is high in humans and low in rodents (Dias *et al.*, 2013). The four Ado receptors (ARs), A_1R , $A_{2A}R$, $A_{2B}R$ and

A₃R, are all metabotropic receptors, i.e., GPCRs (Burnstock, 2017; Cunha, 2019). Typically, A₁R and A₃R couple to the G_{i/o} family inhibiting cAMP production leading to inhibitory actions, whereas A_{2A}R and A_{2B}R stimulate cAMP production via G_s therefore leading to excitatory actions (Abbracchio *et al.*, 2009; Dias *et al.*, 2013; Burnstock, 2017). In addition to this contrary regulation of AC by excitatory vs inhibitory, A₁R, A_{2A} and A₃R can trigger MAP kinase activation. Through activation of PLC, these receptors can promote IP₃ formation, resulting in Ca²⁺ release from intracellular stores; another effect caused by PLC is mediated by DAG accumulation that promotes activation of PKC (Figure 5). A₁R can also inhibit Ca²⁺ conductance and activate K⁺ channels, allowing postsynaptic hyperpolarization. High levels of A₁R occur in the cortex, hippocampus, and cerebellum, which unlike A_{2A}R that is strongly expressed by striatopallidal GABAergic neurons with lower levels present in other brain regions (Dias *et al.*, 2013).

In the CNS, Ado exerts a multitude of functions, including modulation of neuronal and glial functions, of neuron-glia signalling or of neuronal development. Dysregulation of the Ado system is involved in pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions (Abbracchio *et al.*, 2009).

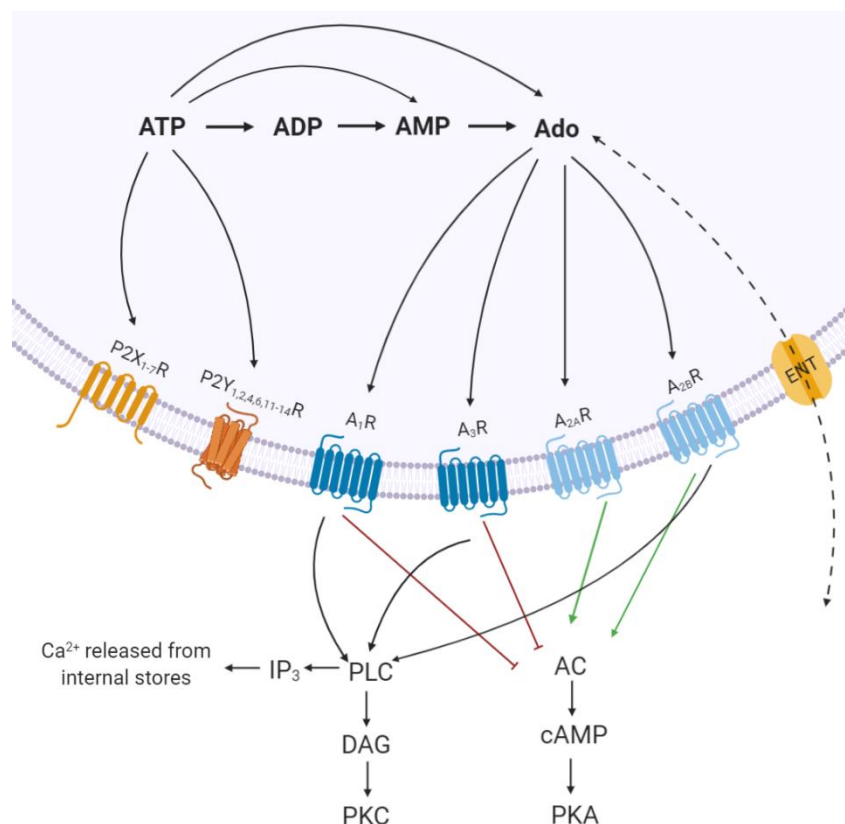


Figure 5: Schematic diagram illustrating ectonucleotidase-mediated metabolism of extracellular ATP to ADP, AMP and Ado, and downstream signalling pathways for these modulators.

1.5. Neurotransmission in the CNS

The two most important neurotransmitters in the brain are glutamate (also named glutamic acid) and γ -aminobutyric acid (GABA). These two neurotransmitters serve dual roles in the brain as metabolites and important neurotransmitters mediating excitatory and inhibitory signals, respectively (Rowley *et al.*, 2012; Schousboe *et al.*, 2014). These amino acids are metabolically intertwined and they are tightly coupled to the intermediary metabolism involving energy homeostasis (Schousboe *et al.*, 2013).

Alteration of the balance between these two amino acids functions in neurotransmission may contribute to increased or decreased seizure activity (Rowley *et al.*, 2012; Schousboe *et al.*, 2014).

1.5.1. Glutamatergic neurotransmission in the nervous system

1.5.1.1. Glutamate

Outside the biomedical community, glutamate is likely better known as “monosodium glutamate” (MSG), which is used as a flavour or taste enhancer in foods. This, however, is not the reason for the enormous scientific interest in glutamate (Zhou *et al.*, 2014). Glutamate was originally speculated to serve a metabolic function in the CNS, as it was found within numerous intracellular compartments and can be synthesized in the body through distinct metabolic pathways. Currently it is well-known that despite its ubiquitous nature, glutamate functions as the *major* excitatory neurotransmitter in the mammalian CNS; the reported intracellular concentrations of glutamate vary between 2 and 20 mM (Newsholme *et al.*, 2003). Glutamate plays a major role in neuronal excitability, synaptic plasticity, immunity and behavioural mechanisms such as learning and memory in the CNS (Yelamanchi *et al.*, 2016).

Glutamate should be present at the right concentrations in the right places at the right time. Both too much glutamate and too little glutamate are dangerous. Excessive activation of glutamate receptors may excite nerve cells to their death in a process now called “excitotoxicity”. This toxicity was perceived initially as a paradox, but it is now clear that glutamate is toxic, notwithstanding its importance (Zhou *et al.*, 2014).

Glutamate is a non-essential amino acid (Yelamanchi *et al.*, 2016) and, as all amino acids, glutamate has a C-terminus and an N-terminus; the C-terminus and carbon backbone derive from glucose. The glutamatergic neurotransmission is tightly controlled through an energy-consuming process, requiring multiple regulatory processes and high-levels of glucose and oxygen.

1.5.1.2. Glutamate metabolism, clearance and cycling

Glutamate plays important roles by linking carbohydrate and amino acid metabolism via the tricarboxylic acid (TCA) cycle, as well as in nitrogen trafficking and ammonia homeostasis in brain (Schousboe *et al.*, 2014). Glutamate metabolism in the brain extends beyond its role as a neurotransmitter, as it serves as the immediate precursor for GABA (Schousboe *et al.*, 2014), a precursor for the biosynthesis of amino acids such as L-proline and L-arginine, and as a component of various amino acid-based derivatives, for instance glutathione, an antioxidant (Niciu *et al.*, 2012; Yelamanchi *et al.*, 2016).

Glucose crosses the blood–brain barrier via astrocytic endfeet and, upon reaching the intracellular compartment it is broken down into pyruvic acid via glycolysis. Pyruvic acid enters the TCA cycle; glutamate dehydrogenase (GDH) catalyzes the reaction between glutamate, α -ketoglutarate (α KG) and ammonia, using NAD^+ or NADP^+ as co-enzyme, with formation of glutamate or α KG and ammonia (Rowley *et al.*, 2012). Consistent with glutamate's key role in multiple aspects of brain physiology, all the glucose that enters the CNS is eventually converted to glutamate (Niciu *et al.*, 2012).

When glutamate enters the cytoplasm, it may undergo further redistribution to mitochondria or synaptic vesicles; (1) Mitochondrial glutamate transport: mitochondria possess four different carriers for glutamate translocation AGC1, AGC2, GC1 and GC2; or (2) glutamate transporters in synaptic vesicles: in glutamatergic nerve terminals, glutamate is transported into synaptic vesicles by vesicular glutamate transporters (VGLUTs) (Zhou *et al.*, 2014).

VGLUTs are multimeric proton/glutamate antiporters; they are divided into three groups - VGLUT1, VGLUT2, and VGLUT3. VGLUT1 and 2 are primarily expressed in glutamatergic neurons and in glial cells; while VGLUT3 has been detected in GABAergic, cholinergic and monoaminergic neurons (Niciu *et al.*, 2012; Rowley *et al.*, 2012).

Ca^{2+} -dependent release of transmitters requires the presence of an exocytotic secretory machinery. Both neurons and astrocytes express the proteins of the soluble N - ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) complex:

synaptobrevin 2 (Sb2), also referred to as vesicle-associated membrane protein 2 (VAMP 2) (Ransom, 2013); glutamate is released into the synaptic cleft to bind to and evoke its effects on postsynaptic receptors (Niciu *et al.*, 2012).

When glutamate levels increase extrasynaptically, they lead to dysregulated excitatory neurotransmission and excitotoxicity. Therefore, it is necessary a rapid removal of extracellular glutamate to avoid cellular damage. Glutamate is actively removed from the synaptic cleft and transported into the cytosol against its concentration gradient via high-affinity excitatory amino acid transporters (EAATs), which are primarily found on astrocytes. These proteins are integral membrane proteins that rely on the lipid environment and are influenced by fatty acids and oxidation (Niciu *et al.*, 2012; Zhou *et al.*, 2014). Five transporters have been identified to date: EAAT1 is abundantly detected in the neocortex and cerebellum, but seems to be confined to astrocytes (Rowley *et al.*, 2012; Zhou *et al.*, 2014); EAAT2, the topmost glutamate transporter in the forebrain, is expressed mostly in astrocytes and in a limited extent, in neurons; EAAT3 is neuron-specific with the highest levels in the brain present in the hippocampus and neocortex (Rowley *et al.*, 2012; Zhou *et al.*, 2014); EAAT4 has been detected in the dendrites of cerebellar Purkinje neurons; lastly, EAAT5 is retina-specific. In rodents, EAAT1-3 homologues are referred to as GLAST, GLT, and EAAC1, respectively (Niciu *et al.*, 2012; Rowley *et al.*, 2012). The location of the EAATs relative to the geometry of synapses places them in a crucial position to prevent glutamate spillover and activation of extrasynaptic glutamate transporters. All the EAATs catalyse coupled transport of 1H^+ , 3Na^+ , and 1K^+ with one substrate molecule. They also function as chloride (Cl^-) channels; EAAT4 and EAAT5 have the largest Cl^- conductance (Zhou *et al.*, 2014).

When in the cytosol, glutamine synthetase (GS), an astrocyte and oligodendrocyte-specific enzyme that is often referred to as phosphate activated glutaminase (PAG) since it is stimulated by phosphate, converts glutamate into glutamine (Gln) by deamidation via phosphate activated glutaminase; this reaction also produces ammonia. GS also catalyses the reverse reaction (Niciu *et al.*, 2012; Rowley *et al.*, 2012; Schousboe *et al.*, 2014). Gln is known to protect the body from nutrient loss, oxidative stress and tumour stress (Schousboe *et al.*, 2014). Both astrocytes and neurons contain Gln transporters that, under proper electrophysiological conditions, lead to the net exchange of Gln from astrocytes to neurons (Figure 6) (Niciu *et al.*, 2012).

Unlike astrocytes, neurons do not have GS neither pyruvate carboxylase enzymes nor are capable to synthesize glutamate from glucose (Niciu *et al.*, 2012; Rowley *et al.*, 2012). As a result, the mitochondrial phosphate-specific enzyme, glutaminase (PAG) in

neurons, hydrolytically deamidates Gln to reconvert it in glutamate, with formation of ammonia, for subsequent repackaging into synaptic vesicles. The cycling of glutamate/Gln in astrocytes and neurons has been termed “the glutamine cycle”. Since this is an open-cycle that actively interfaces with other pathways and loses intermediates to other pathways, the *de novo* synthesis of Gln in astrocytes helps to maintain the operation of this cycle (Newsholme *et al.*, 2003; Niciu *et al.*, 2012; Schousboe *et al.*, 2014). The fine-tuned biochemical specialization of astrocytes allows them to respond to subtle changes in neurotransmission by dynamically correcting their anaplerotic and glycolytic activities (Niciu *et al.*, 2012), and adjusting the oxidation of glutamate for energy relative to direct formation of Gln (Schousboe *et al.*, 2014).

In summary, there are two pathways for production of neuronal glutamate: (1) the production of new glutamate molecules from glucose and amino acid derivatives via energy-costly metabolism, and (2) recycling glutamate from Gln through glutamate uptake, enzymatic activity of glutaminase and the activities of the Gln transporters (Niciu *et al.*, 2012).

The primary and more discussed mechanism of glutamate release from nerve terminals is by Ca^{2+} -dependent exocytosis of synaptic vesicles, but this is not the only mechanism. Glutamate can be released through non-exocytotic mechanisms such as: anion channels, reversal of high-affinity transmembrane glutamate transporters, and glutamate-cysteine antiporter (xCT) (Figure 6). Another mechanism that has been debated is whether mature brain astrocytes *in situ* also have the ability to release glutamate by exocytosis (Niciu *et al.*, 2012; Zhou *et al.*, 2014)

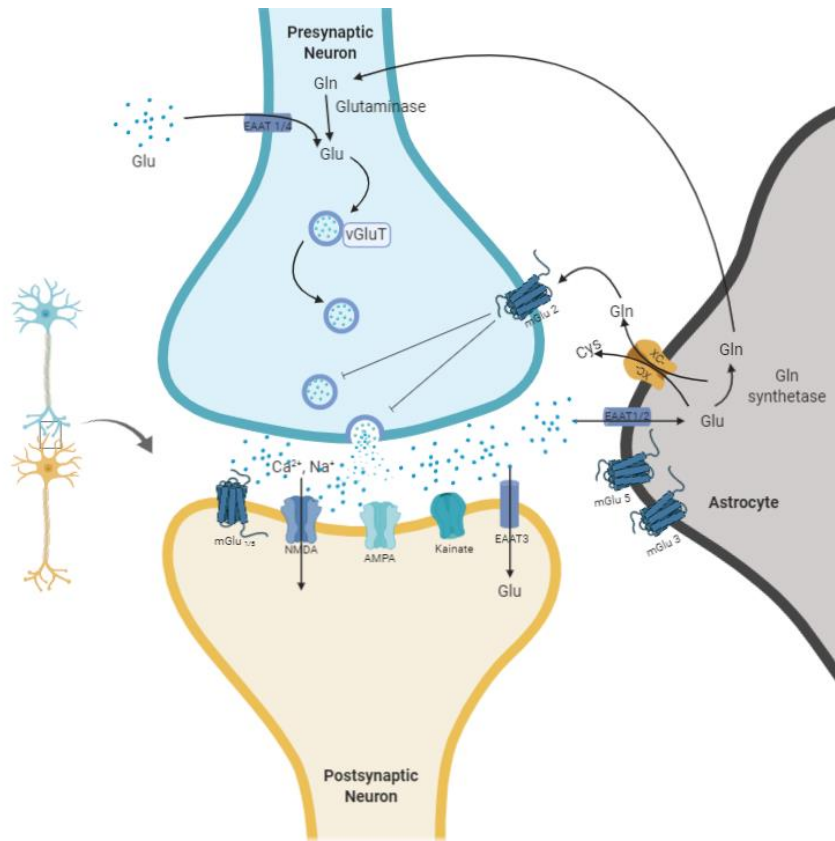


Figure 6: Schematic representation of glutamatergic synapse.

1.5.1.3. Glutamate receptors

Glutamate receptors may be divided into two distinct subtypes: ionotropic (iGluRs) and metabotropic receptors (mGluRs). iGluRs are ion channels that flux cations (i.e. Ca^{2+} , Na^{+}). Conformational changes “open” the channel in response to activation by an agonist. mGluRs, alternatively, activate or inhibit second messenger systems via interactions with associated G-proteins (Zhou *et al.*, 2014).

iGluRs are a group of receptors that are related in their amino acid sequences and belong to the huge superfamily of ion channels containing a P-loop, containing several subunits, as ion-pore-forming segment. iGluRs form tetrameric complexes of individual/heteromeric subunits (Niciu *et al.*, 2012). The P-loops form the channel pore and determine whether the ion channel is selective for monovalent and/or divalent ions (Sprengel, 2016). The iGluRs include four subgroups, which were named based on their agonist selectivity: N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-

isoxazole propionic acid (AMPA), kainate (KA) (Niciu *et al.*, 2012) and orphan receptors (Figure 7) (Sprengel, 2016).

NMDA receptors have received the most attention in the neuroscience community and are among the most tightly regulated in the mammalian brain and exceptional in requiring co-agonists for activation. In the mature brain, the NMDA receptors are necessary for the formation and the extinction of memory (Sprengel, 2016). At least six binding sites have been identified that regulate ion channel opening probability, to wit, sites for two obligatory coligands (glutamate and glycine), polyamines and cations (Mg^{2+} , Zn^{2+} and H^+). NMDA receptor ligands are short-chain dicarboxylic amino acids such glutamate and aspartate (Niciu *et al.*, 2012) though under normal physiological conditions, the receptor cannot be activated by glutamate. At the resting potential, the ion pore of the receptor is physically blocked by Mg^{2+} ions. Immediately when the cell membrane is depolarized, the Mg^{2+} -block ceases, and the NMDA receptor can be activated by glutamate. When the NMDA receptor is activated, Na^+ ions can enter to the postsynaptic cell accompanied by a strong Ca^{2+} -influx. This Ca^{2+} acts as a second messenger and induces many different postsynaptic cellular responses. The complex signalling leads to increased responses of the postsynaptic cell that can last for hours, days, or months (Sprengel, 2016).

KA and orphan receptors have not yet been characterized in detail: the KA receptors form fast channels, but no ion channel function has, so far, been found for the orphan receptors (Sprengel, 2016).

The AMPA receptors are widely expressed in the CNS. They directly mediate fast excitatory neurotransmission in response to glutamate binding which represent the communication points between neurons (Niciu *et al.*, 2012; Sprengel, 2016). AMPA receptor currents are strengthened by NMDA receptors (Niciu *et al.*, 2012).

Unlike iGluRs that depend on cation flux, mGluRs exert their effects via the recruitment and activation of intracellular G-proteins and downstream signal transduction pathways (Niciu *et al.*, 2012). Like all GPCRs, the mGluR consist of seven transmembrane domains joined by three extracellular and three intracellular loops. The N-terminal extracellular domain contains a glutamate-binding Venus flytrap domain and a cysteine-rich domain. The cytoplasmic carboxy-terminal domain modulates receptor signaling, trafficking and G protein coupling (Suh *et al.*, 2018).

To date, eight metabotropic glutamate receptors have been identified; the nomenclature of mGluRs is numeric beginning with mGlu1 (mGluR1-8), which have been further subdivided into three functional groups on the basis of sequence homology, second

messenger coupling, and pharmacological properties - mGluRs are divided into three sub-families: Group I, II and III (Niciu *et al.*, 2012; Suh *et al.*, 2018).

Group I (mGlu1 and mGlu5) receptors are coupled to G_q -like proteins and elicit their downstream effects by two mechanisms: (1) PLC via IP_3 to release Ca^{2+} from intracellular stores and (2) simultaneous binding of elevated Ca^{2+} and DAG activates PKC (Niciu *et al.*, 2012).

Group II and Group III receptors are coupled to $G_{i/o}$ proteins and inhibit AC, which leads to a reduction of cAMP (Niciu *et al.*, 2012) and activates the MAPK-ERK pathway (Suh *et al.*, 2018). Nearly all mGluRs are broadly expressed in the brain preferentially expressed in presynaptic axon terminals, except for mGlu6, which is restricted to the retina. Although these receptors share a common signalling pathway, they differ in their expression patterns and subcellular distribution: Group II mGluRs are positioned away from the glutamate release sites, whereas Group III mGluRs are typically located at the presynaptic active zones (Suh *et al.*, 2018).

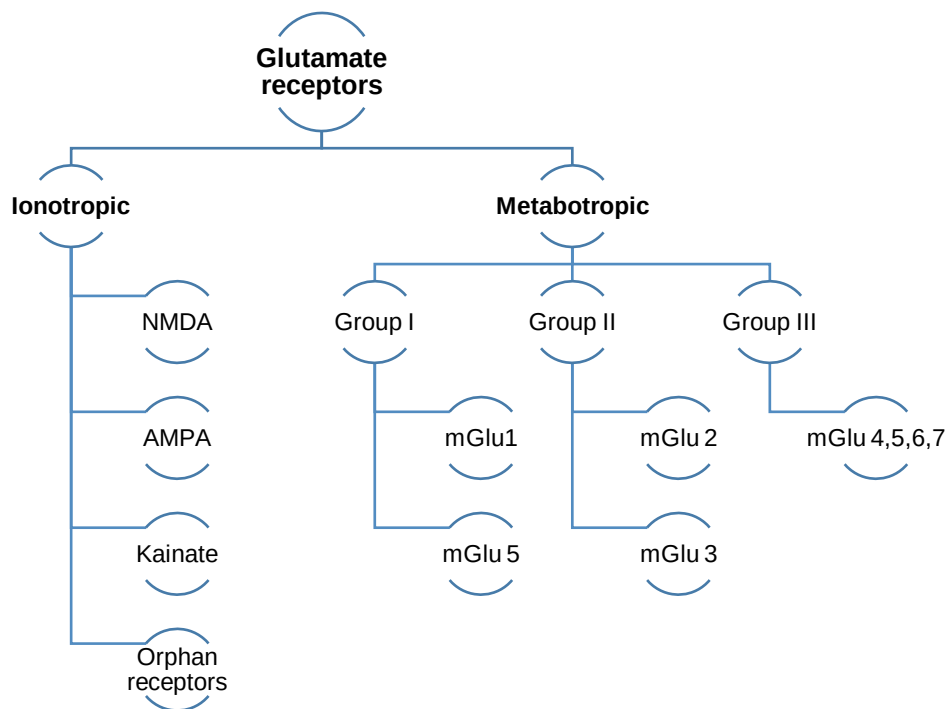


Figure 7: Glutamate receptor subtypes.

1.5.2. GABAergic neurotransmission in the nervous system

1.5.2.1. GABA (γ -aminobutyric acid)

γ -aminobutyric acid (GABA) is a major neurotransmitter expressed since the embryonic stage and throughout life. At an early developmental stage, GABA acts in an excitatory manner and is related to neurogenesis in many processes, such as neuronal proliferation, migration, differentiation, and preliminary circuit building, as well as the development of critical periods (Owens *et al.*, 2002; Wu *et al.*, 2015). In the mature CNS, GABA is widely known for its potent inhibitory role (Owens *et al.*, 2002). This change is termed the “GABA shift” (Wu *et al.*, 2015).

In tandem with the glutamate, GABA modulates the inhibitory-excitatory balance necessary for correct brain function in mature brains. GABA also plays an important role in the development of interstitial neurons of the white matter, as well as in oligodendrocyte development (Wu *et al.*, 2015).

1.5.2.2. GABA metabolism, clearance and cycling

GABA metabolism is less complex than that of glutamate (Schousboe *et al.*, 2007). GABA can be formed either directly from (1) glutamate in the cytosol or (2) from glutamate originating from α KG produced in the TCA cycle involving GDH. Gln released by astrocytes can also function as a precursor of GABA via glutamate, extending the concept to a glutamate/GABA-Gln cycle (Rowley *et al.*, 2012; Walls *et al.*, 2014). This biosynthetic pathway consists of a decarboxylation of glutamate catalysed by the enzyme glutamate decarboxylase (GAD) (Rowley *et al.*, 2012; Arne Schousboe & Waagepetersen, 2007). GAD exists in two isoforms, GAD₆₅ and GAD₆₇, which have different: molecular weights (65 and 67 KDa), catalytic and kinetic properties, and subcellular localization (Schousboe *et al.*, 2007, 2013; Rowley *et al.*, 2012). The extent of TCA cycle metabolism before GABA synthesized from Gln appears to be dependent on the pool (that is, the vesicular or metabolic pool) from which GABA is released (Schousboe *et al.*, 2017). Nevertheless GABA is removed from the extrasynaptic space predominantly by transporters located in the presynaptic neurons, and to a reduced extent into astrocytes (Schousboe *et al.*, 2013).

After its biosynthesis GABA is packed into synaptic vesicles by a vesicular neurotransmitter transporter (VGAT) and is released from nerve terminals by a Ca^{2+} -

dependent exocytosis (Figure 8) (Owens *et al.*, 2002). After depolarization, vesicles containing GABA may fuse with the membrane in a process involving SNARE proteins that are activated and reorganized after elevation of the intracellular Ca^{2+} concentration brought about by opening a number of pharmacologically distinct voltage-gated Ca^{2+} channels; this mechanism leads to a spatially highly localized release (Schousboe *et al.*, 2007, 2013). However, non-vesicular forms of GABA secretion exist, these comprise transport reversal given to the fact that GABA transporters are electrogenic and, therefore, sensitive to depolarization (Owens *et al.*, 2002; Schousboe *et al.*, 2007, 2013). This release is spatially less precise compared to the vesicular release (Schousboe *et al.*, 2007, 2013) and might be particularly important during development before the establishment of synaptogenesis (Owens *et al.*, 2002).

After releasing, GABA diffuses into the narrow synaptic cleft, allowing activation of a set of presynaptic and postsynaptic GABA receptors (Schousboe *et al.*, 2007, 2013). GABA signals cease by reuptake of the neurotransmitter into the presynaptic GABAergic nerve ending and/or into surrounding glial cells by a class of membrane GABA transporters (GATs) (Figure 8) (Schousboe *et al.*, 2017). Hence GABA transporters are a complex component of the inhibitory system with dual roles, both in uptake and release (Sperk *et al.*, 2004).

GATs, like EEATs, belong to a family of neurotransmitter sodium symporters referred as the solute carrier 6 (SLC6) family, a family of Na^+ and Cl^- coupled transporters, in human (Sperk *et al.*, 2004; Scimemi, 2014). In the currently accepted nomenclature, GAT is the designation for human (A1-20) and rat (GAT, BGT, NTT, etc.) (Scimemi, 2014). For simplicity, it will be used the human/rat GATs nomenclature.

The GABA transporters group comprises six transporters: A1/GAT1, A13/GAT2, A11/GAT3, A12/BGT1, A8/CT1, and A6/TauT. Although all members carry GABA, as well as other molecules, only A1/GAT1, A13/GAT2, A11/GAT3 are high-affinity GABA transporters (Sperk *et al.*, 2004; Schousboe *et al.*, 2013, 2017; Scimemi, 2014).

GAT1 is the most prevalent being expressed both in GABAergic neurons and in astrocytes, GAT2 is expressed neonatally in the brain but its expression decreases as a function in postnatal, GAT3 may be considered primarily astrocytic and GAT4 are mainly non-neuronal (Sperk *et al.*, 2004; Schousboe *et al.*, 2013, 2017).

Inactivation of GABA requires GABA-transaminase (GABA-T) action in the GABA shunt. GABA-T is located in the mitochondrial matrix and unlike GAD, is an enzyme that is ubiquitously expressed not only in the brain but also in other organs. GABA-T converts GABA to succinic semialdehyde (SSA) by transamination with the co-substrates glutamate

and α KG. SSA is subsequently oxidized by SSA dehydrogenase (SSADH) to succinate, which enters the TCA cycle (Rowley *et al.*, 2012; Walls *et al.*, 2014; Schousboe *et al.*, 2017). Alternatively, SSA can be reduced by c-OH-butyric acid dehydrogenase (GHBDH) to c-OH-butyric acid which can activate GABA_BR (Rowley *et al.*, 2012).

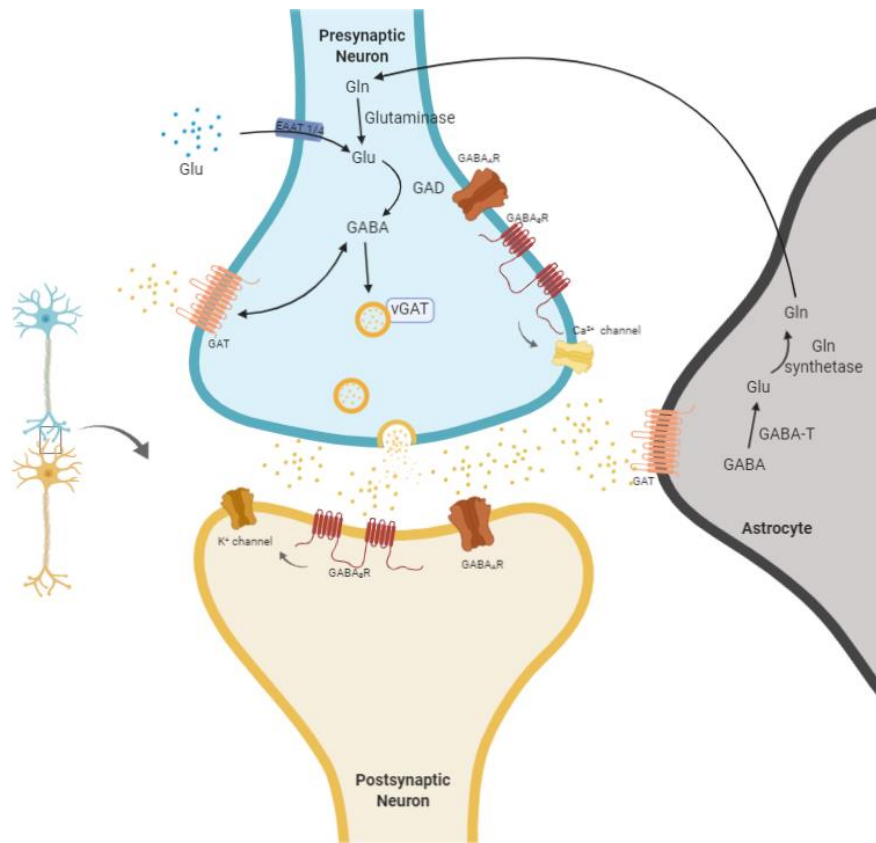


Figure 8: Schematic representation of GABAergic synapse.

1.5.2.3. GABA receptors

GABA receptors (GABAR) are divided into two wide families. It was found that certain GABA responses could be mimicked by the lipophilic GABA analog, baclophen, an effect that could not be blocked by the classical GABAR antagonist bicuculline. This led to the separation into two major classes: GABA_AR and GABA_CR for the Cl⁻ channel-forming receptors (ionotropic receptors); and GABA_BR for this new class of receptor that did not gate an ion channel and are associated with G proteins, which link receptor activation to either K⁺ or Ca²⁺ channels or to AC (metabotropic receptors) (Schousboe *et al.*, 2008, 2017).

Mature neurons typically maintain low concentrations of intracellular Cl⁻, and activation of ionotropic GABA_ARs open these anion channels resulting in the influx of anions

with a permeability ratio of approximately 3:1 regarding Cl^- and HCO_3^- , respectively; the influx of anions result in cells hyperpolarization (Schousboe *et al.*, 2017). In the adult brain, GABA acts primarily through activation of the fast hyperpolarizing GABA_A R (Wu *et al.*, 2015).

During early developmental stages, neurons can accumulate intracellular Cl^- due to deficient efflux carriers causing opposite Cl^- fluxes to occur in immature neurons. Therefore, in selected regions of the CNS, such as the hippocampus or dorsal root ganglia, or under certain circumstances, depolarizing effects of GABA are often observed in the embryonic nervous system, a property of GABA_A ergic neurotransmission that is very likely important for the neurotropic action of GABA during early development stages (Enna *et al.*, 2007; Schousboe *et al.*, 2017).

GABA_A R is comprised of α , β , γ and δ subunits in a heteropentameric structure and are distributed differentially in the mature brain (Enna *et al.*, 2007; Schousboe *et al.*, 2017). The subunit composition greatly influences the pharmacological profile of the receptor and its localization (Schousboe *et al.*, 2017). Extrasynaptic GABA_A R are high affinity receptors implicated in tonic inhibition, while synaptic GABA_A R are those involved in fast, phasic inhibition (Wu *et al.*, 2015). In addition to the agonist/antagonist binding sites, the GABA_A R has multiple binding sites for a variety of modulators i.e. benzodiazepines, barbiturates, steroids, ethanol, and Zn^{2+} , making it a multidrug target (Enna *et al.*, 2007; Wu *et al.*, 2015; Schousboe *et al.*, 2017).

The distinction between GABA_A R and GABA_C R is based only on pharmacological grounds. GABA_C Rs are characterized by their activation by cis-4-aminobut-2-enoate (CACA), whereas classic GABA_A R agonists such as isoguvacine have no effect (Schousboe *et al.*, 2017). GABA_C R has a homopentameric structure composed solely of 3 ρ subunits (Wu *et al.*, 2015).

The GABA_B R belongs to the GPCR superfamily, is associated with $\text{G}_{i/o}$ and it has been shown to form heteromeric complexes of $\text{GABA}_{\text{B}(1)}$ and $\text{GABA}_{\text{B}(2)}$ subunits to be functionally active (Enna *et al.*, 2007; Schousboe *et al.*, 2008, 2017). GABA_B Rs are responsible for the later and slower component of inhibitory transmission. They are expressed both pre and post synaptically and their stimulation reduces the activity of AC or, through coincident signaling, enhances the production of cAMP. The predominant response to GABA_B R activation is an increase in K^+ conductance and reduction of neuronal Ca^{2+} conductance with a consequent hyperpolarization of the neuron (Enna *et al.*, 2007; Schousboe *et al.*, 2017).

Chapter 2

Aim

Epilepsy affects over 70 million people worldwide which makes it one of the most common serious neurological disorder (Moshé *et al.*, 2015; Thijs *et al.*, 2019). Thirty per cent of individuals have uncontrolled seizures, that can be associated with reduced quality of life and mental illness (Moshé *et al.*, 2015; Ali *et al.*, 2019). This disease is biologically characterized by an imbalance between glutamatergic and GABAergic neurotransmission (Bradford, 1995). The most alarming group of childhood epilepsies are the development of epileptic encephalopathies, which include DS, LGS and others. The incidence of these genetic syndromes is rare, about one in 2000 births, however these children have drug-resistant frequent seizures and epileptiform abnormalities that result in severe cognitive and behavioural impairment, and in a high mortality rate before reaching the age of 20. Although difficult to explain, there is an increasing number of children affected by genetic epileptic syndromes over the last few years (Ali *et al.*, 2019).

The ECS participates in the pathophysiology of a range of neurological and psychiatric disorders, including epilepsy. CBD emerged as an attractive therapy candidate because of its comparatively favourable therapeutic index and lack of the undesirable euphoria-inducing properties associated with other cannabinoids, namely Δ^9 -THC (Devinsky *et al.*, 2014; Franco *et al.*, 2019). In fact, CBD (Epidiolex®) was developed as an orphan medicine for DS and LGS approved by FDA (U.S. Food and Drug Administration, 2018) and, more recently, also by EMA (European Medicines Agency, 2019). Despite its approval and apparent efficacy, the precise mechanism of action and long-term side effects are not yet resolved. Available data indicate that multiple actions are probably involved (Ibeas Bih *et al.*, 2015; Franco *et al.*, 2019). Growing evidence point to a proposed mechanism for CBD action involving blockage of the ENT1 adenosine transporter, thus reducing Ado uptake and thereby increasing extracellular Ado accumulation contributing to downregulate neuronal excitability (Carrier *et al.*, 2006; Perucca, 2017; Franco *et al.*, 2019).

Considering the gaps in our knowledge concerning the mechanisms of action of CBD in the immature brain, we set up to investigate whether it could synchronously modulate GABA and glutamate release in the brain of immature juvenile rats compared to adult animals. Our findings may shed light on the mechanism(s) underlying the therapeutic role of CBD in epileptic children. The interplay between CBD and the endogenous roles of Ado (a potent anti-epileptic substance) will also be secondarily addressed in this study.

Chapter 3

Materials and methods

3.1. Animals

Animal care and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and followed the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for Care and Use of Laboratory animals (NIH Publications No. 80-23) revised 1996. The study was approved by the Ethics Committee and the Animal Welfare Responsible Organism of ICBAS-UP (Decision nº 224/2017). All efforts were made to minimize animal suffering and to reduce the number of animals used. Immature (21-28 days) and adult Wistar rats (2-3 months) of either sex (Charles RiverTM, Barcelona, Spain) were kept at a constant temperature (21 °C) and a regular light (06.30–19.30 h)–dark (19.30–06.30 h) cycle, with food and water *ad libitum*.

3.1.1. Hippocampal isolation from immature and adult rats

Hippocampi were obtained from Wistar rats of both gender of 21 to 28 days (immature juveniles) and 2 to 3 months old (adults) (Charles RiverTM, Barcelona, Spain).

Animals were sacrificed by decapitation followed by exsanguination, without using any anaesthetic drug. Their brains were quickly removed and placed in ice-cold Krebs solution (composition in mM: NaCl 136, KCl 3, MgCl₂ 1.2, CaCl₂ 0.5, NaHCO₃ 16.2, glucose 5.5, Na₂HPO₄ 1.2; pH=7.4), oxygenated with 95% O₂ and 5% CO₂. Both hippocampi were dissected out of rat brains and synaptosomes prepared as described below.

3.2. Preparation of synaptosomes from the rat hippocampus

The synaptosomal preparation is enriched in isolated and resealed presynaptic nerve terminals, many of which retain their *in vivo* function and biochemical, morphological and electrophysiological properties (Gray *et al.*, 1962; Whittaker *et al.*, 1964; Weiler, 2009). Synaptosomes (1) maintain the pathways for the synthesis, exocytosis, and reuptake of specific neurotransmitters, (2) utilize glucose, via glycolysis and oxidative phosphorylation, to generate a high cytosolic ATP phosphorylation potential supplying, among other processes, plasma membrane Na⁺/K⁺- and Ca²⁺-ATPases to maintain a stable and

physiological membrane potential and low cytosolic free calcium concentration ($[Ca^{2+}]_c$), and (3) have the ability of distinguishing true releasers from reuptake inhibitors (Raiteri *et al.*, 2000; Nicholls, 2018). Synaptosomes can, therefore, be viewed as autonomous secretory “mini cells.” A major advantage of the preparation is the ability to prepare synaptosomes from animals of any age, free from the neonatal limitation inherent to primary cell cultures (Nicholls, 2018).

Synaptosomes were isolated as previously described by Helme-Guizon *et al.* (1998) and then modified by Bancila *et al.* (2009). Briefly, the brain hemispheres were separated and the hippocampi were isolated and sliced into small cubes with a scalpel, placed in 15 mL falcon tubes in cold oxygenated Krebs solution and gently homogenized, with the use of a P1000 pipette, until there was no resistance. After homogenization, all the procedures were performed at room temperature. Homogenates were filtered through a nylon filter (mesh size 100 μ m). The filtrate was left to sit in Krebs solution during 45-60 min until formation of a pellet, which was re-suspended into new Krebs solution and left to sit for more 30-45 min until newly formation of a pellet (Figure 9).

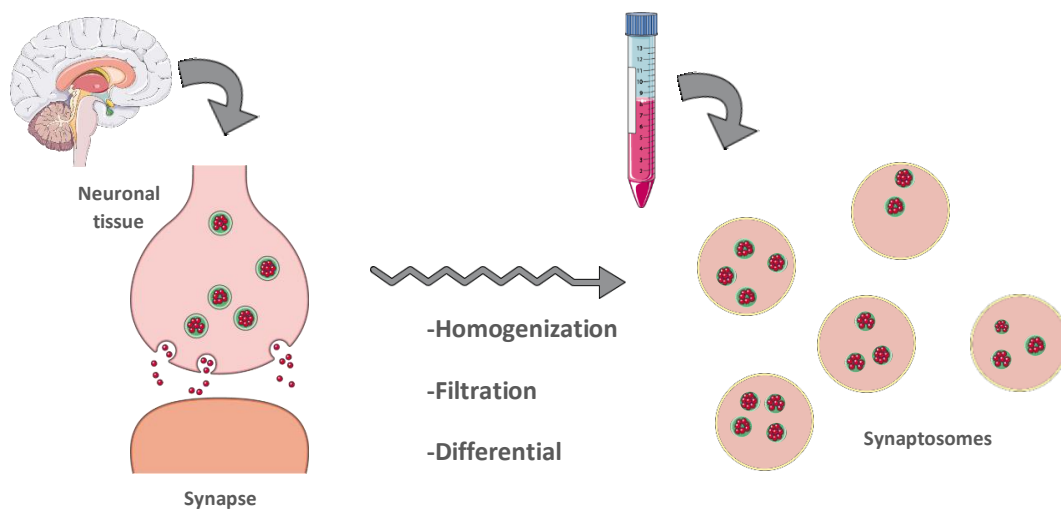


Figure 9: Schematic representation of protocol for preparation of hippocampal synaptosomes.

3.3. Protein quantification

Protein concentration was determined by the bicinchoninic acid method (BCA; Pierce™, ThermoScientific, Rockford USA) (Figure 10) according to the manufacturer instructions. The protein content was calculated by interpolation of a standard curve made with bovine serum albumin (BSA; concentrations 0-1000 µg/mL). The protein concentration of the samples was adjusted to 6.25 mg protein mL⁻¹.

Protein quantification assays were performed in 96-well plates, by adding 200 µL of the working reagent to 50 µL of sample (5x and 10x diluted synaptosomal suspension or BSA solution), per well. All samples were incubated 30 min at 37°C and the absorbance was read at 562 nm in BioTek Synergy™ HT microplate reader.



Figure 10: BioTek Synergy™ HT microplate reader.

3.4. Measurement of [³H]GABA and [¹⁴C]glutamate release by synaptosomes of the rat hippocampus

The release of [³H]GABA and [¹⁴C]glutamate by synaptosomes was measured simultaneously after incubating the synaptosomes with [³H]GABA (0.25 μ Ci mL⁻¹; 70 Ci mmol⁻¹; 0,5 μ M) and [¹⁴C]Glutamate (0.25 μ Ci mL⁻¹; 0.270 Ci mmol⁻¹; 10 μ M) during 10 min, at 37 °C. Aliquots (250 μ L) of a synaptosomal suspension containing 0.5 mg protein mL⁻¹ were layered onto glass fiber filters (Merck Millipore, Cork, IRL), which were mounted in 365 μ L chambers of a semi-automated 12-sample superfusion system (SF-12 Suprafusion 1000, Brandel, Gaithersburg, MD, USA) (Figure 12-A). Filters containing the synaptosomes were superfused at a flow rate of 0.5 mL min⁻¹ with a physiological solution (in mM: NaCl 128; MgCl₂ 1.2; KCl 3; glucose 10; HEPES–Na 0.01 (pH =7.4); CaCl₂ 2.2 and aminooxyacetic acid 0.01) or a Ca²⁺-free physiological solution (in mM: NaCl 128; MgCl₂ 1.2; KCl 3; glucose 10; HEPES–Na 0.01 (pH =7.4); EGTA 0.1 and aminooxyacetic acid 0.01) or a Na⁺-free physiological solution (in mM: NMDG 128; MgCl₂ 1.2; KCl 3; glucose 10; HEPES–Na 0.01 (pH =7.4); and aminooxyacetic acid 0.01) at 37 °C.

The synaptosomes were challenged twice (S1 and S2; 8 and 26 min after beginning fraction collection) with high KCl (15 mM, for 2 min) by changing the inlet tube from one flask to another containing the test drug (Figure 11). When Na⁺-free physiological solution was tested an extra 10 min period was added to the protocol to allow proper equilibrium with the new solution. All test drugs (CBD and receptor agonists) were added to the superfusion solution 15 min before S2; receptor antagonists were added from the beginning of sample collection period and, thus, they were present throughout the experimental protocol.

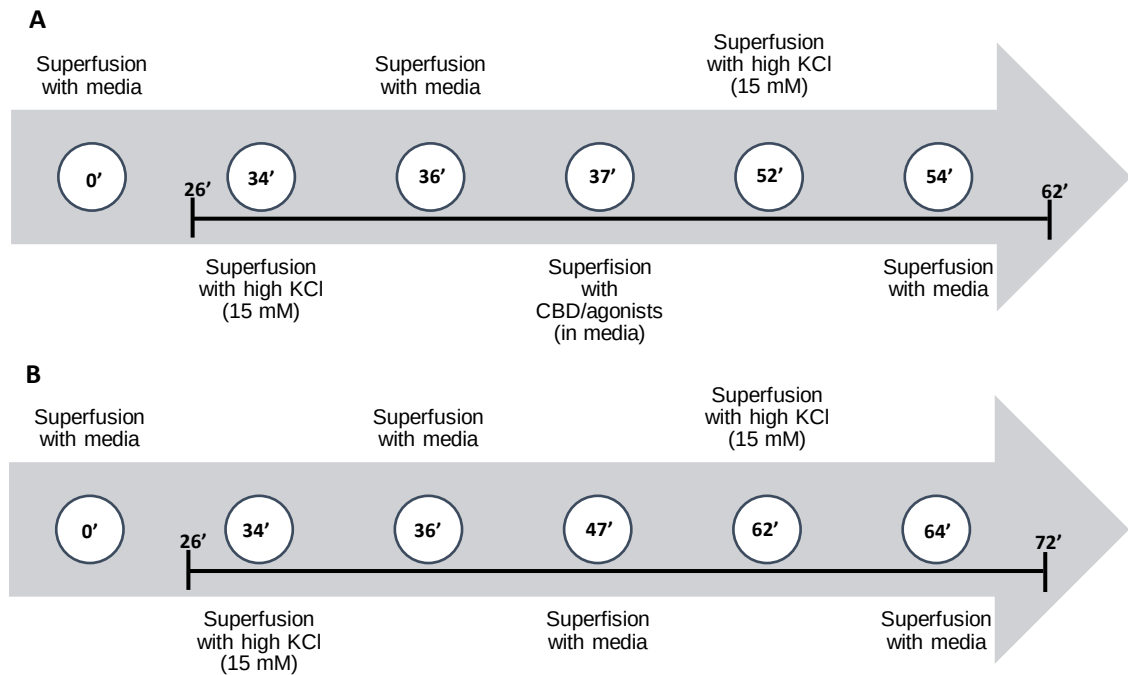


Figure 11: Schematic protocol of release of [^3H]GABA and [^{14}C]glutamate from synaptosomes.

The radioactive content of collected fractions and that remaining in the filters at the end of the experimental protocol were measured by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA) (Figure 12-B).

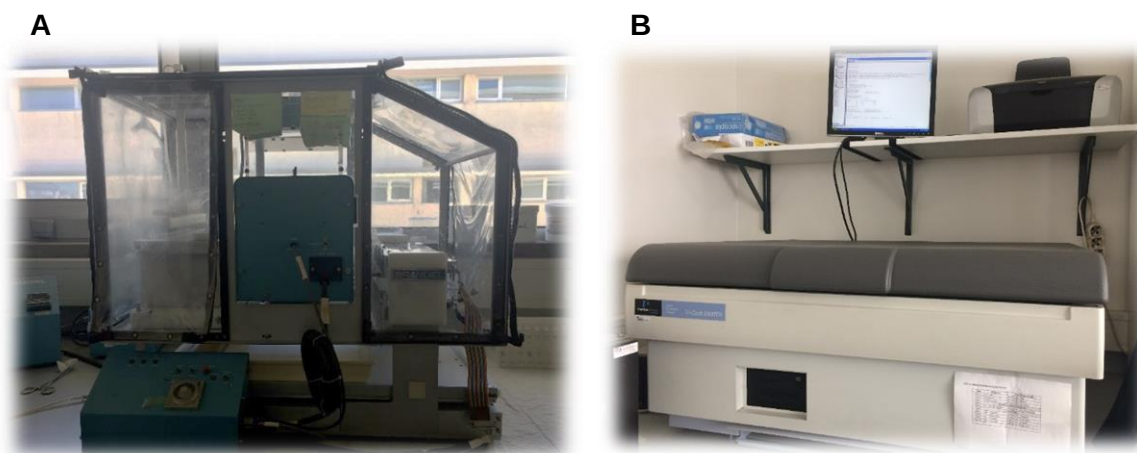


Figure 12: **A:** Semi-automated 12-sample superfusion system (SF-12 Suprafusion 1000, Brandel, Gaithersburg, MD, USA). **B:** TriCarb2900TR Perkin Elmer spectrometer.

3.5. Evaluation of drug-induced cellular damage

3.5.1. Hippocampal slices

Both hippocampi were quickly dissected out and transverse sections (400 μm) were prepared using a Leica VT 1000 S tissue chopper. Hippocampal slices were divided into 5 groups: negative control (physiological solution), positive control (Triton X-100 1%), CBD 3 μM , CBD 10 μM and CBD 30 μM and placed into 96-well culture plates. Following a 30-min equilibrium, drugs were added to the physiological bathing solution (in mM: NaCl 128; MgCl_2 1.2; KCl 3; glucose 10; HEPES-Na 0.01 (pH =7.4); CaCl_2 2.2 and aminooxyacetic acid 0.01) for an additional period of 15 min, at 37 °C. Control and CBD experiments were run in parallel using four hippocampal slices of the same animal for each experimental setting (Cechetti *et al.*, 2007).

3.5.2. Measurement of lactate dehydrogenase activity

Cell damage was quantified by measuring the LDH activity released into the medium. The method used is based on the direct, NADH-coupled assay employing pyruvate as the substrate (Keiding *et al.*, 1974). Two hundred microliter samples were collected from

each plate and the LDH activity was measured at 365 nm using a spectrophotometer (JenWay 630S UV/Vis). The spectrophotometer was calibrated to zero using 1 ml of phosphate buffer and readings were registered 30 s, 1 min, 2 min and 3 min after adding 1 ml of NADH solution (Keiding *et al.*, 1974; Kaja *et al.*, 2017). LDH activity was calculate in U/ml using the following formula.

$$U/ml = \frac{slope \times 10^3 \times 20}{6.2 \times 10^3}$$

Slope is calculated plotting the Abs vs min

6.2x10³ is the Molar extinction coefficient of NADH



Figure 13: Sanyo incubator

3.6. Data presentation and statistical analysis

[³H]GABA and [¹⁴C]glutamate release by synaptosomes was obtained as scintillations per minute (CPMs) in function of time (min). The disintegrations per minute (DPMs) were calculated from CPMs (according to the expressions 1 and 2 presented below) and the results were presented as the area of the peak.

$$(1) \quad C = \frac{N1 - N2 \left(\frac{h1}{h2} \right)}{c1 - c2 \left(\frac{h1}{h2} \right)}$$

$$(2) \quad H = \frac{N2 - N1 \left(\frac{c2}{c1} \right)}{h2 - h1 \left(\frac{c2}{c1} \right)}$$

C = carbon DPMs in sample

H = tritium DPMs in sample

c1 = carbon – 14 efficiency in channel 1

c2 = carbon – 14 efficiency in channel 2

h1 = tritium efficiency in channel 1

h2 = tritium efficiency in channel 2

N1 = CPMs in channel 1

N2 = CPMs in channel 2

The results were expressed as mean $\pm$ SEM, with *n* (showed in graphs) indicating the number of individuals. Statistical analysis of data was carried out using GraphPad Prism 8.0.2 software (La Jolla, CA, USA). Unpaired Student's *t*-test with Welch correction was used for statistical analysis when parametric data was considered. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test were used. $P < 0.05$ was considered to represent significant differences.

Chapter 4

Results

4.1. Comparison of hippocampal synaptosomes from immature juvenile and adult rats

4.1.1. [^3H]GABA and [^{14}C]Glutamate release pattern is similar both in immature and adult rats

Figure 14 shows that there are no significant differences ($P>0.05$) in the outflow of [^3H]GABA and [^{14}C]Glutamate from hippocampal synaptosomes obtained from immature juvenile and adult Wistar rats. This was verified in physiological sodium (NaCl 128 mM) and calcium (CaCl_2 2.2 mM) conditions, as well as after depolarization of hippocampal synaptosomes with high KCl (15 mM, for 2 min); the S2/S1 ratio for [^3H]GABA and [^{14}C]Glutamate release were not significantly different in immature juvenile and adult rat hippocampi (Figure 15).

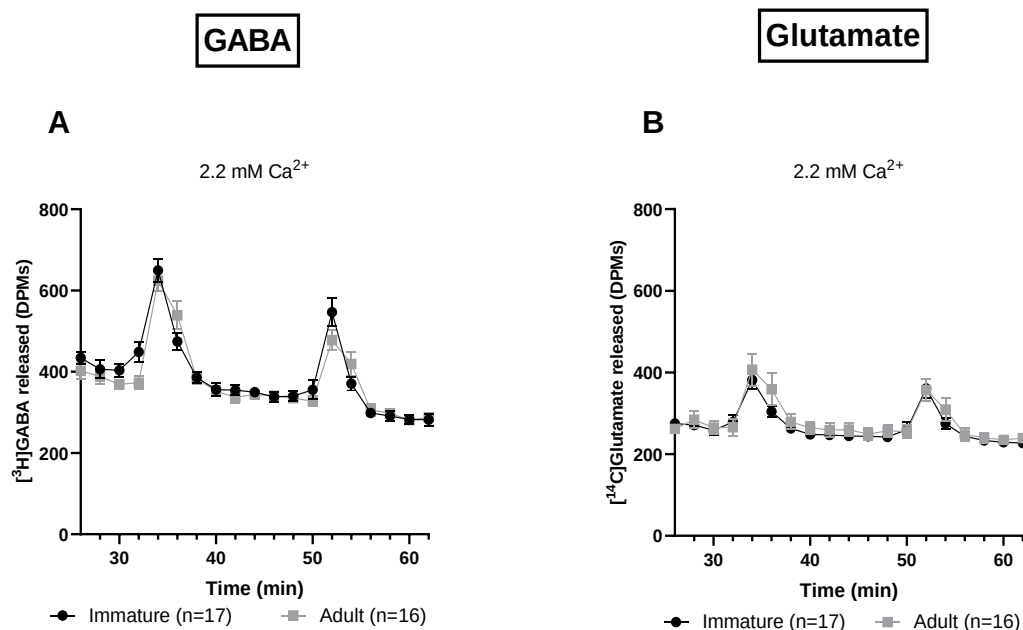


Figure 14: [^3H]GABA (A) and [^{14}C]Glutamate (B) outflow from hippocampal synaptosomes of immature vs adult rats. Graphs represent the outflow of [^3H]GABA and [^{14}C]Glutamate in physiological conditions and after synaptosomal depolarization with high KCl (15 mM, for 2 min), i.e. S1 and S2.

Removal of extracellular Ca^{2+} (plus EGTA 250 mM) 15 min before S2 did not significantly ($P>0.05$) modify depolarization-evoked [^3H]GABA and [^{14}C]Glutamate release, indicated by the observation of similar S2/S1 ratios in control and in Ca^{2+} -free conditions (Figure 15). Notwithstanding this, data obtained by elimination of external Ca^{2+} (plus EGTA

250 mM) from beginning of the experiment revealed that the magnitude of depolarization-evoked [^3H]GABA and [^{14}C]Glutamate release was smaller ($P<0.05$) in Ca^{2+} -free conditions than in the control medium (Figure 16).

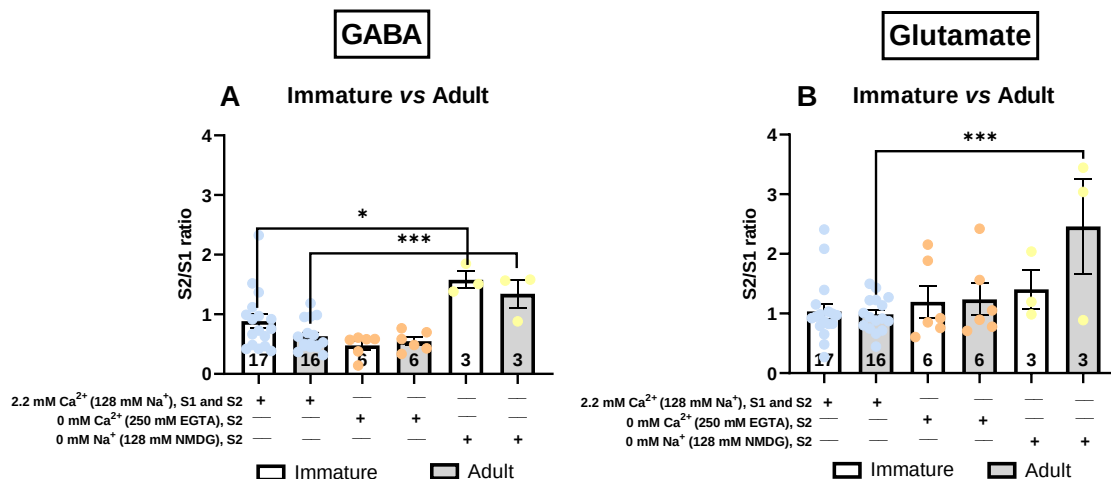


Figure 15: Effect of extracellular Ca^{2+} removal (plus EGTA 250 mM) and Na^+ substitution with NMDG (128 mM) on [^3H]GABA (A) and [^{14}C]Glutamate (B) release from hippocampal synaptosomes of immature vs adult rats depolarized by high KCl (15 mM). Ordinates represent S2/S1 ratios. Test solutions contacted with the synaptosomes 15 min before S2. The results are expressed as mean $\pm$ SEM of an n number of individuals; colour dots represent every datum acquired. * $P<0.05$ and *** $P<0.001$ (one-way ANOVA followed Dunnett's multiple comparisons test) represents statistically significant differences when compared to controls.

On the other hand, replacement of extracellular Na^+ by NMDG $^+$ 15 min before S2 significantly ($P<0.05$) increased depolarization-evoked [^3H]GABA release, both in immature and adult rat hippocampi (Figure 15A), but this modification in the medium only affected the release of [^{14}C]Glutamate in adult animals (Figure 15B). Substitution of extracellular Na^+ with NMDG $^+$, but not removal of extracellular Ca^{2+} , also transiently increased the resting [^{14}C]Glutamate outflow from hippocampal synaptosomes being this effect of higher magnitude in adult compared to immature rats; no changes were observed regarding the release of [^3H]GABA (data not shown).

Overall data indicate that the collapse of the transmembrane Na^+ gradient transiently increases both the resting and the depolarization-evoked [^{14}C]Glutamate outflow preferentially in adult animals, while this effects was only observed when [^3H]GABA release was evoked by high KCl in the two age groups. The smaller and timely-dependent effect of extracellular Ca^{2+} removal (plus EGTA 250 mM) on depolarization-evoked [^3H]GABA and [^{14}C]Glutamate release indicates that vesicle exocytosis is not the main mechanism accounting for the release of both neurotransmitters, strengthening the hypothesis that Na^+ -

dependent high affinity amino acid transporters reversal is preponderant for [^3H]GABA and [^{14}C]Glutamate outflow from depolarized hippocampal synaptosomes of both animal groups.

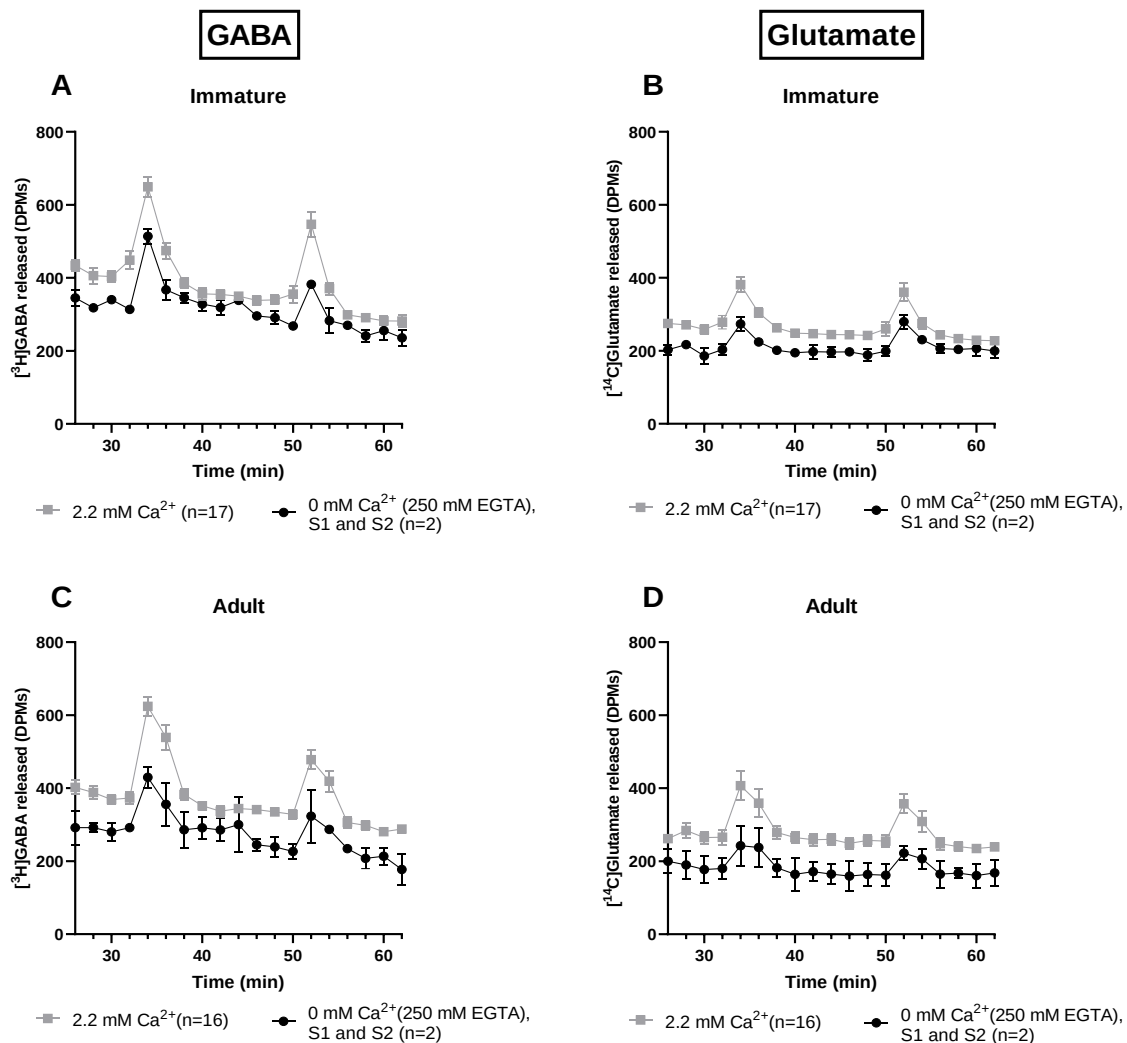


Figure 16: [^3H]GABA (A) and [^{14}C]Glutamate (B) outflow from hippocampal synaptosomes of immature vs adult rats in physiological superfusion media (grey symbols) and in Ca^{2+} -free conditions (plus EGTA 250 mM) (black symbols). Graphs represent the outflow of [^3H]GABA and [^{14}C]Glutamate from hippocampal synaptosomes during resting conditions and after depolarization with high KCl (15 mM, for 2 min) in S1 and S2.

4.2. Effects of cannabidiol on [³H]GABA and [¹⁴C]Glutamate release from hippocampal synaptosomes of immature and adult rats

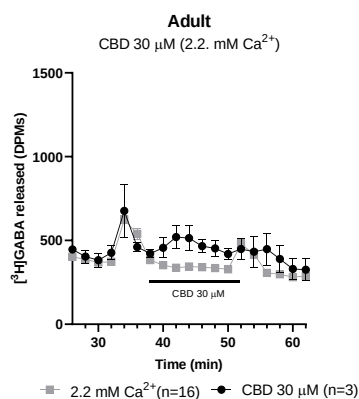
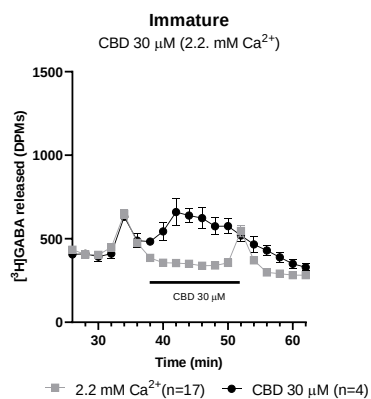
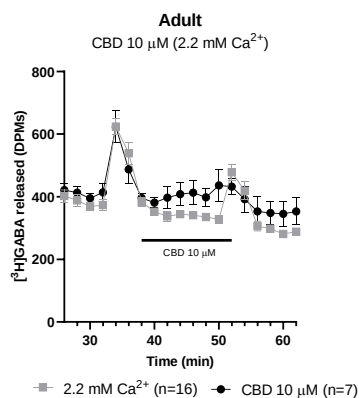
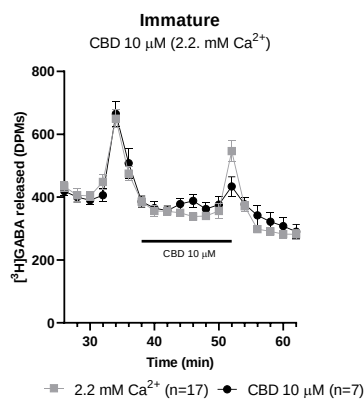
4.2.1. Cannabidiol increases the resting outflow but inhibits depolarization-evoked release of [³H]GABA and [¹⁴C]Glutamate from hippocampal synaptosomes of immature and adult rats

Figure 17 shows the outflow curves concerning [³H]GABA and [¹⁴C]Glutamate release from hippocampal synaptosomes of immature and adult rats depolarised by high KCl (15 mM) obtained either in the absence or in the presence of CBD (3-30 µM, was added to the superfusion medium 15 min before S2).

Immediately after its application, CBD (10-30 µM) concentration-dependently increased the resting outflow of [³H]GABA and [¹⁴C]Glutamate above baseline (Figure 17). The CBD-induced [³H]GABA and [¹⁴C]Glutamate release under resting conditions was more evident ($P < 0.05$) in immature compared to adult animals (data not shown).

In contrast to the facilitatory effect of the cannabinoid on resting [³H]GABA and [¹⁴C]Glutamate outflow, CBD (3-30 µM) significantly decreased the evoked release of the two amino acids from hippocampal synaptosomes depolarized with high KCl (15 mM) (Figure 18 and 19). The inhibitory effect of CBD (3-30 µM) was slightly more potent on the evoked release of [³H]GABA vs. [¹⁴C]Glutamate, but no significant differences ($P > 0.05$) were noticeable when comparing immature vs. adult animals (Figure 18).

GABA



Glutamate

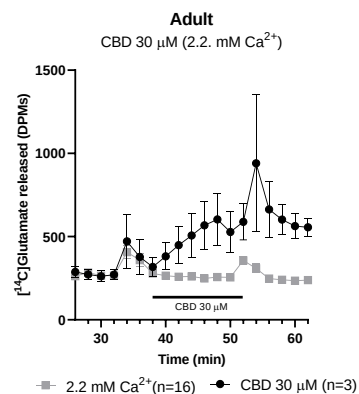
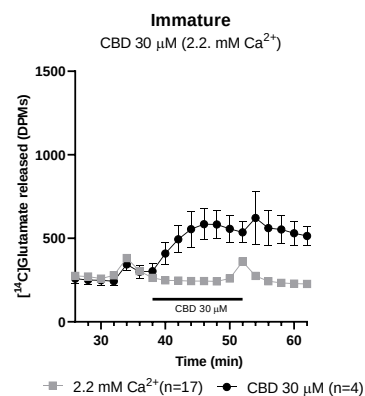
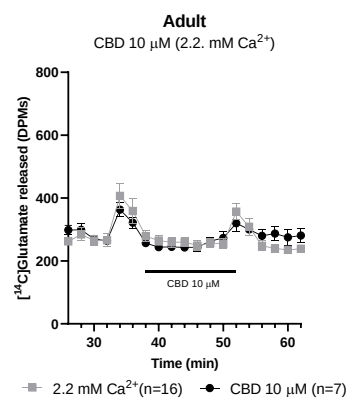
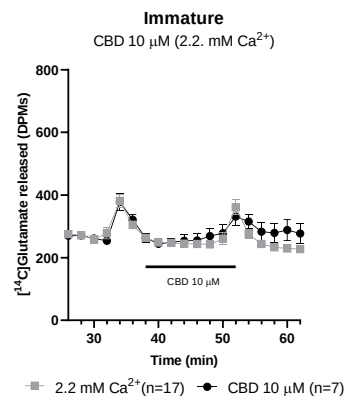


Figure 17: Effects of cannabidiol (CBD, 10-30 μ M) on [^{3} H]GABA (left-hand side) and [^{14} C]Glutamate (right-hand side) release from hippocampal synaptosomes of immature and adult rats. CBD was added to superfusion media 15 min before S2.

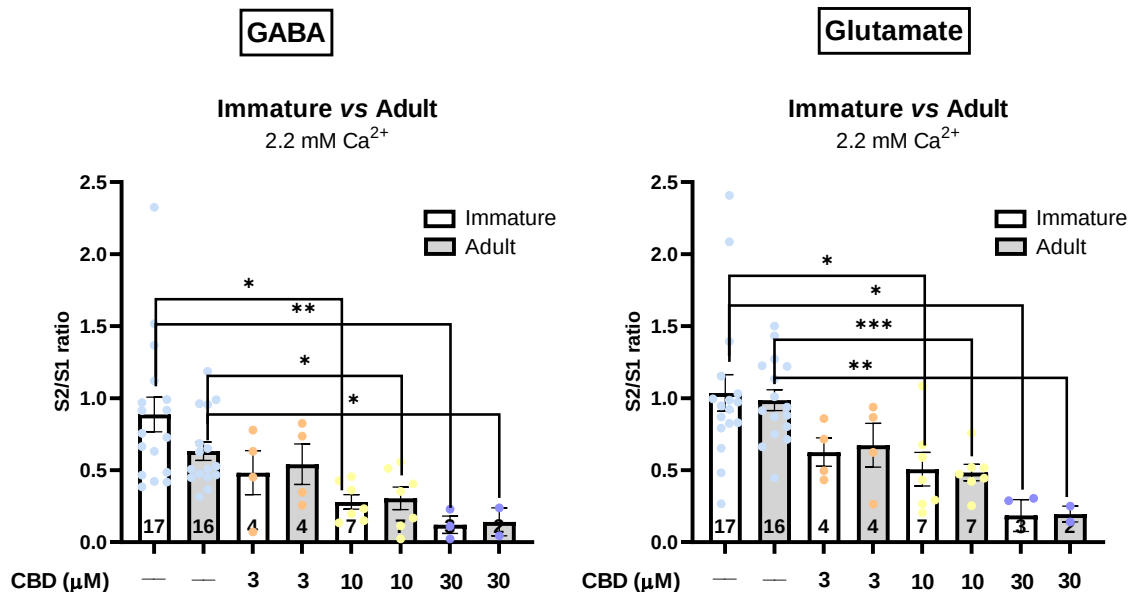


Figure 18: Effects of cannabidiol (CBD, 3-30 μ M) on [^{3} H]GABA (left-hand side) and [^{14} C]Glutamate (right-hand side) release from hippocampal synaptosomes of immature (white bars) and adult (grey bars) rats depolarized by high KCl (15 mM). CBD was added to superfusion media 15 min before S2. Ordinates represent S2/S1 ratios. The results are expressed as mean $\pm$ SEM of an n number of individuals; colour dots represent every datum acquired. *P<0.05, **P<0.01 and ***P<0.001 (one-way ANOVA followed Dunnett's multiple comparisons test) represents statistically significant differences when compared to controls.

4.2.2. Influence of Ca^{2+} on CBD-induced resting [^{3} H]GABA and [^{14} C]Glutamate outflow from hippocampal synaptosomes of immature and adult rats

As mentioned above, CBD (30 μ M) increased the resting outflow of [^{3} H]GABA and [^{14} C]Glutamate above baseline (see Figure 19) and this effect was more evident in immature compared to adult rats (data not shown). Figure 19 shows that removal of external Ca^{2+} (plus EGTA 250 mM) from the beginning of the experimental protocol delayed the outflow of [^{3} H]GABA and [^{14} C]Glutamate caused by CBD (30 μ M) under resting conditions in immature, but not in adult, rats.

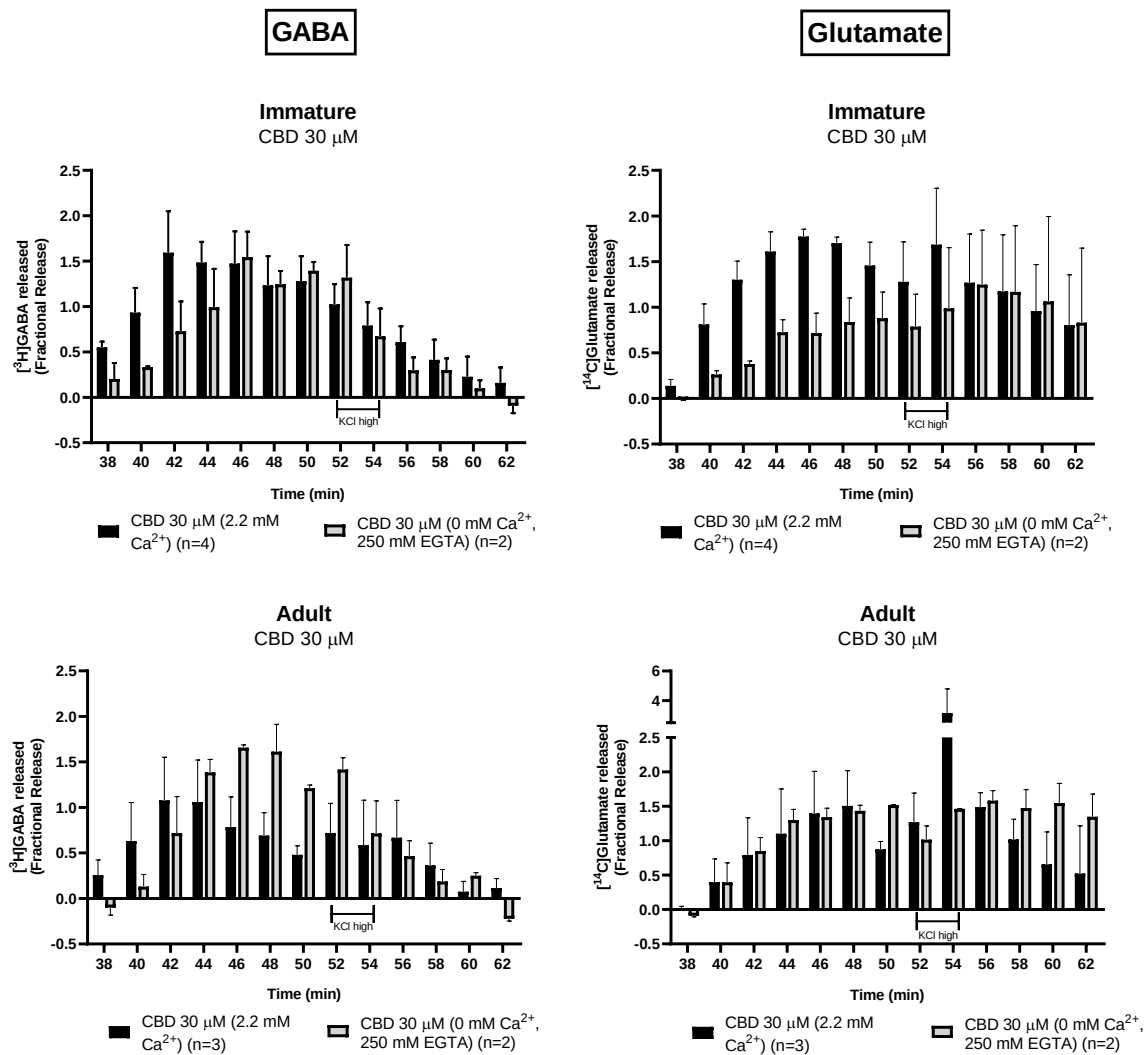


Figure 19: CBD (30 μ M)-induced [^3H]GABA and [^{14}C]Glutamate release under resting physiological conditions and in Ca^{2+} -free media (plus EGTA 250 mM). CBD was added to superfusion media 15 min before high KCl (15 mM, S2).

4.2.3. CBD-induced [^3H]GABA and [^{14}C]Glutamate outflow cannot be attributed to hippocampal cells damage, both in immature and adult rats

To test whether CBD-induced resting [^3H]GABA and [^{14}C]Glutamate release from hippocampal synaptosomes was not due to membrane disruption and/or viability of neuronal cells, we set an experimental protocol to test the activity of the intracellular enzyme, lactate dehydrogenase (LDH), in the incubation medium of hippocampal slices challenged with CBD (3-30 μ M) (Figure 20). The LDH activity of the incubation fluid

collected from hippocampal slices challenged with CBD (3-30 μ M) was not different from that obtained under physiological bathing conditions. As a positive control, we used 1% of Triton X-100, which is a well-known membrane disruptor. No significant differences were detected in the LDH activity using hippocampal slices isolated from immature and adult rats. Data suggest that CBD-induced [^{3} H]GABA and [^{14} C]Glutamate outflow cannot be attributed to damage of hippocampal cell membranes, both in immature and adult rats.

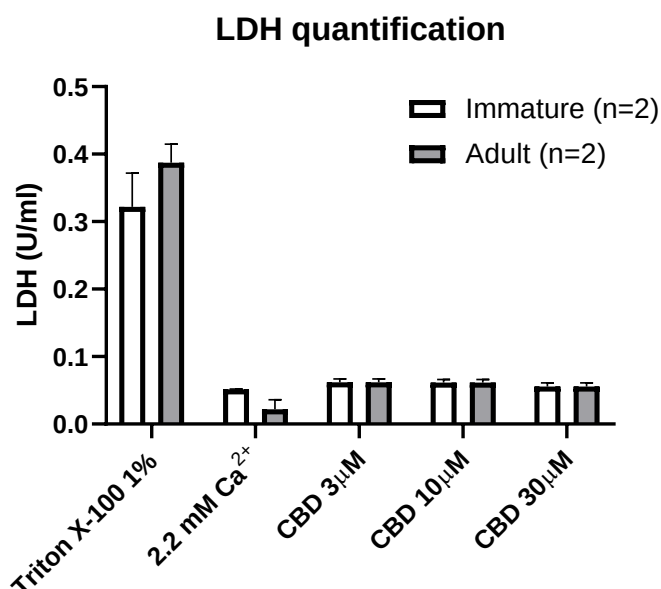


Figure 20: LDH activity in the extracellular fluid collected from hippocampal slices of immature (white bars) and adult (grey bars) rats incubated with CBD (3-30 μ M) during 15 min. As a positive control, we tested the effect of the membrane cells disruptor, Triton X-100 1%.

4.3. Interplay between CBD and adenosine A₁ receptors activation in immature vs. adult rat hippocampal synaptosomes

4.3.1. Adenosine A₁ receptor activation failed to affect [^{3} H]GABA and [^{14} C]Glutamate release from hippocampal synaptosomes of immature and adult rats

Activation of the adenosine A₁ receptor with CPA (30-100 nM, applied 15 min before S2 did not change neither the resting nor depolarization-evoked release of [^{3} H]GABA and [^{14} C]Glutamate from immature and adult rat hippocampal synaptosomes (Figure 21).

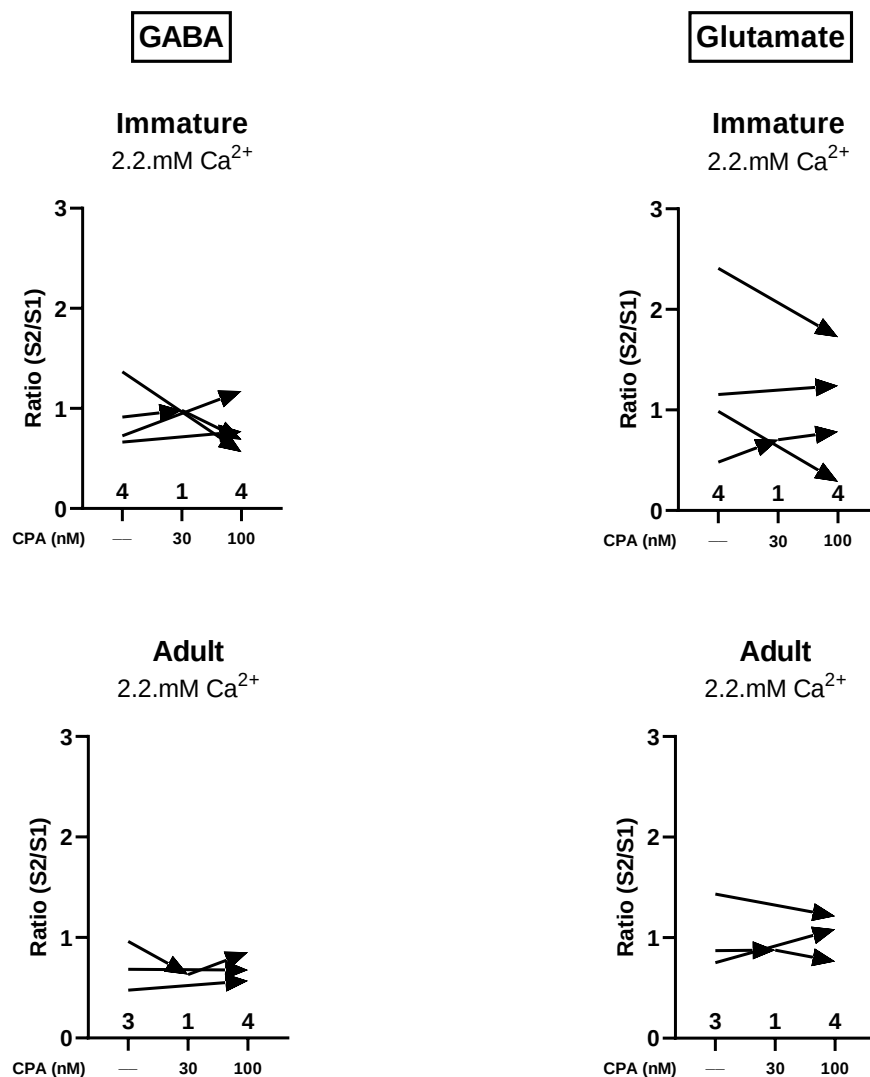


Figure 21: Effects of the adenosine A_1 receptor agonist, CPA (30-100 nM) on [^3H]GABA (left-hand side) and [^{14}C]Glutamate (right-hand side) release from hippocampal synaptosomes of immature (upper panels) and adult (bottom panels) rats depolarized by high KCl (15 mM). CPA was added to superfusion fluid 15 min before S2. Ordinates represent S2/S1 ratios. The results are expressed as datum acquired in each experiment; the n number of animals *per* protocol is shown in the graphs.

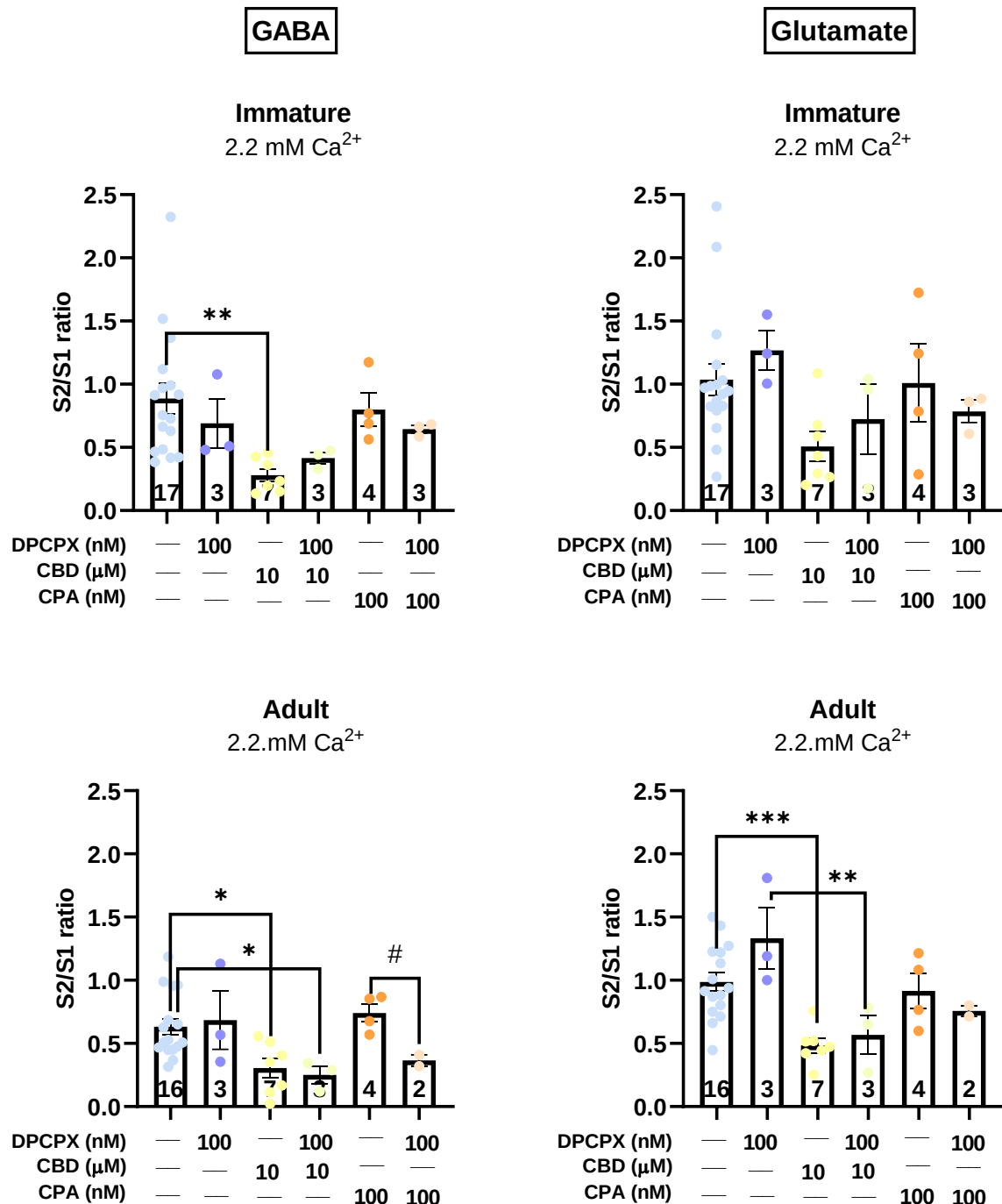


Figure 22: Effects of cannabidiol (CBD, 10 μM) and the adenosine A1 receptor agonist, CPA (100 nM) on [³H]GABA (left-hand side) and [¹⁴C]Glutamate (right-hand side) release from hippocampal synaptosomes of immature (white bars) and adult (grey bars) rats depolarized by high KCl (15 mM) tested in the absence and in the presence of the A1 antagonist, DPCPX (100 nM). CBD and CPA were added to superfusion fluid 15 min before S2; DPCPX was applied from the beginning of the release period, i.e. it was present on S1 and S2. Ordinates represent S2/S1 ratios. The results are expressed as mean ± SEM of an n number of individuals; colour dots represent every datum acquired. *P<0.05, **P<0.01 and ***P<0.001 (one-way ANOVA followed Dunnett's multiple comparisons test) represents statistically significant differences when compared to control or DPCPX alone, respectively; #P<0.05 (Student's t-test with Welch correction) represents statistically significant differences when compared to CPA alone.

Likewise, selective blockage of the adenosine A₁ receptor tone with DPCPX (100 nM), also failed ($P>0.05$) to modify the release of [³H]GABA and [¹⁴C]Glutamate from immature and adult rat hippocampal synaptosomes depolarized by high KCl (15 mM) (Figure 22).

4.3.2. Adenosine A₁ receptor activation does not affect CBD-induced inhibition of [³H]GABA and [¹⁴C]Glutamate release from depolarized hippocampal synaptosomes

Mounting evidence points towards an interplay between CBD and adenosine signalling pathways to explain the antiepileptic effects of the cannabinoid (Carrier et al., 2006; Perucca, 2017; Franco et al., 2019). Therefore, we tested the inhibitory effect of CBD (10 μM) on depolarization-evoked [³H]GABA and [¹⁴C]Glutamate release in the presence of the selective adenosine A₁ receptor antagonist, DPCPX (100 nM; Figure 23). Pre-treatment with DPCPX (100 nM, applied from the beginning of the release period) did not change ($P>0.05$) the inhibitory effect of CBD (10 μM) on [³H]GABA and [¹⁴C]Glutamate release from both immature and adult hippocampal synaptosomes depolarized by high KCl (15 mM) (Figure 22). Results suggest that adenosine A₁ receptor activation does not mediate CBD-induced inhibition of [³H]GABA and [¹⁴C]Glutamate release from depolarized immature and adult rat hippocampal synaptosomes.

Chapter 5

Discussion

Endocannabinoid, phytocannabinoid (e.g. CBD), and synthetic cannabinoid compounds are recognized for their inhibitory action on GABA and glutamate release and their potential to influence a wide range of SNC disorders, including epilepsy. Previous studies described that CBD blocks seizure activity in different animal models of epilepsy (Kaplan et al., 2017; Klein et al., 2017), as well as in children and young adults with drug-refractory epilepsy syndromes (Mellis, 2018; Thiele et al., 2018), despite the fact that only CBD has been approved for DS and LGS affecting epileptic children (U.S. Food and Drug Administration, 2018; European Medicines Agency, 2019). Recently, a patent request was presented to the FDA for a synthetic transdermal formulation of CBD to treat focal epilepsy in adults (Donna Guterman et al., 2019). The use of CBD has been more focused for children medication and, to our knowledge, this is the first study comparing the effects of CBD on synchronous release of GABA and glutamate from the hippocampus of immature (during the weanling period) and adult rats.

Ca^{2+} ions are key sensors in the fusion machinery that triggers vesicle fusion and neurotransmitter release. Depolarization-induced Ca^{2+} influx controls the timing and amplitude of synaptic responses (Squire et al., 2012). Our data show that depolarization-evoked [^3H]GABA and [^{14}C]Glutamate release from hippocampal synaptosomes was only slightly inhibited after removing Ca^{2+} (plus EGTA 250 mM) from the incubation fluid, without much affecting the release of the two amino acids under resting conditions (Leenders et al., 2005; Südhof, 2012). Contrariwise, substitution of extracellular Na^+ by NMDG $^+$ (128 mM) transiently increased the resting outflow of [^{14}C]Glutamate, without affecting [^3H]GABA release, from hippocampal synaptosomes of both immature and adult rats. Depolarization-evoked [^3H]GABA and [^{14}C]Glutamate release from adult rat hippocampal synaptosomes was also significantly augmented after replacement of Na^+ with NMDG $^+$ (128 mM), but only the evoked release of [^3H]GABA was increased under the same experimental conditions in immature animals. Data indicate that Ca^{2+} -dependent vesicle exocytosis plays a minor role on depolarization-evoked release of [^3H]GABA and [^{14}C]Glutamate. The dominant extracellular Na^+ dependency support the involvement of high-affinity amino acid transporter reversal as the main path to release [^3H]GABA and [^{14}C]Glutamate from depolarized hippocampal synaptosomes at least in adult rats, whereas the release of [^{14}C]Glutamate may involve other mechanism in immature rats. Experiments using GAT1 and EAAT transporter blockers may elucidate this hypothesis.

The increase in [^3H]GABA and [^{14}C]Glutamate release secondary to the collapse of the transmembrane Na^+ gradient may be carried out via the neurotransmitter:sodium symport (NSS) family and/or the dicarboxylate/amino acid:cation (Na^+ or H^+) symport (DAACS) family; more exactly, GATs for GABA and EAATs for glutamate, respectively. These transporters are responsible for terminating synaptic transmission and recycling amino acid neurotransmitters by catalysing the (re)uptake of their respective substrates from the extracellular milieu across the plasma membrane of presynaptic neurons against their concentration gradients in a Na^+ -dependent manner (symport) (Shi et al., 2008; S. Napier, 2009; Zhao et al., 2010).

In our hands, CBD transiently increased the resting outflow of [^3H]GABA and [^{14}C]Glutamate, while decreasing depolarization-evoked release of the two amino acids from hippocampal synaptosomes. CBD was more potent at inhibiting the release of [^3H]GABA compared to [^{14}C]Glutamate from depolarized hippocampal synaptosomes, but no significant differences were noted in this regard when comparing immature vs. adult animals. CBD-induced [^3H]GABA and [^{14}C]Glutamate from hippocampal synaptosomes release under resting conditions was more evident in immature compared to adult animals (data not shown).

Previous studies demonstrated that when used in micromolar concentrations CBD may cause damage to the plasma membrane of cultured cells (Hershkowitz et al., 1977; Kinney et al., 2016). Given that plasma membrane damage could explain the release of [^3H]GABA and [^{14}C]Glutamate from resting hippocampal synaptosomes incubated with CBD (10-30 μM), we set an experimental protocol to check for membrane integrity by evaluating the activity of the intracellular enzyme, LDH, in the superfusate of hippocampal slices challenged with CBD (3-30 μM) in comparison with Triton X-100 (1%, a detergent that disrupts plasma membrane lipids). No significant changes were observed in the LDH activity in hippocampal slice superfusates after incubation with CBD compared to the control situation, while Triton X-100 dramatically increase the extracellular LDH activity reflecting plasma membrane cells damage.

CBD can increase Ca^{2+} influx via VGCCs resulting in increases in the probability of transmitters release (Drysdale et al., 2006; Ghovanloo et al., 2018). It can also trigger intracellular Ca^{2+} accumulation by releasing Ca^{2+} from intracellular stores (Drysdale et al., 2006). Hence, we tested the effects of CBD in Ca^{2+} - free media (plus EGTA 250 mM). Removal of Ca^{2+} from the incubation fluid delayed CBD-induced [^3H]GABA and [^{14}C]Glutamate from resting hippocampal synaptosomes of immature rats, without

significantly affecting the evoked release of the two amino acids. Elucidation of Ca^{2+} involvement in the effects of CBD on resting $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{Glutamate}$ release would benefit from using selective drugs targeting intracellular Ca^{2+} , like the fast intracellular Ca^{2+} chelator, BAPTA-AM, and thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA).

At almost forty years ago researchers stated that Ado can act as an endogenous anticonvulsant in the brain (Dunwiddie, 1980) and this concept was validated by several studies since then (Boison, 2016; Weltha et al., 2018). Reports also suggest that CBD increases brain Ado levels in adult rodents by reducing Ado cellular uptake (Castillo et al. 2010). This theory is supported because CBD has the ability to bind to equilibrative nucleoside transporters, namely ENT1, thus preventing Ado uptake and leading to subsequent activation of Ado receptors (Carrier et al., 2006). Adenosine may as well be one of the reasons why CBD decreases the release of neurotransmitters. From the known Ado receptors, the A_1R is the best candidate to inhibit hippocampal neurotransmission since it is extensively expressed in hippocampus and its dysregulation is intrinsically linked to the pathophysiology of epilepsy (Marchi et al., 2002; Ciruela, 2006; Borycz et al., 2007; Dias et al., 2013; Weltha et al., 2018). Despite these evidences, we failed to prove that adenosine A_1 receptors activation mediates the inhibitory effect of CBD on $[^3\text{H}]\text{GABA}$ and $[^{14}\text{C}]\text{Glutamate}$ release from hippocampal synaptosomes depolarized by high KCl. Given the small number of experiments performed in this regard, additional work must be done to dismiss this hypothesis.

Chapter 6

Conclusion

CBD emerged as a new treatment option for childhood epilepsy, but there is a gap in our knowledge regarding its mechanism of action. This study was conceived to seek for answers and possibly confirm (1) that CBD modulates GABA and glutamate synchronous release differently in immature and adult hippocampal nerve terminals, and (2) that this phytocannabinoid might act by increasing adenosine A₁ receptor-mediated antiepileptic effect to control seizures. While the first aim was fulfilled, the interplay between CBD and adenosine signalling requires more work to be done. Further studies are also needed to explore the differences regarding the effects of CBD in the hippocampus of immature vs. adult rats, as for instance the higher sensitivity of immature hippocampal synaptosomes to CBD-induced GABA and Glutamate release in resting preparations and the differences found in the magnitude of the Na⁺-dependent transporter-mediated release of Glutamate from KCl-depolarized hippocampal synaptosomes between immature and adult animals.

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Chapter 7

Bibliographic References

Abbracchio, M. P.; Burnstock, G.; Verkhratsky, A.; Zimmermann, H., 2009: Purinergic signalling in the nervous system: an overview. *Trends in Neurosciences.*, **32**, 19–29.

Adams, R.; Hunt, M.; Clark, J. H.; 62, V.; Clark^, J. H.; Washington, D. C., 1939: Structure of Cannabidiol, a Product Isolated from the Marihuana Extract of Minnesota Wild Hemp. I (IN COLLABORATION WITH THE TREASURY DEPARTMENT, NARCOTICS LABORATORY, **123**, 196–200.

Aizpurua-olaizola, O.; Elezgarai, I.; Rico-barrio, I.; Zarandona, I.; Etxebarria, N.; Usobiaga, A., 2017: Targeting the endocannabinoid system: future therapeutic strategies. *Drug Discovery Today.*, **22**, 105–110.

Aizpurua-Olaizola, O.; Elezgarai, I.; Rico-Barrio, I.; Zarandona, I.; Etxebarria, N.; Usobiaga, A., 2017: Targeting the endocannabinoid system: future therapeutic strategies. *Drug Discovery Today.*, **22**, 105–110.

Ali, S.; Scheffer, I. E.; Sadleir, L. G., 2019: Efficacy of cannabinoids in paediatric epilepsy. *Developmental Medicine and Child Neurology.*, **61**, 13–18.

Araque, A.; Castillo, P. E.; Manzoni, O. J.; Tonini, R., 2017: Synaptic functions of endocannabinoid signaling in health and disease. *Neuropharmacology.*, **124**, 13–24.

Baldwin, S. A.; Beal, P. R.; Yao, S. Y. M.; King, A. E.; Cass, C. E.; Young, J. D., 2004: The equilibrative nucleoside transporter family, SLC29. *Pflugers Archiv European Journal of Physiology.*, **447**, 735–743.

Bancila, V.; Cordeiro, J. M.; Bloc, A.; Dunant, Y., 2009: Nicotine-induced and depolarisation-induced glutamate release from hippocampus mossy fibre synaptosomes: two distinct mechanisms. *Journal of Neurochemistry.*, **110**, 570–580.

Battista, N.; Di Tommaso, M.; Bari, M.; Maccarrone, M., 2012: The endocannabinoid system: an overview. *Frontiers in Behavioral Neuroscience.*, **6**, 1–7.

Ben-Ari, Y.; Bernard, C.; Freund, T. F.; Gozlan, H.; Milh, M.; Morozov, Y. M., 2005: Altering cannabinoid signaling during development disrupts neuronal activity. *Proceedings of the National Academy of Sciences.*, **102**, 9388–9393.

Blum, D.; Lopes, L. V., 2017: *Adenosine receptors in neurodegenerative diseases. Adenosine Receptors in Neurodegenerative Diseases.*

Bodin, P.; Burnstock, G., 2001: Purinergic signalling: ATP release. *Neurochemical Research.*, **26**, 959–969. Boison, D., 2016: Adenosinergic signaling in epilepsy.

Neuropharmacology., **104**, 131–139.

Borycz, J.; Pereira, M. F.; Melani, A.; Rodrigues, R. J.; Köfalvi, A.; Panlilio, L.; Pedata, F.; Goldberg, S. R.; Cunha, R. A.; Ferré, S., 2007: Differential glutamate-dependent and glutamate-independent adenosine A₁ receptor-mediated modulation of dopamine release in different striatal compartments. *Journal of Neurochemistry.*, **101**, 355–363.

Bradford, H. F., 1995: Glutamate, GABA and epilepsy. *Progress in Neurobiology.*, **47**, 477–511.

Burnstock, G., 2006: Historical review: ATP as a neurotransmitter. *Trends in Pharmacological Sciences.*, **27**, 166–176.

Burnstock, G., 2007: Physiology and pathophysiology of purinergic neurotransmission. *Physiological Reviews.*, **87**, 659–797.

Burnstock, G., 2009: Purinergic signalling: Past, present and future. *Brazilian Journal of Medical and Biological Research.*, **42**, 3–8.

Burnstock, G.; Verkhratsky, A., 2012: *Purinergic Signalling and the Nervous System*. *Purinergic Signalling and the Nervous System*. Springer Berlin Heidelberg, Berlin, Heidelberg.

Burnstock, G., 2017: Introduction to the Special Issue on Purinergic Receptors. *Panminerva Medica.*, Vol. 54pp. 1–6.

Burnstock, G., 2018: Purine and purinergic receptors. *Brain and Neuroscience Advances.*, **2**, 239821281881749.

Busquets-Garcia, A.; Bains, J.; Marsicano, G., 2018: CB₁ Receptor Signaling in the Brain: Extracting Specificity from Ubiquity. *Neuropsychopharmacology.*, **43**, 4–20.

Cardoso, A. M.; Schetinger, M. R. C.; Correia-de-Sá, P.; Sévigny, J., 2015: Impact of ectonucleotidases in autonomic nervous functions. *Autonomic Neuroscience: Basic and Clinical.*, **191**, 25–38.

Carrier, E. J.; Auchampach, J. A.; Hillard, C. J., 2006: Inhibition of an equilibrative nucleoside transporter by cannabidiol: A mechanism of cannabinoid immunosuppression. *Proceedings of the National Academy of Sciences.*, **103**, 7895–7900.

Cavalheiro, E. A.; Silva, D. F.; Turski, W. A.; Calderazzo-Filho, L. S.; Bortolotto, Z. A.; Turski, L., 1987: The susceptibility of rats to pilocarpine-induced seizures is age-dependent.

Developmental Brain Research., **37**, 43–58.

Cechetti, F.; Rhod, A.; Simão, F.; Santin, K.; Salbego, C.; Netto, C. A.; Siqueira, I. R., 2007: Effect of treadmill exercise on cell damage in rat hippocampal slices submitted to oxygen and glucose deprivation. *Brain Research.*, **1157**, 121–125.

Chapman, K. E.; Raol, Y. H.; Brooks-Kayal, A., 2012: Neonatal seizures: Controversies and challenges in translating new therapies from the lab to the isolette. *European Journal of Neuroscience.*, **35**, 1857–1865.

Cherubini, E.; Gaiarsa, J. L.; Ben-Ari, Y., 1991: GABA: an excitatory transmitter in early postnatal life. *Trends in Neurosciences.*, **14**, 515–519.

Ciruela, F., 2006: Presynaptic Control of Striatal Glutamatergic Neurotransmission by Adenosine A1-A2A Receptor Heteromers. *Journal of Neuroscience.*, **26**, 2080–2087.

Ciruela, F.; Jacobson, K. A., 2019: Editorial: Purinergic pharmacology. *Frontiers in Pharmacology.*, **9**, 10–12.

Console-Bram, L.; Marcu, J.; Abood, M. E., 2012: Cannabinoid receptors: Nomenclature and pharmacological principles. *Progress in Neuro-Psychopharmacology and Biological Psychiatry.*, **38**, 4–15.

Cottrell, J. R.; Dube, G. R.; Egles, C.; Liu, G., 2000: Distribution, density, and clustering of functional glutamate receptors before and after synaptogenesis in hippocampal neurons. *Journal of Neurophysiology.*, **84**, 1573–1587.

Cunha, R. A., 2019: Signaling by adenosine receptors-Homeostatic or allostatic control? *PLoS biology.*, **17**, e3000213.

Demuth, D. G.; Molleman, A., 2006: Cannabinoid signalling. *Life Sciences.*, **78**, 549–563.

Devane, W. A.; Dysarz, F. A.; Johnson, M. R.; Melvin, L. S.; Howlett, A. C., 1988: Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology.*, **34**, 605–613.

Devinsky, O.; Cilio, M. R.; Cross, H.; Fernandez-Ruiz, J.; French, J.; Hill, C.; Katz, R.; Di Marzo, V.; Jutras-Aswad, D.; Notcutt, W. G.; Martinez-Orgado, J.; Robson, P. J.; Rohrback, B. G.; Thiele, E.; Whalley, B.; Friedman, D., 2014: Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia.*, **55**, 791–802.

Di Marzo, V.; Melck, D.; Bisogno, T.; De Petrocellis, L., 1998: Endocannabinoids:

Endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends in Neurosciences.*, **21**, 521–528.

Di Marzo, V.; Bifulco, M.; De Petrocellis, L., 2004: The endocannabinoid system and its therapeutic exploitation. *Nature Reviews Drug Discovery.*, **3**, 771–784.

Di Marzo, V., 2018: New approaches and challenges to targeting the endocannabinoid system. *Nature Reviews Drug Discovery.*, **17**, 623–639.

Dias, R. B.; Rombo, D. M.; Ribeiro, J. A.; Henley, J. M.; Sebastião, A. M., 2013: Adenosine: Setting the stage for plasticity. *Trends in Neurosciences.*, **36**, 248–257.

Donna Gutterman, R.; Terri Sebree, G.; Theodore Smith, T.; Messenheimer, J., 2019: Patent Application Publication. *United States Patent Application.*

Drysdale, A. J.; Ryan, D.; Pertwee, R. G.; Platt, B., 2006: Cannabidiol-induced intracellular Ca²⁺ elevations in hippocampal cells. *Neuropharmacology.*, **50**, 621–631.

Dunwiddie, T. V., 1980: Endogenously Released Adenosine Regulates Excitability in the In Vitro Hippocampus. *Epilepsia.*, **21**, 541–548.

E. Parkinson, F.; L. Damaraju, V.; Graham, K.; Y.M. Yao, S.; A. Baldwin, S.; E. Cass, C.; D. Young, J., 2011: Molecular Biology of Nucleoside Transporters and their Distributions and Functions in the Brain. *Current Topics in Medicinal Chemistry.*, **11**, 948–972.

Enna, S. J.; Möhler, H., 2007: *The GABA Receptors*. (Enna, S. J. & H. Möhler, Eds.) *The GABA Receptors.*, The Receptors. Humana Press, Totowa, NJ.

European Medicines Agency, 2019: Summary of opinion: Epidyolex cannabidiol.

Fernández-Ruiz, J.; Pazos, M. R.; García-Arencibia, M.; Sagredo, O.; Ramos, J. A., 2008: Role of CB2 receptors in neuroprotective effects of cannabinoids. *Molecular and Cellular Endocrinology.*, **286**.

Franco, V.; Perucca, E., 2019: Pharmacological and Therapeutic Properties of Cannabidiol for Epilepsy. *Drugs.*

Fredholm, B. B.; IJzerman, A. P.; Jacobson, K. A.; Linden, J.; Müller, C. E., 2011: International union of basic and clinical pharmacology. LXXXI. Nomenclature and classification of adenosine receptors - An update. *Pharmacological Reviews.*, **63**, 1–34.

Freund, T. F.; Katona, I.; Piomelli, D., 2003: Role of Endogenous Cannabinoids in Synaptic Signaling. *Physiological Reviews.*, **83**, 1017–1066.

Friedman, D.; Sirven, J. I., 2017: Historical perspective on the medical use of cannabis for epilepsy: Ancient times to the 1980s. *Epilepsy and Behavior.*, **70**, 298–301.

Galve-Roperh, I.; Chiurchiù, V.; Díaz-Alonso, J.; Bari, M.; Guzmán, M.; Maccarrone, M., 2013: Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation. *Progress in Lipid Research.*, **52**, 633–650.

Gaoni, Y.; Mechoulam, R., 1964: Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *Journal of the American Chemical Society.*, **86**, 1646–1647.

Gessi, S.; Merighi, S.; Varani, K.; Borea, P. A., 2011: *Adenosine Receptors in Health and Disease. Advances in Pharmacology.*, Vol. 61.

Ghovanloo, M. R.; Shuart, N. G.; Mezeyova, J.; Dean, R. A.; Ruben, P. C.; Goodchild, S. J., 2018: Inhibitory effects of cannabidiol on voltage-dependent sodium currents. *Journal of Biological Chemistry.*, **293**, 16546–16558.

Gray, E. G.; Whittaker, V. P., 1962: The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. *Journal of anatomy.*, **96**, 79–88.

Haut, S. R.; Velíšková, J.; Moshé, S. L., 2004: Susceptibility of immature and adult brains to seizure effects. *Lancet Neurology.*, **3**, 608–617.

Helme-Guizon, A.; Davis, S.; Israel, M.; Lesbats, B.; Mallet, J.; Laroche, S.; Hicks, A., 1998: Increase in syntaxin 1B and glutamate release in mossy fibre terminals following induction of LTP in the dentate gyrus: A candidate molecular mechanism underlying transsynaptic plasticity. *European Journal of Neuroscience.*, **10**, 2231–2237.

Hershkowitz, M.; Goldman, R.; Raz, A., 1977: Effect of Cannabinoids on Neurotransmitter Uptake, Atpase Activity and Morphology of Brain Synaptosomes. *Biochemical Pharmacology.*, **26**, 1327–1331.

Ibeas Bih, C.; Chen, T.; Nunn, A. V. W.; Bazelot, M.; Dallas, M.; Whalley, B. J., 2015: Molecular Targets of Cannabidiol in Neurological Disorders. *Neurotherapeutics.*, **12**, 699–730.

Interaction, P., 2015: Hot Topics in Neuroscience, **12**, 38–41.

Izzo, A. A.; Borrelli, F.; Capasso, R.; Di Marzo, V.; Mechoulam, R., 2009: Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends in Pharmacological Sciences.*

Jacobson, K. A.; Gao, Z. G., 2006: Adenosine receptors as therapeutic targets. *Nature Reviews Drug Discovery.*, **5**, 247–264.

Jones, N. A.; Glyn, S. E.; Akiyama, S.; Hill, T. D. M.; Hill, A. J.; Weston, S. E.; Burnett, M. D. A.; Yamasaki, Y.; Stephens, G. J.; Whalley, B. J.; Williams, C. M., 2012: Cannabidiol exerts anti-convulsant effects in animal models of temporal lobe and partial seizures. *Seizure.*, **21**, 344–352.

Kaja, S.; Payne, A. J.; Naumchuk, Y.; Koulen, P., 2017: Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes. *Current Protocols in Toxicology.*, **2017**, 1–10.

Kaplan, J. S.; Stella, N.; Catterall, W. A.; Westenbroek, R. E., 2017: Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proceedings of the National Academy of Sciences of the United States of America.*, **114**, 11229–11234.

Keiding, R.; Hörder, M.; Denmark, W. G.; Pitkänen, E.; Tenhunen, R.; Strömme, J. H.; Theodorsen, L.; Waldenström, J.; Tryding, N.; Westlund, L., 1974: Recommended methods for the determination of four enzymes in blood. *Scandinavian Journal of Clinical and Laboratory Investigation.*, **33**, 291–306.

Khakh, B. S.; Burnstock, G., 2009: The Double Life of ATP. *Scientific American.*, **301**, 84–92.

Kinney, W. A.; McDonnell, M. E.; Zhong, H. M.; Liu, C.; Yang, L.; Ling, W.; Qian, T.; Chen, Y.; Cai, Z.; Petkanas, D.; Brenneman, D. E., 2016: Discovery of KLS-13019, a Cannabidiol-Derived Neuroprotective Agent, with Improved Potency, Safety, and Permeability. *ACS Medicinal Chemistry Letters.*, **7**, 424–428.

Klein, B. D.; Jacobson, C. A.; Metcalf, C. S.; Smith, M. D.; Wilcox, K. S.; Hampson, A. J.; Kehne, J. H., 2017: Evaluation of Cannabidiol in Animal Seizure Models by the Epilepsy Therapy Screening Program (ETSP). *Neurochemical Research.*, **42**, 1939–1948.

Koo, C. M.; Kang, H. C., 2017: Could Cannabidiol be a Treatment Option for Intractable Childhood and Adolescent Epilepsy? *Journal of Epilepsy Research.*, **7**, 16–20.

Kumaria, A.; Tolia, C. M.; Burnstock, G., 2008: ATP signalling in epilepsy. *Purinergic Signalling.*, **4**, 339–346.

Ligresti, A.; De Petrocellis, L.; Di Marzo, V., 2016: From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles

Through Complex Pharmacology. *Physiological Reviews.*, **96**, 1593–1659.

Lu, H. C.; MacKie, K., 2016: An introduction to the endogenous cannabinoid system. *Biological Psychiatry.*, **79**, 516–525.

Maa, E.; Figi, P., 2014: The case for medical marijuana in epilepsy. *Epilepsia.*, **55**, 783–786.

MacCarrone, M.; Guzmán, M.; MacKie, K.; Doherty, P.; Harkany, T., 2014: Programming of neural cells by (endo)cannabinoids: From physiological rules to emerging therapies. *Nature Reviews Neuroscience.*, **15**, 786–801.

Marchi, M.; Raiteri, L.; Risso, F.; Vallarino, A.; Bonfanti, A.; Monopoli, A.; Ongini, E.; Raiteri, M., 2002: Effects of adenosine A1 and A2A receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. *British Journal of Pharmacology.*, **136**, 434–440.

Martínez-Orgado, J.; Galve-Roperh, I.; Gutierrez-Rodriguez, A.; Palazuelos, J.; Satta, V.; Sagredo, O., 2018: Cannabinoid signalling in the immature brain: Encephalopathies and neurodevelopmental disorders. *Biochemical Pharmacology.*, **157**, 85–96.

Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I., 1990: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature.*, **346**, 561–564.

Mechoulam, R.; Ben-Shabat, S.; Hanus, L.; Ligumsky, M.; Kaminski, N. E.; Schatz, A. R.; Gopher, A.; Almog, S.; Martin, B. R.; Compton, D. R.; Pertwee, R. G.; Griffin, G.; Bayewitch, M.; Barg, J.; Vogel, Z., 1995: Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology.*, **50**, 83–90.

Mechoulam, R.; Fride, E.; Di Marzo, V., 1998: Endocannabinoids. *European Journal of Pharmacology.*, **359**, 1–18.

Mechoulam, R.; Parker, L., 2013: The Endocannabinoid System and the Brain. *Ssrn.*

Mellis, C., 2018: Cannabidiol for drug-resistant seizures in the Dravet syndrome. *Journal of Paediatrics and Child Health.*, **54**, 101–102.

Moshé, S. L.; Perucca, E.; Ryvlin, P.; Tomson, T., 2015: Epilepsy: New advances. *The Lancet.*, **385**, 884–898.

Munro, S.; Thomas, K. L.; Abu-Shaar, M., 1993: Molecular characterization of a peripheral

receptor for cannabinoids. *Nature.*, **365**, 61–65.

Navarro, G.; Morales, P.; Rodríguez-Cueto, C.; Fernández-Ruiz, J.; Jagerovic, N.; Franco, R., 2016: Targeting cannabinoid CB2 receptors in the central nervous system. Medicinal chemistry approaches with focus on neurodegenerative disorders. *Frontiers in Neuroscience.*, **10**, 1–11.

Newsholme, P.; Procopio, J.; Lima, M. M. R.; Pithon-Curi, T. C.; Curi, R., 2003: Glutamine and glutamate?their central role in cell metabolism and function. *Cell Biochemistry and Function.*, **21**, 1–9.

Nicholls, D. G., 2018: Synaptosomal Bioenergetics and Glutamate Release. *Neuromethods.*, Vol. 141pp. 109–129.

Niciu, M. J.; Kelmendi, B.; Sanacora, G., 2012: Overview of glutamatergic neurotransmission in the nervous system. *Pharmacology Biochemistry and Behavior.*, **100**, 656–664.

O'Connell, B. K.; Gloss, D.; Devinsky, O., 2017: Cannabinoids in treatment-resistant epilepsy: A review. *Epilepsy and Behavior.*, **70**, 341–348.

Ohno-Shosaku, T.; Kano, M., 2014: Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Current Opinion in Neurobiology.*, **29**, 1–8.

Oliveira da Cruz, J. F.; Robin, L. M.; Drago, F.; Marsicano, G.; Metna-Laurent, M., 2016: Astroglial type-1 cannabinoid receptor (CB 1): A new player in the tripartite synapse. *Neuroscience.*, **323**, 35–42.

Owens, D. F.; Kriegstein, A. R., 2002: Is there more to GABA than synaptic inhibition? *Nature Reviews Neuroscience.*, **3**, 715–727.

Patel, R. R.; Barbosa, C.; Brustovetsky, T.; Brustovetsky, N.; Cummins, T. R., 2016: Aberrant epilepsy-associated mutant Nav1.6 sodium channel activity can be targeted with cannabidiol. *Brain.*, **139**, 2164–2181.

Perucca, E., 2017: Cannabinoids in the Treatment of Epilepsy: Hard Evidence at Last? *Journal of Epilepsy Research.*, **7**, 61–76.

Peters, M.; Murillo-rodriguez, E.; Hanus, L. O., 2007: Cannabidiol – Recent Advances, **4**, 1678–1692.

Piomelli, D., 2003: The molecular logic of endocannabinoid signalling. *Nature Reviews*

Neuroscience., **4**, 873–884.

Raiteri, L.; Raiteri, M., 2000: Synaptosomes Still Viable after 25 Years of Superfusion. *Neurochemical Research.*, **25**, 1265–1274.

Ransom, B. R., 2013: *Neuroglia*. (Kettenmann, H., Ed.). Oxford University Press, Vol. 1.

Reddy, D. S.; Golub, V. M., 2016: The Pharmacological Basis of Cannabis Therapy for Epilepsy. *Journal of Pharmacology and Experimental Therapeutics.*, **357**, 45–55.

Reichlin, S., 2005: *Cannabinoids*. *The American Journal of The Medical Sciences.*, Vol. 258.

Rowley, N. M.; Madsen, K. K.; Schousboe, A.; Steve White, H., 2012: Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. *Neurochemistry International.*, **61**, 546–558.

S. Napier, M. B., 2009: *Transporters as Targets for Drugs*. (Napier, S. & M. Bingham, Eds.) Topics in Medicinal Chemistry. Springer Berlin Heidelberg, Berlin, Heidelberg, Vol. 4.

Saroya, A. S., 2017: *The Phytocannabinoids*. *Contemporary Phytomedicines*.

Schousboe, A.; Waagepetersen, H. S., 2007: GABA: Homeostatic and pharmacological aspects. *Progress in Brain Research.*, **160**, 9–19.

Schousboe, A.; Waagepetersen, H. S., 2008: GABA Neurotransmission: An Overview. *Handbook of Neurochemistry and Molecular Neurobiology.*, 213–226.

Schousboe, A.; Bak, L. K.; Waagepetersen, H. S., 2013: Astrocytic control of biosynthesis and turnover of the neurotransmitters glutamate and GABA. *Frontiers in Endocrinology.*, **4**, 1–12.

Schousboe, A.; Scafidi, S.; Bak, L. K.; Waagepetersen, H. S.; McKenna, M. C., 2014: Glutamate Metabolism in the Brain Focusing on Astrocytes, Vol. 11pp. 13–30.

Schousboe, A.; Waagepetersen, H. S., 2017: *Gamma-Aminobutyric Acid (GABA)* ☆. *Reference Module in Neuroscience and Biobehavioral Psychology*. Elsevier.

Scimemi, A., 2014: Structure, function, and plasticity of GABA transporters. *Frontiers in Cellular Neuroscience.*, **8**, 1–14.

Shi, L.; Quick, M.; Zhao, Y.; Weinstein, H.; Javitch, J. A., 2008: The Mechanism of a Neurotransmitter: Sodium Symporter-Inward Release of Na⁺ and Substrate Is Triggered by

Substrate in a Second Binding Site. *Molecular Cell.*, **30**, 667–677.

Soltesz, I.; Alger, B. E.; Kano, M.; Lee, S. H.; Lovinger, D. M.; Ohno-Shosaku, T.; Watanabe, M., 2015: Weeding out bad waves: Towards selective cannabinoid circuit control in epilepsy. *Nature Reviews Neuroscience.*, **16**, 264–277.

Sperk, G.; Furtinger, S.; Schwarzer, C.; Pirker, S., 2004: *Recent advances in epilepsy research. Cell Biochemistry and Function.*, Vol. 23.

Sprengel, R., 2016: Ionotropic Glutamate Receptors. *Neuroscience in the 21st Century*. Springer New York, New York, NY, pp. 61–83.

Squire, L. R.; Berg, D.; Bloom, F. E.; Lac, S. du; Ghosh, A.; Spitzer, N. C., 2012: *Fundamental Neuroscience Fourth*. Academic Press, 2013.

Straiker, A.; Dvorakova, M.; Zimmowitch, A.; Mackie, K., 2018a: Cannabidiol Inhibits Endocannabinoid Signaling in Autaptic Hippocampal Neurons. *Molecular Pharmacology.*, **94**, 743–748.

Straiker, A.; Dvorakova, M.; Zimmowitch, A.; Mackie, K., 2018b: Cannabidiol Inhibits Endocannabinoid Signaling in Autaptic Hippocampal Neurons. *Molecular Pharmacology.*, **94**, 743–748.

Suh, Y. H.; Chang, K.; Roche, K. W., 2018: Metabotropic glutamate receptor trafficking. *Molecular and Cellular Neuroscience.*, **91**, 10–24.

Thiele, E. A.; Marsh, E. D.; French, J. A.; Mazurkiewicz, M. B.; Benbadis, S. R.; Joshi, C.; Lyons, P. D.; Taylor, A.; Roberts, C.; Sommerville, K.; Gunning, B.; Gawlowicz, J.; Lisewski, P.; Mitosek Szewczyk, K.; Steinborn, B.; Zolnowska, M.; Hughes, E.; McLellan, A.; Benbadis, S. *et al.*, 2018: Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet.*, **391**, 1085–1096.

Thijs, R. D.; Surges, R.; O'Brien, T. J.; Sander, J. W., 2019: Epilepsy in adults. *The Lancet.*, **393**, 689–701.

Trezza, V.; Cuomo, V.; Vanderschuren, L. J. M. J., 2008: Cannabis and the developing brain: Insights from behavior. *European Journal of Pharmacology.*, **585**, 441–452.

U.S. Food and Drug Administration, 2018: FDA approves first drug comprised of an active ingredient derived from marijuana to treat rare, severe forms of epilepsy. *Case Medical Research.*, **November**.

Vilela, L. R.; Aguiar, D. C.; Santos, R. P. M.; Gonçalves, B. D. C.; Gobira, P. H.; Vieira, L. B.; Crippa, J. A.; de Oliveira, A. C.; Moreira, F. A., 2015: Cannabidiol, a Cannabis sativa constituent, inhibits cocaine-induced seizures in mice: Possible role of the mTOR pathway and reduction in glutamate release. *NeuroToxicology.*, **50**, 116–121.

Walls, A. B.; Waagepetersen, H. S.; Bak, L. K.; Schousboe, A.; Sonnewald, U., 2014: The Glutamine–Glutamate/GABA Cycle: Function, Regional Differences in Glutamate and GABA Production and Effects of Interference with GABA Metabolism. *Neurochemical Research.*, **40**, 402–409.

Weiler, I. J., 2009: Synaptosomes. *Encyclopedia of Neuroscience*. Elsevier, Vol. 356pp. 815–818.

Weltha, L.; Reemmer, J.; Boison, D., 2018: The role of adenosine in epilepsy. *Brain Research Bulletin.*, **151**, 46–54.

Whittaker, V. P.; Michaelson, I. A.; Kirkland, R. J., 1964: The separation of synaptic vesicles from nerve-ending particles ('synaptosomes'). *The Biochemical journal.*, **90**, 293–303.

Wilson, R. I.; Nicoll, R. A., 2002: Neuroscience: Endocannabinoid signaling in the brain. *Science.*, **296**, 678–682.

Wu, C.; Sun, D., 2015: GABA receptors in brain development, function, and injury. *Metabolic Brain Disease.*, **30**, 367–379.

Yap, M.; Easterbrook, L.; Connors, J.; Koopmans, L., 2015: Use of cannabis in severe childhood epilepsy and child protection considerations. *Journal of Paediatrics and Child Health.*, **51**, 491–496.

Yelamanchi, S. D.; Jayaram, S.; Thomas, J. K.; Gundimeda, S.; Khan, A. A.; Singhal, A.; Keshava Prasad, T. S.; Pandey, A.; Somani, B. L.; Gowda, H., 2016: A pathway map of glutamate metabolism. *Journal of Cell Communication and Signaling.*, **10**, 69–75.

Zhao, Y.; Quick, M.; Shi, L.; Mehler, E. L.; Weinstein, H.; Javitch, J. A., 2010: Substrate-dependent proton antiport in neurotransmitter:sodium symporters. *Nature Chemical Biology.*, **6**, 109–116.

Zhou, Y.; Danbolt, N. C., 2014: Glutamate as a neurotransmitter in the healthy brain. *Journal of Neural Transmission.*, **121**, 799–817.

Zou, S.; Kumar, U., 2018: Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *International Journal of Molecular*

Sciences., **19**, 833.

Zuardi, A. W., 2008: Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action Canabidiol : de um canabinóide inativo a uma droga com amplo espectro de ação. *Revista Brasileira de Psiquiatria.*, **30**, 271–280.