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Tatiana Jorge Torres Vilhena Ventura

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Dissertação de Candidatura ao grau de Mestre em Medicina Legal submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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

O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

Os canabinóides têm sido usados clinicamente em diversas patologias do foro neurológico ou psiquiátrico tais como a esclerose múltipla, esquizofrenia, doença de Parkinson, dor neuropática, anorexia, epilepsia, entre outras. O canabidiol (CBD), sem efeitos psicoativos relevantes, tem sido o principal fitocanabinóide usado no tratamento de epilepsias pediátricas, nomeadamente nos síndromes de Dravet e de Lennox-Gastaut, tendo sido aprovado recentemente pela FDA e pela EMA.

Apesar da evidência clínica das suas propriedades antiepiléticas, o esclarecimento do mecanismo de ação do CBD ainda não foi conseguido, tendo sido aceites três mecanismos propostos: o controlo das oscilações intracelulares de Ca^{2+} via ativação do recetor órfão GPR55 e do influxo deste ião através do canal TRPV1, bem como a modulação da sinalização pela adenosina (antiepilético endógeno).

Em trabalhos anteriores do nosso grupo, o CBD foi testado na libertação síncrona de GABA e glutamato, a partir de sinaptossomas de hipocampo de ratos adultos e imaturos, tendo sido demonstrado que (1): a exocitose não é o principal mecanismo envolvido na libertação destes dois aminoácidos, sugerindo que a inversão dos transportadores do GABA e do glutamato é a principal responsável por essa libertação; (2): o CBD aumentou transitoriamente a libertação espontânea de [^{3}H]GABA e de [^{14}C]glutamato e inibiu a libertação evocada dos dois neurotransmissores, quer em ratos adultos quer imaturos, sem diferenças observadas entre os dois grupos de animais. Observou-se que a libertação espontânea de [^{3}H]GABA e [^{14}C]glutamato induzida pelo CBD foi superior nos animais imaturos; (3): a sinalização mediada pela adenosina não pareceu ser relevante na ação do CBD sobre a libertação de [^{3}H]GABA e [^{14}C]Glutamato a partir de terminais nervosos isolados (sinaptossomas) do hipocampo de ratazana (Caulino, 2019).

Tendo em conta os resultados anteriores, decidimos no presente trabalho avaliar a possível interação entre o CBD e os canais catiónicos não seletivos TRPV1 na libertação de [^{3}H]GABA e [^{14}C]Glutamato em sinaptossomas de ratazanas adultas, uma vez que o influxo de sódio ou de cálcio através dos canais TRPV1 pode aumentar a libertação de neurotransmissores e que a dessensibilização destes canais pelo CBD pode justificar a inibição da libertação destes aminoácidos. Testámos a capsaicina, um dos agonistas do recetor TRPV1, na libertação de [^{3}H]GABA e [^{14}C]Glutamato em sinaptossomas de ratazana adulta, mas este agonista não alterou significativamente a libertação síncrona

dos dois neurotransmissores, apenas se observou uma ligeira inibição, não significativa, da libertação evocada pelo KCl para a concentração de 10 µM. Também a capsazepina (10 µM), antagonista seletivo dos recetores TRPV1, não modificou nem a libertação espontânea nem a libertação evocada de [^{3}H]GABA e [^{14}C]Glutamato de sinaptossomas de ratazana adulta. O pré-tratamento com capsazepina inibiu ligeiramente, embora sem significado estatístico, a facilitação da libertação espontânea de [^{3}H]GABA e [^{14}C]glutamato, induzida pelo CBD, sugerindo, nestas condições experimentais, que a ligação do CBD aos recetores TRPV1 desempenham um ligeiro, ou talvez inexistente, papel na libertação de aminoácidos pelos terminais nervosos do hipocampo de rato adulto.

Seguidamente, fomos confirmar farmacologicamente se a inversão dos transportadores de elevada afinidade dependentes de Na^{+} para aminoácidos está envolvida na libertação induzida pelo CBD de [^{3}H]GABA e [^{14}C]glutamato a partir de sinaptossomas em repouso ou após despolarização pelo KCl. Como esperado, o inibidor não seletivo do transporte de glutamato (EAAT), DL-TBOA, aumentou gradualmente o efluxo de [^{14}C]glutamato, aplicado após o primeiro estímulo (S1), sem modificar os níveis de [^{3}H]GABA, medido nas mesmas amostras. Pelo contrário, o inibidor do transportador de GABA (GAT-1), SKF 98876A, não modificou o efluxo de [^{3}H]GABA e/ou [^{14}C]glutamato, nos sinaptossomas do hipocampo de rato adulto.

Tendo sido contraditórios os resultados obtidos com os dois inibidores do transporte de aminoácidos, DL-TBOA e SKF 98876A, fomos testar se o colapso do gradiente de Na^{+} transmembranar, trocando iso-osmoticamente o Na^{+} pelo NMDG (128 mM) no meio de perfusão, poderia inverter o sentido dos transportadores de elevada afinidade de aminoácidos localizados na membrana celular. A remoção do Na^{+} do meio de superfusão aumentou progressivamente a libertação extracelular de [^{3}H]GABA sem modificar a libertação de [^{14}C]glutamato. Estes resultados sugerem que os transportadores de elevada afinidade do [^{3}H]GABA localizados nos terminais nervosos do hipocampo são mais sensíveis ao colapso do gradiente transmembranar de Na^{+}, quando comparados com os transportadores de glutamato, concordando com resultados publicados pelo nosso grupo (Barros-Barbosa *et al*., 2015; 2018).

O pré-tratamento com DLT-BOA e SKF98876A, preveniu significativamente a facilitação induzida pelo CBD na libertação espontânea de [^{3}H]GABA e de [^{14}C]glutamato, sugerindo que o CBD aumenta a libertação destes neurotransmissores por inversão dos

transportadores de GABA e de glutamato. Estes resultados foram reforçados pela potenciação observada da facilitação da libertação espontânea destes dois aminoácidos mediada pelo CBD, quando o Na^+ foi retirado do meio de perfusão.

Em trabalhos publicados pelo grupo (Barros-Barbosa *et al*., 2015; 2018), ficou demonstrado que o transporte de [^{3}H]GABA a partir de terminais nervosos de hipocampo é mais suscetível ao colapso de Na^+, pelo menos em ratos adultos. Pelo contrário, no presente trabalho, o efeito potenciador do CBD (30 µM) na libertação de [^{3}H]GABA e [^{14}C]glutamato em meio de perfusão sem Na^+, foi de magnitude superior nos sinaptossomas de ratos imaturos do que nos de adultos.

As alterações ontogénicas da sensibilidade do hipocampo ao CBD detetadas no presente trabalho vêm de encontro aos resultados publicados por outros grupos, que sugerem que o cérebro imaturo é muito mais excitável do que o cérebro adulto. Deficiências na inibição mediada pelo GABA, juntamente com a sobre expressão transitória de recetores de glutamato, podem favorecer a suscetibilidade do cérebro imaturo às convulsões.

Para consolidar os resultados desta tese serão necessários mais estudos para (1) esclarecer qual o alvo/gatilho que é ativado pelo CBD quando aumenta a libertação de [^{3}H]GABA e [^{14}C]glutamato, e (2) se o controlo fino da libertação em repouso de GABA pareado com o glutamato poderá contribuir para a relevância do CBD no tratamento dos síndromes epiléticos pediátricos.

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Abstract

Cannabinoids have been clinically used to treat several neurological and psychiatric disorders such as multiple sclerosis, schizophrenia, Parkinson's disease, neuropathic pain, anorexia, epilepsy among others. Free from significant psychoactive effects, cannabidiol (CBD) has been largely recommended for the treatment of pediatric epilepsies, namely Dravet and Lennox-Gascault syndromes, being clinically approved since 2018 by FDA and in 2019 by EMA.

Although with proven antiepileptic properties, the mechanism of action of CBD continues to be a matter of debate and global research interest. Three main mechanisms of anticonvulsant action of CBD include: modulation of intracellular Ca^{2+}, resulting from the activation of the orphan GPR55 receptor and the influx of this ion through the TRPV1 channel, as well as the modulation of adenosine-mediated signaling (an endogenous antiepileptic).

In previous work from our group CBD was tested on [^{3}H]GABA and [^{14}C]glutamate synchronous release from rat hippocampal synaptosomes obtained from both immature and adult rats (Caulino, 2019). In this work it was clarified that (1): exocytosis is not the main mechanism mediating these neurotransmitters release, strengthening the hypothesis that the reversal of Na^{+}-dependent high affinity amino acid is the main driving force to the outflow of [^{3}H]GABA and [^{14}C]glutamate from depolarized hippocampal synaptosomes of both animal groups; (2): CBD transiently increased the resting outflow of [^{3}H]GABA and [^{14}C]glutamate, while decreasing depolarization-evoked release of the two amino acids from hippocampal synaptosomes of immature and adult rats, without no changes observed on depolarization-evoked amino-acids release among the two animal groups. On the other hand, CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from hippocampal synaptosomes under resting conditions was more evident in immature compared to adult animals; (3) the increase on adenosine (Ado) levels by CBD as a possible mechanism of inhibition of neurotransmitters release does not seem to have any relevant action in [^{3}H]GABA and [^{14}C]glutamate release from isolated nerve terminals (synaptosomes) of rat hippocampus (Caulino, 2019).

Therefore, we set to investigate in this thesis the interplay between CBD and the nonselective cation channel TRPV1 receptor, as in theory Na^{+} and/or Ca^{2+} influx into nerve terminals via TRPV1 channels may increase neurotransmitters release and also

that the fast desensitization of TRPV1 currents following CBD binding could also explain depolarization-evoked amino-acid release inhibition by the cannabinoid.

Besides CBD, here we tested the effect of capsaicin (0.1-1 µM), one agonist of TRPV1 receptor, which did not significantly affect the release of [^{3}H]GABA and [^{14}C]glutamate from synaptosomes isolated from hippocampi of adult rats, although a minor inhibitory effect was observed on depolarization-evoked [^{14}C]glutamate release when capsaicin was used at the highest concentration tested (10 µM). Likewise, the competitive TRPV1 antagonist, capsazepine (10 µM), also failed to modify the release of both amino-acids, either under resting or after KCl depolarization of hippocampal synaptosomes. Pretreatment with capsazepine slightly, yet not significantly, decreased CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from rat hippocampal synaptosomes under resting conditions, suggesting that binding of CBD to TRPV1 receptor channels plays a minor, if at all, effect on the outflow of [^{3}H]GABA and [^{14}C]glutamate induced by the cannabinoid from hippocampal synaptosomes of adult rats under these experimental conditions.

Next we set to pharmacologically confirm that reversal of Na^{+}-dependent high-affinity amino acid transporters is in fact mediating CBD-induced [^{3}H]GABA and [^{14}C]glutamate outflow from depolarized hippocampal synaptosomes. As expected, the EAAT glutamate transporter inhibitor, DL-TBOA, increased progressively the extracellular amount of [^{14}C]glutamate after the first depolarization stimulus (S1) delivered to adult rat hippocampal synaptosomes, without affecting the [^{3}H]GABA outflow measured in the same samples. On the contrary, the selective GAT-1 transport inhibitor, SKF 98876A, failed to modify the outflow of [^{3}H]GABA and/or [^{14}C]glutamate from hippocampal synaptosomes of adult rats.

Given the discrepancy in the results obtained with the two amino-acid transport inhibitors, DL-TBOA and SKF 98876A, we tested if the collapse of the Na^{+} gradient across the plasma membrane, by removing this cation from the extracellular milieu while keeping osmolality by replacing it by NMDG (128 mM), could reverse to the outward mode the function of GABA and glutamate transporters. Removal of external Na^{+} from the superfusion fluid progressively increased the release of [^{3}H]GABA from rat hippocampal synaptosomes, without concurrent accumulation of [^{14}C]glutamate in the extracellular fluid. Overall, these results indicate that high-affinity GABA transporters located in hippocampal nerve terminals are more vulnerable to the collapse of the

transmembrane Na^+ gradient compared to the glutamate transporters, as previously reported (Barros-Barbosa et al., 2015; 2018).

Pre-treatment with SKF 98876A and DL-TBOA significantly attenuated CBD-induced release of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats under resting conditions, respectively. These findings strengthen our hypothesis that the reversal of GABA and glutamate transporters to the outward mode is mandatory to promote the outflow of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats triggered by CBD, reinforced by the present observation that CBD-induced [^{3}H]GABA and [^{14}C]glutamate release was significantly potentiated when hippocampal synaptosomes were superfused in Na^+-free conditions.

As mentioned before, high-affinity [^{3}H]GABA transport reversal seems to be more sensitive to removal of extracellular Na^+ by replacing it with NMDG than [^{14}C]glutamate release (Barros-Barbosa et al., 2015; 2018), at least in the hippocampus of adult rats compared to that of immature animals. This renders the adult hippocampus more prone to the GABAergic inhibitory control. By contrast, the potentiating effect of CBD (30 µM) on [^{3}H]GABA and [^{14}C]glutamate release in Na^+-free conditions had a higher magnitude in hippocampal synaptosomes of immature rats than in those isolated from adult animals. The ontogenic changes in hippocampal sensitivity to CBD detected in the present study agree with data from previous reports suggesting that the immature brain is by far more excitable than the adult brain. Deficient GABAergic-mediated inhibition, together with transient overexpression of glutamate receptors, may boost susceptibility to seizures in the immature brain.

In order to strength the data reported in the present work, further studies are required (1) to elucidate the molecular trigger underlying CBD-induced increases in [^{3}H]GABA and [^{14}C]glutamate release from hippocampal nerve terminals and (2) whether fine-tuning control of resting GABA vis a vis glutamate release might contribute to the antiepileptic relevance of CBD in the treatment of pediatric epilepsy syndromes.

Content

List of abbreviations

2-AG, 2-arachidonoyl glycerol

Ado, Adenosine

AEA, Anandamide

AEDs, Antiepileptic drugs

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxozole proprionic acid

ATP, Adenosine triphosphate

BCA, Bicinchoninic acid

BSA, Bovine serum albumin

Ca^{2+}, Calcium

cAMP, cyclic adenosine mono phosphate

CB_1, Type 1-cannabinoid receptor

CB_2, Type 2-cannabinoid receptor

CBD, cannabidiol

Cl^-, Chloride

CNS, central nervous system

DAG, diacylgycerol

DEEs, Developmental and epileptic encephalopathies

DS, Dravet syndrome

EAATs, excitatory amino acid transporters

EMA, European Medicines Agency

ENT, Equilibrative nucleotide transporter

EPSPs, Excitatory postsynaptic potentials

FDA, Food and Drug Association

GABA, γ-aminobutyric acid

GAD, Glutamate decarboxylase

GATs, GABA transporters

Gi, G-proteins

GPCR, G-protein-coupled receptors

GS, Glutamine synthetase

HEPES, 2-[4-(2-Hydroxyethyl)piperazine-1-yl]ethanesulfonic acid

HCO_3^-, Bicarbonate

IP3, inositol-1,4,5-triphosphate

K^+, Potassium

KA, Kainite

LGS, Lennox-Gastaut syndrome

Mg^{2+}, Magnesium

mGluRs, Metabotropic glutamate receptors

min, Minutes

Na^+, Sodium

NBQX, 6-nitro-7-sulphamobezo(f)quinoxaline-2,3-dione

NMDA, *N*-methyl-D-aspartate

NMDG, *N*-methyl-D-glucamine

PAG, phosphate-activated glutaminase

THC, Δ9-tetrahydrocannabinol

TRPA, transient receptor potential ankyning

TRPM, transient receptor potential melastantin

TRPV1, transient receptor potential vanilloid 1

VGLUTs, vesicular glutamate transporters

VGSC, Voltage-gated sodium channel

SNARE, N-ethylmeleimide-sensitive factor altachment protein receptor

xCT, Cystine/glutamate antiporter

Zn^{2+}, Zinc

List of figures and tables

Chapter 1: Introduction

1.1 Cannabinoids

Cannabis sativa plant has been used to treat epilepsy for centuries. Cannabis plants contain several therapeutic cannabinoids, with relevance to cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), for their anticonvulsant effects, although only CBD has a favourable side-effect profile, without significant psychoactive effects (Devinsky *et al*., 2014). Both can prevent seizures and reduce mortality in animal models of seizure with low toxicity and high tolerability (Rosenberg *et al*., 2015). Cannabinoids have been used in many neurological and psychiatric disorders, such as multiple sclerosis, schizophrenia, Parkinson's disease, neuropathic pain, anorexia, epilepsy and others. CBD has been considered as the major cannabinoid for paediatric epilepsy treatment (Ali *et al*., 2019). In June 2018, the US Food and Administration (FDA) approved a pharmaceutical preparation of highly purified, plant-derived CBD (Epidiolex®) for the treatment of Dravet and Lennox-Gastaut syndromes, which approval was followed one year later by the European Medicines Agency (EMA) (Morano *et al*., 2020; Williams and Stephens, 2020).

Epilepsy is the most prevalent global neurological conditional, affecting over 50 million people in the world. Characterized by recurrent spontaneous seizures, epilepsy results from multiple disorders caused by different aetiologies, such as genetic syndromes, stroke, infection, traumatic brain injury among others. Epileptic patients also have sensorimotor, cognitive, psychological, psychiatric and social impairments, a major impairment of their quality of life and an increased risk of premature death (Ali *et al*., 2019).

Seizures can be limited by antiepileptic drugs (AEDs) drugs, but none of these drugs can prevent the original cause or the development of epilepsy in patients who are at risk and live with uncontrolled seizures. The molecular targets of most of these AEDs are restricted to voltage-gated Na^+, K^+ and Ca^{2+} channels, $GABA_A$ receptors, the GAT-1 GABA transporter, glutamate, GABA transaminase and synaptic proteins involved in neurotransmitter release (Iannotti *et al.,* 2014).This disease is characterized by an imbalance between glutamatergic and GABAergic neurotransmission (Bradford, 1995). To many patients, epilepsy is a progressive disorder associated with ongoing loss of brain neurons and function (Rosenberg *et al*., 2015; Williams and Stephens, 2020).

Around one third of patients with epilepsy do not respond to current AEDs and therefore live with uncontrolled seizures (Janmohamed *et al*., 2020).

The most severe group of childhood epilepsies are the developmental and epileptic encephalopathies (DEEs). Dravet syndrome is a prototype DEE that presents in typically developing children at ages between 3 months and 15 months, with prolonged hemiclonic and/or generalized tonic-clonic seizures, often associated with fever (Ali *et al.*, 2019).

The incidence of paediatric 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, thus favouring excitability (Cottrell *et al*., 2000; Chapman *et al*., 2012). Additionally, GABA exerts a net excitatory effect in immature neurons due to developmental 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, a characteristic of seizures (Chapman *et al.*, 2012).

CBD revealed to be an effective anticonvulsant in Phase 3 clinical trials for the treatment of Dravet and Lennox Gastaut syndromes and in 2018 received FDA approval in the United States for seizures associated with these disorders (*Devinsky et al*., 2017; FDA, 2018; Thiele *et al*., 2018).

The administration of CBD as adjunct therapy decreases seizure frequency in patients with Dravet and Lennox–Gastaut syndromes. The most well-known case using CBD is about a little girl named Charlotte Figi, with Dravet syndrome, at the age of 5 years was having nearly 50 seizures per day. After the administration of an oil containing a high CBD/THC ratio, seizures decreased significantly with an improvement of her development. The disclosure of information about this occurrence had a major impact in other families with children who have intractable epilepsy (Ali *et al*., 2019).

This new trend using medical cannabis in children show some risks because of a lack of standardization and regulation, imprecise dosing, possible adverse side effects and medication interactions. Some parents report that they use cannabis extracts, purchased from a dispensary or directly from a medical cannabis grower. The quality and composition of these products lead to a lack of regulation and standardization. These cannabis extracts not only have variable levels of CBD but also of THC, which has poor

clinical potential due to its psychoactive and significant proconvulsive properties, that may limit their chronic use (Porter and Jacobson, 2013; Patra *et al*., 2018)

Studies designed to assess safety, optimal dosing, tolerability and efficacy of a standardized CBD preparation in different populations of children and adults with epilepsy will guide to an optimal composition with impact in the treatment of epilepsy (Porter and Jacobson, 2013).

1.2 Dravet and Lennox-Gastaut Syndromes

Among children with treatment-resistant epilepsy, those with early onset and severe epilepsies such as Dravet syndrome (DS) and Lennox-Gastaut syndrome (LGS) have the greatest neurodevelopmental problems, including intellectual disability and autism (Devinsky *et al.*, 2014).

In DS, which often results from a genetic disease due to a voltage-gated sodium channel (VGSC) *SCN1A* gene mutation, healthy, developmentally normal children present in the first year of life, usually around 6 months, with compulsive status epilepticus (SE) commonly triggered by fever (Devinsky *et al*., 2014; Kaplan *et a.l*, 2017, Williams and Stephens, 2020). Further episodes of SE, hemiclonic or generalized, tend to occur and other seizure types are developed, including focal dyscognitive seizures, absences, and myoclonic seizures, after the first year of life. From the second year of life, frequent seizures (refractory to standard AEDs) lead to the development of epileptic encephalopathy, resulting in cognitive, behavioral and motor impairment in these children. In most patient's outcome is generally poor, with intellectual disabilities and ongoing seizures (Devinsky *et al.*, 2014).

DS symptoms result from the loss-of-function of $Na_v1.1$ channels, which selectively reduces sodium current and excitatory drive in many types of GABAergic interneurons. Accordingly, targeting the *SCN1A* mutation to $Na_v1.1$ channels in forebrain GABAergic interneurons recapitulated DS phenotype and established that hypoexcitability of those interneurons is sufficient to cause the epileptic phenotype and autistic-like behaviors (Kaplan *et al*., 2017).

Effective treatment of DS could potentially be achieved through enhancing GABAergic signaling by inhibitory neurons (Han S, et al., 2012) or selectively decreasing electrical excitability in excitatory neurons (Anderson et al., 2017).

LGS is a rare but devastating childhood epilepsy syndrome that can result from diverse etiologies, including structural, metabolic, and many genetic disorders, but in many cases the cause is unknown. LGS presents in children ages 1 to 8 years, being more usual between the ages 3 to 5 years. Most patients with LGS experience multiple refractory seizures everyday despite multiple AEDs and nonpharmacologic treatment including ketogenic diet, vagus nerve stimulation and epilepsy surgery. The prognosis remains poor with current therapies. The morbidity is significant: head injuries are common making the patients wear helmets; some patients have even become wheelchair-bound as a result of violent drop attacks (Kaplan *et al*., 2017).

Effective treatments for both DS and LGS are needed. Some clinical trials are ongoing and show promising results, as it is shown in the tables below (Tables 1 and 2).

Patients with DS and LGS are good candidates for a CBD trial given the need for more effective and better tolerated therapies for these epilepsies, due to the high rate of seizure frequency, and the relative homogeneity of specific syndromes (Devinsky *et al*., 2014).

Table 4 Summary of some preclinical antiepileptic trials in rat and mouse (Ali et al., 2019)

Preclinical antiepileptic trial	Results	Reference
Rat hippocampal slices	CBD attenuates epileptiform activity	Jones *et al*., 2010
In vivo: rats with CBD before pentylenetetrazole administration	Increases the amount of pentylenetetrazole required to initiate a seizure Reduces the incidence of tonic-clonic seizues Decreased mortality	Jones *et al*., 2010 Shirazi-Zand *et al*., 2013
In vivo: *SCN1A* knockout mouse model of Dravet syndrome treated with CBD	Decreased the number of spontaneous seizures as well as the duration and severity of thermally-induced seizures	Kaplan *et al*., 2017

Table 5 Summary of some randomized, placebo-controlled, double blind trials of pharmaceutical grade neutral CBD in children with DEEs (98% CBD) (Ali et al., 2019)

Preclinical antiepileptic trial	Results	Adverse events	References
Children with DS aged 2 to 18 years Children had to be on one or more AEDs, with minimum of 4 convulsive seizures per month n=120 61 - 20 mg/kg/day CBD 59 - Placebo	• Greater than 50% convulsive seizure reduction: CBD – 43%; placebo – 27%; • Seizure free: CBD – 5%; placebo – 0%; • No difference in non-convulsive seizure frequency between groups Improvement of children: CBD – 62%; placebo – 34%	CBD: somnolence (36%), diarrhea (31%), decrease appetite (28%), fatigue (20%); Placebo: somnolence (10%), diarrhea (10%), pyrexia (8%), upper respiratory track inflammation (8%).	Devinsky *et al*., 2017
Individuals aged to 2 to 55 years who had LGS who had had two drop seizures per week during a 28-day baseline period n = 225: 76 - 20 mg/kg/day CBD 73 - 10 mg/kg/day CBD 76 – placebo Drop seizures included tonic, atonic and tonic-clonic	• Seizure reduction in more than 50%: 20 mg/kg/day CBD: 39%; 10 mg/kg/day CBD: 36%; Placebo: 14% • Seizure reduction in more than75%: 20 mg/kg/day CBD: 25%; 10 mg/kg/day CBD: 11%; Placebo: 3%	CBD 20 mg/kg/day: somnolence 21%; decrease appetite: 16%; diarrhea:8% CBD 10 mg/kg/day: somnolence 30%; decrease appetite: 30%; diarrhea:15% Placebo: somnolence 5%; decrease appetite: 8%; diarrhea:8%	Devinsky *et al*., 2018
Individuals aged to 2 to 55 years who had LGS evidence of more than one type of generalized seizure for at last 6 months, at last two drop seizures per week during the 4-week baseline period, and had not responded to treatment with at last two AEDs n =171: 86 - 20 mg/kg/day CBD 85 - placebo Drop seizures included tonic, atonic and tonic-clonic	Median percentage reduction in monthly drop seizure frequency from base lines: 43.9% CBD 21.8% placebo	CBD 20 mg/kg/day: Diarrhea: 13% Somnolence: 14% Decreased appetite: 9% Vomiting: 7% Placebo: Diarrhea: 4% Somnolence: 8% Decreased appetite: 9% Vomiting: 5%	Thiele *et al*., 2018

1.3 Pharmacokinetics and Mechanism of Action of CBD

The pharmacokinetics of CBD show extensive variability in relation to the route of administration, type of product administrated, concomitant food intake, drug-drug interactions, and many other factors (Franco and Perucca, 2019).

Smoke inhalation is the most common administration path of cannabis used for recreational or medicinal purposes, although this approach is unsuitable for medical drug delivery, despite the fact that the lungs are a very efficient drug delivery path (Devinsky *et al*., 2014). In some human trials, CBD has been administrated orally using an oil-based capsule. CBD has a low water solubility and low absorption from the gastrointestinal system, resulting in low bioavailability mostly due to first-pass metabolism in the liver (Devinsky *et al*., 2014). To avoid this, some products have been delivered effectively via oromucosal spray or transdermal gel. Other transdermal approaches, eye drops, intranasal spray, and rectal suppositories are being evaluated as CBD delivery methods (Devinsky *et al*., 2014; Ali *et al*., 2019).

CBD is highly lipophilic and, therefore, exhibits a high distribution volume leading to high penetration in the brain, adipose tissue and other organs. CBD is eliminated in feces with a terminal half-life of 18 to 32 hours (Devinsky *et al*., 2014; Ali *et al*., 2019). Some authors claim that the efficacy of CBD may also be due to its impact on the pharmacokinetics of co-administrated AEDs. CBD interacts with some CYP isozymes and may inhibit other AEDs metabolism that are inactivated by these pathways, thus increasing their bioavailability in plasma (Devinsky *et al*., 2014; Ali *et al*., 2019). The pharmacokinetics and toxicity of CBD in children is far from being completely understood (Ali *et al*., 2019).

The mechanism of action of CBD still remains a mystery, mostly because CBD binds to more than 65 molecular targets, does not activate cannabinoid receptors (either CB1 or CB2) and its plausible targets against epilepsy include voltage-dependent anion channel 1, Cav3.x channel, 5-HT1A and glycine receptors, the G protein-coupled receptor 55, and modulation of the endogenous levels of adenosine, a wide-recognized endogenous anticonvulsant (Ibeas *et al*., 2015).

Three main mechanisms of action of CBD against epilepsy have gain significant consensus among the scientific community: modulation of intracellular Ca^{2+} (including changes on neuronal Ca^{2+} mobilization via GPR55 and influx via TRPV1) and modulation of adenosine-mediated signaling (e.g. Grey and Whalley, 2020).

Adenosine is as a well-known negative modulator of excitatory transmission and consequent seizure termination in the CNS through the activation of most expressed A_1, A_{2a} and A_3 in the CNS. CBD reduces adenosine reuptake by inhibiting equilibrative nucleotide transporters (ENTs). This suggests that CBD might decrease seizures occurrence in DS and LGS by enhancing the endogenous adenosine levels at central synapses and, consequent, activation of presynaptic inhibitory A_1 receptors resulting in the inhibition of neurotransmitters release (Franco and Perucca, 2019).

1.4 The endocannabinoid system

Cannabinoids activate cannabinoid receptors (mainly CB_1 and CB_2). These receptors are G-protein-coupled receptors (GPCR) activated endogenously by endocannabinoids, but also by phytocannabinoids or synthetic cannabinoids (Di Marzo and Piscitelli, 2015).

The CB_1 receptor subtype is expressed in the brain, mostly in hippocampus, olfactory bulb, basal ganglia and cerebellum, and in peripheral tissues. The CB_2 receptor is expressed in peripheral organs with immune function, cells such macrophages and leukocytes, lungs, testis and central nervous system (Felder *et al.*, 1995).

Other receptor proteins have been identified as possible targets for the endogenous cannabinoids such as GPR55, GPR119 and transient receptor potential vanilloid 1 (TRPV1) (Di Marzo and Piscitelli, 2015).

The endogenous activators of CB_1 and CB_2 receptors comprise N-arachidonoyl-ethanolamine (AEA, anandamide) and 2-arachidonoylglycerol (2-AG), which are derivatives of the arachidonic acid.

CBD exhibits low affinity for both CB_1 and CB_2, but it has the ability to antagonize CB_1/CB_2 receptor agonists at low concentrations (lower than 1µM). At CB_2 receptors, CBD may act as an inverse agonist, because it is uncertain about whether the antagonism is non-competitive. In addition, CBD may also antagonize THC effects via non-CB_1/CB_2 receptors, like the GPR55, which activation by THC is prevented by CBD.

The endocannabinoid system might play a crucial role in modulating neuronal excitability by regulating neurotransmitters release at the synaptic level. Endocannabinoids are retrograde messengers, synthesized “on demand” in response to increased neuronal activity, probably due to the contribution of group 1 metabotropic

glutamate receptors (mGluRs). Indeed, in excitatory synapses, $mGlu_5Rs$, that are typically located peri-synaptically are activated by glutamate "spill over" occurring during hyper-excitable states (like epileptic seizures), so producing a feed-forward positive mechanism (Olmo *et al*., 2016).

1.5 CBD and its influence on TRP channels activation

Transient receptor potential (TRP) channels are a group of membrane proteins involved in the transduction of chemical and physical senses. These channels modulate the influx of ions, mediating several neural signalling processes implicated in temperature sensation, pressure and pH, as well as smell, taste, vision and pain perception. TRP channels are also involved in the regulation of cardiovascular function and neurotransmitters release, embryological development, and immune function. TRP channels dysfunction are involved in many diseases, such as neuropathic pain, inflammation and respiratory disorders. Some cannabinoids can modulate certain subfamilies of these channels (Muller *et al*., 2019, Caterina, 2014).

Humans express 27 TRP channels which are divided according to their primary structures into 6 subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPV (vanilloid). TRP channels are invariably cation selective, however, a few TRP channels are highly calcium or sodium selective, most of which can also be permeated by a range of monovalent and divalent cations (Caterina, 2014).

TRPV1 is a nonselective cation channel that is permeable to many cations, such as Ca^{+}, Na^{+} and Mg^{2+}; it is activated by capsaicin, low pH, noxious temperatures (≥ 42 °C), voltage change, AEA and various lipid metabolites (Nazıroğlu, 2015; Reggio *et al*, 2018). Activation of TRPV1 tends to facilitate seizures rather than to reduce. Experimental results suggest that TRPV1 blockers may exert antiepileptic effects (Asth *et al*., 2019). Activation of TRPV1 results in Ca^{2+} influx through the channel pore. Depending on the duration and influx magnitude, increases in intracellular Ca^{2+} can desensitize the channel leading to a protective negative feedback mechanism (Gray *et al*., 2019).

The TRP vanilloid (TRPV), TRP ankyrin (TRPA), and TRP melastatin (TRPM) subfamilies comprise ligant-activated channels that can be modulated by several endogenous, phytogenic, and synthetic cannabinoids. Up to date, six TRP

subfamilies have been reported to mediate cannabinoid activity: TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8 (Muller *et al*, 2018).

In 2001, Bisogno *et al*., were the first to describe that CBD has affinity for the TRPV1 receptor, which agonistic action may be responsible for this receptor rapid desensitization (Bisogno *et al*., 2001). Interestingly, TRPV1 expression is increased in human epilepsy and unsurprisingly plays a role in the regulation of cortical excitability. The putative involvement of the TRPV1 channel in the anticonvulsive activity of CBD is based on studies showing that CBD (at low micromolar concentrations) can activate and rapidly desensitize TRPV1 receptors in recombinant cell systems and in "*in vitro"* experimental models of epilepsy. Patch clamp recordings in human TRPV1-expressing HEK293 cells showed rapid, concentration-dependent, activation and desensitization of TRPV1 by CBD. Both effects were sensitive to capsazepine, a selective TRPV1 channel inhibitor, thus confirming that CBD was acting specifically on this receptor (Lannotti *et al*., 2019; Gray *et al*., 2019), thus strengthening the concept that CBD may exert an antiepileptic action by modulating TRPV1 activity (Grey and Whalley., 2020).

1.6 Endocannabinoids: Anandamide (AEA)

Anandamide (AEA) is the main activator of metabotropic CB_1 and CB_2 receptors in the brain. AEA is an endocannabinoid, but it is also an endogenous agonist of TRPV1 channels when used in high concentrations (Asth *et al*., 2019). The concentration-dependent effects of AEA on TRPV1 channels is affected by many factors, including phosphorylation, voltage, temperature changes, pH and CB_1 receptor activation (Nazıroğlu, 2015). A possible role of AEA at both the CB_1 receptor and the TRPV1 channel can be exploited for the development of new drugs with a dual mechanism (Asth *et al*., 2019). Concentration-dependent dual effects of AEA can be explained as it binds to the CB1 receptor inhibiting neuronal activity, while its binding to TRPV1 depolarizes neurons and promotes neurotransmitters release (Asth *et al*., 2019).

1.7 Endocannabinoids: 2-Arachidonoylglycerol (2-AG)

The endocannabinoid 2-AG activates cannabinoid receptors CB_1 and CB_2, and it is the most abundant endogenous CB_2 agonist in the brain (Long *et al.*, 2009, Schloss *et al.*, 2019). With levels up to 170 times higher than those of AEA (Stella *et al.,* 1997), 2-AG acts as an important signalling molecule and as an intermediate in lipid metabolism (Baggelaar *et al.*, 2018).

It is well established that 2-AG is synthesized "on demand" as a retrograde messenger inhibiting neurotransmitter release at both inhibitory and excitatory synapses, resulting in conspicuous activities affecting both short- and long-term plasticity (Baggelaar *et al*., 2018; Fagundo *et al.*, 2013). Activation of CB_1 and CB_2 receptors by 2-AG is associated with many physiological processes, including inflammation, food intake, locomotor activity, learning and memory, epileptogenesis, neuroprotection, pain sensation, mood, stress and anxiety, addiction, and reward (Baggelaar *et al.*, 2018).

1.8 Amino-acid neurotransmission in the brain

γ-aminobutyric acid (GABA) and glutamate are the major inhibitory and excitatory neurotransmitters in the mammalian central nervous system, respectively. These amino acids are involved directly or indirectly in most aspects of normal brain function including cognition, memory and learning. They are released from depolarized nerve terminals by exocytosis of synaptic vesicles (Zhou and Danbolt, 2013, 2014). Translocation of these amino acids to the extracellular space may also occur through uptake transport reversal by collapsing the sodium gradient across the plasma membrane (Zhou and Danbolt, 2013).

GABA and glutamate exert their actions through activation of plasma membrane-bound receptors. The concentration of amino acids in the plasma membrane surroundings regulate their effects at synaptic and non-synaptic levels. It is, therefore, highly important to keep the extracellular concentrations of these two amino acids low to ensure that their effects are synaptic-mediated. Low extracellular levels of both GABA and glutamate are sustained by cellular uptake, as no extracellular amino acid metabolism has been recognized at the synaptic level (Zhou and Danbolt, 2013).

Intercellular distribution of glutamine and glutamate pools corresponding to astrocytes and neurons, respectively, led to the suggestion of the existence of a glutamate/glutamine cycle among neurons and astrocytes. Glutamate released into the synaptic cleft from glutamatergic neurons is (1) taken up by surrounding astrocytes, (2) converted into glutamine by the astrocyte-specific enzyme glutamine synthetase (GS), which is (3) released into the extracellular space. Released glutamine is, then, taken up by neurons to be recycled into glutamate by the phosphate-activated glutaminase (PAG). In the GABAergic synapse, released GABA is taken up into astrocytes and catabolized by the tricarboxylic acid (TCA) cycle into a succinate intermediate via the concerted

action of GABA transaminase and succinate semialdehyde dehydrogenase. Glutamine may be synthesized from succinate via the TCA cycle, including condensation of oxaloacetate and acetyl-CoA to form citrate and subsequently synthesis of alpha-ketoglutarate and glutamate. Glutamate formed by the PAG activity in GABAergic neurons is converted by glutamate decarboxylase (GAD) into GABA. GABAergic neurons most likely exhibit a larger proportion of reuptake of the released neurotransmitter compared to their glutamatergic counterparts, as a result of the supply of glutamine to GABAergic neurons being quantitatively less significant (Bak et al., 2006).

Glutamine synthetase, an astrocyte and oligodendrocyte-specific cytosolic enzyme converts glutamate into glutamine in an ATP-requiring reaction with ammonia. Under appropriate electrophysiological conditions, other astrocytes and neurons contain glutamine transporters leading to a net exchange of glutamine from astrocytes-to-neurons. Inside neurons, the mitochondrial phosphate-specific enzyme, glutaminase, reconvert inert glutamine-to-glutamate for subsequent repackaging into synaptic vesicles. The cycling of glutamate/glutamine in astrocytes and neurons has been termed “the glutamine cycle”. Thus, there are two pathways to produce neuronal glutamate: 1) the *de novo* production of glutamate from glucose and amino-acid derivatives via energy metabolism, and 2) the recycling of glutamate via glutamate reuptake, enzymatic activity of glutaminase and the activities of the glutamine transporters (Niciu *et al.*, 2012).

1.8.1. GABA

GABA is the main inhibitory neurotransmitter in the brain. It modulates the inhibitory/excitatory balance necessary to proper brain function in mature brain (Wu and Sun, 2015). The uptake of GABA is electrogenic, as it is driven by the transmembrane gradients of Na^+ and Cl^- (Zhou and Deadbolt, 2013).

The first neurotransmitter transporter identified was the GABA transporter, known as GAT1 (Zhou and Dandbolt, 2013). Other transporters were also identified and are known as GAT2, GAT3 and BGT1 (GAT4) (Liu *et al.*, 1993; Zhou and Dandbolt, 2013; Eskandari *et al.*, 2017). GATs regulate the extracellular concentration of GABA during resting and active conditions in synaptic, as well as extrasynaptic, regions in the brain. Thus, GATs can modulate both tonic and phasic inhibitory GABAergic signaling in the nervous system. These transporters belong to the neurotransmitter/Na^+ symporter (Eskandari *et al.*, 2017).

GAT1 and GAT3 are both coupled to sodium and chloride. GAT1 is present in neuronal cell bodies for a short time during development, which are later on concentrated in nerve terminals. GAT3 is selectively expressed in astrocytes throughout the CNS. The highest levels of GAT1 are in the cerebral cortex while the highest GAT3 levels are in the brainstem. Meanwhile, GAT2 and BGT1 are expressed in hepatocytes of liver and in the kidney (Zhou and Dandbolt, 2013).

Potentiation of GABAergic neurotransmission via inhibitor or reversal of GATs is believed to have therapeutic value in treating epileptic seizures. GAT inhibitors are known to increase GABA levels in the brain and exhibit anticonvulsant activity (Eskandari *et al.*, 2017).

GABA transport was determined not to rely directly on metabolism, but instead is driven by ions gradients. Na^+ and Cl^- play and important role in GABA transport. This transport, across the plasma membrane mediated by GATs, is coupled to the contranslocation of Na^+ and Cl^-. GABA transport is absolutely Na^+ dependent, while the dependence on Cl^- is less strict. So, GABA transporters can be referred to as Na^+-dependent and Cl^--facilitated transporters (Eskandari *et al.*, 2017).

GABA transporters can work in the forward (GABA uptake) or the reverse (GABA release) modes. The forward mode mediates the cotransport of Na^+/Cl^-/GABA into the cell, resulting in the movement of net positive charges into the cell. The reverse mode leads to Na^+/Cl^-/GABA cotransport out of the cell, resulting in the movement of net positive charges out of the cell (Eskandari *et al.*, 2017). The Na^+/Cl^-/GABA coupling stoichiometry is a fundamental property of the transporter. An accurate knowledge of the stoichiometry is essential for a better understanding of the role that GABA transporters play in GABAergic signaling. They define if the mode of transport is forward or reverse, depending on the transmembrane concentration and electrochemical gradients (Eskandari *et al.*, 2017).

There are three main types of GABA receptors: the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptor (Wu and Sun, 2015). GABA acts primarily through activation of fast hyperpolarizing $GABA_A$ receptors. When GABA binds to these receptors at post-synaptic location, the ion channel opens and chloride diffuses into the cell favored by its concentration gradient, therefore hyperpolarizing the post-synaptic neuron (Wu and Sun, 2015). $GABA_B$ receptors are found both pre and post synaptically. Activation of these receptors is coupled to K^+ and/or Ca^{2+} channels via G-

protein mediated pathways, leading either to activation of postsynaptic K^+ channels or inhibition of presynaptic Ca^{2+} influx (Wu and Sun, 2015).

There is a third type of GABA receptor, the $GABA_C$, which is identified in the central nervous system. It is present in many parts of the brain including the superior colliculus, cerebellum, hippocampus, and, most prominently, in the retina. $GABA_C$ receptors are not blocked by traditional $GABA_A$ receptor antagonists (e.g. bicuculline) nor by a range of $GABA_A$ receptor ligands, including benzodiazepines, although they exhibit a similar structure and function, a similar degree of variation in subunit pharmacology as that found among $GABA_A$ subunit types, and a great potential to couple with other $GABA_A$ subunits (Johnson, G. 1986; Wu and Sun, 2015).

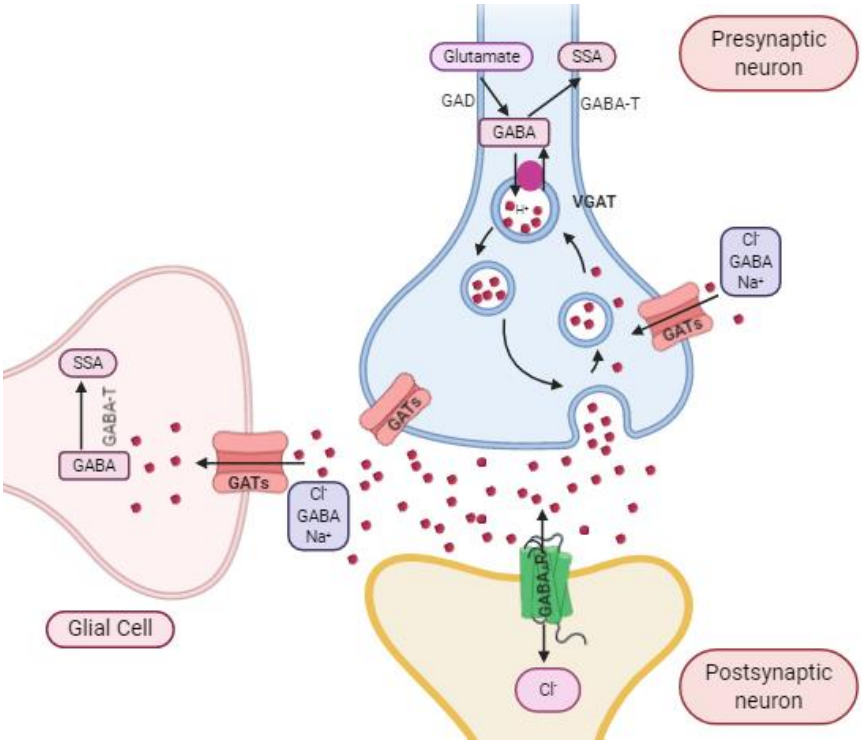


Figure 1 GABAergic synapse and GABA transporters (adapted from Eskandari et al., 2017)

1.8.2. Glutamate

Glutamate is a non-essential amino acid that can be synthesized in the body through distinct metabolic pathways. Being the major excitatory neurotransmitter in the central nervous system of mammals, glutamate functions both as substrate and product in many distinct reactions. Glutamate plays a significant role in neuronal excitability, synaptic

plasticity, immunity and behavioral mechanisms, such as learning and memory in the central nervous system (Yelamanchi *et al.*, 2015).

Cytosolic glutamate crosses the vesicular membrane via the activity of vesicular glutamate transporters (VGLUTs). The VGLUTs are multimeric proton/glutamate antiporters. There are three types of VGLUTs: VGLUT1 and VGLUT2 that are highly expressed in glutamatergic neurons; and VGLUT3, which is detected in GABAergic, cholinergic and monoaminergic neurons. The loss of these transporters expression via target knockout strategies results in the loss of glutamate packaging into synaptic vesicles causing toxic neuropsychiatric sequelae (Niciu *et al.*, 2012).

Glutamate is regularly released to extracellular fluid, and its uptake inhibition leads to extracellular buildups of glutamate within seconds. Most of the focus has been on the synaptic release of glutamate from nerve terminals by exocytosis of synaptic vesicles, but there are others mechanisms that show some importance too, such as (1) through anion channels, (2) via reversed operation of glutamate transporting proteins at the plasma membrane, (3) via cystine/glutamate antiporter (xCT) and (4) by exocytosis from *in situ* mature brain astrocytes (Zhou and Dandbolt, 2014).

Glutamate levels are finely-tuned by a family of transporter proteins located at the cell surface of both astrocytes and neurons (Niciu et al., 2012). The glutamate uptake processes are electrogenic and it is driven by ion gradients of K^+ and Na^+ (Zhou and Dandbolt, 2013). All the excitatory amino acid transporters (EAATs) catalyze coupled transport of $1H^+$, $3Na^+$ and $1K^+$ with one substrate molecule (Zhou and Danbolt, 2014). The first glutamate transporter to be isolated in the active form was the EAAT2. The three human counterparts were quickly identified as EAAT 1-3. Two more glutamate transporters were found later, the EAAT-4 and EAAT-5, which catalyze Na^+ and K^+-coupled transport of L-glutamate, as well as L- and D-aspartate. EAAT-1 and EAAT-2 are the glutamate transporters expressed in astrocytes, while EAAT-3 and EAAT-4 are neuronal (Zhou and Dandbolt, 2013).

EAAT-2 is highly expressed in astrocytes in all parts of the brain and spinal cord, being the highest levels found in the hippocampus and the neocortex. Meanwhile the EAAT-1 is found in the astrocytes throughout the CNS. These two transporters are targeted to the plasma membranes. The EAAT-3 is a neuronal transporter, and is expressed widely through CNS, targeting somata and dendrites avoiding axon terminals. Its highest concentration is found in the hippocampus. EAAT-4 is located in the cerebellar

Purkinje cells, targeting dendrites, spines, but there is also EAAT-4 in a subset of forebrain neurons. EAAT-5 is expressed in the retina, but scarcely expressed in the brain (Zhou and Dandbolt, 2013).

Most cells in the nervous system express at least one type of glutamate receptor (Zhou and Danbolt, 2014). Glutamate receptors are grouped in ionotropic and metabotropic receptors. Ionotropic glutamate receptors are ligant-activated cation channels (Ca^{2+} and Na^{+}), while metabotropic receptors exert their effects via the recruitment and activation of intracellular trimeric G-proteins and downstream signal transduction pathways (Niciu *et al.*, 2012).

There are three classes of ionotropic glutamate receptors identified, named N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainate (KA), where most of the cells in the nervous system express at least one type of these receptors (Niciu *et al.*, 2012; Zhou and Danbolt, 2014).

NMDA receptors have the highest affinity for glutamate. Three families of these receptors' subunits have been identifying as NR1, NR2A-D and NR3A-B. NR1 expression seems to always appear in the brain, and they are critical for neurodevelopment; NR2A expression predominates in the neocortex and hippocampus, and NR2B is expressed in the forebrain; on the other hand, NR2C and NR2D are expressed in the cerebellum and lower brain stem; NR3A is expressed in neocortex and displays neurodevelopmental regulation; NR3B mRNA expression is shown in the brainstem and alpha motor neurons of spinal cord; NR3B has been detected in the cerebellum and hippocampus (Niciu *et al.*, 2012).

NMDA receptors are among the most tightly regulated in the mammalian brain and unique in requiring co-agonists for activation. At least, six binding sites have been identified that regulate the probability of ion channel opening sites for two obligatory co-ligands, polyamide and cations (Mg^{2+}, Zn^{2+} and H^{+}). Extracellular Mg^{2+} acts as an open-channel, voltage-dependent "pore blocker" to prevent cation flux. Zn^{2+} is an important allosteric modulator of some glutamate receptors; it is stored inside synaptic vesicles and co-released with glutamate in selected populations of synaptic vesicles, which possibly provides an additional mechanism to regulate glutamate receptor activation. H^{+} ions are also critical endogenous allosteric modulators of glutamate receptors.

AMPA receptors are also widely expressed in the mammalian CNS and mediate fast excitatory neurotransmission in response to glutamate binding. AMPA receptor subunits are called GluR1-4; kainate receptor subunits are GluR5-7 and KA1-2. GluRs contain an unusually large extracellular N-terminus. Upon forming a tetrameric complex of GluR1-4s, AMPA receptors mediate fast excitatory neurotransmission that can be blocked by specific quinoxalinediones including 6-nitro-7-sulphamobezo(f)quinoxaline-2,3-dione (NBQX), a potent and selective AMPA receptor antagonist. Kainate receptors are also tetrameric complexes of GluR5-7 and KA1-2 subunits. The release of a small and brief concentrations of glutamate into synaptic cleft generates robust excitatory postsynaptic potentials (EPSPs). AMPA-mediated currents generate a fast upstroke and rapid current decay while NMDA-receptor activation provides a more prolonged phase of depolarization (Niciu *et al.*, 2012).

Too much or too little glutamate is harmful to the neurons. Excessive activation of ionotropic glutamate receptors dangerously increases intracellular Ca^{2+} levels, leading to their death in a process referred as “excitotoxicity” (Niciu *et al.*, 2012). Glutamatergic neurotoxicity is increasingly thought to be mediated by the differential activation of extrasynaptic relative to synaptic NMDA receptors (Niciu, 2012).

Unlike ionotropic glutamate receptors that depend on cation flux, metabotropic glutamate receptors exert their effects via the recruitment and activation of intracellular trimeric G-proteins and downstream signal transduction pathways. Like all G-protein coupled receptors, metabotropic glutamate receptors are seven transmembrane domain spanning receptors with an extracellular N-terminus and intracellular C-terminus, and, like AMPA receptors, they possess an especially large N-terminus. The metabotropic receptors (except mGluR8) localize primarily to perisynaptic and extrasynaptic localization on neurons and glial cells and modulate synaptic activity and plasticity.

To date, eight metabotropic glutamate receptors have been identified (mGluR1-8), which have been further subdivided into three functional groups based on amino-acid homology, agonist binding and activated downstream signal transduction cascades. Group I metabotropic glutamate receptors are mGluR1 and mGluR5. They elicit their downstream effects by two mechanisms: (1) phospholipase C via inositol-1,4,5-triphosphate (IP3) to release Ca^{2+} from intracellular stores and (2) diacylgycerol (DAG) to stimulate protein kinase C. Group II metabotropic glutamate receptors (mGluR2 and mGluR3). Group III metabotropic glutamate receptors (mGluR4-8) are coupled to

inhibitory G-proteins (Gi) that decrease intracellular cyclic adenosine monophosphate (cAMP) via inhibition of the adenylyl cyclase/protein kinase A pathway. Glutamate activates metabotropic glutamate receptors with varying degrees of affinity/avidity, and selective agonists, antagonists and modulators have been identified and developed for the various receptor classes and subtypes. Postsynaptic activation of metabotropic glutamate receptors has been demonstrated to modulate ion channel activity, and whether agonist binding to metabotropic glutamate receptors potentiates or inhibits channel activity depends on whether their cognate downstream signal transduction cascades (Niciu *et al*., 2012).

Metabotropic glutamate receptors localized on presynaptic membranes have been demonstrated to decrease both excitatory glutamatergic and inhibitory GABAergic neurotransmission. Although the precise mechanisms mediating presynaptic modulation has not been conclusively demonstrated, metabotropic glutamate receptors appear to elicit their diverse effects via the modulation of voltage-dependent presynaptic Ca^{2+} channels, thereby influencing quantal neurotransmitter release in a N-ethylmeleimide-sensitive factor attachment protein receptor (SNARE)-dependent manner. There is presently intense effort to develop both positive and negative modulators of presynaptic group II and III metabotropic glutamate receptors to treat a plethora of neuropsychiatric illnesses (Niciu et al., 2012).

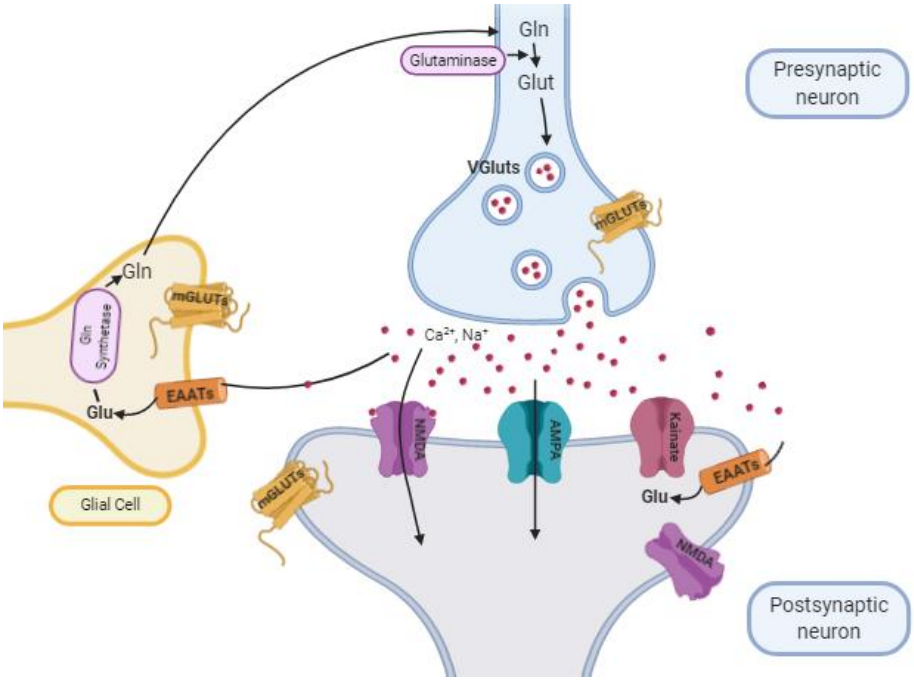


Figure 2 Glutamatergic neurotransmission (adapted from Niciu *et al*., 2012)

Chapter 2: Aim

Around one third of patients with epilepsy do not respond to current AEDs and therefore live with uncontrolled seizures (Janmohamed *et al*., 2020).The most severe group of childhood epilepsies are the developmental and epileptic encephalopathies (DEEs). Dravet syndrome is a prototype DEE that presents in typically developing children at ages between 3 and 15 months, with prolonged hemiclonic and/or generalized tonic-clonic seizures, often associated with fever (Ali *et al.*, 2019). Cannabis has been used to treat epilepsy throughout history, due to its anticonvulsant effects mediated mainly by their phytoccanabinoids THC and CBD. Considering the negative psychotropic effects of THC, the main research focus is on CBD. CBD revealed to be an effective anticonvulsant in Phase 3 clinical trials for the treatment of Dravet and Lennox Gastaut syndromes and in 2018 received FDA approval in the United States for seizures associated with these disorders (*Devinsky et al*., 2017; FDA, 2018; Thiele *et al*., 2018). Several particularities of paediatric epilepsy suggest a possible age-specific susceptibility to seizures, being immature brain more susceptible to seizures. ECS plays different roles according to the course of brain development, participating actively in neurogenesis in embryonic stages but is involved mainly on gliogenesis, myelination and circuit refinement in the perinatal and adolescent brain, while modulating neuronal homeostasis in the adult.

Previous work from our group was engaged to study in parallel and under the same experimental conditions, the mechanisms underlying [^{3}H]GABA and [^{14}C]glutamate release from rat hippocampal synaptosomes obtained from immature and adult rats, in the presence and absence of CBD (Caulino, 2019).The main findings of this work were: (1) 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 [^{3}H]GABA and [^{14}C]glutamate outflow from depolarized hippocampal synaptosomes of both animal groups; (2) CBD transiently increased the resting outflow of [^{3}H]GABA and [^{14}C]Glutamate, while decreasing depolarization-evoked release of the two amino acids from hippocampal synaptosomes of immature and adult rats; (3) CBD-induced [^{3}H]GABA and [^{14}C]Glutamate release from hippocampal synaptosomes under resting conditions was more evident in immature compared to adult animals.

There are, at least, three main mechanisms of action of CBD as an anticonvulsant drug: on the one hand, CBD can modulate intracellular Ca^{2+} by changing GPR55-induced neuronal Ca^{2+} mobilization and/or by affecting its influx via TRPV1 channels, and on the

other hand, it can modulate adenosine-mediated inhibitory signals by interfering with the nucleoside transport system (e.g. Grey and Whalley, 2020). Preliminary data from our group suggest that crosstalk between CBD and the adenosinergic system did not have a significant impact on CBD-induced modifications in [^{3}H]GABA and [^{14}C]Glutamate release from isolated nerve terminals of the rat hippocampus (Caulino, 2019).

In the present work we set up to investigate the interplay between CBD and the nonselective cation channel TRPV1 receptor, as in theory Na^{+} and/or Ca^{2+} influx into nerve terminals via TRPV1 channels may increase neurotransmitters release and also that the fast desensitization of TRPV1 currents following CBD binding could also explain depolarization-evoked amino-acid release inhibition by the cannabinoid.

We also intend to confirm if the reversal of Na^{+}-dependent high-affinity amino acid transporters is in fact mediating CBD-induced [^{3}H]GABA and [^{14}C]glutamate outflow from depolarized hippocampal synaptosomes.

To reinforce our results, we pretend to collapse the Na^{+} gradient across the plasma membrane, of hippocampal synaptosomes from adult and immature rats, by removing this cation from the extracellular milieu while keeping osmolality by replacing it by NMDG (128 mM), to check for the reversal to the outward mode the function of GABA and glutamate transporters, as the main mechanism of CBD modulation of the release of these neurotransmitters.

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. Female immature (21-28 days) and female adult Wistar rats (2-5 months) (Charles RiverTM, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30–19.30 h) dark (19.30–06.30 h) cycle, with food and water *ad libitum*.

3.2. Hippocampal isolation from adult and immature rats

Hippocampi were obtained from female Wistar rats of 21 to 28 days (immature juveniles) and 2 to 5 months old (female 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.

3.3. Preparation of synaptosomes from rat hippocampal

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 Ca^{2+}-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 µm). 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 45-60 min until newly formation of a pellet (Figure 1).

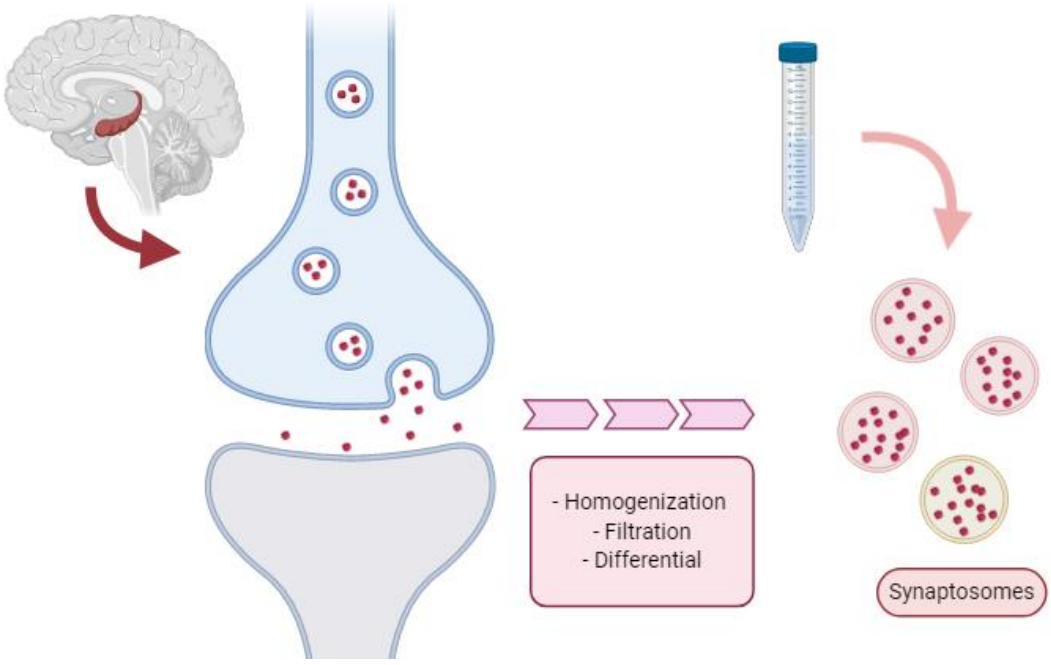


Figure 3 Schematic representation of protocol for preparation of hippocampal synaptosomes.

3.4. Protein Quantification

Protein concentration was determined by the bicinchoninic acid method (BCA; PierceTM, Thermoscientific, Rockford USA) 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 or 12.5 mg protein mL^{-1}. Protein quantification assays wereperformed 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), in a well. All samples were incubated 30 min at room temperature and the absorbance was read at 562 nm in BioTek SynergyTM HT microplate reader.

3.5. Measurement of [^{3}H]GABA and [^{14}C]glutamate release by synaptosomes from the rat hippocampus

The release of [^{3}H]GABA and [^{14}C]glutamate by synaptosomes was measured simultaneously after incubating the synaptosomes with [$_{3}$H]GABA (0.25 µCi mL-1; 70 Ci mmol-1; 0,5 µM) and [^{14}C]glutamate (0.25 µCi mL-1; 0.270 Ci mmol-1; 10 µM) during 10 min, at 37 ºC. Aliquots (250 µL) 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 µL chambers of a semi-automated 12-sample superfusion system (SF-12 Suprafusion 1000, Brandel, Gaithersburg, MD, USA). 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); $CaCl_2$ 2.2 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); EGTA 0.1 and aminooxyacetic acid 0.01) at 37 ºC.

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. 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.

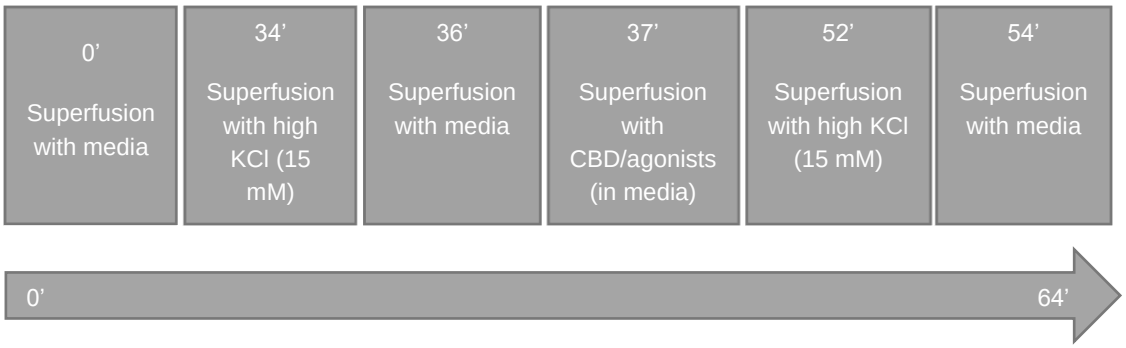


Figure 4 Schematic protocol of release [^{3}H]GABA and [^{14}C]glutamate from synaptosomes

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).

3.6 Drugs, Antagonists/Agonists and Inhibitors

All drugs, agonists, antagonists and inhibitors were prepared at the concentrations represented in the following table:

Table 6 Concentrations of drugs, antagonists, agonists and inhibitors used in the protocol of [^{3}H]GABA and [^{14}C]glutamate release

Drug	Concentration	Supplier
Cannabidiol	10 µM 30 µM	Abcam
Antagonist	**Concentration**	**Supplier**
Capsazepine	1 µM 3 µM 10 µM	Sigma/Abcam
Agonist	**Concentration**	**Supplier**
Capsaicin	0.1 µM	Sigma

	0.3 µM	
	1 µM	
	10 µM	
Inhibitors	**Concentration**	**Supplier**
DL-TBOA	100 µM	Tocris
SKF 98876A Hydrochloride	40 µM	Tocris

3.7 Data presentation and statistical analysis

[^{3}H]GABA and [^{14}C]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.

$$(1)\quad C = \frac{N1 - N2\left(\frac{h1}{h2}\right)}{c1 - c2\left(\frac{h1}{h2}\right)} \qquad (2)\quad H = \frac{N2 - N1\left(\frac{c2}{c1}\right)}{h2 - h1\left(\frac{c2}{c1}\right)}$$

$$C = carbon\ DPMs\ in\ sample$$

$$H = tritium\ DPMs\ in\ sample$$

$$c1 = carbon - 14\ efficiency\ in\ channel\ 1$$

$$c2 = carbon - 14\ eficiency\ in\ channel\ 2$$

$$h1 = tritium\ efficiency\ in\ channel\ 1$$

$$h2 = tritium\ efficiency\ in\ channel\ 2$$

$$N1 = CPMs\ in\ channel\ 1$$

$$N2 = CPMs\ in\ channel\ 2$$

The results were expressed as mean ± SEM, with *n* (showed in graphs) indicating the number of individuals. Statistical analysis of data was carried out using GraphPad Prism 6.01 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 Cannabidiol (CBD) increases the resting outflow of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats

Figure 5 represents the outflow of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats under resting conditions and after depolarization by high KCl (15 mM, during 2 min, applied at the 34th and 52nd min, S1 and S2, respectively). CBD (10 and 30 µM) was added to the superfusion medium 15 minutes before S2.

CBD (10 and 30 µM) concentration-dependently increased the resting outflow of [^{3}H]GABA (Figure 5A) and [^{14}C]glutamate (Figure 5B).

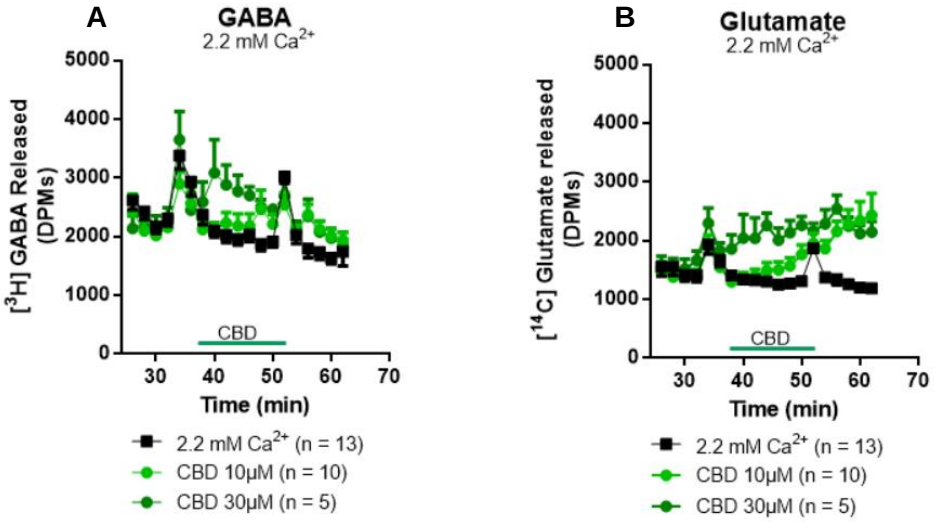
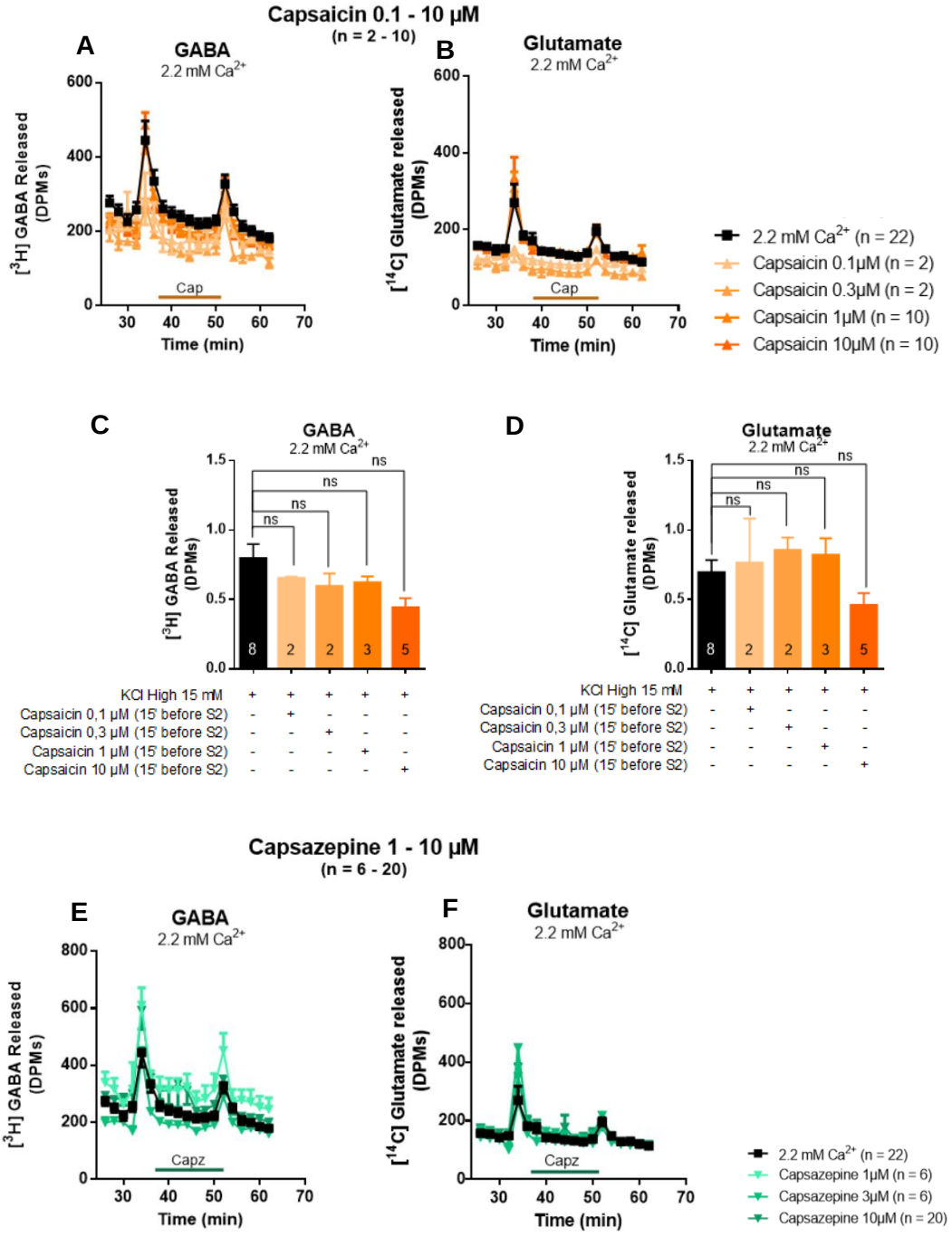


Figure 5 Effects of cannabidiol (CBD 10 and 30 µM) on [^{3}H]GABA (left-hand side - **A**) and [^{14}C]glutamate (right-hand side - **B**) release induced by high KCl [15 mM, applied for 2 min, at 34' (S1) and 52' (S2) of the perfusion period] from hippocampal synaptosomes of adult rats. CBD was added to the superfusion media 15 min before S2 (green horizontal line). The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph.

4.2 Selective activation of the TRPV1 receptor had no effect on [^{3}H]GABA and [^{14}C]glutamate release from hippocampal synaptosomes of adult rats

Activation of the TRPV1 receptor with the agonist capsaicin (0.1-1 µM, applied 15 min before S2) had no effect on [^{3}H]GABA and [^{14}C]glutamate release both during rest and after depolarization of rat hippocampal synaptosomes with high KCl (15 mM) (Figure 6A and 6B). No significant effect was observed on the release of [^{3}H]GABA nor [^{14}C]glutamate for any concentration of capsaicin when compared to the controls (Figure 6C and 6D).

On its own, the TRPV1 receptor antagonist, capsazepine (1-10 μM, applied 15 min before S2), did not modify [^{3}H]GABA and [^{14}C]glutamate release both from resting and upon depolarization of synaptosomes of the adult rat hippocampus (Figure 6E, 6F, 6G and 6H).



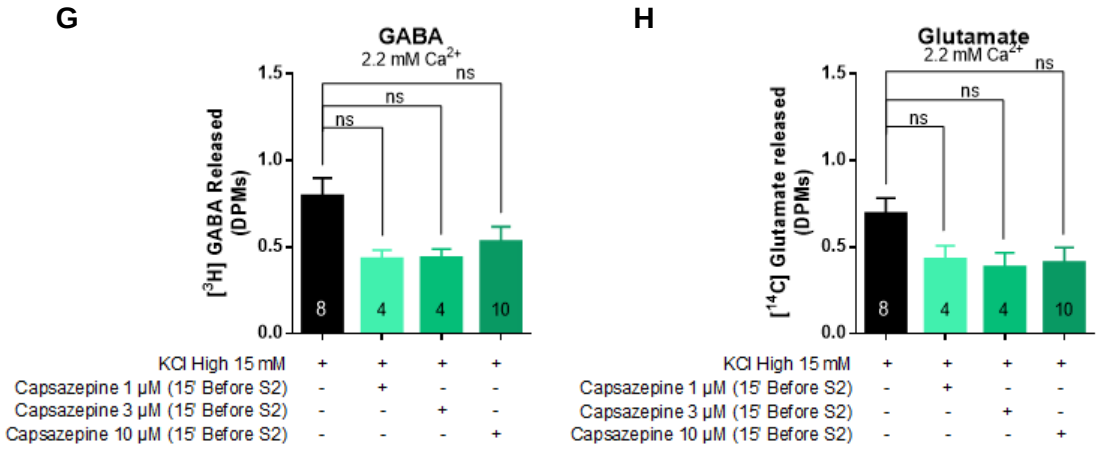


Figure 6 *Effects of capsaicin and capsazepine, the agonist and the antagonist of TRPV1 receptor, respectively, on [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) release from hippocampal synaptosomes of adult rats.* **[A and B]** *represent the effect of capsaicin (0,1 – 10 µM, applied 15 min before S2, orange horizontal line), on [^{3}H]GABA and [^{14}C]glutamate release induced by high KCl [15 mM, applied for 2 min, at 34' (S1) and 52' (S2) of the perfusion period];* **[C and D]** *bars represent S2/S1 ratios of A and B efflux charts.* **[E and F]** *represent the effect of capsazepine (1 – 10 µM, applied 15 min before S2, blue horizontal line), on [^{3}H]GABA and [^{14}C]glutam*ate release induced by high KCl. **[G and H]** *bars represent S2/S1 ratios of E and F efflux charts. The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph or bar. ns – no significance (one-way ANOVA followed Dunnett's multiple comparisons test) represents statistically no significant differences when compared to controls.*

4.3 Effect of TRPV1 receptor blockage by capsazepine on CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from hippocampal synaptosomes of adult rats

The facilitatory effect of CBD (30 µM) on [^{3}H]GABA and [^{14}C]glutamate outflow from resting rat hippocampal synaptosomes was not significantly attenuated by blockade of TRPV1 receptors with capsazepine (10 µM) (Figure 7). Likewise, pretreatment with capsazepine (10 µM, applied since the beginning of the infusion period) did not significantly modify the effect of CBD (30 µM) on [^{3}H]GABA release from KCl-depolarized rat hippocampal synaptosomes (Figure 8A), but it slightly decreased the levels of [^{14}C]glutamate release following synaptosomal depolarization (Figure 8B).

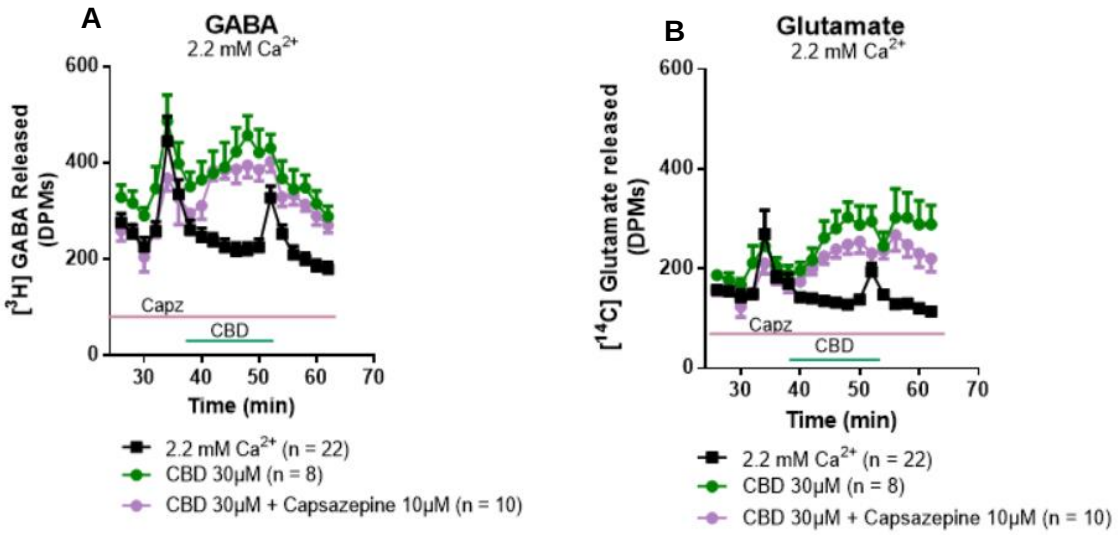


Figure 7 Interplay between CBD and capsazepine, a TRPV1 receptor antagonist, on [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) release induced by high KCl [15 mM, applied for 2 min, at 34' (S1) and 52' (S2) of the perfusion period] from hippocampal synaptosomes of adult rats. Panels **[A and B]** represent [^{3}H]GABA and [^{14}C]glutamate outflow from synaptosomes over time. CBD (30 μM) was applied to the superfusion fluid 15 min before S2 (green horizontal line), in the absence or in the presence of capsazepine (10 μM), applied from the beginning of the release period, i. e. it was present on S1 and S2 (purple horizontal line). The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph.

4.4 Inhibition of high affinity glutamate transport prevented CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from resting synaptosomes of adult rats

The nonselective glutamate transporter inhibitor, DL-TBOA (100 μM, applied 15 min before S2), increased both resting and depolarization-evoked [^{14}C]glutamate release from hippocampal synaptosomes of adult rats, without affecting the release of [^{3}H]GABA (Figure 8A and 8B).

Pretreatment with DL-TBOA (100 μM), applied since the beginning of the superfusion period, decreased CBD (10 μM)-induced [^{14}C]glutamate release from resting synaptosomes of the hippocampus of adult rats (Figure 8D), without much affecting the outflow of [^{3}H]GABA (Figure 8C).

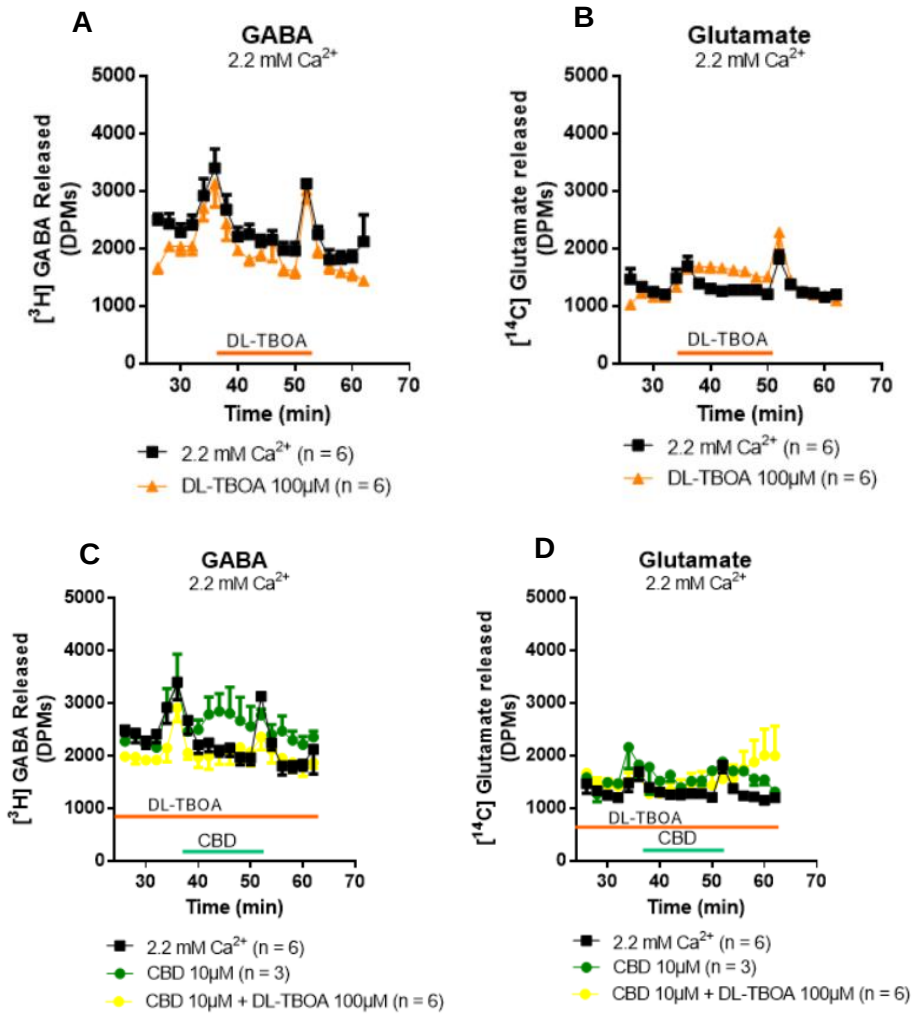


Figure 8 Interplay between DL-TBOA and CBD on [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) release from synaptosomes of adult rats. Synaptosomes were challenged twice by high KCl [15 mM, applied for 2 min, at 34' (S1) and 52' (S2) of the perfusion period]. Panels **[A and B]** represent [^{3}H]GABA and [^{14}C]glutamate outflow from synaptosomes over time. DL-TBOA (100 µM) was applied to the superfusion fluid 15 min before S2 (orange horizontal line); **[C and D]** represent [^{3}H]GABA and [^{14}C]glutamate outflow from synaptosomes over time. CBD (10 µM) was applied to the superfusion fluid 15 min before S2 (green horizontal line), in the absence or in the presence of DL-TBOA (100 µM). DL-TBOA was applied from the beginning of the release period, i. e. it was present on S1 and S2 (orange horizontal line). The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph.

4.5 Inhibition of GAT-1 GABA transporter prevented CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from synaptosomes of adult rats in resting conditions

On its own, the selective GAT-1 transport inhibitor, SKF98876A (40 µM), applied since the beginning of the superfusion period, did not significantly affect [^{3}H]GABA and

[^{14}C]glutamate release from hippocampal synaptosomes of adult rats (Figure 9A and 9B, respectively).

Pretreatment with SKF98876A (40 µM) prevented CBD (10 µM)-induced [^{3}H]GABA outflow from resting synaptosomes of adult rats (Figure 9C), without affecting the sincronous release of [^{14}C]glutamate (Figure 9D), at least until the second stimulation period (S2).

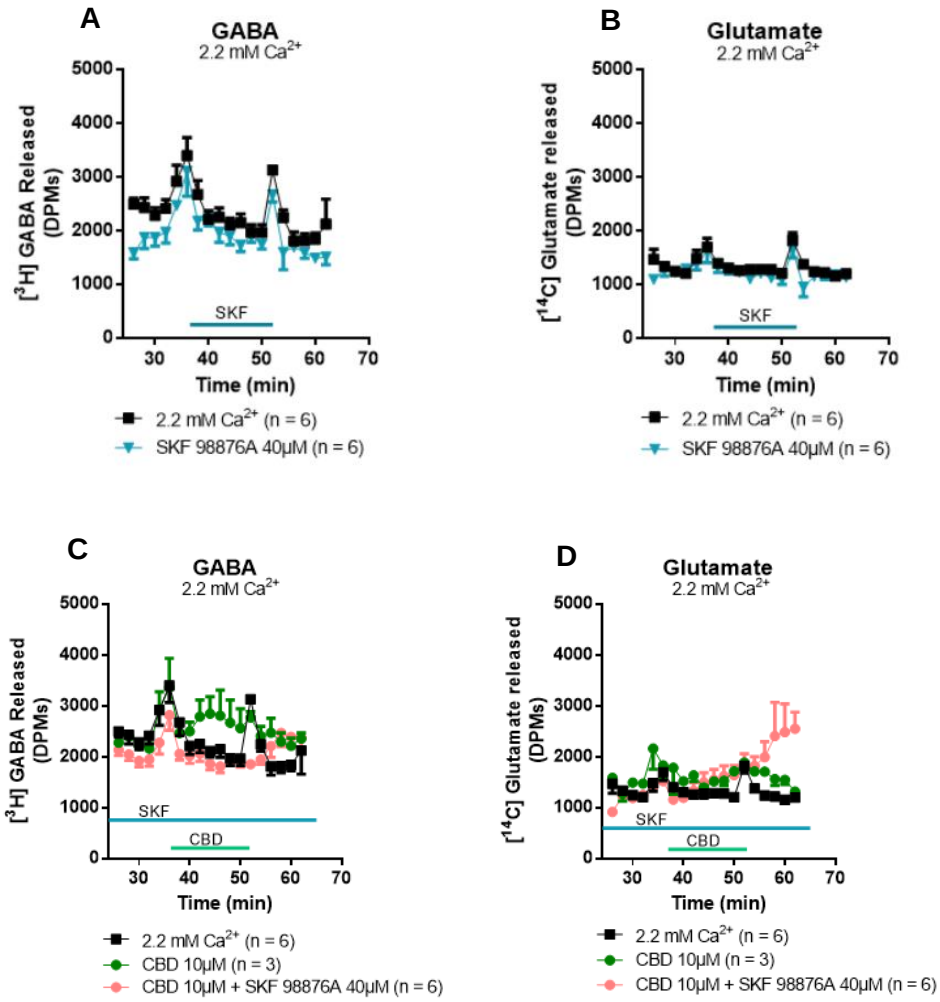
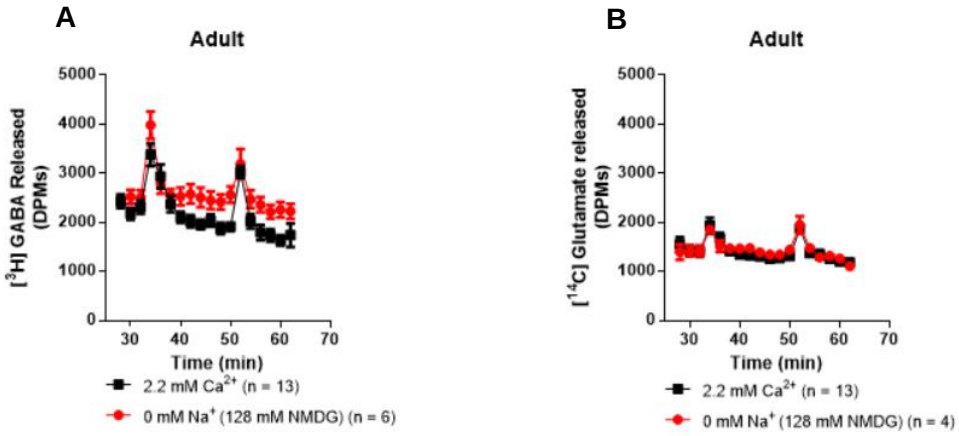


Figure 9 Interplay between SKF 98876A and CBD on [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) release from synaptosomes of adult rats. Synaptosomes were challenged twice by high KCl [15 mM, applied for 2 min, at 34' (S1) and 52' (S2) of the perfusion period]. Panels **[A and B]** represent [^{3}H]GABA and [^{14}C]glutamate outflow from synaptosomes over time. SKF 98876A (40 µM) was applied to the superfusion fluid 15 min before S2 (blue horizontal line); **[C and D]** represent [^{3}H]GABA and [^{14}C]glutamate outflow from synaptosomes over time. CBD (10 µM) was applied to the superfusion fluid 15 min before S2 (green horizontal line), in the absence or in the presence of SKF 98876A (40 µM). SKF 98876A was applied from the beginning of the release period, i. e. it was present on S1 and S2 (blue horizontal line). The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph.

4.6 CBD-induced spontaneous [^{3}H]GABA and [^{14}C]glutamate release is increased in immature and adult rats at low extracellular Na^+ conditions

Removal of external Na^+ (osmotically substituted by NMDG 128 mM) from the superfusion media progressively increased extracellular [^{3}H]GABA amounts from the first KCl (15 mM) depolarization period (S1) onwards, but the same did not occur regarding the release of [^{14}C]glutamate (Figure 10). Increases in extracellular [^{3}H]GABA normalized by hippocampal synaptosomal protein amounts, caused by removal of external Na^+ (plus NMDG 128 mM) were of higher magnitude in adult compared to immature rats.

In the absence of Na^+ in the superfusion fluid, the facilitatory effect of CBD (30 µM) on resting [^{3}H]GABA and [^{14}C]glutamate release was significantly higher than that obtained in normal sodium conditions (Figure 11). This was observed in synaptosomes obtained from the hippocampus of both adult (Figure 11A and 11B) and immature rats (Figure 11C and 11D). Interestingly, the magnitude of the facilitatory effect of CBD (30 µM) on resting [^{3}H]GABA and [^{14}C]glutamate release in Na^+-free conditions was much higher in hippocampi from immature rats compared to adult animals.



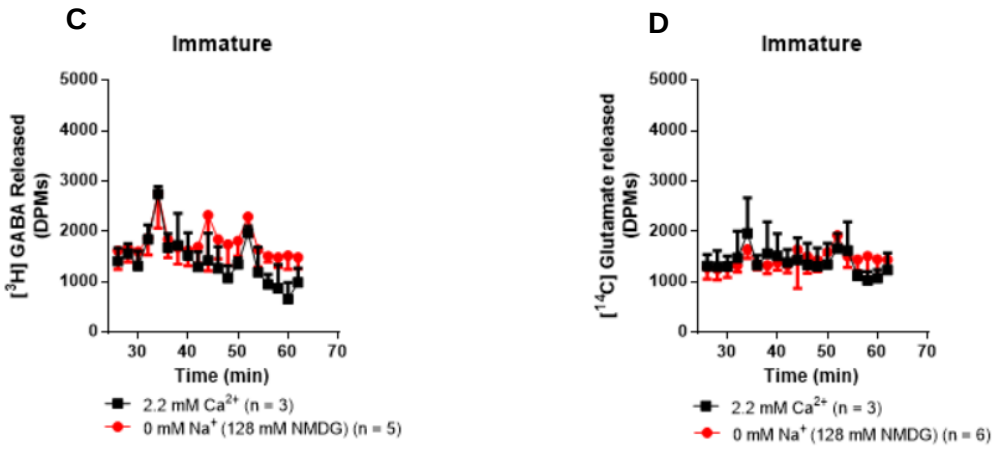
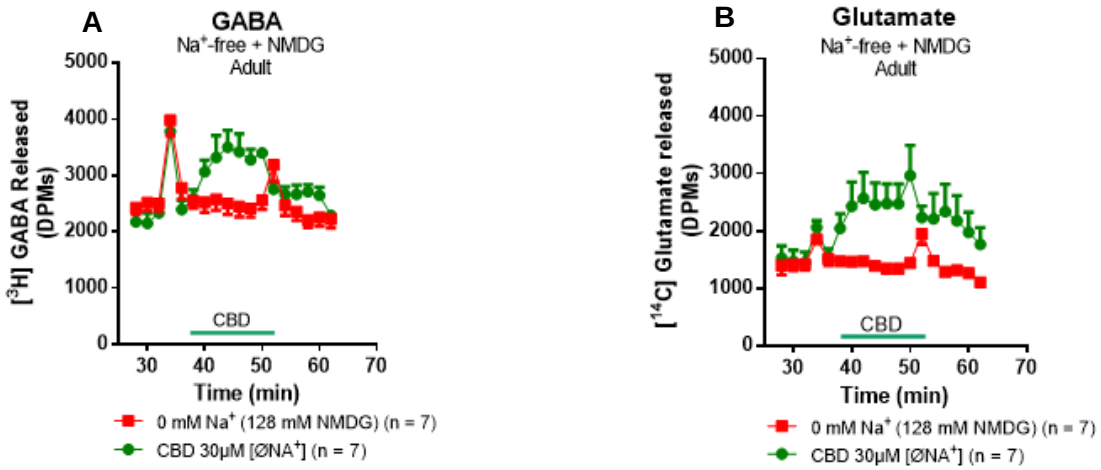


Figure 10 [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) outflow from hippocampal synaptosomes of adult **[A and B]** vs immature rats **[C and D]** in physiological superfusion media (black symbols) and in Na^{+}-free conditions (plus NMDG 128 mM) (red symbols). Graphs represent the outflow of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes during resting conditions and after depolarization with high KCl [15 mM, for 2 min, at 34' (S1) and 52' (S2)]. The results are expressed as mean ± SEM of an n number of individual experiments per condition, shown below each graph.



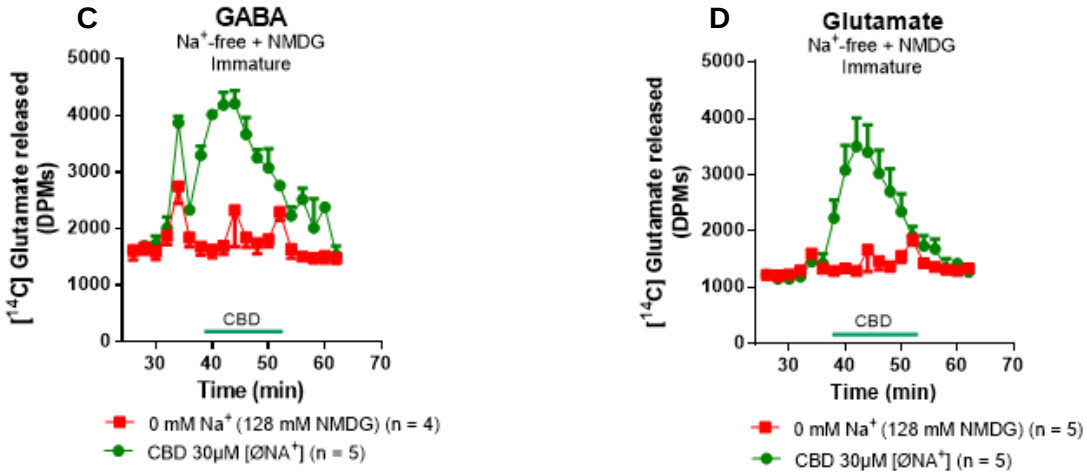


Figure 11 [^{3}H]GABA (left-hand side) and [^{14}C]glutamate (right-hand side) outflow from hippocampal synaptosomes of adult **[A and B]** vs immature rats **[C and D]**, in the presence of CBD 30 μM in Na^{+}-free conditions (plus NMDG 128 mM). Graphs represent the outflow of [^{3}H]GABA and [^{14}C]Glutamate from hippocampal synaptosomes during resting conditions and after depolarization with high KCl [15 mM, for 2 min, at 34' (S1) and 52' (S2)]. CBD was added to superfusion media 15 min before S2 (green horizontal line). The results are expressed as mean ± SEM of an n number of individual experiments per condition is shown below each graph.

Chapter 5: Discussion and Conclusions

Cannabinoids have been used in many neurological and psychiatric disorders, such as multiple sclerosis, schizophrenia, Parkinson's disease, neuropathic pain, anorexia, epilepsy and others. Devoid of psychoactive effects, cannabidiol (CBD) has been considered the major cannabinoid derivative for pediatric epilepsy treatment (Ali *et al*., 2019). In June 2018, the US Food and Drug Administration (FDA) approved a pharmaceutical preparation of highly purified plant-derived CBD (Epidiolex®) for the treatment of Dravet's and Lennox-Gastaut's epilepsy syndromes, as it passed Phase 3 clinical (Devinsky et al., 2017; FDA, 2018; Thiele et al., 2018). The same decision was taken one year after by the European Medicines Agency (EMA) (Morano *et al.*, 2020; Williams and Stephens, 2020). .

Although with proven antiepileptic properties, the mechanism of action of CBD continues to be a matter of debate and global research interest. CBD binds to more than 65 molecular targets, but surprisingly it does not seem to activate CB1 and CB2 cannabinoid receptors. Plausible targets for the antiepileptic effect of CBD include voltage-dependent anion channel 1, voltage-sensitive $Ca_v3.x$ channels, TRPV1 channels, 5-HT_{1A} and glycine receptors, G protein-coupled receptor 55 (GPR55), and modulation of the amount and activity of the endogenous anticonvulsant adenosine (Ibeas *et al.*, 2015). Among these, three modes of action of CBD in epilepsy have gained increasing interest among the scientific community: modulation of intracellular Ca^{2+} (including changes on neuronal Ca^{2+} mobilization via GPR55 and Ca^{2+} influx via TRPV1 channels) and control of adenosine-mediated signaling pathways (*e.g.* Grey and Whalley, 2020).

As a matter of fact, CBD may exert anticonvulsant effects by modulating the extracellular amount and activity of adenosine (Ado) (Carrier *et al*., 2006; Perucca, 2017; Franco *et al.*, 2019). Many years elapsed since Ado has been considered an important endogenous anticonvulsant in the brain (Dunwiddie, 1980). This concept was further validated by countless studies (reviewed in Boison, 2016; Weltha *et al*., 2018). It has been demonstrated that CBD can increase Ado levels in the brain of adult rodents by reducing the cellular uptake of the nucleoside (Castillo *et al.*, 2010). This theory is strengthen by findings showing that CBD may bind to equilibrative nucleoside transporters, namely ENT1, thus preventing Ado uptake which fosters activation of membrane-bound Ado receptors (Carrier *et al.*, 2006). Ado-mediated signaling may as well be one of the reasons why CBD decreases depolarization-evoked neurotransmitters release. Among Ado receptors, the A_1 receptor is the best candidate to inhibit

hippocampal neurotransmission, since it is highly expressed in the 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).

In a previous work from our group we investigated, in parallel and under the same experimental conditions, the molecular mechanisms underlying the effect of CBD on [^{3}H]GABA and [^{14}C]glutamate release from rat hippocampal synaptosomes obtained from both immature and adult rats (Caulino, 2019). The main findings were that vesicle exocytosis is not the main mechanism accounting for the release of both amino-acid neurotransmitters, strengthening the hypothesis that reversal of Na^{+}-dependent high affinity amino acid transporters is paramount for [^{3}H]GABA and [^{14}C]glutamate outflow from depolarized hippocampal synaptosomes of both animal groups. Moreover, it was demonstrated that CBD transiently increased the resting outflow of [^{3}H]GABA and [^{14}C]Glutamate, while decreasing depolarization-evoked release of the two amino acids from hippocampal synaptosomes of immature and adult rats. While no changes were apparently observed on depolarization-evoked amino-acids release among the two animal groups, CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from hippocampal synaptosomes under resting conditions was more evident in immature compared to adult animals.

Using a pharmacological approach, Caulino (2019) also showed that Ado does not seem to play a significant role on CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from synaptosomes isolated from the rat hippocampus. Therefore, we set to investigate in this thesis the interplay between CBD (like the endocannabinoid, anandamide) and the nonselective cation channel TRPV1 receptor channels, as in theory Na^{+} and/or Ca^{2+} influx into nerve terminals via TRPV1 channels may increase neurotransmitters release. This can be observed either by promoting Ca^{2+}-dependent vesicle exocytosis and/or by reversing the high affinity amino-acid transport to the outward mode upon collapsing the Na^{+} gradient across the plasma membrane. On the other hand, paradoxical Ca^{2+}-dependent fast desensitization of TRPV1 currents following CBD binding could also explain depolarization-evoked amino-acid release inhibition by the cannabinoid.

The best-known activators of TRPV1 are: temperature greater than 43 °C (109 °F); acidic conditions; capsaicin (the irritating compound in hot chili peppers); and allyl isothiocyanate, the pungent compound in mustard and wasabi. Fatty acid conjugates, including endocannabinoids found in the CNS (e.g. N-Arachidonoyl dopamine,

anandamide) and several phytocannabinoids (like CBD), are also potent activators of TRPV1 receptor channels. Besides CBD, here we tested the effect of capsaicin (0.1-1 μM) which did not significantly affect the release of [^{3}H]GABA and [^{14}C]glutamate from synaptosomes isolated hippocampi from adult rats, if one excludes a minor inhibitory effect on depolarization-evoked [^{14}C]glutamate release when capsaicin was used at the highest concentration tested (10 μM). Likewise, the competitive TRPV1 antagonist, capsazepine (10 μM), also failed to modify the release of both amino-acids, either under resting or after KCl depolarization of hippocampal synaptosomes. Pretreatment with capsazepine slightly, yet not significantly, decreased CBD-induced [^{3}H]GABA and [^{14}C]glutamate release from rat hippocampal synaptosomes under resting conditions. Altogether, these results suggest that binding of CBD to TRPV1 receptor channels plays a minor, if at all, effect on the outflow of [^{3}H]GABA and [^{14}C]glutamate induced by the cannabinoid from hippocampal synaptosomes of adult rats under these experimental conditions.

Next we set to pharmacologically confirm that reversal of Na^{+}-dependent high-affinity amino acid transporters is in fact mediating CBD-induced [^{3}H]GABA and [^{14}C]glutamate outflow from depolarized hippocampal synaptosomes. As expected, the EAAT glutamate transporter inhibitor, DL-TBOA, increased progressively the extracellular amount of [^{14}C]glutamate after the first depolarization stimulus (S1) delivered to adult rat hippocampal synaptosomes, without affecting the [^{3}H]GABA outflow measured in the same samples. On the contrary, the selective GAT-1 transport inhibitor, SKF 98876A, failed to modify the outflow of [^{3}H]GABA and/or [^{14}C]glutamate from hippocampal synaptosomes of adult rats.

Given the discrepancy in the results obtained with the two amino-acid transport inhibitors, DL-TBOA and SKF 98876A, we tested if the collapse of the Na^{+} gradient across the plasma membrane, by removing this cation from the extracellular milieu while keeping osmolality by replacing it by NMDG (128 mM), could reverse to the outward mode the function of GABA and glutamate transporters. Removal of external Na^{+} from the superfusion fluid progressively increased the release of [^{3}H]GABA from rat hippocampal synaptosomes, without concurrent accumulation of [^{14}C]glutamate in the extracellular fluid. Overall, these results indicate that high-affinity GABA transporters located in hippocampal nerve terminals are more vulnerable to the collapse of the transmembrane Na^{+} gradient compared to the glutamate transporters, as previously reported (Barros-Barbosa *et al*., 2015; 2018).

Pre-treatment with SKF 98876A and DL-TBOA significantly attenuated CBD-induced release of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats under resting conditions, respectively. These findings strengthen our hypothesis that reversal of GABA and glutamate transporters to the outward mode is mandatory to promote the outflow of [^{3}H]GABA and [^{14}C]glutamate from hippocampal synaptosomes of adult rats triggered by CBD. As a matter of fact, CBD-induced [^{3}H]GABA and [^{14}C]glutamate release was significantly potentiated when hippocampal synaptosomes were superfused in Na^{+}-free conditions, a situation that favors high-affinity amino-acid transport reversal in hippocampal nerve terminals.

As mentioned before, high-affinity [^{3}H]GABA transport reversal seems to be more sensitive to removal of extracellular Na^{+} by replacing it with NMDG than [^{14}C]glutamate release (Barros-Barbosa *et al.*, 2015; 2018), at least in the hippocampus of adult rats compared to that of immature animals. This renders the adult hippocampus more prone to the GABAergic inhibitory control. This scenario is fully reversed in the presence of CBD. That is, the potentiating effect of CBD (30 µM) on [^{3}H]GABA and [^{14}C]glutamate release in Na^{+}-free conditions had a higher magnitude in hippocampal synaptosomes of immature rats than in those isolated from adult animals. The ontogenic changes in hippocampal sensitivity to CBD detected in the present study agree with data from previous reports suggesting that the immature brain is by far more excitable than the adult brain. Deficient GABAergic-mediated inhibition, together with transient overexpression of glutamate receptors, may boost susceptibility to seizures in the immature brain.

Despite the advances reported in the present work, further studies are required (1) to elucidate the molecular trigger underlying CBD-induced increases in [^{3}H]GABA and [^{14}C]glutamate release from hippocampal nerve terminals (which apparently is not related to Ado signaling and TRPV1 receptors), and (2) whether fine-tuning control of resting GABA vis a vis glutamate release might contribute to the antiepileptic relevance of CBD in the treatment of pediatric epilepsy syndromes.

Chapter 6: Bibliographic References

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