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Antiseizure Effects of *Cannabis Sativa* L. Derivatives in Rodent Models: Targeted Hippocampal Regions and Hypothesized Mediators

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“Focal Seizures” reprinted from John Bramblitt with permission.
This painting was inspired because at the time John Bramblitt was having more seizures and was feeling disconnected from the world. Even after coming out of the seizures, it took a bit for his brain to orient, which is depicted in the painting. After 5 years of losing his sight due to seizures and a combination of Lyme disease, he drew this art piece.

- This thesis is dedicated to all the souls suffering from epilepsy -

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Abbreviations

ABC	ATP-binding cassette
ASMs	Anti-seizure medications
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BCSFB	Blood-cerebrospinal fluid barrier
CA1/3	Cornu ammonis 1/3
CBZ	Carbamazepine
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
C. sativa	*Cannabis sativa* L.
CNS	Central nervous system
CSF	Cerebrospinal fluid
DBS	Deep brain stimulation
DG	Dentate gyrus
DRE	Drug-resistant epilepsy
ECoG	Video-electrocorticography
EEG	Electroencephalography
FDA	Food and Drug Administration
GABA	γ-Aminobutyric acid
GPCRs	G-protein coupled receptors
HS	Hippocampal sclerosis
Hz	Hertz
ILAE	International League Against Epilepsy
IL-1β/6	Interleukin 1 beta/6

i.p.	Intraperitoneal
i.v.	Intravenous
KA	Kainic acid
KAR	Kainic acid receptor
mA	Milliampere
MCT	Medium chain triglyceride
MES	Maximum electroshock
MRP1/2	Multidrug resistance associated protein 1/2
ms	Milliseconds
mTLE	Mesial temporal lobe epilepsy
NINDS	National Institutes of Neurological Disorders and Stroke
p-ERK1/2	Phosphorylated extracellular signal-regulated kinases 1/2
P-gp	P-glycoprotein
PPARγ	Peroxisome proliferator-activated receptor gamma
PTZ	Pentylenetetrazole
SNP	Single nucleotide polymorphism
SRSs	Spontaneous recurrent seizures
SUDEP	Sudden unexpected death in epilepsy
s.c.	Subcutaneous
THC	(−)-*trans*-Δ^9-tetrahydrocannabinol
TLE	Temporal lobe epilepsy
TMS	Transcranial magnetic stimulation
TNFα	Tumor necrosis factor alpha
tVNS	Transcutaneous vagus nerve stimulation
UHPLC	Ultra-high-performance liquid chromatography
VNS	Vagus nerve stimulation

ZO	Zona occludens

Abstract (English Version)

Epilepsy affects approximately 1% of the overall population worldwide. Currently, 25%-30% of patients suffering from epilepsy are pharmacoresistant to all types of anti-seizure medication. For this reason, in the last decades alternative medicine has received more attention in the search for novel therapeutics. Recently, compounds found in *Cannabis sativa* L. have received great interest in the treatment of drug-resistant epilepsy. Cannabidiol (CBD) is successfully used as an add-on medication for infantile epilepsy including Lennox-Gastaut and Dravet syndrome and is thought to be a safe compound with few side effects. In addition, CBD-rich Cannabis extracts seem to present a beneficial effect on epileptic seizures. In detail, *C. sativa* extracts display a promising anti-seizure profile due to the synergistic effects of terpenes and other phytocannabinoids. In literature, an interaction was described between CBD and peroxisome proliferator-activated receptor gamma (PPARγ). The role of PPARγ has been well documented in metabolic disorders but has lately been studied for its involvement in neurological diseases. The neuroprotective activity of CBD in ischemic stroke and neurological disorders, including epilepsy, is hypothesized to be mediated by PPARγ.
In my thesis, I aimed to 1) evaluate the anti-seizure effects of CBD in the kainic acid model of temporal lobe epilepsy in rats, and if CBD effects were associated with PPARγ regulation. Additionally, I investigated the presence of regional specific effects in the hippocampal formation. and 2) determine the anticonvulsant activity of *C. sativa* extracts in the repeated 6-Hz corneal stimulation model in mice.
Particularly, in the first approach, the role of CBD was assessed in epileptic rats previously treated with kainic acid. Sixty-seven days after the induction of *status epilepticus* and the appearance of spontaneous recurrent seizures in all rats, CBD was administered subcutaneously (s.c.) twice a day for three days. In both approaches, the animals were evaluated by video-electrocorticographic recordings, behavioral analysis based on a modified Racine scale, and immunohistochemical staining. I secondly tested two hemp oils with different concentrations of terpenes in the modified repeated 6-Hz corneal stimulation model, which were administered by gavage 1h before the stimulation.
CBD abolished the seizures in 50% of rats and reduced total seizure duration and occurrence. After the administration of CBD at 12 and 120 mg/kg, PPARγ levels increased in the hippocampal CA1 subfield and subiculum. However, only the highest dose also increased the immunoreactivity in the hippocampal CA3 subfield, perirhinal cortex, and amygdala Further, both *C. sativa* extracts significantly diminished the percentage of mice experiencing convulsive seizures in comparison to olive oil-treated mice, but only the oil enriched with a high concentration of terpenes significantly accelerated full recovery from the seizure. These effects occurred in the presence of reduced power of delta rhythm, and, instead, increased power of theta rhythm, along with a lower FosB/ΔFosB expression detected in the subiculum.
Overall, these findings suggest that the antiseizure effects of CBD are characterized by a regional specificity and are associated with an upregulation of PPARγ in the hippocampal CA3 region. Finally, both cannabinoids and terpenes in oil extracts should be considered as potential therapeutic agents against epileptic seizures and epilepsy.

Key words: *Cannabis sativa* L.; cannabidiol; peroxisome proliferator-activated receptor gamma; drug-resistant epilepsy; anti-seizure medication

Abstract (Italian version)

Titolo in italiano: Effetti anticonvulsivanti dei composti presenti in *Cannabis sativa* L. in modelli sperimentali con roditori: Regioni ippocampali coinvolte e possibili mediatori

L'epilessia colpisce circa l'1% della popolazione mondiale. Attualmente, circa il 30% dei pazienti affetti da epilessia è farmacoresistente a qualsiasi classe di farmaci antiepilettici. Per questo motivo, negli ultimi decenni composti alternativi e di potenziale rilevanza terapeutica hanno ricevuto maggiore attenzione nella ricerca di nuove terapie. Recentemente, i composti presenti nella *Cannabis sativa* L. hanno riscosso un grande interesse nel trattamento dell'epilessia farmacoresistente. Il cannabidiolo (CBD) è usato con successo come farmaco aggiuntivo per il trattamento di pazienti in età pediatrica affetti da sindrome di Lennox-Gastaut e Dravet, in quanto si pensa che sia un composto sicuro con pochi effetti collaterali. Inoltre, gli estratti di Cannabis ricchi di CBD sembrano avere un effetto benefico sulle convulsioni epilettiche. Infatti, si ipotizza che gli estratti di Cannabis presentino possibili proprietà anticonvulsivanti grazie all'effetto sinergico dei terpeni e di altri fitocannabinoidi. In letteratura, è stata descritta una interazione tra CBD e il recettore gamma attivato dai proliferatori dei perossisomi (PPARγ). Il ruolo di PPARγ è stato ben documentato nei disordini metabolici e più recentemente è stato studiato il suo coinvolgimento nelle malattie neurologiche. L'attività neuroprotettiva del CBD nell'ictus ischemico e nei disturbi neurologici, tra cui l'epilessia, si suggerisce essere mediata da PPARγ.
Nella mia tesi di dottorato di ricerca, l'obiettivo è quello di: 1) valutare gli effetti antiepilettici del CBD in ratti affetti da epilessia del lobo temporale, precedentemente esposti ad acido cainico, e se gli effetti di CBD siano associati alla regolazione di PPARγ. Inoltre, ho studiato la presenza di effetti regionali specifici nella formazione dell'ippocampo e 2) determinare una possibile attività anticonvulsivante degli estratti di *C. sativa* in un modello in cui le crisi epilettiche sono indotte da ripetute stimolazioni elettriche corneali alla frequenza di 6 Hz nei topi.
In particolare, è stato valutato il ruolo del CBD nei ratti epilettici precedentemente trattati con acido cainico. Sessantasette giorni dopo l'induzione dello stato di male epilettico e la comparsa di convulsioni spontanee e ricorrenti in tutti i ratti, il CBD è stato somministrato per via sottocutanea due volte al giorno per tre giorni. In entrambi gli approcci, gli animali sono stati valutati mediante registrazioni video-elettrocorticografiche, analisi comportamentali basate sull'utilizzo di una scala di Racine modificata, e analisi immunoistochimiche. Nel secondo approccio, ho testato due oli di canapa con diverse concentrazioni di terpeni nel modello in cui le crisi epilettiche sono indotte da ripetute stimolazioni elettriche corneali alla frequenza di 6 Hz, somministrandoli mediante sonda gastrica 1 ora prima della stimolazione.
l CBD ha abolito lo sviluppo delle convulsioni epilettiche nel 50% dei ratti e ha ridotto la durata e il numero totale delle crisi di tipo convulsivo. Dopo la somministrazione di 12 e 120 mg/kg di CBD, i livelli di PPARγ risultano essere aumentati nella regione CA1 dell'ippocampo e nel subiculum. Oltre a ciò, la dose più elevata di CBD si è dimostrata efficace nell'indurre un aumento dei livelli di PPARγ nella regione CA3 dell'ippocampo, nella corteccia peririnale e nell'amigdala.

Inoltre, entrambi gli estratti di *C. sativa* hanno ridotto significativamente la percentuale di topi che hanno avuto crisi di tipo convulsivo rispetto ai topi trattati con il solo olio d'oliva, ma solo l'olio arricchito con un'alta concentrazione di terpeni ha accelerato significativamente il pieno recupero delle normali attività dopo la crisi epilettica. Questi effetti si sono verificati in presenza di una riduzione delle onde cerebrali di tipo delta e, al contrario, un aumento delle onde di tipo theta, insieme a una ridotta espressione di FosB/ΔFosB nel subiculum.
In conclusione, questi risultati suggeriscono che gli effetti antiepilettici del CBD sono caratterizzati da una specificità regionale e, infine, possano essere associati ad un aumento dei livelli di PPARγ nella regione CA3 dell'ippocampo. Infine, si suggerisce che sia i cannabinoidi che i terpeni negli estratti di olio di canapa dovrebbero essere considerati come potenziali agenti terapeutici contro le crisi epilettiche e l'epilessia.

Parole chiavi: *Cannabis sativa* L.; cannabidiolo; recettore gamma attivato dai proliferatori dei perossisomi; epilessia farmacoresistente; farmaci antiepilettici

Chapter 1

Epilepsy: General Introduction

The history of epilepsy is as old as human existence. The first documentation of an epileptic seizure appeared 2000 BC in a text on an Akkadian tablet found in the region of Mesopotamia. The engraving describes a person with seizure-like symptoms: "*his neck turning left, hands and feet are tense, and his eyes wide open, and from his mouth froth is flowing without him having any consciousness*" [1]. Nearly one thousand years later Neo-Babylonians further described epilepsy in a manuscript named BM47753. This tablet is part of a diagnostic handbook, the Akkadian medical diagnostic series named Sakikku, which are composed of 40 tablets divided into six chapters. With the 26th tablet the description and categorization of seizure types and epilepsy based on their appearance began. Further, it is thought that the Babylonian already had a way of understanding prognostics, documenting changed behavior after different seizure types and poor cure after experiencing a *status epilepticus* (SE). It was believed that the observed uncontrolled contractions were brought on by evil and divine spirits infiltrating the body. Therefore, the practice of exorcism and spiritual healing was frequently used as a treatment [2]. Later accounts for epilepsy could be found in the Edwin Smith surgical papyrus captured in 1700 BC in ancient Egypt. A person with an open wound on the head was described to exert seizure-like effects as to "*shudder exceedingly*", making the ancient Egyptians the first culture who recognized the fact that seizures can be caused by brain damage and hence distinguishing them from the Mesopotamians who still were in belief that the origin of seizures lied in the punishment of evil spirits and gods. In fact, this report may be the first documentation of focal seizures caused by cortical irritation resulting from an injury [3]. Ancient manuscripts of all different civilizations include numerous allusions to epilepsy, but especially Greek medical texts form the basis of our present knowledge about the disease. The word epilepsy derives from the Greek verb *epilambanein* (επιλαμβανεΐνη), which means to seize, take hold of or attack. The father of medicine, Greek physician Hippocrates, was one of the first to link epilepsy to the brain and proposed that it is inherited rather than a communicable disease, as described in the book *On the Sacred Disease*. Hippocrates believed that the sacred disease (epilepsy) was "*no more divine than any other*" but was given the epithet "sacred" because of its peculiar and enigmatic physical characteristics. He further suggested that seizures themselves could be cured, but once they will develop to the chronic phase there is no remedy [4]. Hippocrates was one of the first to describe a non-spiritual cause for epilepsy, but rather a dysfunction of the brain, however, his theory had little impact on the belief in the supernatural for the following hundreds of years.

The idea of epilepsy being a neurological disorder gained again attraction in the 18th century with Samuel Tissot, William Cullen and Jules Maisonneuve representing physicians who first accepted the principles of Hippocrates in the Age of Enlightenment. The necessity of hospitalization of patients suffering from seizures or epilepsy and adequate neurological treatment were first recognized by these physicians [5–7]. Later in 1849, Dr. Robert Bentley Todd proposed that the brain uses electricity to function and proposed that seizures might result from "electrical discharges" in the brain [8]. Using the magnetoelectric rotation apparatus of Michael Faraday on

rabbits, he was able to prove his theory [9]. In 1870, John Hughlings Jackson published his pivotal treatise "Study of Convulsion" stating his findings about the physiology of focal epilepsy and the location of lesions that could cause seizures - creating the scientific basis for epileptology [10,11]. In 1924, Hans Berger developed the electroencephalogram (EEG), enabling him to confirm that convulsions were caused by aberrant electrical activity in the brain [12]. The discovery of EEG was a historical breakthrough allowing a new neurological diagnostic tool which is almost 100 years later still one of the most frequently used and indispensable instrument to monitor seizure activity [13].

Now, in 2022, we know that epilepsy is a noncommunicable chronic neurological disorder which is characterized by the recurrence of spontaneous unprovoked seizures [14]. This minimal criterion is reflected by the often-quoted clinical rule: "A single seizure does not epilepsy make" [15]. Notably, its prevalence rate of 0.5% to 1.0% within the world's population ranks epilepsy in fifth place among neurological disorders after stroke, migraine, dementia, and meningitis [16,17]. The incidence of epilepsy occurs predominantly in younger age groups (infancy and early childhood) and older age groups (circa more than 50-60 years of age). But the prevalence of epilepsy shows no trend towards age. No sex differences in incidence or prevalence can be proved. However, some studies have shown a male preponderance, possibly as a result of women under-reporting the disease, especially in areas where a woman with epilepsy would be marginalized or considered ineligible for marriage if her diagnosis would be public [18–20]. Besides, epilepsy displays a high impact in psychological, social and economic conditions affecting patients and their families tremendously apart from the problems of the disease itself [21]. Notably, an individual is considered to have epilepsy if there is an appearance of any of the following conditions proposed by the International League Against Epilepsy (ILAE) task force: *"(i) at least two unprovoked (or reflex) seizures occurring > 24 h apart; (ii) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; and (iii) diagnosis of an epilepsy syndrome*". A person with epilepsy is considered cured when in the last 10 years no seizures occurred and no need of anti-seizure medication (ASM) intake was necessary for the last 5 years, or suffered from an age-dependent epilepsy syndrome, but have now passed the relevant age [22]. Seizures are specified as paroxysmal electrical discharges from diverse brain regions that cause molecular, physiological, cognitive, and social impairment. The imbalance of the activation of excitatory and inhibitory synapses due to a variety of factors, such as hereditary abnormalities, stroke, infections, traumas, etc. is regarded as a possible origin of seizures [15]. Finally, comorbidities display a significant negative impact on patients with epilepsy. Several diseases, such as depression, anxiety, dementia, migraine, heart disease, and arthritis are up to eight times more frequent in people suffering from epilepsy than in the healthy population. Epilepsy and comorbid conditions are linked by a variety of mechanisms, including i.e., reciprocal influences and common risk factors [23]. Therefore, patients are in desperate need of finding the most adequate therapy approach to achieve the ultimate goal – freedom from seizures.

1. Classification of seizure and epilepsy

In 2017, the task force of ILAE updated the terminology and classification of seizures and epilepsies [24,25]. The new revision is based on the classification of seizures and epilepsies published in 1981 and 1989, respectively [26,27]. This current classification was modified to create a better understanding for clinicians as well as patients, changing several terminologies, as i.e., partial to focal, grand mal to generalized tonic-clonic, and psychomotor and dyscognitive to focal impaired awareness. Moreover, it has been improved to be more logically structured and to include new seizure types, as well as the addition of the latest discovered mechanisms underlying epilepsies and, therefore, displaying enhanced approaches to diagnose and treat individuals [28]. In this current classification, clinical characteristics of epilepsy are divided into three levels: seizure types, epilepsy types and epilepsy syndrome (Figure 1). Finding the type of seizure is always the first step in the diagnosis of epilepsy, which is then followed by identification of the type of epilepsy. Epilepsy might subsequently be categorized as epilepsy syndrome based on further clinical data and available research findings [28].

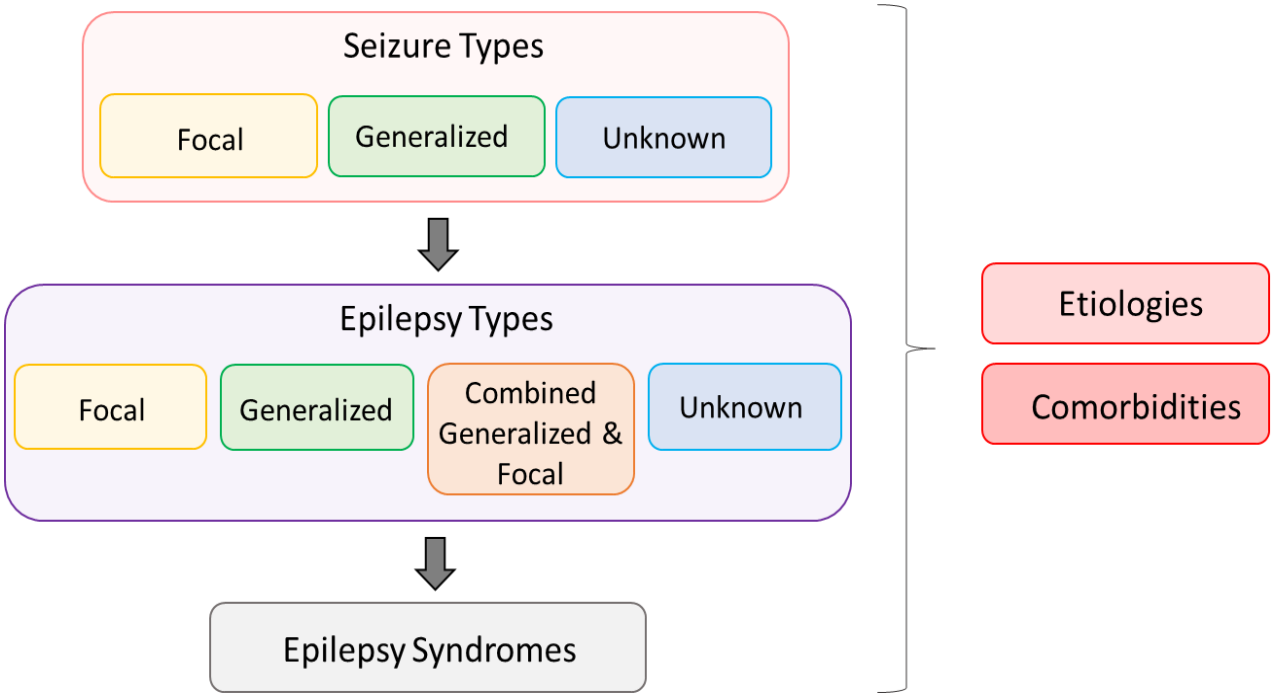

Figure 1. 2017 ILAE classification of epilepsies. Modified from Scheffer et at. (2017) [28].

The early classification of epilepsies has an impact on every clinical consultation but is also significant for basic and clinical epilepsy research as well as the creation of new treatments. The classification displays numerous benefits, including giving a framework to understand the patient's particular type of seizure, the other seizure types that are more likely to occur in that person, the causes of the seizures, as well as the prognosis. The risk of comorbid conditions such as learning and intellectual disability, mental/psychiatric characteristics such as autism spectrum disorder

(ASD), and the risk of mortality such as sudden unexpected death in epilepsy (SUDEP) are also determined by this classification. It is noteworthy that classification frequently guides the choice of anti-seizure therapies [28].

- *Seizure Types*

The seizure classification begins with the evaluation of whether the initial manifestation of the seizure is of focal, generalized, or unknown origin, which is presented in the revised classification of seizures by the ILAE (Figure 2). When a patient's EEG exhibiting epileptiform activity or an epileptogenic abnormality can be observed during brain imaging, the likelihood of experiencing recurrent seizures can be considered [24,25,29].

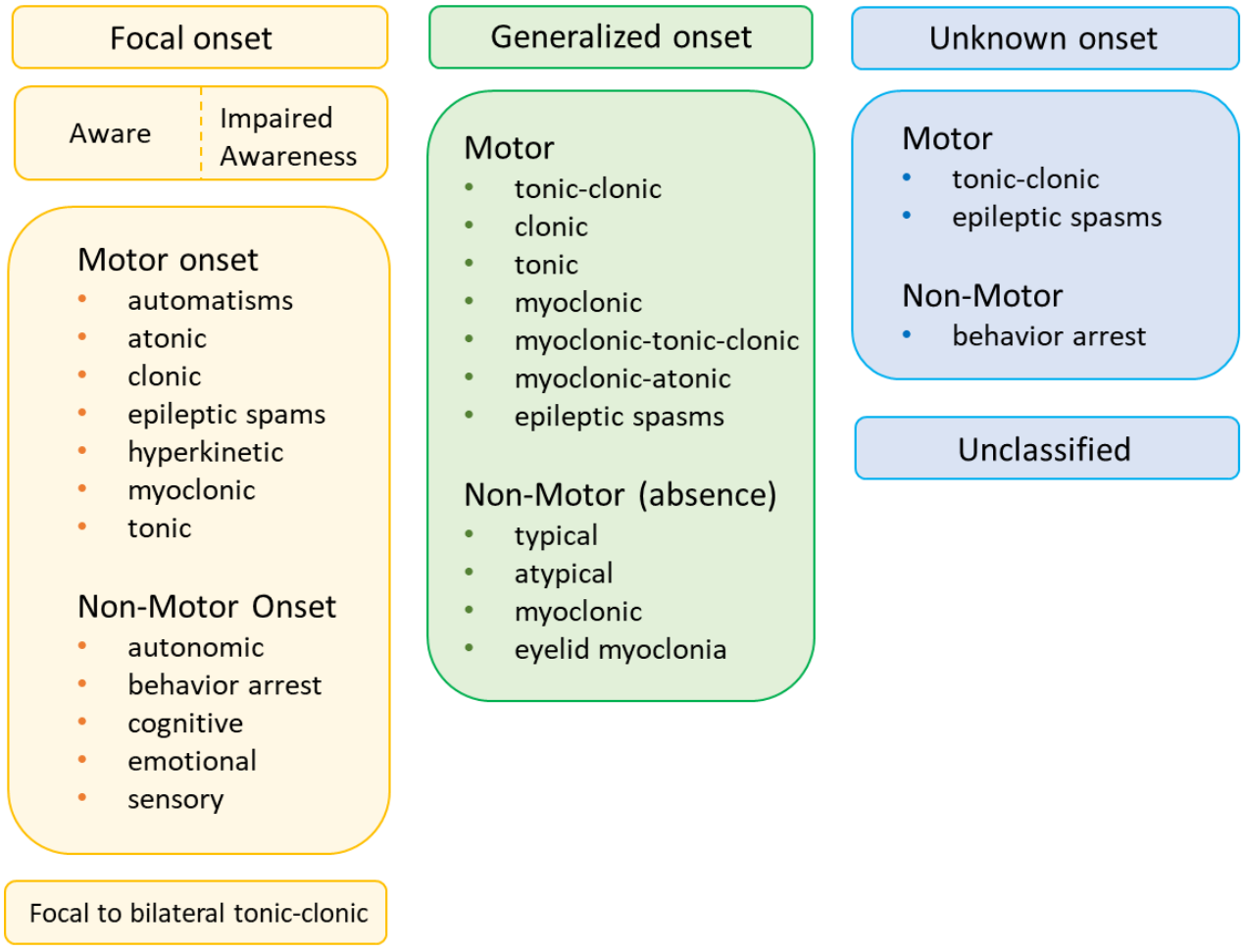

Figure 2. The expanded ILAE 2017 operational classification of seizure types. Modified from Fisher et al. (2017a, 2017b) [24,25].

Seizures are described as *focal* when abnormal electrical activities arise from one side of the cerebral hemisphere, which can spread to the other side. Focal seizures can be classified by the level of awareness. In this context, awareness represents the knowledge of self and environment.

In detail, it refers to the fact that a patient is aware during the seizure, and not aware if a seizure occurred or not. Focal aware or focal impaired awareness can further be classified into motor onset or non-motor onset. The seizure type *focal to bilateral tonic-clonic* replaces the old term *secondarily generalized tonic-clonic* and is considered as a propagation pattern of seizure activity rather than a single seizure type, therefore presented in a special category. The types of *focal motor* seizures are described in the following table 1.

Table 1. Types and glossary of focal motor seizures [24,25,28–30].

Automatisms	coordinated and repeated motor activity usually occurs when cognition is impaired and is followed by amnesia afterwards. This often resembles a voluntary movement and may consist of an inappropriate continuation of preictal motor activity.
Atonic	a sudden loss or diminution of muscle tone that occurs suddenly without apparent preceding myoclonic or tonic event lasting approx. 1-2 sec, involving head, trunk, jaw, or limb musculature and possible only on one side of the body.
Clonic	rhythmical jerking, either symmetric or asymmetric, that is regularly repetitive and involve the same muscle groups.
Epileptic Spasms	sudden und controlled and often painful flexion, extension, or mixed extension–flexion of predominantly proximal and truncal muscles. Limited forms may occur including grimacing, head nodding or subtle eye movements. Epileptic spasms may frequently occur in clusters and can be focal, generalized, or of unknown onset. Infantile spasms are the best-known form but can also occur at all ages.
Hyperkinetic	exaggerated, and often uncontrolled muscles activities such as agitated kicking, trashing and peddling during the seizure.
Myoclonic	similar to clonic seizures but sudden and brief (< 100 msec) involuntary single or multiple contraction(s) of muscles(s) or muscle groups of a variable topography (axial, proximal limb, distal) and is less regularly repetitive and less sustained than clonus is.
Tonic	sustained increase in muscle contraction that lasts for a few seconds or minutes and presenting clinically as stiffening of a limb or the neck.

Types of *focal non-motor* seizures are structured in table 2.

Table 2. Types and glossary of focal non-motor seizures [24,25,28–30].

Autonomic	a distinct alteration of autonomic nervous system function involving cardiovascular, pupillary, gastrointestinal, sudomotor, vasomotor and thermoregulatory functions and presenting symptoms such as rising sensation in the stomach, hot and cold feelings, a strange taste or smell.

Behavior arrest	arrest of activities, freezing, immobilization and unresponsiveness for the entire duration of the seizure.
Cognitive	impaired language or other cognitive domains or positive features such as déjà vu, hallucinations, illusions, or perceptual distortions.
Emotional	beginning with an emotion such as fear, panic, anxiety, spontaneous joy or euphoria, laughing and crying.
Sensory	a perceptual abnormal experience including visual, olfactory, auditory and gustatory sensations, which are not caused by stimuli of the environment.

Generalized-onset seizures are classified by motor and non-motor (absence) seizures. The categorization of aware and impaired awareness is not used for generalized seizures since the majority of generalized seizures are associated with impaired awareness. Generalized seizures are caused by abnormal neuronal activity that starts in a widespread distribution over both hemispheres at the same time. Some types of motor seizures have already been defined under focal motor seizures. Additional types of generalized motor seizures are specified below in table 3.

Table 3. Types and glossary of generalized motor seizures [24,25,28–30].

Tonic-clonic	a sequence consisting of a tonic followed by a clonic phase.
Myoclonic-tonic-clonic	one or a few jerks of limbs bilaterally. Characterized by arms jerking, then tonic stiffening, and lastly, clonic rhythmical jerking. Common in juvenile myoclonic epilepsy.
Myoclonic-atonic	a generalized seizure type with a myoclonic jerk leading to an atonic motor component. This type was previously called myoclonic–astatic. Often seen in patients with the Doose syndrome.

Further, types of non-motor seizures have already been presented in table 2. Additional types of generalized non-motor (absence) seizures are listed in the subsequent table 4.

Table 4. Types and glossary of generalized non-motor (absence) seizures [24,25,28–30].

Absence, typical	a sudden onset, interruption of ongoing activities, a blank stare, possibly a brief upward deviation of the eyes. Usually, the patient will be unresponsive when spoken to. Duration is a few seconds to half a minute with very rapid recovery. EEG would show generalized epileptiform discharges during the event.
Absence, atypical	changes in tone that are more pronounced than in typical absence or the onset and is often associated with slow, irregular, generalized spike-wave activity.
Eyelid myoclonia	jerking of the eyelids at frequencies of at least 3 per second, commonly with upward eye deviation, usually lasting < 10sec with possible loss of awareness.

Seizures could be classified as unknown and are categorized as motor, including tonic–clonic, non-motor, or unclassified. Seizures of unknown onset are those that might happen while the patient is alone, or that occur in sleep, or in a situation that cannot be reported by a person witnessing the seizure.
Finally, both seizures with patterns that do not match the criteria of other categories and seizures with inadequate information to allow classification fall under the umbrella term unclassified [24,25].

- *Epilepsy Types*

Epilepsy can be divided into four main types: 1. focal; 2. generalized; 3. combined generalized and focal and 4. unknown (Figure 1).
Focal epilepsy can manifest as any type of focal seizure and is a continuum of seizures, unifocal and multifocal disorders involving one hemisphere. It includes a variety of seizure types such as focal aware seizures, focal impaired awareness seizures, focal motor seizures, focal non-motor seizures, and focal to bilateral tonic–clonic seizures. The diagnosis is based on clinical factors, however, an interictal EEG typically showing focal epileptiform discharges supports the right diagnosis.
Similarly, a patient with generalized epilepsy can have several types of seizures including absence, myoclonic, atonic, tonic, and tonic–clonic seizures. The diagnosis of generalized epilepsy is confirmed by generalized spike-wave activity seen on the EEG, besides the clinical manifestations.
The new category defined by combined generalized and focal epilepsy implies the appearance of both generalized and focal epilepsy in one patient and is usually identified by video electroencephalography (VEEG). This combined epilepsy type usually occurs in severe childhood and infantile epilepsy such as Lennox-Gastaut and Dravet syndrome. The interictal EEG may show both generalized spike-wave and focal epileptiform discharges; however, the epileptiform activity is not necessary for the diagnosis.

The epilepsy type is referred to as unknown when the clinician, due to a lack of information and data, is unable to formulate a precise diagnosis [28].

- *Epilepsy syndrome*

The third level of classification of epilepsies is an epilepsy syndrome diagnosis. An epilepsy syndrome is a collection of factors that includes seizure types, EEG patterns, and imaging features that frequently co-occur. Often, it displays age-dependent characteristics such as age onset and remission, seizure triggers, diurnal variation, and sometimes prognosis [27,31]. Along with different EEG and magnetic resonance imaging (MRI) findings, an epilepsy syndrome could also show distinct comorbidities such intellectual and mental impairment. Further, associated etiologic, prognostic, and treatment implications cannot be excluded. For instance, childhood absence epilepsy, West syndrome, and Dravet syndrome are a few well-known epilepsy syndromes. Notably, a formal classification of epilepsy syndromes has never been established by the ILAE. In the majority of cases though, diagnosis of an epilepsy type is the final level of diagnosis achievable [28,32].

2. Etiology

The etiology of epilepsy is a significant factor affecting the clinical course and prognosis, yet epilepsy has no identifiable cause in circa 50% of patients. Causes of epilepsy are unique and different for each individual, while some people have no identifiable explanation. In others, doctors can trace epilepsy directly to the six major etiologic categories defined by the ILAE task force: 1. structural; 2. genetic; 3. infectious; 4. metabolic; 5. immune and 6. unknown. Multiple etiologic categories may apply to a patient's epilepsy [28].

Structural etiology is used to describe abnormalities that can be seen on structural neuroimaging when the electroclinical evaluation together with the imaging findings support a reasonable conclusion that the imaging abnormality is the most probable cause for the patient's seizures [28]. However, adequate MRI tests employing particular procedures to detect even small structural lesions is necessary [33]. Structural etiologies can be genetic, including numerous anomalies of cortical development, or induced, such as in the case of stroke, trauma, and infection [32]. A key association of structural etiology with epilepsy can be often seen in findings of mesial temporal lobe seizures with hippocampal sclerosis (HS) [34].

Genetic etiology occurs when epilepsy directly results from a genetic mutation and when seizures are a primary symptom of the disease. A family history of an autosomal dominant condition alone may be sufficient to establish a genetic origin. In other cases, the contribution of a genetic etiology is supported by clinical research in populations with the same syndrome such as childhood absence epilepsy or juvenile myoclonic epilepsy. Lastly, it can be based on the molecular evidence that links a single gene or copy number variation to a significant effect [35]. Until now, there are several genes known to be associated with epilepsy, including i.e., *KCNQ2*, *SCN9A* and *CLCN2* [36].

Infectious etiology is the most common cause for epilepsy worldwide. Infectious etiology results directly from the infection in which seizures are a core symptom of the disorder and refers to a

patient with epilepsy rather than the cause of the seizure itself following acute infection such as meningitis or encephalitis. Some typical examples include neurocysticercosis, tuberculosis, HIV, cerebral malaria, subacute sclerosing panencephalitis, cerebral toxoplasmosis and congenital infections such as Zika virus [28].

Metabolic etiology refers to the fact that a metabolic disorder can cause epilepsy in a patient. Metabolic disorders associated with epilepsy include glucose transporter member 1 (GLUT1) deficiency, adenylosuccinate lyase (ADSL) deficiency, and serine biosynthesis defects [18,28,37]. Importantly, most cases of metabolic epilepsies are due to genetic defects [38].

Immune etiology is used to explain that an auto-immune disorder is the cause of new-onset epilepsy. A variety of immune epilepsies, secondary to multiple sclerosis (MS) and auto-immune encephalitis, have been described showing a characteristic presentation in both children and the elderly. Both neuronal intracellular antibodies (GAD65, ANNA-1, and Ma) and neuronal cell surface antibodies (VGKC complex, NMDAR, AMPA, GABA-B, and GluR5) have been connected to auto-immune encephalitis and epilepsy [28,39,40].

Epilepsy of unknown etiology refers to the fact that its cause cannot be identified. Available tools and the extent to which a cause can be evaluated depends on the patient itself, the health care system, and country of examination. In resource-constrained countries the percentage of registered epilepsies of an unknown cause are higher than in developed and richer countries [28].

3. Temporal lobe epilepsy (TLE)

Temporal lobe epilepsy (TLE) is a partial form of epilepsy that originates in the temporal lobe, and can spread throughout a network of neuronal interconnections to the adjacent brain tissue [41]. TLE is the most common form of refractory focal epilepsy, which applies to approx. 40% of epilepsy patients [42]. In 1989, TLE was included in the classification of the ILAE under the group of localization-related symptomatic epilepsies [27]. It distinguishes two main types of TLE: the first and most common type is the mesial temporal lobe epilepsy (mTLE), in which the crisis originates principally in temporal medial structures including the hippocampus, entorhinal cortex, amygdala, and parahippocampal gyrus. The second and less frequent type is lateral or neocortical epilepsy (lTLE; nTLE), where the onset of seizures is localized in the temporal neocortex. The structures involved in lTLE/nTLE include the superior, medial, and inferior temporal circumvolutions, the temporal-occipital and temporal-parietal junctions, and the associative sensorial areas for hearing, visual, and language functions [27,43]. Furthermore, the ILAE divides TLE by ictal and interictal EEG recordings, plus classifies three forms of seizures that develop in TLE: 1. simple partial seizures (SPS), characterized by retention of consciousness; 2. complex partial seizures (CPS), showing a disruption of normal awareness, and 3. secondarily generalized tonic-clonic seizures. SPS are common in mTLE and are usually associated with HS which is a common neuropathological finding in mTLE patients who exhibit hippocampal cell loss in the cornu ammonis 1 and 3 (CA1 and CA3) hippocampal regions and hilus of the dentate gyrus (DG) [26,44]. Moreover, patients suffering from

mTLE have an increased incidence of genetic history of epilepsy in the family and display higher possibilities of developing febrile seizures and SE [45].
SE is defined as "*a seizure that lasts for at least 5 minutes, or a seizure that consists of two or more sequential seizures without full recovery between seizures*" [46]. After an initial insult, usually after several years (latent period) EEG alterations and spontaneous recurrent seizures (SRSs) might occur. In patients affected by mTLE, SRSs often start in late infancy or early adolescence and these patients might often be declared as pharmacoresistant [47]. Studies of patients with chronic TLE have shown to manifest characteristic emotional changes, which occur only during the seizures, the so-called "ictal phenomena." This phase is characterized by feelings of unreality, déjà vu and visual or auditory hallucinations. Ictal-EEG is identified by focal rhythmic activity in the theta range (5-9 Hz) with maximum amplitude in the basal temporal electrodes, preceded or not by bilateral hypersynchronous slowdown. Whereas more enduring emotional alternations are evident when the patient is not having a seizure, the so-called "interictal phenomena", that come mainly with an increase in aggression and extreme emotions. In this phase the interictal-EEG indicates more frequently unilateral spike-wave, normally located in the anterior temporal region [48]. Besides neurological characteristics, TLE is often associated with psychiatric comorbidities. In particular, the prevalence of mental conditions, such as anxiety, depression and cognitive, learning, and behavioral impairment, is higher in patients with TLE than in generalized epilepsy, especially in children (79 vs. 47%) [49].

4. Pharmacoresistance

In 1881, the well-known book "Epilepsy and other chronic convulsive diseases" by Sir William R. Gowers has already provided an early clue that pharmacoresistance is a dynamic objective that requires continuous development of a variety of ASMs [50]. The term "pharmacoresistant epilepsy", is equally referred to as "drug-resistant epilepsy" (DRE), "refractory epilepsy" or "medical intractable epilepsy", implying the often chronic, lifelong failure of ASMs. Due to the lack of a uniform and precise definition of DRE in the last decades, the ad hoc task force of ILAE published a consensus proposal in 2009. Accordingly, DRE is categorized in two levels: Level 1 defines the result of every therapeutic treatment as seizure free or intervention failure. According to this plan, the patient was adequately treated, indicating that the ASMs were suitably chosen, well tolerated, and appropriately administered. Level 2 defines the lack of efficacy of two therapies, whether as monotherapies or in combination to achieve sustained seizure freedom [51]. Even tough significant progress was made in the development of novel ASMs in the last twenty years, approximately 30% of patients diagnosed with epilepsy remain drug-resistant [52]. Seventy percent of patients diagnosed appropriately achieve seizure freedom, from which 50% after the initial monotherapy, 10% using alternative medications and 10% with first add-on drugs or polytherapy. Failure of the first two ASMs decreased the likelihood to succeed to 5-10% [53].
Inappropriate triage and/or dose of ASMs and misdiagnosis of DRE are common issues for treatment failure. To start an adequate therapy for patients suffering from refractory epilepsy,

pseudopharmacoresistance, defined as persistent seizures resulting from inappropriate or inadequate treatment, should be ruled out [54]. Another cause that could lead to “false pharmacoresistance” is the inability to correctly classify the seizure or epilepsy type and misdiagnosis of psychogenic non-epileptic events as seizures, which make up to 25% of treated patients [55].

In this regard, invasive treatments are often considered as the last resort for patients suffering from refractory epilepsy. There are diverse approaches of resective surgeries for different types of epilepsy [56]. Execution of early surgery of DRE patients have shown to lead to an higher success rate of post-surgery remission [57]. Even tough clinical studies are limited to randomized control trials of TLE, resection of lesions have shown to be more effective regarding seizure freedom than the continuous administration of ASMs [58]. For patients not eligible for surgery, neuromodulation treatments could be considered as a promising option. Up to date, there are several neurostimulation techniques available for pharmacoresistant epilepsy, some of which are non-invasive with no use of a permanent implant, or invasive, which require the surgical implantation of a device. The most investigated invasive neurostimulation strategies are vagus nerve stimulation (VNS) and deep brain stimulation (DBS). Non-invasive options are for example transcutaneous vagus nerve stimulation (tVNS) and transcranial magnetic stimulation (TMS) [59]. The moderate outcomes of VNS, for example, can range from a 50% reduction of seizure severity and frequency to a 15% rate of total seizure freedom [60–62]. However, some patients suffered from respiratory side effects during sleep, hoarseness, couching and throat pain [63,64]. Another alternative could be the ketogenic diet (KD), a dietary modification to a high-fat/low-carbohydrate food intake used mostly in children with juvenile DRE [65]. Over 50% reduction in seizures occurred in almost half of adults and children who switched to KD [66]. The compliance of children in strictly following this dietary plan in their normal daily routine, as well as adverse side effects such as hyperlipidemia, vomiting, bloating and cold reveals just a few challenges concerning this therapy [67].

Notably, in patients with pharmacoresistant epilepsy the probability is 27-fold higher to die from sudden death compared to the general population, independent of age and sex [68]. Sudden unexpected death in epilepsy (SUDEP) is described as sudden and unexpected death of a person suffering from epilepsy who is otherwise healthy and without a trauma or toxicological cause of death [69]. SUDEP is the most prevalent cause of mortality among patients with chronic refractory epilepsy ranging from 1.2 to 6.3 cases per 1000 individuals annually [70–72]. In addition, persons with DRE are susceptible to other factors of premature death, as for example seizure-related accidents, cerebral neoplasms, cardiovascular, gastrointestinal, and pulmonary disorders, which may be directly linked to the cause of epilepsy or comorbidities [73,74].

4.1. Possible Mechanisms of Pharmacoresistance

The knowledge of the underlying mechanisms of pharmacoresistance is - without doubt - crucial to design new medications to potentially overcome drug-refractoriness in epilepsy patients. The pathogenesis of DRE is likely variable and multifactorial, dependent on environmental and genetic factors, which might explain different responses to ASMs in treated patients, who suffer from the same type of seizures or epilepsy [54,75]. There are several hypothesized mechanisms of

pharmacoresistance. In the section below, the following hypotheses will be analyzed: (i) the transporter hypothesis, (ii) the target hypothesis, (iii) the gene variant hypothesis, (iv), the intrinsic severity hypothesis, (v) the pharmacokinetic hypothesis and (vi) the neural network hypothesis (Figure 3) [76]. Although there is evidence supporting the data of the aforementioned hypotheses, conflicts, and poor implementation of the results in clinical care should be considered as major limitations.

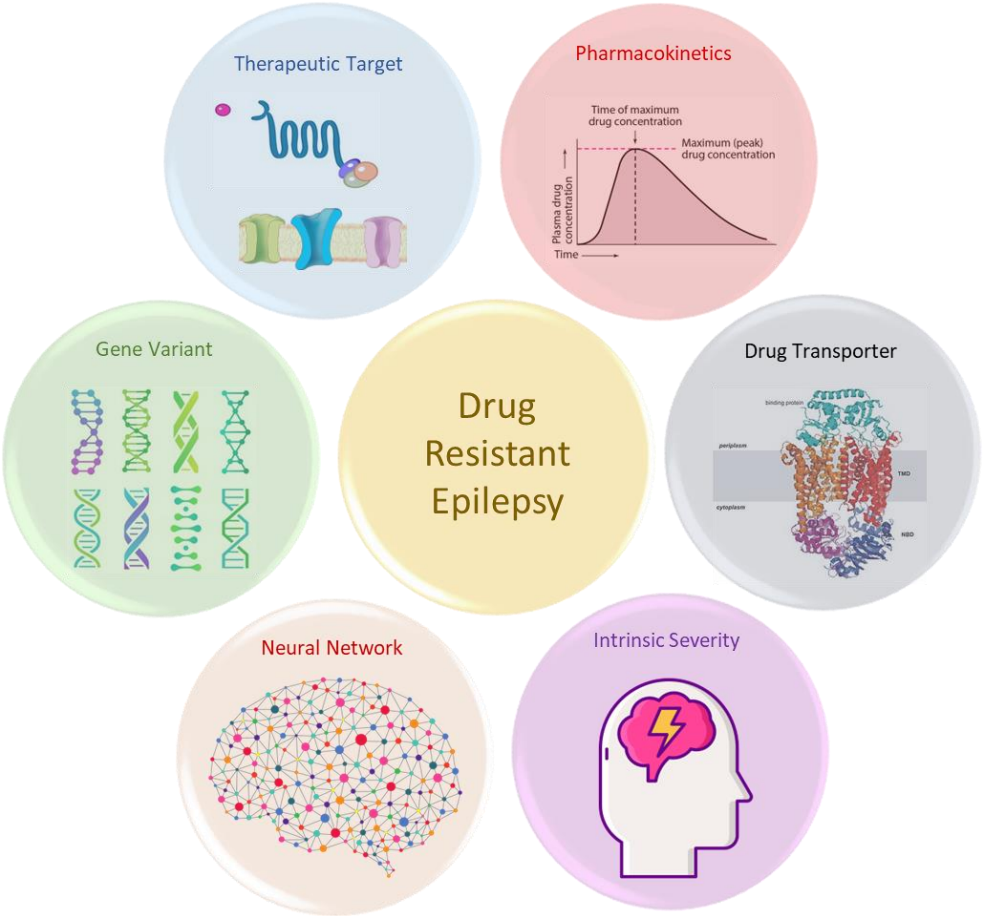

Figure 3. Illustration of the six major hypotheses for potential mechanisms of drug resistant epilepsy. Modified from Pérez-Pérez et al. (2021) [76].

The *multidrug transporter hypothesis* is based on the idea that excessive efflux transporter expression occurs across the BBB and therefore reducing the level of ASMs that arrives to the sites of action [77]. The best studied efflux transporters are members of the ABC (adenosine triphosphate (ATP)-binding cassette) superfamily subfamilies B, C, and G, specifically P-glycoprotein (P-gp, ABCB1 or MDR1), the multidrug resistance associated proteins (MRP1, ABCC1; MRP2, ABCC2), and breast cancer resistance protein (BCRP, ABCG2) [78,79]. P-glycoprotein and other members of the ABC transporter family are ATP-dependent pumps, that are expressed in the BBB or the blood-cerebrospinal fluid (CSF) barrier (BCSFB) [80]. These transporters are known to actively

mediate the transport of molecules against their concentration gradient out of cells and tissues to limit the penetration of substrates in the brain and protect from intoxication [81]. This process underlies the failure of treatment for a variety of brain infections, brain tumors, and other neurological disorders [82]. A result of ABC transporter overexpression can be seen in cancer patients, in which P-gp, BCRP, and several other MRPs prevent the entry of chemotherapeutic substances into cancer cells, leading to a poor prognosis for those patients [83]. The first evidence of *MDR1* mRNA overexpression was shown by Tishler et al. in 1995, in resected brain tissue from patients with refractory focal epilepsy. 11 out of 19 brain specimens exhibited a more than 10 times higher *MDR1* mRNA levels compared to healthy brain tissue [84]. Numerous studies followed aiming to reproduce this outcome and could as well discover that P-gp showed overexpressed functions in capillary endothelial cells of epileptic brain tissue in individuals with refractory epilepsy [85–88]. Hence, the overexpression of P-gp at the BBB hinders the ASMs to diffuse into the epileptic brain resulting in DRE similar to drug-resistance in chemotherapy [89]. Moreover, P-gp overexpression was also found in astrocytes and dysplastic neurons in people with HS, focal cortical dysplasia (FCD), and dysembryoplastic neuroepithelial tumors (DNT), which are one of the primary pathogenic causes of DRE [85]. Due to the difficulty of obtaining a reliable healthy control for comparison with the P-gp expression in surgically resected tissue from epileptic patients, animal models have been broadly used to study the effects of ABC transporter overexpression in the epileptic brain. The results of these models revealed that P-gp can be transiently induced by seizure activity and that P-gp overexpression arises in a localized manner, suggesting its limitation to brain regions involved in generating and spreading seizures [90,91]. Despite that, there is ambiguous evidence that pharmacoresistance of ASMs correlates with the overexpression of efflux transporters or whether it is just an epiphenomenon of the disease. In this regard, no change of P-gp expression rate was detected in rodents treated 7 or 14 days with the ASMs carbamazepine, phenytoin, or phenobarbital [92,93], whereas after a 21-day administration elevated P-gp concentrations could be found in brain capillary endothelial vessels. Conflicts persist, whether the overexpression in epileptic patients is of intrinsic matter or results from seizure activity or long-term ASMs administration [94]. However, P-gp transport a large variety of chemically varied substances, notably hydrophobic and amphipathic molecules [95]. Since the majority of ASMs are lipophilic compounds, in theory many of them should be P-gp substrates [96]. The controversial documentation that AMSs are transported by efflux transporters raises many doubts in the transporter hypothesis. The three major techniques used in these documentations include *in vitro* approaches in cell lines overexpressing or inhibiting transporters and *in vivo* experiments, which examine transporter gene knockout to analyze the impact of genetic deficiencies. However, all these study designs reveal strengths and limitations. For instance, the overexpression of transporters lacks the feasibility in *in vivo* analysis, transporter inhibitors are imprecise and may interact with other transporters and knockout animals may display compensatory mechanisms, as the upregulation of different transporters. In conclusion, the obtained data could show that some ASMs are substrates of P-gp, while others failed to reproduce this result [97–100]. To finally obtain conclusive and accurate findings, all methods need to be employed in a single survey. In a different study, a modified transport assay was implemented and showed the ability of P-gp in transporting

phenytoin, phenobarbital, lamotrigine, levetiracetam, and topiramate, but not carbamazepine (CBZ) in MDR1-transfected LLC-PK1 cells [101]. Additionally, this outcome could be confirmed in an *in vivo* study, suggesting that different ASMs are substrates of different transporters [102]. At present, the transporter hypothesis is still ambiguous and further investigations are required to better understand the importance of the overexpression of efflux transporters at the BBB.

The *target hypothesis* is based on the assumption that activation and therefore alteration in structure and functionality of voltage-gated ion channels and receptors by ASMs leads to an increased insensitivity and thus results in a decrease of response to the treatment. In this regard, studies have been mainly focused on evaluating the properties of carbamazepine in hippocampal neurons. The alteration of sodium channel currents by carbamazepine is characterized by voltage-dependent inhibition shifting the inactivation curve in a hyperpolarized direction [103]. The target theory is established by studies with CBZ on voltage-gated ion channels in hippocampal CA1 and dentate granule cells of patients with mesial TLE. Blockade of use-dependent voltage-sodium channels in dentate granule cells by CBZ was absent in hippocampi of patients with CBZ-resistant mesial TLE, compared with neurons from healthy patients, suggesting that a loss of sodium channel sensitivity to ASMs could explain the development of refractoriness [91]. In addition, in DRE a fast recovery from inactivation of the Na^+ current was detected to have CBZ-insensitive properties [104]. The same outcomes were described for CBZ and phenytoin using the pilocarpine rat models, but no other ASMs could produce a similar result, leaving open for discussion the selectivity for carbamazepine and phenytoin [105]. Another group reported that in the kainic acid model sodium channel voltage-dependence of activation and steady-state inactivation shifted to more hyperpolarizing potential, resulting in a reduced membrane density of sodium currents but showing no signs of channel resistance to carbamazepine [106]. Furthermore, other sites of action for ASMs, namely the γ-aminobutyric acid type A ($GABA_A$) receptors, have been studied, which are suggested to be altered in animal models and patients of DRE [91]. In a pilocarpine model of TLE a decreased sensitivity of $GABA_A$ receptors to substances that bind to benzodiazepine binding site has been described. Also, $GABA_A$ receptors showed a change in expression of its subunits, which correlates with receptor function and affinity [107]. Interestingly, in epileptic rats an increased $GABA_A$ sensitivity towards zinc was reported. Associated with these modifications, $GABA_A$ receptors exhibited a lack of sensitivity to the benzodiazepine site modulator zolpidem [108]. Supporting findings were obtained in a immunohistochemical study involving brain specimens of TLE patients with hippocampal sclerosis [109]. Recapitulating the mentioned studies and the fact that ASMs affect different targets or even exhibit multitarget actions, makes the target hypothesis less relevant as a strong hypothesis, which can be applied to all ASMs.

The *gene variant hypothesis* suggests that substance efficacy underlies variations in genes encoding enzymes that display key functions in the pharmacodynamics and kinetics of AMSs or genes associated with the epileptic phenotype. In DRE, the most frequently studied polymorphisms are those associated with multidrug resistance genes: ABCB1 or MDR1, ABCC2 or MRP2, SCN α subunits and metabolizers of endogenous and xenobiotic substances: cytochromes P450 families 2 and 3

(CYP2 and CYP3). For instance, phenytoin is metabolized by CYP2C9 and CYP2C19. In two different studies, an association between decreased expression of alleles of *CYP2C9* and a reduced dose requirement of phenytoin has been reported [110,111]. In addition, patients responding to their drug treatment had more of heterozygous *CYP2C8*4* and *CYP2C9*3* variant allele carriers than patients refractory to first-line anticonvulsants [112]. Further evidence of the importance of gene variants in pharmacoresistance was also found in voltage-dependent sodium channels, which are primary targets of numerous AMSs such as carbamazepine, phenytoin, lamotrigine, valproate, oxcarbazepine and zonisamide [91]. These channels consist of an α subunit and two β subunits whereby four of the α subunits, namely Nav1.1, 1.2, 1.3, and 1.8 encoded by *SCN1A*, *2A*, *3A*, and *8A*, respectively, are expressed at high levels in the brain [113]. Mutations in the *SCN2A* gene are associated with a range of neurological diseases including epilepsy, intellectual disability, and autism [114–116]. An intronic single nucleotide polymorphism (SNP) in the *SCN1A* gene may modify the channel's functional characteristics by changing gene splicing. In a UK study, a correlation between this polymorphism and the maximum required dose prescribed for carbamazepine and phenytoin was described. However, the authors did not provide information about the association with the drug-response [110]. In a follow-up study with Chinese epilepsy patients, the outcomes could not be reproduced, claiming no correlation between SNP and maximum used phenytoin dose [117]. Moreover, polymorphisms of SCN1A and CYP3A4 were found to be involved in the mechanism of DRE in Chinese children treated with valproic acid, while polymorphisms in the SCN1A gene alone were found to be correspondent to refractoriness in Italian pediatric patients with epilepsy [118,119]. Numerous other studies have proven that patients treated with ASMs and with ABCB1 polymorphism seemed to have a significantly higher chance to develop drug-resistance, compared to epileptic patients without the gene variant [120–126]. Moreover, mutations of the GABA receptors have also been implicated in the pathogenesis and severity of TLE. Polymorphisms in the $GABA_B$ receptor highly increased susceptibility to TLE and seem to modulate the severity of seizures [127], while the gene variant C588T in the $GABA_A$ receptor γ2 subunit is linked a higher probability of developing childhood idiopathic generalized epilepsy (IGE) and may account for the drug-resistance in those patients [128]. Concluding the above stated results, a higher number of cohorts are needed to confirm the genetic association to DRE, plus due to the fact that certain mentioned alleles occur with low frequency may question their impact pharmacoresistance. Finally, genomic technologies need to be further explored such as next-generation sequencing, which has been shown to act as an effective diagnostic tool to identify specific genetic variations, hence improving treatment efficacy [129].

The *intrinsic severity hypothesis* proposes that epilepsy-related neurobiological factors contribute to the severity of the disease and response to ASMs, implying that an increased severity is associated with drug-resistance [75]. It was proposed that drug-resistance is not due to specific pharmacoresistant features but is an inherent property of epilepsy related to disease severity, which can range from mild to severe [130]. High seizure frequency seen before the start of a drug treatment could be an important factor in DRE [131]. A study on children with epilepsy came to a similar finding, showing that anticonvulsant medication given within the first ten episodes had no

effect on seizure control or early remission effect [132]. A randomized controlled research of 1847 epileptic patients, demonstrated that patients receiving immediate anti-seizure treatment compared to those who had a deferred start of therapy, displayed a reduction of seizure recurrence in the first 1-2 years. However, long-term remission rate did not differ between the two groups [133]. Contrarily, a clinical study in which epileptic patients immediately started medication therapy after the first experienced tonic-clonic seizure did not show an improvement of the prognosis, suggesting that long-term remission is not influenced by treatment of the first seizure [134]. To date, the neurobiological factors that might cause seizure severity are poorly understood.

The *pharmacokinetic hypothesis* states that peripheral upregulation of efflux transporter expression occurring in the liver, intestines and kidney, plus accelerated drug metabolism leads to decreased circulating plasma rates of an ASM and hence a reduced availability to cross the BBB [135]. In fact, the expression and alteration of efflux transporters in DRE is not limited to the brain only, but can as well be found in peripheral organs, where P-gp is assumed to act as a barrier of substances entering the bloodstream from the intestinal lumen, and consequently reducing the drugs' bioavailability [136]. In this regard, neuronal MDR-1 gene expression with persistent low levels of carbamazepine, phenytoin, and valproic acid could be evaluated in a child with refractory epilepsy. In the resected brain specimen of the same patient, endothelial cells, astrocytes, and neurons presented increased levels of P-gp [137]. In a different case report of drug intractability in a child, reduced phenytoin plasma levels coincided with augmented P-gp protein expression shown via immunohistochemical analysis of the resected epileptic brain [138]. Furthermore, in eight patients with epilepsy, increased plasma levels of the P-gp substrate 99mTc-hexakis-2-methoxyisobutylisonitrile was detected, which increased hepatic clearance that might affect the ASMs resistance [139]. However, low levels of ASMs in refractory patients could rather be due to metabolizing enzymes, than to the alteration in functionality of multidrug transporters. Liver cytochrome P450 is primarily responsible for the metabolism of ASMs. Some of the cytochromes in this category involve allelic types that encode isoforms with different properties, which can impact the level of ASMs present in the blood serum. The BBB's endothelial cells and brain parenchyma both contain cytochrome P450 metabolic enzymes, which enhances the function of the barrier. A reciprocal role in drug resistance is played by changes in the cerebrovascular hemodynamic conditions, which can also impact the expression of cytochrome P450 enzymes and efflux transporters [140]. On the other hand, a weak correlation between the increased intestinal P-gp concentration and low circulating carbamazepine plasma levels was found and increased intestinal MRP2 expression levels were poorly related to the high carbamazepine doses administered to patients with DRE. Nevertheless, in both studies the question if P-gp expression changes the drug response was not addressed [141,142]. Interestingly, the severity of side effects evoked by ASMs was the same in drug-responders and in drug-resistant epileptic patients, assuming similar drug levels in both groups. This result could either indicate that P-gp overexpression is exclusive to the epileptic lesion or that similar plasma concentrations are related to similar levels of enzyme and transporter expression in peripheral organs. Studies in animal models do not support the pharmacokinetic hypothesis. Neither a difference of ASM plasma level, nor different extents of

adverse effects between ASM responders and non-responders have been observed in rat models of refractory epilepsy [143,144]. Overall, the evidence for the pharmacokinetic evidence is quite limited. As ASM plasma concentrations vary among patients, plus there is no evidence that pharmacokinetic variability is a major contributor to drug intractability, this hypothesis is difficult to verify.

The *neural network hypothesis* claims that epilepsy-associated structural alterations as neurodegeneration, axonal sprouting, synaptic reorganization, neurogenesis, and gliosis contribute to the formation of an abnormal neural network, therefore inhibiting the drugs to access the epileptic foci and reducing the efficacy of ASMs. Electrophysiological, neuroimaging, and clinical evidence assumes that epilepsy and especially DRE might be a network disorder. In many patients with refractory mesial TLE, hippocampal mossy fiber sprouting and HS were found suggesting they might be a factor to reduce the drug efficacy on the side of action [145]. Resection of the affected lesion in combination with a continued medical therapy could result in an up to 60% success rate in achieving seizure freedom [146]. In an epilepsy model in rats, the neuronal loss in the area CA1, CA3 and hilus was significantly higher in drug-non-responders compared to the animals which responded to ASMs. In addition, the hippocampal morphology did not differ between most of the responders and non-epileptic rats [147]. These findings suggest that the neurodegenerative functional changes in hippocampal pyramidal neurons and in the DG that result from the loss of hilar cells play a significant role in the mechanisms behind DRE. Furthermore, these network alterations can also change the function of the targets, as described previously. Moreover, malformations of cortical development (MCDs) are common causes of DRE in children [148]. But the exact mechanism behind linking malformation to drug refractoriness is still unknown. Another study supporting the neural network hypothesis claims that the growth cone at the tip of an axon displays overly stimulated signaling molecules in the epileptic brain, as well as progressive sprouting leading to a formation of new excitatory circuits, as seen in TLE patients [149]. In summary, clinical evidence of network alterations, as for example changes in cytoskeleton, synaptic plasticity or cellular reorganization directly leading to drug refractoriness is still not available. The fact that not all patients affected with network modulations end up being ASMs refractory makes this hypothesis not very strong.

In the last years, a further explanation for pharmacoresistance evolved. Based on the transporter hypothesis, there are also different neuroinflammatory factors which can induce BBB dysfunction and overexpression of efflux transporters resulting in loss of responsiveness to ASMs. For instance, activation of a neuroinflammatory response introduced by an increased expression of interleukin-1 beta (IL-1β) or tumor necrosis factor alpha (TNFα) in the rodent brain that causes a higher BBB permeability to blood macromolecules such as albumin, which are normally kept isolated from the brain. It is known that serum albumin binds many ASMs and engages in their transport within the blood stream. BBB dysfunction and the extravasation of serum albumin into the brain are thought to contribute to drug refractoriness [150]. Further, it was shown that IL-1β promotes the breakdown of tight junctions, such as zonula occludens (ZO)-1, by activating IL-1 receptor type 1 (IL-1R1) expressed by endothelial cells, even if the underlying processes connecting

neuroinflammation to BBB failure have not yet been fully understood [151–153]. In a different study using extracellular field potential recordings from acute rat entorhinal cortex–hippocampus slices, it was reported that phenytoin and carbamazepine failed to suppress a so-called "seizure-like events" (SLE) in the presence of albumin. This outcome might be due to a "buffering-effect" of albumin binding to ASMs in the brain's extracellular space so to reduce the circulation of free ASMs. Hence, the authors suggested that ASMs with a low binding affinity to albumin may be more effective [154]. There is evidence that certain inflammatory mediators, including cyclooxygenase-2 (COX-2) and IL-1β, released by astrocytes and neurons due to hyperexcitability during the ictal state, may generate a higher P-gp expression in astrocytes and endothelial cells, but clinical studies which can support that activation of these mediators contribute directly to pharmacoresistance is still not documented [155,156].

Overall, all the above-mentioned hypotheses have their own strengths and weaknesses. Putting together the information gathered so far, it is very likely that pharmacoresistance results from more than one factor and possibly changing from person to person.

4.2. Tolerance

Another form of pharmacoresistance in epilepsy is depicted in the tolerance to ASMs. Tolerance is defined as a loss of efficacy due to a chronic administration of a drug, after an initial positive response to a newly administered substance. Development of tolerance is an adaptive reaction to a long-term exposure of xenobiotics that affect the central nervous system (CNS), as well as peripheral organs. Some patients develop a faster tolerance to certain ASMs than others who suffer from the same seizure or epilepsy type. However, the extent of the tolerance varies between different drugs, which could further lead to an aggravation of adverse effects and failure of response to the medication. In animal as well as clinical studies, it was reported that the decrease of efficacy of a ASM can initially occur by induction of pharmacokinetic tolerance that changes the bioavailability of the drug [157]. For example, the often-used ASMs carbamazepine, phenytoin and phenobarbital share the same breakdown pathways by activating the cytochrome P450 enzymes [158]. In this regard, patients receiving those medications are more likely to develop a drug tolerance or are in need of higher doses. Further crucial factors in the bioavailability of ASMs are other comorbid diseases and hence additional drug intake, which can impact the hepatic, renal clearance and gut absorption function [159,160]. With regards to the target hypothesis, pharmacodynamic changes of $GABA_A$ receptors may occur not only after a prolonged, but also acute exposure to benzodiazepines. Despite many years of intense efforts, benzodiazepines tolerance remains poorly understood, and may involve a variety of different mechanisms [161,162]. Due to the development of tolerance and addicting properties, benzodiazepines are not suitable for a long-term treatment of epilepsy. This statement has already been proposed in an amygdala-kindled rat model published in 1989 [163]. In another study, also using amygdala-kindled rats, administration of a different ASM called vigabatrin, which is known to increase GABA availability, resulted in tolerance already in the first two weeks [164]. This result of tolerance seen immediately with vigabatrin is not aligned with studies in patients, in which vigabatrin is used as a successful long-term treatment [165,166].

Furthermore, in many patients a cross-tolerance can be observed, which is described as the loss of response to a new ASM drug that has not yet been tested in patients, thus contributing to the problem of patients with multi-drug refractoriness. It is assumed that such cross-tolerances are caused by substances which share the same pathway, as for example applying different benzodiazepines together, which can cause enhanced potentiation of GABA-mediated inhibition and internalization of the receptor [167]. However, valproic acid lost its anticonvulsant properties in more than 50% of mice, which were pre-treated twice a day for three days with either clobazam, clonazepam, diazepam, or lorazepam, but no cross-tolerance was shown between benzodiazepines and phenobarbitone. In addition, after treatment with valproic acid, no difference in seizure activity was achieved by any of the benzodiazepines, nor by valproic acid itself. In this study it was first reported that unidirectional cross-tolerance can occur for ASMs, which appears to be mediated through different pathways. However, some evidence indicate that valproic acid, for example, may also be involved, at least in part, in the GABAergic transmission [168,169]. The exact mechanisms behind the cross-tolerance of co-administered ASMs acting at different targets still need more clarification.

Another theory is simply that the lack of efficacy of the second administered ASMs is independent of the already given first medication or that that is simply results from the progression of the disease [157]. There are also other forms of tolerance, as for example conditioned tolerance, which is due to associative learning mechanisms of the brain, i.e., drug administration always goes in line with the same smell of the hospital, or when the same situation with the responsible nurse who greets the patients always the same way. Therefore, these special cues lead to anticipation of the effects of the medication even before the drug reaches the site of action. This phenomenon by which conditioned tolerance develops adheres to the well-known learning principles of “Pavlov”. When the medication is administered in new or unexpected conditions, conditioned tolerance does not take place, and drug efficacy is enhanced [170,171]. There is evidence that habitual, conditioned, or behavioral factors play a crucial role in drug tolerance. In a classic study of tolerance, rats received for a longer period amphetamine before having access to food, which resulted in drug tolerance. When the same rats were subsequently given food after the drug administration, tolerance could not be detected, suggesting the importance of a contingent relationship between drug intake and a special routine which is related to the administration [172]. Tolerance - regardless of its type - is reversible after discontinuation of the ASM [157,173].

4.2.1. Rebound Effect

The rebound effect occurs often after the rapid withdrawal of various classes of drugs, that act in favor to the disorder. Discontinuation often worsens the condition to levels higher than prior to the treatment [174]. The rebound effect is the outcome of the body’s natural attempt to return to its baseline state following the primary impact of medications, which modify the organism. In epilepsy it implies that after discontinuation of one or more ASMs, generalized seizures during a brief period or longer-lasting partial seizures can aggravate immediately, which could lead to serious and even life-threatening consequences, such as developing a SE or experiencing SUDEP [174–176]. In a chronic rat model of epilepsy discontinuation of levetiracetam, seizures increased rapidly up to

700% in the first week [177]. Vigabatrin is presently used as a successful add-on medication, as well as first-line monotherapy for infantile spams. In children tolerant to vigabatrin, withdrawal of the drug caused extreme seizure relapse associated with severe mental retardation. Whether the severity of the rebound effect was correlated to the drug refractoriness could not be evaluated [178]. In amygdala-kindled rats, relapse of hyperexcitability was seen 24h after the last injection of clobazam and diazepam, whereas in rats treated with clonazepam, no rebound hyperexcitability could be detected (even though a trend towards a slow rebound effect cannot be excluded from the data). Due to the fact that clobazam and diazepam lost more quickly their anticonvulsant activities than clonazepam, the authors suggested that the missing rebound effect of clonazepam could be related to a slower tolerance to the drug [163]. The same phenomenon was confirmed in a study using genetically epilepsy prone rats [179]. This effect might be caused by a higher binding affinity to benzodiazepine sites of clonazepam than of diazepam and clobazam [180]. Another hypothesis could be that ASMs are from the same class of drugs but exhibit different half-life times. For example, the half-life of diazepam and clobazam is about 35 hours on average after a single dose, whereas the half-life of lamotrigine is about 25 hours [181]. The metabolism that carbamazepine significantly promotes results in higher clearance, a shorter serum half-life, and a gradual decline in serum level. After 10–20 days of multiple administration, the half-life falls circa down to 50%. Consequently, a higher dose would be needed to maintain the serum levels. A rapid decrease of serum levels can be observed using carbamazepine, while with lamotrigine, using the same withdrawal protocol, a slower decline in serum levels can be detected [182]. Both ASMs block the use-dependent sodium channels, but lamotrigine is additionally able to block glutamate release [183,184]. This multifactorial mechanism may possibly explain the smaller impact of lamotrigine discontinuation on seizure frequency. Though to specifically determine if the reduced glutamate release following lamotrigine administration is an independent effect or whether it is dependent on the sodium channel inhibition, will require additional investigations [185,186]. It is still not clear why some ASMs, such as oxcarbazepine, phenytoin, lamotrigine, and carbamazepine, which are all sodium channel blockers and exert the same mechanism of action, differ in respect to seizure relapse [187]. Long-term investigations on the rebound effect of ASMs are needed in animal models because of the risk related to the development of uncontrolled seizures, which could further end in brain hypoxia and neurodegeneration [188].

5. In vivo models of refractory epilepsy

In epilepsy the use of animal models has always played a vital role regarding three main categories: 1. Investigation of the mechanisms behind seizures and epilepsy, 2. Understanding of the underlying factors of pharmacoresistance in patients with epilepsy, and 3. Identification of new anti-seizure medications. In the following section, different *in vivo* models of DRE will be described, with the main focus on 6-Hz corneal stimulation and the kainic acid model.

Most animal models in epilepsy are models of epileptic seizures rather than epilepsy itself. In rodents, acute seizures can be induced in wild type animals to screen for novel anti-seizure

medications or to investigate the mechanisms of SRSs. There are also chronic models of epilepsy, in which SRSs are induced chemically or by means of genetic modifications. While the animal models of acutely induced seizures are mainly utilized to screen novel ASMs and to understand the single seizure functions, chronic models are closer to the actual disease and clinical manifestation in patients. Some models are more than 60 years old, for example the maximal electroshock (MES) and s.c. pentylenetetrazole (PTZ, a GABA antagonist) test, which are both used to test new ASM. In the MES, a corneal shock is delivered with 60-Hz of alternating current (50 mA in mice and 150 mA in rats) for a duration of 0.2 sec, generating generalized-tonic clonic seizures. In another acute seizure model, the PTZ test, rats are injected subcutaneously with PTZ resulting in generalized absence and myoclonic seizures [189]. Even though they are suitable models to screen for new ASMs, they are not suitable models to study pharmacoresistance [190]. The amygdala kindling rat model of TLE is one of the most well characterized chronic epilepsy models [191]. The rat's basolateral amygdala receives repetitive electrical stimulation delivered by a depth electrode resulting in focal to bilateral tonic–clonic seizures. Since this model displays a high rate of non-responding ASMs, it was the first proposed model of drug resistant partial epilepsy [192]. Furthermore, to assess post-SE TLE, the pilocarpine model is the most frequently used. Pilocarpine acts on muscarinic receptors and intraperitoneal (i.p.) administration in rodents produces a sequence of automatisms and motor limbic seizures evolving into SE [193]. Circa after two weeks post-treatment, pilocarpine-treated animals showed spontaneous seizures after the initial SE, establishing this as a widely accepted model of TLE. The same phenomenon can be achieved using the intrahippocampal kainate model in mice, which is another chronic model of mesial TLE [194,195].

5.1. The kainic acid model

Kainic acid (KA) was one of the first substances used to mimic TLE in rodents [196]. KA can be injected by different routes: systemically via i.p., intravenous (i.v.) and s.c. injections, or directly in the ventricle, hippocampus, or amygdala. It is a cyclic analog of L-glutamate and binds with a higher affinity to the ionotropic kainic acid receptors (KARs) than glutamate in the brain [197]. In 1953, KA was first extracted from the red algae *Digenea simplex* Aq. present in tropical and sub-tropical waters [198]. KA was first used to eliminate ascariasis, a disease induced by the parasitic worm Ascaris lumbricoides. But in 1970, it was shown that intracerebral KA injections into the rat ventricle, amygdala, or hippocampus causes cortical neurons to exhibit prolonged excitatory responses and damage to hippocampal pyramidal neurons [199,200]. Ben-Ari and coworkers first introduced KA as a model of epilepsy by injecting KA directly into the amygdala of rats. Focal seizures developing a SE and further neuronal degeneration and gliosis in the CA3 field of the hippocampus were found to be the main effects of intra-amygdala injections, suggesting KA as a suitable tool to mimic TLE in rats [201,202]. Over the last thirty years, extensive molecular studies have successfully identified the localization of KARs. These receptors can be found at different levels of expression in the mammalian brain, such as the entorhinal cortex [203], cerebellum [204], basal ganglia [205], amygdala [206] and in particularly high levels in the hippocampus [207]. Similar to AMPA and NMDA receptors, KAR belongs to the ionotropic glutamate receptor family. KAR have

been found on presynaptic terminals, where they act in a bidirectional manner, having excitatory functions through their ionotropic activity and inhibition via a “non-canonical” metabotropic signaling [208,209]. They are also located on postsynaptic terminals contributing to excitatory neurotransmission [210]. In addition, KARs are able to pre- and postsynaptically control GABAergic neurotransmission [211,212]. There are five known subunits of KAR: KA1 (GluK4), KA2 (GluK5), GluR5 (GluK1), GluR6 (GluK2), and GluR7 (GluK3). KA1 is mainly found in the hippocampal CA3 field, while little expression was seen in CA1 [213]. KA2 is instead highly expressed in both CA1 and CA3 pyramidal cells [204,213]. The fact that both KA1 and KA2 display high affinities to glutamate and that they are highly expressed in CA3 may explain the excitotoxic damage induced by KA found in this region and suggests that they might be responsible for neuronal cell death, therefore designating the hippocampus as the seizure onset region [214,215]. In patients suffering from TLE, increased expression of KA1 was found, whereas KA1-KO mice showed robust neuroprotection in the CA3 field following KA administration, but also displayed impairments in memory acquisition [216,217]. While GluR6 is highly expressed in both CA1 and CA3, GluR5 has just been found in CA3 [207,213]. Even though both subunits have low affinities for KA, GluR5 KO mice demonstrated an enhanced sensitivity to the epileptogenic effect of KA, while GluR6 deletion could prevent the generation of epileptiform discharges [218]. Interestingly, GluR5 shows an high abundance in hippocampal interneurons and has been associated with acute seizure induction, which is thought to be mediated through modulation of inhibition [219,220]. Furthermore, GluR6-KO mice were also reported to have a decreased responsiveness to the epileptogenic effects of KA [221]. Moreover, GluR7, which possesses the lowest glutamate affinity, has been demonstrated to have a long-lasting downregulation following KA-induced seizures [222].

The first systemic injections of KA were performed in the late 1970s to investigate the damage via axonal connections. In this study, rats were injected i.p. with 12 mg/kg KA and epileptiform EEG patterns were observed after 30-90 minutes. After this time, interictal spikes were detected in the entorhinal cortex, that correlated to "wet dog shaking", and 88% of them progressed to secondary generalized tonic-clonic convulsions. Ictal discharges then began to emerge in the CA3 region and in the amygdala, which further spread to the thalamus, the CA1 region, and the frontal cortex. However, ictal discharges are not present in patients. It was later detected that EEG obtained from the hippocampus in the KA model is characterized by gamma oscillations (30–40 Hz) and subsequent sporadic spikes before the appearance of behavioral symptoms [223]. The first signs of neuronal damage were detected three hours after the injection with a steadily aggravation in the following three weeks [224]. A loss of CA1, CA3 and CA4 pyramidal cells was observed, besides a reduction in GABAergic interneurons in the basolateral segment of the amygdala, with somatostatin-expressing interneurons being the mostly affected [225,226]. Interestingly, it was demonstrated that 10 mg/kg KA injected s.c. resulted in similar behavioral and neurochemical modifications as i.p., a suitable approach as post-SE TLE model [227,228]. In adult rats, s.c. administration is often preferred over i.p., due to slower absorption rates and since unintended injection of a substance into the intestines and abdominal fat can frequently occur [229]. Contrary to i.p. administration, s.c. injection does not require the animal to be turned on its back, potentially causing increased stress. As for every approach, the systemic administration of KA displays

advantages as well as disadvantages. Notably, systemic injections allow simple, non-surgical administration of a high number of animals in a brief time, without the demand for anesthesia. On the other hand, there is no control of bioavailability or penetration into the brain. Moreover, a high mortality rate was seen in strains including Long-Evans and Wistar rats [230,231]. After approximately 5-60 minutes from the KA administration, 93% of the animals develop a SE, which can be inhibited by the co-administration of diazepam (20 mg/kg) and ketamine (50 mg/kg) [196,232,233]. Acute seizures are rated using a Racine scale, which rank behavioral abnormalities in different stages: stage 1 and 2 implies immobility followed by facial clonus (head nodding), masticatory movements and wet dog shakes; stage 3 stands for uni- or bilateral forelimb clonus; stage 4 is characterized by rearing and finally the most severe stage: stage 5 includes rearing and loss of posture [234–236].

5.1.1. Latent period post SE

Approximately 5-30 days after treatment, in 60-80% of rats that receive systemic injections non-convulsive SRSs appear [237–239]. The latent phase is usually the interval of time between the initial brain injury (SE in the KA model of TLE) and the onset of chronic epilepsy, which appears as early SRSs [240]. In other cases of, i.e., meningitis- or stroke-induced epilepsy, the latency can be one week short [241]. In rodents, abnormalities in the EEG of KA-treated animals can occur in few hours following SE, in particular interictal-like spikes, that can appear from the ipsilateral and contralateral hippocampus, and in extrahippocampal brain regions [242]. Systemic injections induce a latent phase characterized by interictal spikes, which can be divided into two distinct types: type 1 is identified by a spike followed by a long-lasting wave and type 2 presents a spike but without a wave. The authors described that the number, amplitude, and duration of type 1 spikes started to decrease during the latent period, whereas the occurrence of type 2 spikes increased, achieving their minimum/maximum values just before the onset of the chronic phase, suggesting that the pattern of interictal spikes may reflect epileptogenic mechanisms [243]. In the KA rat model, the seizure-free period can range from 5-30 days, but in some rare cases can extend up to five months [237,244]. During this period, animals might not show signs of behavioral seizures, therefore electrophysiological monitoring is crucial for the ability to detect non-convulsive seizures. The average number of SRSs in the KA model is eight per day (± 5.4) and a single seizure lasts circa 40 seconds on average [228,245]. SRSs mainly occur as non-convulsive seizures, but can develop to convulsive ones during the late chronic phase of epileptogenesis [246].

5.2. The 6-Hz corneal stimulation

The 6-Hz corneal stimulation mouse model was first described in 1951 by Toman as an assay procedure and has been designated as a psychomotor seizure test, which is characterized as seizures evoked by low frequency stimulation [247]. Early then, it has already been proposed that the induced convulsions differ from other types of induced seizures demonstrated in other experimental approaches including the MES or PTZ models of generalized seizures, as both its characteristics and EEG pattern reflect psychomotor epilepsy seen in patients [248]. Although the approach was in theory just another method to evaluate the brain's electrical convulsive threshold,

it has been suggested that the assay distinguishes from other assays due to its possible beneficial characteristics for evaluating prospective treatments for patients with psychomotor epilepsy [247,249]. Later in 2001, White and his group first proposed the 6-Hz corneal stimulation as a model for refractory partial seizures [250]. The identification of levetiracetam as a clinically effective anticonvulsant is an important example of the significance of discovering new ASMs. Importantly, the National Institutes of Neurological Disorders and Stroke (NINDS) has included the 6-Hz corneal stimulation model in the anticonvulsant screening project [250,251].

Compared to the well-established high-frequency (50 or 60 Hz) MES test, the 6-Hz model uses low-frequency (6 Hz) rectangular pulses of a 0.2 ms duration generated by corneal electrodes for a relatively long duration of 3 seconds. Provoked behavioral abnormalities can be described as short forelimb clonus, automatic masticatory movements and facial clonus, followed by an immobility referred to as stunned position [252]. Using a current intensity of 22 mA, which is for example required to produce psychomotor seizures in 97% of mice (CC_{97}), phenytoin, lamotrigine, ethosuximide, levetiracetam, and valproic acid were all able to inhibit the induced seizures, revealing that this model does not discriminate between clinically-employed classes of ASMs at this current. If the current is increased to 32 mA, phenytoin and lamotrigine lose their efficacy, but the 6-Hz seizures remain sensitive to ethosuximide, levetiracetam, and valproic acid. By further increasing the stimulus to 44 mA, the induced seizures become insensitive to ethosuximide and levetiracetam and valproic acid, but new ASMs such as brivaracetam and retigabine allow a complete protection against the 6-Hz seizures [250]. Based on these results, this model was proposed as an ideal and inexpensive screening test for pharmacoresistant psychomotor seizures evoked in wildtype mice.

Recently, a modified approach of the 6-Hz corneal stimulation test was proposed to reduce the number of tested animals, to mimic seizure aggravation in the same animals, as well as to screen new ASMs after repeated seizure exposure. In detail, the mice were stimulated four times every 72 h with a fixed current of 32 mA to evaluate seizure severity and duration. According to the video-EEG, provoked seizures exhibited both a motor component, which is depicted as typical epileptiform discharge and a non-motor component which is characterized by a flattening of the EEG, for instance a postictal depression. Although seizures always emerged in the frontal cortex, they only started to also affect the hippocampus just at the last stimulation, which suggests the onset of an epileptogenic process. After the second stimulation, the non-motor component of seizure duration steadily declined but convulsive seizures remained constant. Moreover, after the second stimulation, a more severe seizure phenotype with tonic-clonic generalized convulsions occurred [253]. In a different study, as well as using the repeated 6-Hz corneal stimulation to achieve seizure aggravation, changed levels of phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2) were used as a biomarker for neuronal activation [254]. In this protocol mice were stimulated just three times with an inter-stimulation interval of 72 hours. A progressive increase in p-ERK1/2 immunoreactivity all over the hippocampus, especially in CA1, could be detected, especially peaking in the last and third stimulation. Seizure severity was ranked according to the following behavioral scores: 1, stunned posture and eye blinking; 2, head nodding, Straub tail and repetitive rhythmic movements (stereotypies) such as chewing; 3, unilateral or alternating

forelimb clonus; 4, generalized tonic-clonic convulsions without loss of posture, or rearing; and 5, generalized tonic-clonic convulsions with loss of posture [255].

6. Biomarkers in pharmacoresistant epilepsy

Despite ongoing trials to discover new potent ASMs, approximately 30% of epilepsy patients still have no control of their disease. Until now, there are no clinical tools that neither signal the appearance of epilepsy in a patient nor allow the prediction of the disease. In addition, there are no indicators that can anticipate the response of patients to a drug, leaving no other choice than trying out different classes of available ASMs. For this reason, precise and reliable biomarkers for epilepsy and epileptogenesis in humans are essential for the development of potent ASMs, but especially for the prevention or modification of the disease. In particular, the major obstacles in the discovery and development of accurate biomarkers might be i.e., the translatability and applicability of animal-discovered biomarkers to humans. In this case, the major burden is illustrated in the difficulty of translating successful functional biomarkers in rodent strains, which are not influenced by environmental or social factors etc., to humans with epilepsy. Secondly, a further challenge is the use of specific biomarkers for the same type of epilepsy applicable in individuals. There are more factors to a patient with a disease than the disease itself. Treatment is in many cases adapted to an individual, as age, sex, psychological and physiological state differ in every person. Moreover, gathering a large cohort of patients displaying an elevated risk for developing epilepsy is essential to obtain a reduction of the costs of clinical trials of potential ASMs or non-invasive and invasive treatments. In addition, the prognosis to treatment response is another crucial hurdle to overcome, given that following an initial epileptogenic trigger, potential biomarkers can have different time courses, complicating the interpretation of the latent period of biomarker activation [256,257]. In particular, the continuous expression of a biomarker, which begins and maintains as the condition worsens to its full extent, may differ. Some other biomarkers could just appear temporarily during the epileptogenic course. While there are further biomarkers which arise in a delayed manner and occur when the disease is already fully developed. As a result, each temporal trend will presumably require a different class of biomarkers. Finally, the severity and type of trigger, inflammation, or developmental anomaly will be expressed in early biomarkers. Biomarkers during the latent and fully established epileptic episodes, however, could be performed more effectively with a variety of focal epilepsy possibly linked to synchronized activity, synaptic plasticity, and network changes in the brain [258]. In the case of epilepsy, the spectrum of biomarkers can vary from electrophysiological measurements (EEG monitoring) to neuroimaging (magnetic resonance imaging; MRI), and molecular and biological alterations (modifications in gene expression and metabolites in blood or tissues) [257]. In the following table 5, diverse molecular and biological biomarkers for seizures and epilepsy are listed.

Table 5. Molecular and biological biomarkers for seizures and epilepsy. Modified from Dixit et al. (2016) [259].

Synaptic molecules	
GluR3B	Serum and CSF GluR3B antibody levels in patients with DRE were significantly increased compared with those in patients with drug-responsive epilepsy [260].
GABA receptors	An increased GABA receptor activity has been reported in severe CD cases and not in non-CD or mild-CD. GABA receptor activity may be specific for specific epilepsy pathology [261,262].
Glutamate receptors	NMDA receptors are involved in epileptogenesis and in epileptic tissues upregulation of NMDA receptor subunits has been shown to contribute to hyperexcitability. Mutations in the gene encoding GluN2A have been identified in patients with idiopathic epilepsy and haplotypes of gene encoding GluN1 have also been associated with epilepsy. Decreased GluN2B expression in pyramidal neurons has been shown in TLE patients and upregulation of this receptor subunit was reported in the pyramidal neurons in HS [263–265].
Nicotinic acetylcholine receptors	Nicotinic receptors enhance the release of glutamate in case of nicotine-induced seizures through activation of NMDA receptors. Mutations in the genes encoding for a7 and a4b2nAChR subtypes are known to be related to various forms of epilepsy. a7 nAChR antagonists prevents electroshock-induced seizures in mice and kindling-induce seizures in rats [266–268].
Kinases	
CDK5	Deregulation of CDK5 activity has been found in HS and in various forms of neuronal diseases. CDK5 levels were significantly higher in the anterior temporal neocortex of intractable epilepsy patients [269].
CK2	*in vivo* treatment with the casein kinase 2 inhibitor 4, 5, 6, 7-tetrabromotriazole augments the slow after hyperpolarizing potential and prevents acute epileptiform activity [270].
TrκB	Transient inhibition of TrκB kinase after SE prevents development of temporal lobe epilepsy in animal models [271].
ADK	ADK is over expressed in a variety of rodent models of TLE, but also in epileptogenic tissues resected from human TLE patients [272].
m-TOR	mTOR has been linked with many epilepsy syndromes, raising the possibility that second messengers in this signaling cascade may be useful biomarkers of epilepsy [273,274].
MEK1	Transgenic mice with elevated MEK1 activity display spontaneous convulsions [275].
pERK1/2	Dephosphorylation of pERK1/2 in the Subiculum of the hippocampus exhibit resilience to audiogenic seizures [276].
MMP	During different stages of epileptogenesis, the expression of MMP-2, MMP-3, MMP-9, and MMP-14 in the brain of rats is increased and dynamically regulated [277].
Inflammatory molecules	
C-reactive protein	CRP serum concentration was significantly higher in patients with refractory focal epilepsy than in controls. The most important predictor of increase in CRP level was secondarily generalized tonic-clonic seizure [278].

IL-1β	Increased mRNA levels of IL-1β have been observed in experimentally induced seizures. IL-1β levels rise in the acute phase during and shortly after SE; they fall during the latent period between a brain insult and chronic epilepsy onset, and then rise again once epilepsy develops [279,280].
IL-6	Elevated in serum and resected tissue within malformation of cortical development (MCD)/FCDs in TS patients [281,282].
COX-2	Increased in neurons after traumatic brain injury and SE in animal models, and prevents CA1 pyramidal and interneuron excitotoxicity, suggesting a neuroprotective role [283,284].
TGF-βR	Blocking TGF-βR in vivo reduces the likelihood of epileptogenesis in albumin-exposed rat brain, TGF-βR acts as a possible therapeutic target and biomarker [285,286].
HMGB1	Elevated in patients with sepsis. It is an important proinflammatory cytokine that is triggered in febrile seizures and SE. The translocation HMGB1 and its release occur in various models including pilocarpine-induced SE, PTZ-induced seizure, KA-induced epilepsy, and experimental febrile SE [287–289].
Epigenetic markers	
miR-132	miR-132 increased in the CA3 in an animal model of SE. In vivo microinjection of antagomirs against miR-132 depleted miR-132 in CA3. A functional role of miR-132 in anti-inflammation has been identified. Since inflammation and BBB opening are implicated in epileptogenesis, it is thought that increased miR-132 may contribute to epileptogenesis [290].
miR-34a	Rapid upregulation of miR-34a after SE. Experiments with the miR-34a antagomir revealed that targeting miR-34a led to an inhibition of activated caspase-3 protein expression, which may contribute to increased neuronal survival and reduced neuronal death or apoptosis [291,292].
miR-146a	Significantly increased expression in lesions compared, negative feedback regulator of inflammatory processes in human glial cell culture. Increased levels during latent period following SE in pilocarpine model [293,294].
H19	H19 is the first identified lncRNA, is found to be upregulated in the latent period of TLE. In KA-induced epilepsy rats, inhibition of H19 causes protection from SE-induced cellular injury [295].
CREB, MeCP2, REST	Control transcription patterns of a set of genes involved in epigenetic modifications leading to altered neuronal excitability [296].
Reelin	Epilepsy-associated susceptibility for promoter methylation in a specific cohort of MTS patients that affects the Reelin gene [297].
Metabolites	
Myoinositol	Increased levels in hippocampus during the latency phase in the pilocarpine and kainate models of epilepsy, and that this increase can be detected with H-MRS [298].
Glutathione	Decrease early after SE but subsequently increase gradually. Glutathione levels in the hippocampus, measured with H-MRS during epileptogenesis, correlate negatively with neuronal cell loss and with the frequency of recurrent seizures observed in the chronic epileptic stage [298–300].

N-acetyl aspartate	Level is decreased early following SE and in the latency phase, possibly reflecting neuronal loss and/or changed neuronal metabolism [298,300].
Hemovanilic acid	Significantly lower in CSF from patients with epilepsy [301,302].
Hydroxyindolacetic acid	Significantly reduced levels in CSF from patients with epilepsy [301,302].
Hormone	
Ghrelin	Increase ghrelin and des-acyl ghrelin plasma levels in children with epilepsy and shows correlation to VPA treatment and KD [303].
Transcription Factor	
FosB/ΔFosB	FosB/ΔFosB antigens, which are known to be markedly induced in seizure and epilepsy models. FosB/ΔFosB isoforms were directly involved in epileptogenesis since their deletion was associated with adult-onset spontaneous epilepsy [304–306].

Abbreviations: ADK, adenosine kinase; CA3, cornu ammonis 3; CD, cortical dysplasia; CDK5, cyclin dependent kinase 5; CK2, casein kinase 2; CSF cerebrospinal fluid; COX-2, cyclooxygenase-2; CREB, cAMP Response Element-Binding Protein; CRP, c-reactive protein; GABA, γ-Aminobutyric acid; GluN1/2A, NMDA receptor subunit; H-MRS, proton magnetic resonance spectroscopy; HMGB1, high mobility group box-1; HS, hippocampal sclerosis; IL-1β, 6, interleukin-1 beta, 6; KD, ketogenic diet; lncRNA, long non-coding RNA; nAChR, nicotinic acetylcholine receptors; NMDA, n-methyl-d-aspartate; m-TOR, mammalian target of rapamycin; MeCP2, methyl-CpG binding protein 2; MEK1, mitogen-activated kinases 1; MMP, metalloproteinase; miR, micro ribonucleic acids; MTS, mesial temporal sclerosis; pERK1/2, phosphorylated extracellular signal-regulated kinases 1/2; REST, repressor element 1-silencing transcription factor; SE, status epilepticus; TGF-βR, transforming growth factor-b receptor; TrκB, tropomyosin receptor kinase B; TS, tuberous sclerosis; VPA, valproic acid.

7. Development of antiseizure medications

7.1. A brief historical résumé of the early progression of ASMs

One of the first documentation of treating epilepsy was discovered circa 3000 BC by Yin Yang Wu Xing and Shen Nung, which followed the fundamental principles of Chinese medicine including the use of herbal drugs, acupuncture and massage to revive a balanced state of the body and mind [307]. On exactly the 11th of May 1857, the documentation of modern history of drug treatment for epilepsy began. Sir Charles Locock discovered - by chance - potassium bromide as the first single substance to treat “hysterical” women with epilepsy [308]. Knowing that potassium bromide causes impotence, it was nonetheless used to counteract catamenial seizures. Numerous inorganic salts of bromide were tested in the following years and were included in clinical therapies. Due to its approximately 50% efficacy rate in epilepsy patients, and the fact that there was no comparable compound on the market at that time, bromide has been used for the next fifty years as the only drug to control seizures, even though severe side effects as severe skin eruption of acne, hallucinations, self-neglect, depression, and psychosis were frequently observed [309]. In 1864 barbituric acid was first synthesized. Due to its not sufficient lipophilic properties to penetrate the

CNS, different barbituric acid derivatives including barbital and phenobarbital (PB) were synthesized in 1911. A significant milestone was reached with the serendipitous discovery by Alfred Hauptmann in 1912 of the anti-seizure activity of PB, which has been in use as a hypnotic and tranquilizer in patients suffering from epilepsy [310]. He noticed that patients had a reduction of seizure occurrence the following day. Furthermore, clinical observations and studies have not only proven the PB anti-seizure efficacy, but also its higher potency and less toxic effects than bromide. In this regard, PB replaced bromide as the second single compound successfully treating seizures [311]. In the early 1950s PB was used as first-line medication to treat neonatal seizures, febrile seizures, and SE. Finally, in 1979 its action on $GABA_A$ receptors was first described, allowing a better understanding of its mechanism [312]. Mephobarbital, which was marketed in 1935, was the last ASM used in clinical therapy before the introduction of seizure and epilepsy animal models. After the establishment of one of the first screening test, using the electroshock threshold test (EST) in cats, phenytoin (a barbituric acid derivative) was discovered serendipitously to exert anti-seizure effects [313]. In 1938, the first clinical test to prove the anticonvulsant properties of phenytoin was performed in 200 patients, which seemed to be more effective than bromide and phenobarbital [314]. Hence, phenytoin was approved and marketed the same year. Further ASMs that are classified as first generation include i.e., borax and other barbiturates derivatives, such as primidone and ethosuximide, which were used from 1857 to 1958 [315]. ASMs which differed from the barbiturate structure and exert a better tolerability are categorized as second generation ASMs and were launched between 1960 and 1975. Those involve benzodiazepines, carbamazepine, and valproate, etc. [316]. In 1975, the National Institute of Neurological Disorders and Stroke of the U.S. National Institutes of Health (NIH) started focusing on a strategy to encourage the improvement of ASMs in the preclinical identification phases, which eventually led to the introduction of the Anticonvulsant Screening Project (ASP) [317]. The aim of the ASP was mainly to encourage industry and research to share compounds for testing their effectiveness to expand the development of new ASMs. In 2015, ASP changed their name to Epilepsy Therapy Screening Program (ETSP), especially with the aim to specialize on the research of compounds against DRE. To this date, using diverse animal models (6-Hz, MES, PTZ) more than 32000 substances have been screened and resulted in numerous FDA-approved medications [318]. In the last two decades, drugs such as topiramate, levetiracetam, lacosamide, cannabidiol etc. classified as third generation ASMs, have made an important contribution to the treatment of epilepsy [319].

7.2. New approaches in the development of antiseizure medications

The currently approved ASMs aim to counteract the excitatory and inhibitory imbalance by regulating voltage-gated ion channels, receptors, transporters, and enzymes involved in neurotransmission as well as diminishing the symptomatic impact. All the above-mentioned substances can be categorized in distinct groups regarding their routes of action to exert beneficial anticonvulsant effects. Some ASMs, such as barbiturates, vigabatrin and valproate enhance GABAergic transmission by interacting with $GABA_A$ receptors or by altering the activity of enzymes and transporters involved in the turnover of GABA. Whereas the main mechanism of carbamazepine, lacosamide and phenytoin is to block sodium channels. In addition, zonisamide acts

both as a sodium and calcium channel blockers. Lamotrigine shows similar mechanisms of action with the additional inhibition of glutamatergic transmission [320]. In the following graph a few ASMs are listed including their sites of action (Figure 4). The molecular targets of Cannabidiol, *Cannabis sativa* L. extracts and PPARγ agonists will be discussed separately and in detail in the review section 4 and 5.

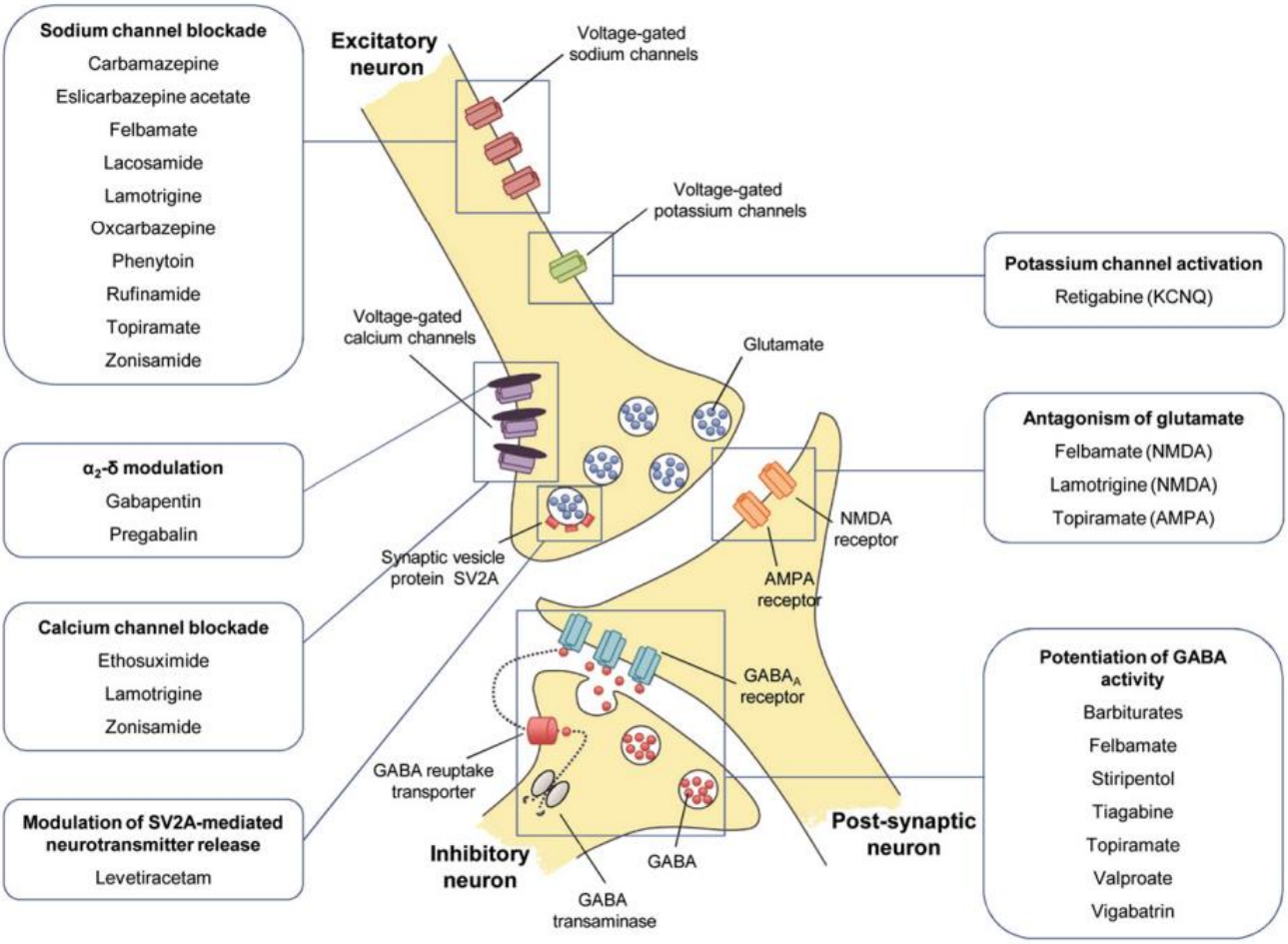

Figure 4. Sites of action of different ASMs [320,321].

In the last decade, significant improvement in the discovery of innovative approaches to find novel AMSs has been achieved. Using enhanced technology and molecular methods, novel therapeutic strategies have been explored to widen the horizon in treating patients with pharmacoresistant epilepsy.

The beneficial effects of stem cell therapy present just one of numerous new treatment plans. Significant loss of inhibitory GABAergic interneurons and their axon terminals is linked to the persistence of seizures in developed epilepsies [322]. According to these findings, providing additional GABAergic interneurons to epileptic foci would improve inhibitory synaptic neurotransmission and reduce the likelihood of spontaneous seizures. In fact, it has been shown in diverse studies using a variety of animal epilepsy models that grafting GABAergic precursor cells

derived from fetal brain into epileptic foci can lower seizure frequency. Interestingly, the precursor cells derived from the medial ganglionic eminence (MGE) of the embryonic brain exhibited the most efficacy for controlling seizures. MGE cells are currently regarded as the most adequate donor cell types for treating epilepsy due to their ability for pervasive migration, to differentiate into a variety of subclasses of GABAergic interneurons, to integrate into synaptic connectivity, to improve inhibitory neurotransmission in the hippocampus, and to significantly reduce the hyperexcitability and seizures in TLE [323–327]. A further study using human MGE-like (hMGE) cells generated from human-induced pluripotent stem cells (hiPSCs) were grafted into the hippocampi of rats that developed SE, resulting in a significant reduction of frequency and intensity of spontaneous seizures as well as decreased EEG power in interictal phases. In addition, hMGE were integrated in the hippocampal circuitry and differentiated mainly into GABAergic interneurons, of which the axons created synaptic connections on the soma and dendrites of the host DG granule cells and CA1 pyramidal cells [328]. Major limitations of the use of grafted cells are the limited survival rate, differentiation, and ability to fully integrate in the hippocampal circuitry on a long-term scale as a result of constant changes of the epileptic lesion.

Another approach to treat DRE is the employment of chemogenetics. Chemogenetics is a term used to describe the practice of expressing a receptor by gene transfer that is non-responsive to endogenous neurotransmitters but sensitive to administered synthetic medications. Successful results have been demonstrated applying an inhibitory Designer Receptor Exclusively Activated by a Designer Drug (DREADD) derived from the human M4 muscarinic receptor hM4Di [329]. This inhibitory GPCR shows no responsiveness to acetylcholine but exerts a high sensibility to synthetic exogenous compounds such as clozapine-n-oxide (CNO) resulting in diminished hippocampal seizures for at least 15 hours in DREADD-expressing animals [330]. Besides, hM4Di has been documented to suppress neuronal activity by inhibition of the presynaptic release of neurotransmitters (used as inhibitory DREADDs), demonstrating an attenuation of focal seizures in a rat model of pilocarpine-induced seizures [331,332].

A further proposal for new anti-seizure targets applying gene therapy could lie in the extraordinary effects of clustered regularly interspaced short palindromic repeats (CRISPR), which is a family of DNA sequences discovered in the genomes of archaea and bacteria. The enzyme CRISPR-associated protein 9 (Cas9) utilizes CRISPR sequences to identify and cleave specific DNA strands which are complementary to the CRISPR sequence using a synthetic guide RNA (gRNA). This allows desired genes to be removed and replaced or added with new ones *in vivo*. This revolutionary gene editing tool displays high hopes to cure many diseases, especially those caused by genetic mutations [333]. For instance, *Kcna1* is the gene that encodes potassium voltage-gated channel subfamily A member 1 (Kv1.1), modulates the neuronal action potential firing and synaptic transmission. Overexpressing *Kcna1* by the promoter CRISPR activation (CRISPRa) has been demonstrated to diminish spontaneous generalized tonic-clonic seizures in a model of TLE and rescued cognitive impairment and transcriptomic alterations associated with chronic epilepsy. CRISPRa uses an inactive Cas9 gene variant dCas9 (nuclease-dead Cas9) that has the ability to specifically target DNA of interest without cleaving it [334]. Furthermore, neurons transduced with Kcna1-dCas9A displayed reduced firing rates and an elevated threshold compared to neurons with Ctrl-dCas9A hypothesizing Kcna1-

dCas9A to be a promising tool in the treatment of epilepsy [335]. Nevertheless, this gene editing tool is still in its early days with respect to its potential use as a therapeutic strategy and demands a high number of studies executed in animal models before its translation into the clinical practice. Targeting the proinflammatory cytokine high mobility group box-1 (HMGB1) represents another potential therapeutic target. HMGB1 is a nuclear protein that exerts regulatory functions on transcription and was shown to be increased in patients experiencing sepsis [336]. Furthermore, HMGB1 expression was found to be elevated in febrile seizures and SE in the intrahippocampal KA mouse model [289]. Anti-HMGB-1 mAb produces acute anti-seizure effects in PTZ-induced acute seizure and MES models as well as diminishes the severity of seizures triggered by kindling or KA. Anti-HMGB-1 mAb-treated mice did not display any signs of insensitivity up to two weeks after the administration [337]. Additionally, HMGB1 antibodies markedly lowered seizure-induced hippocampal damage and prevented apoptosis in the hippocampus in the pilocarpine-induced mouse epilepsy model [338]. However, the possibility of conformational shifts in the tertiary structure of the Ab-recognized area is one restriction of HMGB1 targeting utilizing antibodies [339]. Some research groups emphasize the role of mammalian target of rapamycin (mTOR) in chronic epilepsy and epileptogenesis [340,341]. mTOR is involved in processes regulating cell growth, and differentiation and synaptic plasticity by modulating cell metabolism and protein synthesis in the mammalian brain [342,343]. Several studies suggest mTOR dysregulation in the pathogenesis of different types of epilepsy, including tuberous sclerosis complex (TSC) patients with epilepsy, focal cortical dysplasia and TLE [344,345]. In addition, reports were published documenting that the mTOR inhibitor rapamycin reduces epilepsy in a mouse epilepsy model of TSC [346]. Recently, different mTOR inhibitors showed consistent protective effects in diverse rodent models of absence epilepsy and post-SE, suggesting rapamycin as a potential anticonvulsant target [347–349]. Besides, the PI3K/Akt/mTOR pathway regulates each other in different diseases, including epilepsy, and mTOR was shown to block caspase-1 activation and IL-1β production. Furthermore, rapamycin was demonstrated to suppress astrocytes and microglia activation, to reduce blood-brain vessel density, and mossy fiber sprouting in models of chronic epilepsy [350,351]. Interestingly, mycophenolate mofetil, which is a frequently used immunosuppressant reduces seizures and improves neurobehavioral comorbidities, by downregulating IL-2 and IL-1β mediated PI3K/AKT/mTOR pathway hyperactivation in a rat model of TLE [352]. Moreover, the new ASM everolimus might exert its anticonvulsant properties via the selective inhibition of the mTOR pathway [353]. However, rapamycin displays a low tolerability and severe side effects including, confusion, hallucinations, ear ringing, vomiting, bloody stools and even seizures, which questions the targeting of mTOR as a safe strategy to reduce seizures, in the absence of important adverse effects [354].

There is a plethora of approaches that try to identify the most accurate treatment for each seizure type. Unfortunately, most commonly used ASMs exert a broad spectrum of side effects ranging from mild to severe. For epilepsy patients and their caregivers, not only the control of seizures and the disease itself is desired, but also the freedom to follow their personal routine and have a social life, without the burden of major side effects. In this regard, drug tolerability should be seen as one important feature of new ASMs, especially for children and juveniles. Therefore, using single compounds or extracts of the *Cannabis sativa* L. plant may represent a highly promising solution

for DRE. The use of *C. sativa* for several neurological disorders, as well as pain, insomnia and rheumatism goes back to thousands of years first described in the Chinese culture [355]. In the last 50 years, a boom in the research on cannabis extracts and derivatives has been observed, though the knowledge of the exact molecular composition and downstream signaling pathways is still at the beginning. Recently, the role of PPARγ has received great interest because of its neuroprotective activity observed in diverse neurological disorders. Particularly, in view of the interaction of PPARγ with synthetic ASMs and naturally occurring compounds found in the Cannabis plant, PPARγ agonists, including thiazolidinedione, have been suggested as novel therapeutic agents to treat pharmacoresistant patients suffering from seizures and epilepsy [356,357]. In the following subsections 4 and 5, the impact of Cannabis derivatives and PPARγ in the treatment of seizures and epilepsy will be discussed in detail.

8. Receptors and Channels Possibly Mediating the Effects of Phytocannabinoids on Seizures and Epilepsy[1]

Abstract

Epilepsy contributes to approximately 1% of the global disease burden. By affecting especially young children as well as older persons of all social and racial variety, epilepsy is a present disorder worldwide. Currently, only 65% of epileptic patients can be successfully treated with antiepileptic drugs. For this reason, alternative medicine receives more attention. Cannabis has been cultivated for over 6000 years to treat pain and insomnia and used since the 19th century to suppress epileptic seizures. The two best described phytocannabinoids, (−)-*trans*-Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are claimed to have positive effects on different neurological as well as neurodegenerative diseases, including epilepsy. There are different cannabinoids which act through different types of receptors and channels, including the cannabinoid receptor 1 and 2 (CB_1, CB_2), G protein-coupled receptor 55 (GPR55) and 18 (GPR18), opioid receptor μ and δ, transient receptor potential vanilloid type 1 (TRPV1) and 2 (TRPV2), type A γ-aminobutyric acid receptor ($GABA_AR$) and voltage-gated sodium channels (VGSC). The mechanisms and importance of the

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interaction between phytocannabinoids and their different sites of action regarding epileptic seizures and their clinical value are described in this review.

Keywords: phytocannabinoids; epilepsy; anticonvulsant; cannabis; seizure

Introduction

Epilepsy is a chronic neurological disease affecting approximately 50 million people of all ages and sexes worldwide. This spectrum disorder not only impairs neuronal circuits, but also leads to social burden and severe morbidity showing highest incidence in young children and the elderly [358]. Epileptic seizures are defined as paroxysmal electrical discharges originated from various brain regions, leading to molecular, physiological, cognitive and social dysfunction [15]. The origin is thought to lie in the imbalance of the activation of excitatory and inhibitory synapses due to several causes, including genetic disorders, stroke, infections, injuries etc. According to the International League Against Epilepsy (ILAE), seizures can be classified in two groups: focal (or partial) and generalized. Focal seizures initiate in small groups of neurons in one hemisphere of the brain, or of one lobe, resulting in jerks and clonic movements. Generalized seizures involve both hemispheres from the onset and might lead to tonic-clonic movements and loss of consciousness and posture. Furthermore, epilepsy can be split into primary and secondary epilepsy. Primary epilepsy is of unknown cause without any previous physiological or molecular damage of the brain, whereas secondary epilepsy might occur as a result of neurological etiologies including those structural, genetic, infectious, metabolic, or immune [26,359].

Epileptic seizures can be caused by an imbalance of inhibitory and excitatory activity as shown by drugs able to block inhibitory type A y-aminobutyric acid receptor ($GABA_AR$) and the corresponding synaptic currents [360], or by activating ligand-gated and voltage-gated excitatory synaptic currents [361,362]. Thus, seizures might be limited by an increase of inhibitory currents [363] or decrease of excitatory currents [364,365]. However, in recent years many researchers have investigated this disease, but the exact mechanisms and ultimate cure still remains to be elucidated.

With the notable exception of absence seizures, anticonvulsants used for different epilepsy types act by enhancing inhibitory drive or by counteracting excitatory activity. Antiepileptic drugs (AEDs) have been used as a successful treatment for approximately 65% of suffering patients [366]. For instance, lorazepam and other benzodiazepines increase $GABA_AR$-mediated inhibition and thus display a decent medication for symptomatic seizures [367,368]. Other medications such as phenytoin and carbamazepine prevent voltage-gated Na^+ channels from activation and therefore reduce the firing of action potentials [369,370]. Nevertheless, these first-line medications are linked to strong-side effects and tolerability. In 1886, the first surgical approach for epilepsy was done by Horsley Victor, which expanded the therapeutic possibilities for epileptic seizures. He resected cortical tissue adjacent to a depressed skull fracture and healed a patient suffering from focal motor seizures [371]. Nowadays, for 35% of drug-resistant patients with refractory epilepsy, invasive treatments including surgical resection or neurostimulation have been demonstrated to be the only chance for cure. Thus, invasive treatments often appear as the ultimate prospect for these patients.

In the last years, the need for therapies for refractory seizures has largely arisen, which leads researchers to expand their mind to investigate more in alternative eligible treatments. In recent studies phytocannabinoids have been tested as an alternative approach for patients suffering from refractory seizures.

Cannabis sativa L. and Its Major Derivatives

Cannabis sativa L. has shown its medical potential for more than 6000 years, during which it spread from Northwestern Asia to Europe and finally to all over the world. The first historical evidence of medicinal use of *C. sativa* was recorded in the herbal medicine *Pên-ts'ao Ching* described by the Emperor Shen Nung around 2000 BC [372]. Furthermore, in ancient Greece and Rome the plant was used to treat pain, spasm and cramps [373]. The genus *Cannabis* belongs to the family of Cannabaceae. The taxonomy proposed by Small and Cronquist combining morphological and chemical description, considered Cannabis as monospecific (*Cannabis sativa* L.) with two subspecies (*Cannabis sativa* L. subsp. *sativa*, and *Cannabis sativa* L. subsp. *indica*) and four varieties (*Cannabis sativa* L. subsp. *sativa* var. *sativa*; *Cannabis sativa* L. subsp. *sativa* var. *spontanea*; *Cannabis sativa* L. subsp. *indica* var. *indica*; *Cannabis sativa* L. subsp. *indica* var. *kafiristanica*) [374]. Cannabis contains a characteristic class of isoprenylated resorcinyl polyketides compounds called phytocannabinoids to distinguish them from synthetic and endogenous cannabinoids. Notwithstanding phytocannabinoids are more characteristic of Cannabis, there are reports in the literature that phytocannabinoids also occur in other plants such as Helichrysum [375]. One hundred and fifty phytocannabinoids have been recorded for *C. sativa* to date and can be classified into 11 general types: (−)-*trans*-Δ^9-tetrahydrocannabinol (THC), (−)-*trans*-Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT), and miscellaneous types [375]. THC and CBD are the most important and studied plant cannabinoids. In 1940, CBD was first isolated from the plant [376]. In 1963 its structure was first described [377] and followed by its first identification as a crystal structure in 1977 [378]. The major breakthrough in cannabinoid research was achieved by Mechoulam and Gaoni in 1964 with the identification of the chemical structure of the first described psychoactive phytocannabinoid THC [379]. Cannabis varieties can be classified into five different chemotypes depending on the concentration of the main phytocannabinnoids. Drug-type cannabis varieties that have a high THC/CBD ratio ($\gg$1.0) are classified as chemotype I; varieties with an intermediate ratio (0.5–2.0) are classified as chemotype II; fiber-type varieties that have a low THC/CBD ratio ($\ll$1.0) are classified as chemotype III; chemotype IV are cannabis varieties that contain CBG as the main cannabinoid; and chemotype V cannabis fiber-type varieties that contain almost no cannabinoids [380].

Although CBD and THC have long been considered authentic natural products of cannabis, these molecules are not enzymatically synthesized in the plant, which instead produces cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA) (Figure 5).

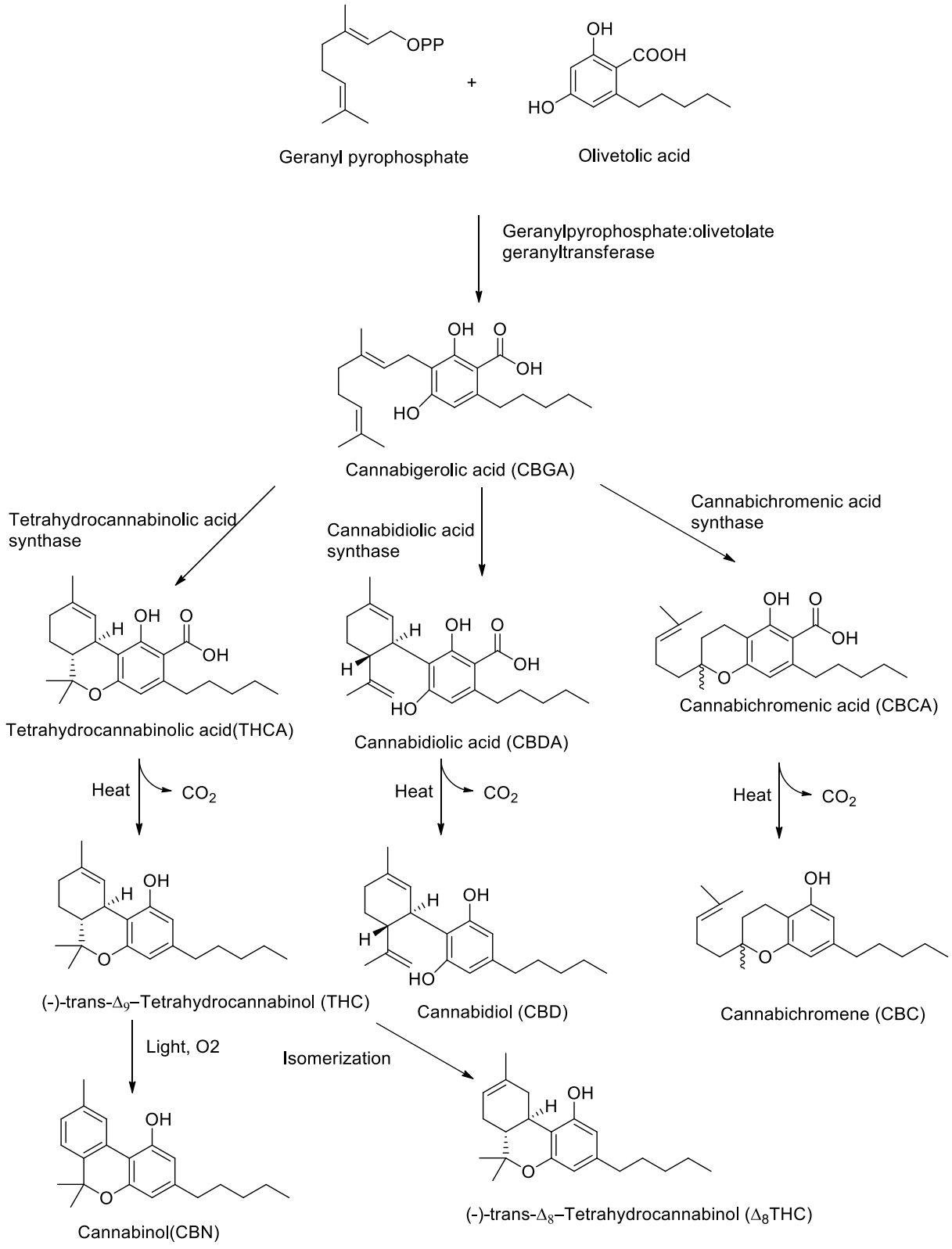

Figure 5. Biosynthetic pathway of major phytocannabinoids.

Today it is accepted that CBD and THC are an unnatural artifact of the corresponding acid precursors CBDA and THCA, produced via a temperature-catalyzed reaction. The different phytocannabinoids in plants originate from a common precursor, which is cannabigerolic acid (CBGA), in turn obtained by the alkylation of olivetolic acid with geranyl pyrophosphate (Figure 5) [381,382]. The other

phytocannabinoids are biosynthesized from CBGA by the action of oxidoreductase enzymes, such as THCA-synthase, CBDA-synthase and cannabicromenic acid (CBCA)-synthase, which lead to the formation of phytocannabinoids such as THCA, CBDA and CBCA [383]. These carboxylated cannabinoids, so-called "acids", are easily decarboxylated into the corresponding "neutral" derivatives, such as THC, CBD and CBC, with a non-enzymatic reaction of decarboxylation catalyzed by heat [384]. There are also different phytocannabinoids resulting from oxidation or isomerization of THC and CBD, such as CBN or Δ^8-THC.

THC is the main phytocannabinoid of cannabis which is responsible for the psychoactive properties such as psychotropic effects, including euphoria, appetite enhancement and alteration of sensory perception. Since the chemical structure of THC was elucidated by Mechoulam in 1964, a large number of scientific papers have been published concerning its mechanism of action. Furthermore, its structure was taken as a lead compound for the development of increasingly active synthetic cannabinoids. Such synthetic cannabinoids have different chemical structures like classical (e.g., nabilones) [385], non-classical (e.g., WIN55212-2) [386], aminoalkylindoles (e.g., JWH-018) [387] and endogenous arachidonic acid derivatives including endocannabinoids such as 2-arachidonoylglycerol (2-AG) and N-arachidonylethanolamide (AEA) [388–390]. THC does not only exhibit positive effects in the treatment of severe pain and nausea [391] but was also shown to inhibit T-cell immune-response, as well as to diminish inflammatory cytokine and chemokine release in rat microglia [392]. However, the use of cannabis is associated with abuse potential leading to behavioral changes and psychological impairment, because of the pharmacological properties of THC [393,394]. Therefore, THC is still not considered as a reliable, predictable and safe long-term derivative to treat neurological diseases such as epilepsy or depression [395]. Alternatively, to THC, CBD represents a promising tool against refractory epilepsy as it lacks psychoactive properties and presents few side effects. A reduced occurrence of seizure discharges in rats has been demonstrated in the hippocampus treated with CBD [396]. It has been shown that CBD exerts beneficial effects to restore the activity of hippocampal neurons [397] and prevents neuronal cell death in temporal lobe epilepsy (TLE) models [398]. It is furthermore observed that CBD, as THC, shows high anti-inflammatory [399], antioxidant, and anticonvulsant activity [400,401]. The cannabis-derived product Epidiolex® (GW Pharmaceuticals, Cambridge, UK) was approved in 2018 by the US Food and Drug Administration (FDA) for the treatment of the rare pediatric onset refractory epilepsy disorders Dravet syndrome (DS) and Lennox-Gastaut syndrome (LGS) [402]. Currently, Epidiolex®, which is composed of 100 mg/mL of CBD in sesame oil, has been tested in clinical trials with children, obtaining a 36.5% median reduction in monthly motor seizures during a 12-week treatment period. Adjunctive CBD could even raise the number to approximately 50% of seizure reduction during a total of 96-weeks. Even though this open-labeled study observed very few side effects (5%) and promising outcomes, one should be reminiscent about the placebo effect of this medication associated with parental expectation and media attention put on the children. Children, who moved across the US country to receive the treatment were twice as likely to decrease seizure events/month compared to children who happened to be living in the area of the medical center (47% versus 22%) [403,404].

Until now over 150 compounds of *Cannabis sativa* could be identified as phytocannabinoids of which a few numbers have already been tested to reveal healing properties (Figure 6). Δ^9-tetrahydrocannabutol (Δ^9-THCB), which is the butyl homologue of THC showed possible anti-inflammatory and analgesic activity in a model of acute inflammatory pain [405]. As THC, Δ^8-THC presents psychoactive effects and in the first experiments promisingly diminished the growth of lung adenocarcinoma both *in vitro* and *in vivo* [406,407]. The propyl analogue of THC, Δ^9-tetrahydrocannabivarin (THCV) is an antioxidant [408] and shows symptom-relieving and neuroprotective effects in animal models of Parkinson's disease [409]. As well as the propyl analogue of CBD named cannabidivarin (CBDV) offers medical advantages: CBDV is able to rescue motor impairment, cognitive dysfunction and brain atrophy in a mouse model of Rett syndrome [410]. Further compounds of the cannabis plant, which count to the most abundant cannabinoids are CBG and CBC: several studies observed that both CBG and CBC could be used against neuroinflammation, oxidative stress and exhibit analgesic effects [411–415]. As CBD, CBN has been detected to have many valuable responses against inflammation [416], convulsions [417] and pain [418]. The structures of the phytocannabinoids are presented in Figure 6. There are still many cannabinoids which have been isolated and described in the last years, but still remain to be experimentally explored.

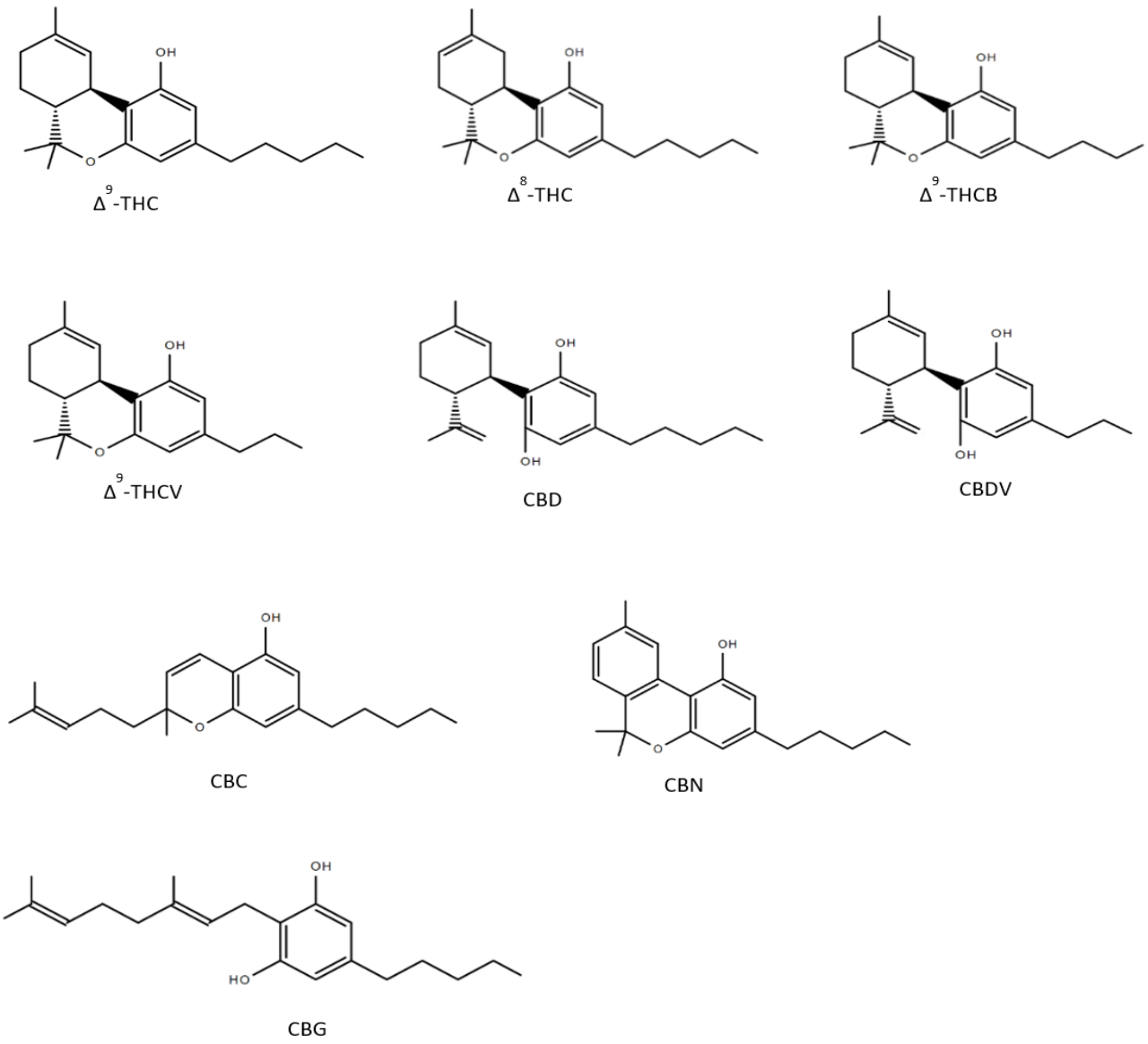

Figure 6. Structures of nine phytocannabinoids showing anticonvulsant activity; Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), Δ^9-tetrahydrocannabutol (Δ^9-THCB), Δ^9-tetrahydrocannabivarin (THCV), cannabidiol (CBD), cannabidivarin (CBDV), cannabichromene (CBC), cannabinol (CBN), cannabigerol (CBG).

A Brief Summary of the Endocannabinoid System (ECS)

The ECS is a key modulatory system involving the cannabinoid receptor 1 (CB_1) and 2 (CB_2), their endogenous ligands and the enzymes responsible for their biosynthesis and inactivation. It has been suggested that the ECS plays an important role in the neuroprotection of acute neurological diseases, such as epilepsy, as well as chronic neurodegenerative diseases such as Parkinson's disease [419,420].

The discovery of CB_1 and CB_2 receptors in the central nervous system (CNS) opened the field for the exploration of endogenous regulating systems and compounds associated with physiological processes and neurological disorders involving the endocannabinoids. CB_1 is mostly sited on

presynaptic inputs in several parts of the brain, including the olfactory bulb, the cerebral cortex and corpus striatum, and is highly expressed also in the hippocampus. In the dentate gyrus and CA3 hippocampal subfield, especially in the stratum oriens, highly dense receptor binding sites have been observed [421]. Conversely, CB_2 receptors are mainly expressed in cells of the immune and hematopoietic system, but they have been previously discovered in neurons of the brain stem [422]. In 1992, the first endogenous ligand of CB_1 was described and named anandamide (i.e., AEA) [389]. Three years later, 2-AG also was identified [388]; both are described as the most abundant endogenous ligands for CB_1 and CB_2. Anandamide primarily targets CB_1, while 2-AG shows agonistic effects on both CB_1 and CB_2 receptors [423]. Anandamide and 2-AG are important mediators of synaptic plasticity and are synthesized by the lipid precursors N-arachidonoyl phosphatidylethanolamine (NAPE) and diacylglycerol (DAG) in the cell membrane [424]. They are released "on demand" by physiological or pathological stimuli and act as retrograde messengers [425]. Their signaling pathway also may be initiated by the depolarization of a postsynaptic neuron, which opens voltage-gated calcium channels (VDCCs) leading to increased cytoplasmic calcium so to trigger endocannabinoid synthesis and release from the postsynaptic cell by a yet unknown mechanism. Endocannabinoids diffuse retrogradely to a presynaptic bouton and bind to receptors reducing the likelihood of release of the excitatory and inhibitory neurotransmitter [426,427]. Anandamide is hydrolyzed to arachidonic acid (AA) and ethanolamine by fatty acid amide hydrolase (FAAH), while 2-AG is hydrolyzed to AA and glycerol by monoacylglycerol lipase [428,429]. Both these endocannabinoids have been found to play a significant role in the regulation of excitatory synapses suggesting the impairment of endocannabinoid signaling being linked to epilepsy. It has been described that CB_1 and diacylglycerol lipase α are downregulated in epileptic human hippocampi [430]. The extracellular accumulation of 2-AG or anandamide was related to an anticonvulsant effect in the rat model of pentylenetetrazole-induced tonic-clonic seizures [431]. Additionally, in patients suffering from TLE lower concentrations of anandamide were found in the cerebrospinal fluid [432]. Therefore, inhibition of the breakdown of 2-AG and especially anandamide has been investigated lately as a new pharmaceutical target against epileptic seizures. The FAAH inhibitor URB597 was able to prevent or diminish alterations evoked by seizures in a kainic acid mouse model of TLE [433]. According to a study on cocaine-induced seizures in mice, URB597 inhibited seizure activity and showed a neuroprotective activity against seizure-related cell death [434]. However, the mechanism and function of inhibition of endocannabinoid hydrolysis in epileptogenesis requires further investigation.

Anticonvulsant Effects of Phytocannabinoids on Diverse Targets

The use of cannabis in neurological and neurodegenerative disorders is controversial and, thus, still under consideration. THC and CBD have been shown in numerous preclinical studies to diminish epileptic seizures, thus increasing their medical interest. They are able to regulate the excitability of neuronal circuits involving the ECS and associated ligands and receptors. CBD has been proved to act as a reuptake inhibitor of anandamide, changing the excitatory and inhibitory dynamics of synapses [435]. The fact that phytocannabinoids not only exhibit agonist and antagonist actions leads to the understanding that the wide range of targets could reveal opposing and unpredictable

effects. Therefore, the major exploration to specify the pharmacological targets of cannabinoids is crucial for the development of medicines for specific disorders. The promising beneficial health effects encourages many researchers in testing the possible therapeutic properties on seizures using phytocannabinoids with a chemical structure similar to THC and to CBD, such as Δ^8-THC, Δ^9-THCB, Δ^9-THCV, CBDV, CBN. The anticonvulsant properties of cannabinoids acting through different receptors and channels are depicted and visualized (Figure 7) as follows.

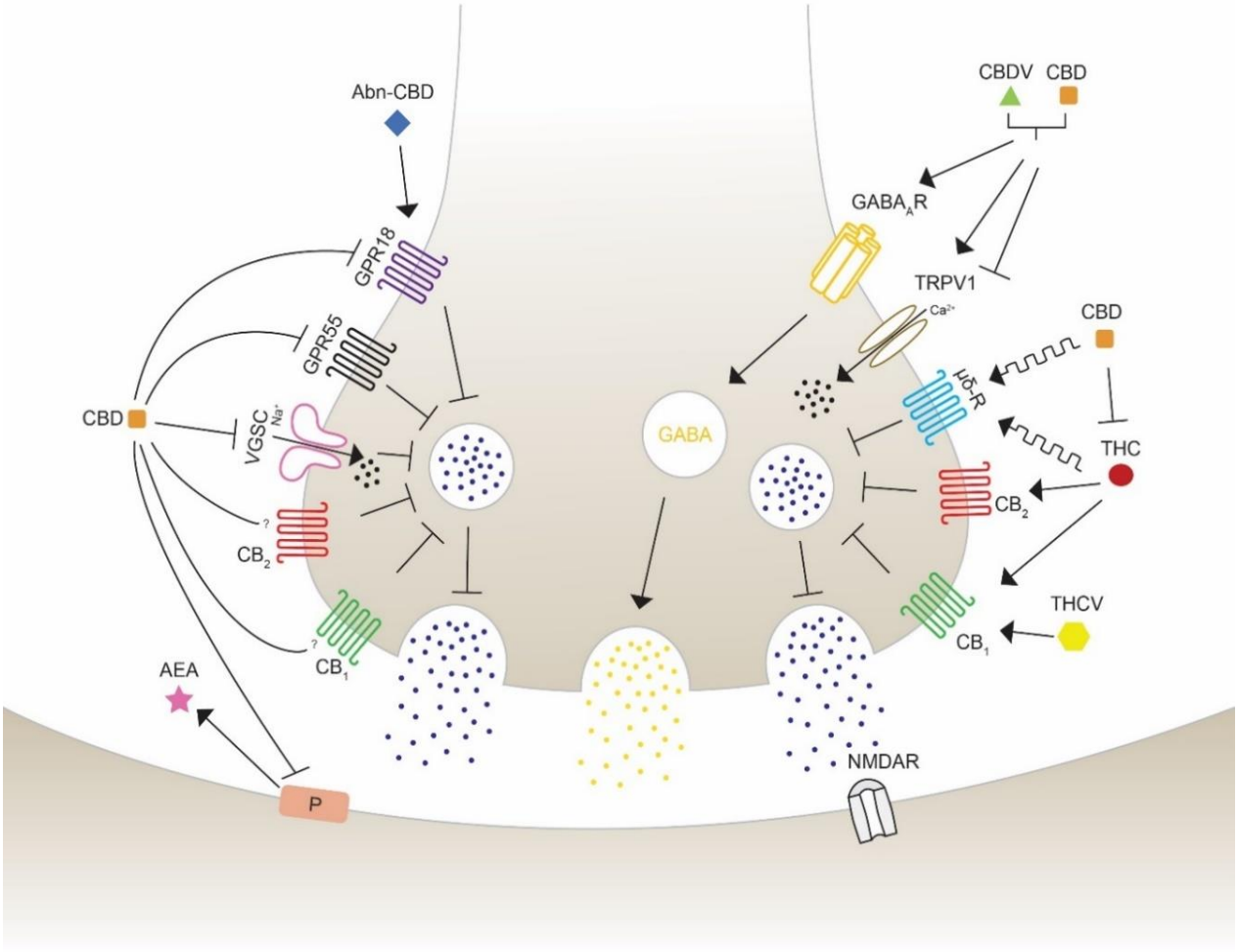

Figure 7. Schematic overview of the action of different phytocannabinoids possibly able to modulate seizures and epilepsy. Cannabidiol (CBD) inhibits the synthesis and mobilization of *N*-arachidonoylethanolamide (AEA) from the postsynaptic synapses, therefore acting in an independent and indirect antagonist manner with respect to the cannabinoid receptors CB_1 and CB_2. CBD antagonizes the activity of G protein-coupled receptor 18 (GPR18) and 55 (GPR55) and Na^+ influx of voltage-gated sodium channels (VGSC) to block neurotransmission activity. Abnormal-CBD (Abn-CBD) acts through GPR18 to decrease intracellular Ca^{2+} release (left side). CBD and CBDV are proposed to show positive modulation and agonist effects on the type A γ-aminobutyric acid receptor ($GABA_AR$), leading to an activation of GABA mobilization on inhibitory synapses. CBD and cannabidivarin (CBDV) activate and desensitize the transient receptor potential vanilloid

type 1 and 2 (TRPV1/2), reducing extracellular Ca^{2+} influx and decreasing Ca^{2+} concentration. (−)-*trans*-Δ^9-tetrahydrocannabinol (THC) and CBD are allosteric modulators of the opioid receptor type μ and δ, which inhibits the release of neurotransmitters to activate the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor leading to a seizure reduction. THC activates CB_1 and CB_2 leading to an inhibition of glutamate release. This action can be blocked by CBD, which is able to inhibit THC-CB_1 interaction. Δ^9-tetrahydrocannabivarin (THCV) is suggested to induce anticonvulsant activity in a concentration- and CB_1-mediated manner. The exact mechanisms of activation, inhibition or modulation are still under consideration. Furthermore, the different potency of the indicated molecules should be taken into account to correctly interpret the illustrated effects, as in the case of THC and CBD which bind to CB receptors with affinity, respectively, in the nM and mM range.

G protein-Coupled Receptors

Cannabinoid Receptors CB_1 & CB_2

In 1991, the cannabinoid receptor CB_1 was first described in the brain by receptor autoradiography and was identified as a G protein-coupled receptor [436,437]. The majority of CB_1 receptors are located on presynaptic boutons of GABAergic interneurons [438,439], but can also be found in glutamatergic synapses [440] Their main task is to reduce the likelihood of neurotransmitter release through various mechanisms, such as inhibition of calcium influx and adenylyl cyclase activity, or activation of the presynaptic potassium channels [441]. CB_1 receptors are the most abundant receptors in the human and murine brain, including the olfactory bulb, hippocampus, amygdala, cerebellum, neocortex and basal ganglia, but are also found in peripheral tissues and cells [421,442]. Sequence analysis showed that CB_1 sequence identity of humans and mice matches 97%, indicating the mouse as a reliable model for researching the CB_1 receptor [443]. In 2005, CB_2 receptors were first observed in the CNS, but their density is much lower than CB_1 and they are mainly located on microglia and specific neurons [422,444,445]. CB_2 receptors are primarily found in hematopoietic and immune cells including B-cells, T-cells and macrophages [446], having their major task in regulating the cytokine release [447]. CB_1 and CB_2 are the main targets of the endogenous ligands anandamide and 2-AG; whereas anandamide mainly binds CB_1, 2-AG shows agonist effects on both receptors [423]. Interestingly, a small number of phytocannabinoids have demonstrated to possess seizure-diminishing effects acting through both CB_1 and CB_2 receptors.

CBD is suspected to act also by interacting with targets different from CB_1 and CB_2 receptors, suggesting the involvement of alternative transduction mechanisms [448–450]. Due to the inhibition of breakdown of anandamide, CBD may have an indirect mechanism to reveal its anticonvulsant activity [435]. Moreover, CBD has even shown to have CB_1/CB_2 antagonist properties and appears to decrease the THC-CB_1 agonist activity in vitro [450]. However, the precise mechanism of the efficacy of CBD signaling is not fully understood yet. Similarly, CB_1 antagonist effects of Δ^9-THCV were observed, while high concentration appeared to be agonistic in a model of antinociception [448,451]. Other data showed that Δ^9-THCV exerts antiepileptic and anticonvulsant activities, suggesting a CB_1-mediated effect [452]. CB_1 and CB_2 represent important but not exclusive agonistic targets for the compounds Δ^8-THC and THC, whereas CB_1 plays the major role for psychoactivity [453]. There have been many controversial studies about the effects of THC on

seizure activity: some studies have shown anticonvulsant properties of THC in maximal electroshock rat model, while in other experiments the opposite effect of THC was observed, initiating seizures in Fischer rats and B6C3F1 mice [454,455]. It was also reported that both Δ^8-THC and THC significantly reduced the incidence of seizures on the first and second day of a 7-day administration in cobalt-epileptic rats [456]. CBN is another cannabinoid, which has shown its effectiveness to reduce seizures in a mouse model of maximal electroshock [457]. As a full agonist for both cannabinoid receptors, as well as inverse agonist for CB_2 (depending on the concentration), it has though not been proven if the anticonvulsant effects of CBN depend on one of the cannabinoid receptors or rely on other targeting [444,458]. Both phytocannabinoids (CBG and CBC) are partial CB_1/CB_2 agonists, but with no anticonvulsant effects [459,460].

G Protein-coupled Receptor 55 (GPR55)

The orphan receptor GPR55, which was identified in 1999, is expressed in regions of the CNS, including the caudate-putamen, and peripheral tissue such as the intestines, spleen and adrenals. This receptor is also located in the hippocampus, in particular the dentate gyrus excitatory neurons where it is suggested to be a regulator of spatial learning and memory, and synaptic plasticity. The rat GPR55 is composed of 319 amino acids that share an amino acid identity of 67% with the human GPR55 [461,462]. This putative cannabinoid receptor is involved in anti-inflammatory effects in microglial cells, and proliferation of pancreatic cells and tumor growth in mice [463,464]. GPR55 activates intracellular Ca^{2+} release in neurons, which can alter neuronal excitability by stimulating glutamate release [465]. For this reason, antagonist activity could result in the shift of excitatory and inhibitory balance. Notably, CBD was shown to increase inhibitory transmission by blocking GPR55, which leads to an attenuation of epileptic seizures as seen in a mouse model of DS, a severe form of childhood epilepsy [466]. GPR55 antagonism has been evaluated in several studies as a potential treatment for refractory epilepsy. Besides, it has been observed that THC, Δ^9-THCV, CBD, CBDV and CBG are able to block the response generated by the main endogenous GPR55 ligand lysophosphatidylinositol (LPI) and endocannabinoids [465,467]. However, THC and Δ^9-THCV, which are partial and weak GPR55 agonists, might act through different targets and, for this reason, they could involve a variety of mechanisms.

G Protein-coupled Receptor 18 (GPR18)

Discovered in 1997, GPR18 is, as GPR55, described as putative and orphan cannabinoid receptor and was shortly considered to be named CB_X and CB_3 receptor, respectively. Due to some missing criteria of the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR), these receptors were officially declared as orphan cannabinoid receptors [468,469]. In the mouse, GPR18 is located in a broad range of tissues including the cardiovascular system and the gastrointestinal tract, where it is involved in obesity/diabetes-associated inflammation and lymphoid system regulation. In humans, this receptor has not been as well studied as in rodents, but shows expression in the brainstem, hypothalamus, testis, spleen and lymph nodes [469–472]. GPR18 is composed of 331 amino acids and overlaps in amino acid identity with a similarity of 86% (mouse) and 85% (rat) to humans [473]. The downstream signaling of GPR18

is activated by *N*-arachidonoyl glycine (NAGly), which is a mixed agonist/antagonist endogenous ligand shown to activate intracellular Ca^{2+} mobilization [474]. The receptor is found on the cell surface of macrophages and microglial cells, as well as intracellularly, so to modulate the downstream signaling [475–477]. There is evidence that it plays a vital role in apoptosis of inflammatory leukocytes and is engaged in the reduction of intraocular pressure in mice [478,479]. As a GPR18 antagonist, CBD inhibits NAGly and was suggested to lower the effects of excitation to restrain the action potential firing. Abnormal cannabidiol (Abn-CBD) is a synthetic regioisomer of CBD, since it has the same functional group on a different position. It is a selective ligand and agonist for GPR18 able to induce a reduction in calcium release [480]. The selective and concentration-dependent ligand THC is able to induce proconvulsant effects by activating the calcium mobilization and, therefore, excitation by activating GPR18. Moreover, CBD was able to block the effects of THC in a simultaneous treatment. The evidence for the ability of CBD and THC to regulate the activity of GPR18 is still poor and does not allow a clear interpretation; however, there is a strong interest for the possible therapeutic use of these molecules [475].

Opioid Receptor μ and δ

Opioid receptors are membrane receptors located in multiple regions of the CNS, including various hypothalamic nuclei, amygdala, hippocampus, substantia nigra, dorsal root ganglia, spinal cord, etc.; they are also peripherally found, as in the gastrointestinal apparatus [481]. Due to their broad range of involvement in numerous neurological modulations, such as mood disorders, pain perception and drug abuse, opioid receptors are widely explored [482–484]. THC and CBD might act as allosteric modulators of the opioid receptor subtypes μ and δ [485]. Antagonists of selective δ receptors were shown to diminish *N*-methyl-D-aspartate (NMDA) receptor-mediated seizures in vivo [486]. CBD revealed in multiple studies beneficial effects on massive uncontrolled glutamatergic firing, especially mediated by NMDA receptors. A study in 2018 proposed that CBD may act either by an unknown mechanism or as antagonist-like agent towards δ receptors to reduce NMDA receptor-induced seizures in vivo [487].

Transient Receptor Potential Vanilloid

In 2001, phytocannabinoids were first observed to interact and modulate the transient receptor potential vanilloid (TRPV) type 1 and 2 [488]. TRPV represents a subtype of the transient receptor potential channel (TRP), consisting of six transmembrane helices, a cation-permeable pore with intracellular N- and C-termini, allowing a calcium influx into the cell. TRPV1 (capsaicin-sensitive) and TRPV2 (capsaicin-insensitive) are widely located on distinct dorsal root ganglia neurons, trigeminal ganglia, peripheral afferent fibers and especially on nociceptive sensory endings, where they transduce pain, temperature, proinflammatory stimuli, and can be also activated by chemical substances, such as anandamide, vanilloids and cannabinoids [489]. As TRPV1 agonists, CBD rapidly dephosphorylates and desensitizes TRPV1 channels leading to a decrease in calcium influx and therefore reduced neurotransmission. There is evidence that TRVP1 channels are overexpressed in models of TLE and patients suffering from epilepsy [490]. Consistent with these observations, CBD presented reduced anticonvulsant properties in TRPV1 knock-out mice [491]. Patch-clamp analyses

performed in HEK293 cells revealed that CBD and CBDV activated and desensitized TRPV1 and TRPV2 in a dose-dependent manner. In addition, CBDV was able to significantly decrease the amplitude and duration of epileptiform neuronal spikes [492]. In a model for juvenile seizures, CBDV was able to suppress seizures induced by pentylenetetrazole at postnatal day 10 (P10) in rats. Otherwise, in P20 rats CBDV decreased seizures induced by pentylenetetrazole or methyl-6,7-dimethoxyl-4-ethyl-β-carboline-3-carboxylate administration, and also by maximal electroshock stimulation, in agreement with the results obtained in P20 TRPV1 knockout mice with the same drug. These findings show that the effects of CBDV in different ages and epilepsy models are TRPV1-dependent [493]. Other phytocannabinoids including CBN, CBG, CBC and Δ^9-THCV show TRPV type 1-4 agonistic activity, but a correlation to epileptic behavior has not yet been found [494,495] . These results will lead to further basic research on targeting TRPV1 and TRPV2 to test their promising potential in clinical treatment of epilepsy.

$GABA_A$ Receptors

The $GABA_AR$ was first detected in the brain in 1950 and is a member of the pentameric ligand-gated ion channels superfamily [496]. To date, there are 19 different subunits present in the mammalian brain: six α (α1–6), three β (β1–3), three γ (γ1–3), and δ, ϵ, θ, π and ρ_1 to ρ_3, which form the heteromeric $GABA_A$ receptors providing a wide spectrum of pharmacological and physiological characteristics [497]. When GABA, which mediates the main inhibitory neurotransmission in the brain and spinal cord, binds to $GABA_AR$, the rapid influx of Cl^- is activated resulting in hyperpolarization and inhibition of the cell [498,499]. Those currents can be regulated by several positive and negative allosteric modulators such as benzodiazepine, neurosteroids, zinc and phytocannabinoids. The composition of the subunits of $GABA_AR$ is responsible for the sensitivity to those modulators, meaning that a shift in subunit composition could alter the functioning of $GABA_AR$ and lead to unexpected neuronal impairment [500]. $GABA_A$ receptors are expressed on synaptic and extrasynaptic sites of the most, if not all, neurons in the CNS and were previously found to be also located on human peripheral blood mononuclear cells (PBMC) [501]. They are considered as being the most crucial receptors for pharmacological and physiological alteration and contribute to the etiology of numerous neurological and mental diseases such as epilepsy, schizophrenia and Angelman's syndrome [502–504]. Multiple studies proved that a change of $GABA_AR$ subunit composition occurred in a model of kainic acid induced TLE, as well as in hippocampal tissues from patients affected with TLE [109,502,505]. At a concentration of 100 µM, CBD acts as a positive allosteric modulator of $GABA_AR$ as showed by voltage clamp electrophysiological measurements in Xenopus oocytes. Interestingly, coadministration of CBD and clobazam resulted in a greater anticonvulsant potency than clobazam only, because it significantly enhanced $GABA_AR$-mediated transmission in a mouse model of DS [506,507]. An additional study examined patients with DS who donated their brain tissue to be transplanted in Xenopus oocytes to prove that a low dose of CBD was able to significantly enhance $GABA_A$ currents [508]. CBDV is yet an unknown $GABA_AR$ agonist but demonstrated in a similar preclinical study for TLE its anticonvulsant properties mediated by $GABA_AR$. TLE tissue with hippocampal sclerosis was derived from pharmaco-resistant TLE patients and showed, after a prolonged incubation with CBDV, a

recovery of the current rundown of $GABA_AR$ [509]. However, there is still little evidence about the anticonvulsant effects of CBDV mediated by $GABA_AR$.

Voltage-Gated Sodium Channel (VGSC)

VGSC (Na_VS) were discovered by Hodgkin and Huxley in 1952, when examining the excitation and conductance in axons of giant squids and were first isolated from the eel electroplax [510,511]. They are members of the cation channel superfamily and are responsible for the Na^+ conduction through the cell plasma membrane. Mammalian VGSC are composed of a large pore-forming α-unit that associates with one or two β subunits and have been found in almost every type of neuron examined. VGSC subunit α has nine known subtypes, which show tissue specific expression properties. The subtypes $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$ are primarily located in the CNS and peripheral neurons, whereas the other members are expressed in skeletal, cardiac muscles and interstitial cells of Cajal [512]. Mutation in the gene SNC1A, which encodes $Na_v1.1$ results in cognitive impairment and causes due to the deficient sodium channel shift in neuronal excitability, resulting in phenotypes known as generalized epilepsy with febrile seizures. Furthermore, this mutation—occasionally in combination with $GABA_AR$ impairment—is carried by the majority (70–80%) of patients with DS [513,514]. Further mutations in $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ have been linked to the occurrence of epileptic seizure in patients of all ages [515–517]. Acting as an agonist, CBD appeared to inhibit and block the opening of $Na_v1.1$ to $Na_v1.7$ with low µM potencies, measured in human cell culture and rat brain slices [459]. In a Hodgkin-Huxley model of cortical neuron, CBD could decrease and stabilize neuronal excitability [518]. A different study showed that CBD was able to preferentially target and inhibit aberrant and increased resurgent currents in mutations in $Na_v1.6$. Moreover, CBD demonstrated to diminish overall action potential firing of murine striatal neurons, assuming the high potential of treating drug-resistant children affected from DS who carry gene mutations in VGSC with CBD [519].

Isolated Phytocannabinoid versus Cannabis Extract: the "Entourage" Effect

The application of individual phytocannabinoids isolated from cannabis extract in therapy has recently sparked widespread debate [520], in spite that Mechoulam elucidated the structure of THC in 1964 and a drug based on the stereoisomer produced by cannabis, THC (Dronabinol), was approved by the FDA as safe and effective drug for HIV/AIDS-induced anorexia and chemotherapy-induced nausea and vomiting [521]. This is because the employment of cannabis or its medicinal extracts is still widespread, since anecdotal evidence indicates that cannabis extracts are more potent and with less side effects than THC itself. Several recent works indicate that other components of the cannabis extract may somehow interact with THC [520,522–525]. Studies in humans and animals suggest high potential for CBD to attenuate the effects of THC, in particular in decreasing the effects of THC on cognition/memory [526–528]. Conversely, there are preclinical studies that indicate that CBD may potentiate some effects of THC [519,529–534]. However, the few studies about the CBD-THC interaction represent a profound lack of research respecting the manner how CBD may affect behavioral and physiological effects of THC.

As THC, CBD was also formulated as a single active substance drug [535]. But also, in this case the use of hemp (cannabis for fiber of chemotype III) extract is widely considered for both nutraceutical and medicinal purposes. A recent study proposes that hemp extract requires a dose of CBD four times less than the drug containing the single CBD molecule to achieve the same therapeutic effect [536]. In this article it is clearly indicated how the use of hemp extract leads to a lower incidence of the adverse effects observed when administering the purified CBD. E. Russo refers to this synergy as an "entourage" effect [520]. The main problem with the use of hemp extracts is the poor knowledge of the entire chemical composition. Over 500 compounds and over 200 terpenes have been identified in cannabis so far [537]. Each of these compounds could be present in the hemp extract and could influence the pharmacological activity of CBD. Furthermore, the non-compliance of strict rules of drug preparation could lead to high concentrations of THC in the extract with side effects typical of the latter compound.

It is challenging to identify the absolute chemical composition of the hemp extract even if, thanks to recent sophisticated analytical techniques, it is possible to determine a large quantity of substances present even at very low concentrations.

In conclusion, the use of CBD extract certainly offers advantages over isolated CBD, but it becomes difficult, if not impossible, to standardize it for each of its chemical components with consequent variability in pharmacological action.

Conclusions

For thousands of years the cannabis plant has represented a significant medical and economic value and is used all over the world. With the first preclinical experiments solely using selected compounds widened the enormous impact on alternative treatments for neurological diseases. *Cannabis sativa L*. stepped in the main focus of present research and reached an approximately 2400-fold publication rate since the first official cannabis report in 1939. While the first experimental approaches of THC and CBD have revealed possible beneficial health effects in general, previous studies focus more on distinct targets as well as signaling cascades in diverse models of specific diseases. Therefore, more and more derivatives of the plant were isolated and tested on a molecular and behavioral level, leading to the knowledge that most phytocannabinoids act through a broad spectrum of targets, which complicates the understanding of their exact pathway and action. THC and CBD have shown in numerous preclinical studies to diminish duration, severity, and incidence of epileptic seizures in combination with remote adverse effects. With the approval of the first cannabis-derived medical drug in 2018, Epidiolex® has fought its way from the laboratory to patients of all ages suffering from refractory epilepsy depicting a high success rate. The severe forms of pharmaco-resistant childhood epilepsy, DS and LGS respond to Epidiolex® with a 36–50% amelioration rate; a milestone in epilepsy research [423,538]. Even though the anticonvulsant efficacy of different phytocannabinoids including CBD, THC, Δ^8-THC, Δ^9-THCB, Δ^9-THCV, CBDV, CBN have been proven, the precise mechanism and modulation of targets depicted in this review opens many questions. Most phytocannabinoids are not only restricted to the modulation of a single receptor, but rather have the ability to regulate various receptors and channels and might therefore change the entire circuitry. In the past years, researchers have

identified further targets of cannabinoids such as serotonin receptor ($5HT_{1A}$, $5HT_{2A}$, $5HT_{3A}$), GPR12, glycine receptor, acetylcholine receptor, peroxisome proliferator-activated receptors, α_2 adrenergic receptor, equilibrative nucleoside transporter and VGCCs. Until now there is no evidence that cannabinoids have beneficial effects on epilepsy acting through those receptors or channels and still remain to be examined.

Recently, two new compounds have been first isolated from the cannabis plant: the butyl and heptyl homologs of THC: Δ^9-THCB and Δ^9-THCP, respectively. Regarding their characteristics showing high CB_1 affinity and overall high cannabimimetic activity, those new derivatives represent promising tools in the research of neurological diseases and especially epilepsy, which needs to be established in future studies [405,539][2].

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9. Is the peroxisome proliferator-activated receptor gamma a putative target for epilepsy treatment? Current evidence and future perspectives[3]

Abstract

The peroxisome proliferator-activated receptor gamma (PPARγ), which belongs to the family of nuclear receptors, has been mainly studied as an important factor in metabolic disorders. However, in recent years the potential role of PPARγ in different neurological diseases has been increasingly investigated. Especially in the search of therapeutic targets for patients with epilepsy the question of the involvement of PPARγ in seizure control has been raised. Epilepsy is a chronic neurological disorder causing a major impact on the psychological, social, and economic conditions of patients and their families, besides the problems of the disease itself. Considering that the world prevalence of epilepsy ranges between 0.5%-1.0%, this condition is the fourth for importance among the other neurological disorders, following migraine, stroke, and dementia. Among others, temporal lobe epilepsy (TLE) is the most common form of epilepsy in adult patients. About 65% of individuals who receive anti-seizure medications (ASMs) experience seizure independence. For those in whom seizures still recur, investigating PPARγ could lead to the development of novel ASMs. This review focuses on the most important findings from recent investigations about the potential intracellular PPARγ-dependent processes behind different compounds that exhibited anti-seizure effects. Additionally, recent clinical investigations are discussed along with the promising results found for PPARγ agonists, and the ketogenic diet (KD) in various rodent models of epilepsy.

Keywords: Antiseizure medications; Anticonvulsant; epilepsy; Peroxisome proliferator-activated receptor gamma; Seizure; Thiazolidinediones.

Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ACA, acetoacetate; AEA, anandamide; AF-1/2, activation function 1/2; aP2, adipocyte fatty-acid-binding protein; AME, Aegle marmelos L. leaf extract; ASM, anti-seizure medication; Bax, b-cell lymphoma protein 2 (Bcl-2)-associated X; BBB, blood-brain

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barrier; BDNF, brain-derived neurotrophic factor; BHB, β-hydroxybutyrate; BSA, body surface area; CA3, cornu ammonis 3; CBD, cannabidiol; CB1, cannabinoid receptor type 1; CBP, CREB binding protein; CD36, cluster of differentiation; CNS, central nervous system; CO, carbon monoxide; COX-2, cyclooxygenase-2; CPT-1, carnitine palmitoyltransferase I; CYP4A, cytochrome P450 4A; DBA, dibenzylamine; FAAH, fatty acid amide hydrolase; FABPs, fatty-acid binding proteins; FMOC-L-leucine, N-[9-fluorenylmethoxycarbonyl]-)-L-leucine; GABA, gamma-aminobutyric acid; HO-1, heme oxygenase-1; IL-1β,6,10, interleukin-1 beta,6,10; l-NAME, N-nitroarginine methyl ester; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; i.v., intravenous; KA, kainic acid; KD, ketogenic diet; LBD, lipid binding domain; MCAD, medium-chain acyl-CoA dehydrogenase; MMP-2/9/13, metalloproteinase-2/9/13; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; nuclear receptors, NRs; Nrf2, nuclear factor erythroid-2 related factor 2; MMP-2/9/13, metalloproteinase-2/9/13; NADPH, nicotinamide adenine dinucleotide phosphate; PI3K, phosphatidylinositol 3-kinase; p.o., per os; PPARγ, peroxisome proliferator-activated receptor gamma; PGC1α/β, PPARγ coactivator 1 α and β; PPREs, PPAR-responsive elements; PTEN, phosphatase and tensin homolog; PTZ, pentylenetetrazole; PUFAs, polyunsaturated fatty acids; RXR, 9-cis-retinoic x receptor; SE, status epilepticus; SOD, superoxide dismutase; SRSs, spontaneous recurrent seizures; SRC1/2/3, steroid receptor coactivator 1/2/3; SUDEP, sudden unexpected death in epilepsy; TLE, temporal lobe epilepsy; TNF-α, tumor necrosis factor alpha; TrkB, tropomyosin-related kinase B; TRIM27, tripartite motif family 27; TZDs, thiazolidinediones; UCP-2, uncoupling protein 2; VEGFR2, vascular endothelial growth factor (VEGF) receptor 2.

Introduction

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors (NRs) for fatty acids (FAs) and important regulators of lipid metabolism. In 1990, Isseman and Green first cloned PPARs in rodents as orphan members of the steroid hormone superfamily, which includes steroid, retinoid and thyroid hormone receptors [540]. As other members of the nuclear receptor gene family, PPARs are ligand-activated transcription factors. After binding with specific agonist ligands the receptor gets translocated to the nucleus, where the expression level of mRNAs encoded by PPAR target genes changes: a process known as “transactivation” [541]. These receptors are involved in the regulation of gene expression in response to different physiological stimuli, such as metabolism, inflammation, adipogenesis, cell differentiation, and migration [542–549]. In the last years, PPARs have emerged as targets in various clinical conditions including diabetes, cancer, hypertension, inflammation, atherosclerosis, allergic diseases, as well as psychiatric and neurological disorders such as, respectively, depression, Parkinson’s disease, Alzheimer’s disease, Huntington’s disease and epilepsy [550–560].

In general, epilepsy is defined as a chronic neurological disease that is characterized by recurrent spontaneous seizures (SRSs) induced by temporary disruptions of brain functions. Particularly, epileptic seizures are determined by an imbalance in the activation of excitatory and inhibitory synapses caused by a variety of factors such as genetic abnormalities, stroke, infections, and traumas, among others [561]. Besides affecting about 50 million individuals globally, epilepsy is ranked fourth among the most prevalent neurological illnesses, behind the group of headache and migraine disorders, dementias, and stroke [17]. This spectrum disorder not only alters neural

circuits, but also causes social burden and significant morbidity, with small children and the elderly being the most affected [360,562,563]. Importantly, the diagnosis of epilepsy has implications in all parts of daily life, including educational prospects, family arrangement, and career. Nowadays, more than one-third of patients with epilepsy experience inadequate seizure control with anti-seizure medications (ASMs), raising the demand to develop new drugs and targets involved in the mechanisms of epilepsy [366]. Targets for ASMs participate in various mechanisms of action, including (i) voltage-gated ion channels (calcium, potassium, and sodium channels), (ii) reinforcement of synaptic inhibition (e.g., γ-aminobutyric acid, GABA), (iii) inhibition of synaptic excitation (e.g., glutamate), (iv) modulation of neurotransmitter release (e.g., the synaptic vesicle protein SV2A) [167,564]. Benzodiazepines, for example, potentiate the GABA type A ($GABA_A$) receptor-mediated inhibition, therefore appearing to be a recommended treatment for most types of seizures [368]. Other ASMs, such as phenytoin and carbamazepine, reduce the activation of voltage-gated sodium channels, preventing paroxysmal activation from being triggered [369,370]. However, these first-line pharmaceuticals are associated with a long list of severe side effects such as nausea, insomnia, psychomotor slowing, anxiety, tremor, diplopia, ataxia, among many others [565,566].

Notably, targeting the isotype PPARγ showed neuroprotective functions by inhibiting oxidative stress and inflammation in epilepsy [567]. For instance, the PPARγ agonists thiazolidinediones (TZDs), applied in different models of epilepsy, displayed protection against apoptotic neuronal cell death in the hippocampus following *status epilepticus* (SE), which is a true medical emergency with an up to 20% mortality rate [568,569]. In addition to this, we recently demonstrated that PPARγ is involved in the anti-seizure properties of EP-80317, a ghrelin analogue tested in a model of repeated seizure induction [570]. By using the systemic kainic acid (KA) rat model, we also found that the anti-seizure effects of cannabidiol (CBD) might lead, at least in part, to increased PPARγ levels in hippocampal and extrahippocampal regions, and especially in the CA3 hippocampal region [571]. Taken together, the beneficial effects of the activation of PPARγ signaling cascades should be considered in the development of novel pharmacological treatment options for epilepsy.

This review outlines the most significant results of the last decades about the possible intracellular PPARγ-dependent mechanisms behind different molecules which displayed anti-seizure effects (listed in Table 6), including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tropomyosin-related kinase B (TrkB), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), cluster of differentiation 36 (CD36), cyclooxygenase-2 (COX-2), and caprylic acid (C8). Moreover, the promising findings observed for various ASMs, PPARγ agonists and the ketogenic diet (KD) in different animal models of epilepsy, along with recent clinical studies, are summarized.

PPARγ: structure, localization, pathway

PPARs are members of the ligand-activated nuclear receptor superfamily that are involved in regulation of genes crucial in cell differentiation and biochemical processes. They play a key role in the lipid and glucose homeostasis, by serving as detectors in managing the lipid metabolism and insulin sensitivity [572]. Targeting PPARs in clinical treatments has its origin in the therapy of type

2 diabetes using PPAR agonists. For instance, TZDs activate genes that favor triglyceride storage, reducing circulating free fatty acid concentrations [573,574]. One mechanism by which these medicines optimize insulin sensitivity in peripheral tissues is the transition from free fatty acids to glucose as an energy source, therefore preserving pancreatic β-cell functions [575]. In general, there are three different isoforms of PPAR that have been identified, which are encoded by three different genes: PPARα (NR1C3), PPARβ/δ (NR1C1) and PPARγ (NR1C2).

PPARα, the major PPAR subtype, reveals its highest expression in metabolically active tissue, as in the heart, brain, kidney, and liver, where it stimulates the breakdown of fatty acids and cholesterol, promoting gluconeogenesis and lowering triglyceride levels [576–578]. It is necessary for the stimulation of peroxisomal and microsomal enzymes such medium-chain acyl-CoA dehydrogenase and cytochrome P450 4A (CYP4A), as well as carnitine palmitoyltransferase I, the rate-limiting enzyme for β-oxidation of fatty acids, and fatty acid translocases and transport proteins, mechanisms that can be mediated for example by the PPARα agonist clofibrate [579,580]. Moreover, numerous studies showed that the use of PPARα agonists in epilepsy, such as fenofibrate, can raise the seizure threshold in an acute pentylenetetrazole (PTZ)-induced kindling mouse model, pilocarpine-induced seizures in rats, and gain seizure control in nicotine-induced seizures in mice and rats, as well as in genetic models of epilepsy [560,581–585]. PPARβ/δ is found in the lungs, brain, skin and skeletal muscles, where it interacts with fatty acids produced from low density lipoproteins and eicosanoids [578,586]. Interestingly, valproic acid, which represents one of the most efficient ASMs, has been shown to activate PPARβ/δ [587,588]. Furthermore, the PPARδ agonist GW0742X has demonstrated to suppress the expression of inflammatory mediators and adhesion molecules, implying that it may play a role in atherogenesis prevention [589]. Due to either alternative transcription start sites or alternative splicing, there are four different mRNA isoforms termed PPARγ1, PPARγ2, PPARγ3, and PPARγ4 [590–592]. In particular, the genes of PPARγ use separate promoters and 5’ exons; therefore, mRNA of PPARγ1, PPARγ3, and PPARγ4 code the same protein, whereas a different protein is produced from PPARγ2 mRNA, containing 30 extra amino acids at the NH_2 terminus [593]. They are distinguished by differences in expression activity and location: PPARγ1 displays the broadest expression pattern (heart, intestines, kidney, spleen, cardiac muscles, white and brown adipose tissue), while mRNA of PPARγ3 is found only in adipose tissue, macrophages, and colon epithelium. PPARγ4 is expressed in endothelial cells [586], whereas PPARγ2 is exclusively expressed in adipose tissue [594,595]. PPARγ plays a key role in adipocyte differentiation, resulting from an improvement of adipocyte sensitivity to insulin [596]. In the brain, PPARγ is detectable in specific regions of the frontal cortex, hippocampus, nucleus accumbens, amygdala, hypothalamus, ventral tegmental area, thalamic paraventricular, nucleus reuniens, substantia nigra, thalamus, and granular layers of the cerebellum [597–600]. As known, these brain structures are involved in several neuroanatomical changes during epileptic seizures [601]. For instance, cortical and subcortical areas are involved in generation, maintenance, and propagation of epileptic activity. In this regard, the piriform cortex and amygdala can generate seizures in response to chemical and electrical stimulation and amplify the epileptic activity when seizures are generated in other brain regions [602]. The hippocampus is also well-known to be affected by epilepsy, such as TLE in adult patients [45]. Moreover, thalamic lesions seem to be

associated with atrophy within the limbic system, and the thalamus might be involved in the evolution and propagation of partial seizures [603]. In the substantia nigra, sustained seizures may induce damage [604].

Reportedly, PPARγ regulates hundreds of genes, many of which are engaged in glucose and lipid metabolism, microglial activation, inflammation, and mitochondrial biogenesis and many more [543,595,605]. Lines of evidence suggest that PPARγ is able to significantly alter various intracellular pathways during an epileptic seizure so to be evaluated as an important modulator of epilepsy (a list of the regulated genes is provided in Table 1), which will be discussed in detail below in further subsections. Common to other nuclear receptors, PPARγ has a modular structure containing functional domains [606]. Specifically, the A/B domain in the amino (N)-terminal contains a weak ligand-independent trans activation function 1 (AF-1), which is a sequence that varies greatly among nuclear receptors. The N-terminal is followed by a highly conserved carboxy (C) domain, which corresponds to the DNA-binding domain (DBD). This domain includes two zinc fingers that bind to certain DNA sequences which are hormone response elements (HRE) known as PPAR-responsive elements (PPREs) [607]. PPREs are direct repeat elements (DR-1) consisting of two hexanucleotides long core recognition motives (AGGTCA) that are partitioned by one single nucleotide spacer. Each zinc finger has four cysteine residues that allow the zinc ion to be coordinated. The inclusion of zinc fingers distinguishes nuclear receptors from other DNA binding proteins. DBD is connected to the ligand binding domain (LBD) via a flexible hinge region and is a target of post-translational modifications such as phosphorylation and sumoylation [608]. LBD is found in the C-terminus of the receptor containing a ligand-dependent AF-2 [609]. Formed by 12 α helices and 4 β sheets, this domain, also known as E domain, has in comparison to other nuclear receptors a large binding pocket (1300-1440 $Å^3$), allowing interaction with a wide range of endogenous, natural, and synthetic ligands [610]. Because of AF-1 low transcriptional activity, it synergizes with AF-2 to provide a stronger upregulation of gene expression [611]. Finally, the F domain carboxy-terminal seems to display a modulatory function on the LBD domain (Figure 8).

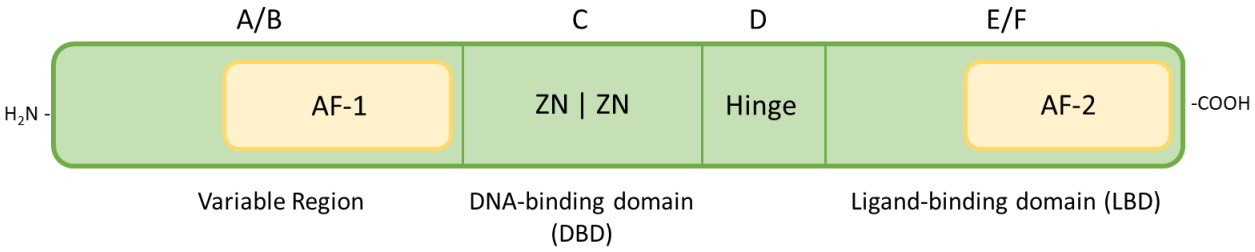

Figure 8. Schematic illustration of the functional PPARγ domains.

All isoforms of the nuclear receptors, including PPARγ, bind to DNA either as a hetero- or homodimer. In the case of PPARγ, binding as a heterodimer with 9-cis-retinoic x receptor (RXR) is

obligatory to proceed further actions [612,613]. Binding of a ligand to one of the receptors of the heterodimer provokes a conformational change. PPAR/RXR complex subsequently binds to PPRE located in the 5' end region of their target genes with the cooperation of cofactors. PPRE and sequence variants have already been identified in several PPARγ-induced genes such as acyl-CoA oxidase (AOX) and adipocyte fatty-acid-binding protein (aP2) (Table 6) [614]. PPARγ coactivator 1 α and β (PGC1α and PGC1β), CREB binding protein (CBP) and steroid receptor coactivator (SRC1, SRC2, SRC3) are just few coactivators of PPARγ, which display many different molecular mechanisms as, for example, the induction of changes in chromatin structure and recruitment of the transcriptional machinery to promote gene transcription (Figure 9) [615]. In Table 6, PPARγ induced genes and their involvement in epilepsy and seizure development are summarized.

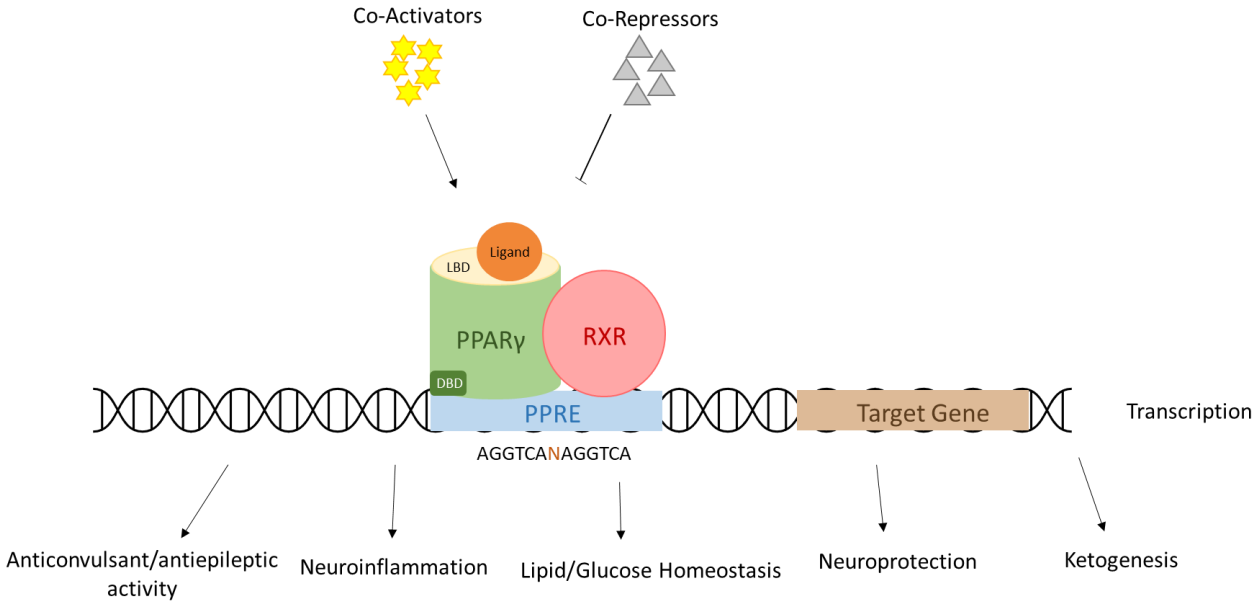

Figure 9. PPARγ DNA binding and transcriptional activation of target genes in the cell nucleus, influencing anticonvulsant and antiepileptic activities, neuroinflammation, lipid and glucose homeostasis, neuroprotection and ketogenesis.

Table 6. PPARγ regulated genes and their involvement in epilepsy and seizure development. Note that upregulated genes are indicated by an upward arrow, instead downregulated genes are indicated by a downward arrow. The gene involvement in seizures or epilepsy is indicated by plus symbol; the lack of information is instead indicated as not determined (ND).

Genes	Function	Seizures	Epilepsy	References
GFAP ↓	Neuroinflammation	+	+	[616]
IL-10 ↓	Neuroinflammation	+	+	[568,617,618]
MMP-9 ↓	Neuroinflammation	+	+	[619,620]
NF-κB ↓	Neuroinflammation, Ketogenesis, Antioxidant	+	+	[608,621,622]
TNF-α ↓	Neuroinflammation, Apoptosis, Ketogenesis, Antioxidant, Lipid/Glucose Homeostasis	+	+	[622–624]
COX-2 ↓	Neuroinflammation, Prooxidant, Ketogenesis	+	+	[622,625,626]
iNOS ↓	Neuroinflammation, Prooxidant	+	ND	[627–629]
IL-1β ↓	Neuroinflammation, Ketogenesis	+	+	[624,630]
IL-6 ↓	Neuroinflammation, Lipid/Glucose Homeostasis	+	+	[631,632]
aP2 ↓	Neuroinflammation, Lipid/Glucose Homeostasis	ND	ND	[633]
ACOX1 ↑	Neuroinflammation, Lipid/Glucose Homeostasis	+	ND	[634–637]

CD36 ↑	Neuroinflammation, Apoptosis, Lipid/Glucose Homeostasis	+	+	[570,638,639]
BDNF ↑	Neuroprotection	+	+	[567,640]
TrkB ↑	Neuroprotection	+	+	[567,641]
Nrf2 ↑	Antioxidant	+	+	[642,643]
HO-1 ↑	Antioxidant	+	+	[644,645]
UCP-2 ↑	Antioxidant	+	+	[569,646]
SOD ↑	Antioxidant	+	+	[644,647,648]
eNOS ↑	Antioxidant	+	+	[649,650]
GPx3 ↑	Antioxidant	ND	ND	[651]
Catalase ↑	Antioxidant	+	+	[652–654]
Caspase-3 ↑	Apoptosis	+	+	[568,655]
Bax ↑	Apoptosis	+	+	[656,657]
PARP-1 ↑	Apoptosis	+	+	[658,659]
Fas ↑	Apoptosis	+	+	[660,661]
Bcl-2 ↑	Apoptosis	+	+	[656,657,661,662]
PTEN ↑	Apoptosis	ND	ND	[663,664]
GLUT4 ↑	Lipid/Glucose Homeostasis	ND	ND	[665]
GyK ↑	Lipid/Glucose Homeostasis	+	ND	[666,667]
Leptin ↓	Lipid/Glucose Homeostasis	+	+	[668,669]
LPL ↑	Lipid/Glucose Homeostasis	ND	ND	[670]
CAP ↑	Lipid/Glucose Homeostasis	ND	ND	[671]
IRS2 ↑	Lipid/Glucose Homeostasis	ND	ND	[672]
cdc2 ↑	Inhibition of cell growth	ND	ND	[673]
cdc25c ↑	Inhibition of cell growth	ND	ND	[673]

CITED2 ↑	Inhibition of cell growth	ND	ND	[674]
p21 ↑	Inhibition of cell growth	+	+	[292,675]
p27 ↑	Inhibition of cell growth	+	+	[676,677]
Cyclin D1 ↓	Inhibition of cell growth	+	+	[678–680]
PAI-1 ↑	Inhibition of metastasis	ND	ND	[681]
TIMP 3 ↑	Inhibition of metastasis	ND	ND	[682]
MMP-2, 9, 13 ↓	Inhibition of metastasis	+	+	[682–686]
HPSE ↓	Inhibition of metastasis	ND	ND	[682]

Abbreviations: PPARγ, peroxisome proliferator-activated receptor gamma; GFAP, glial fibrillary acidic protein; IL-1β, 6, 10, interleukin-1β, 6, 10; MMP-2, 9, 13, matrix metalloproteinase 2, 9, 13; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; iNOS/eNOS, inducible/endothelial nitric oxide synthase; aP2, adipocyte fatty-acid-binding protein; ACOX1, acyl-CoA oxidase; CD36, cluster of differentiation 36; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; Nrf2, nuclear factor erythroid-2 related factor 2; HO-1, heme oxygenase-1; UCP-2, uncoupling protein 2; SOD, superoxide dismutase; GPx3, glutathione peroxidase 3; Bax, b-cell lymphoma protein 2 (Bcl-2)-associated X; PARP-1, poly(ADP-ribose) polymerase 1; Fas, fatty acid synthase; PTEN, phosphatase and tensin homolog; GLUT4, glucose transporter 4; GyK, glycerol kinase; LPL, lipoprotein lipase; CAP, c-Cbl-associated protein; IRS2, insulin receptor substrate 2; cdc2/cdc25c, cell division cycle protein 2/25c; CITED2, Cbp/P300 Interacting transactivator with glu/asp rich carboxy-terminal domain 2; p21/p27, cyclin-dependent kinase inhibitor 1/1b; PAI-1, plasminogen activator inhibitor-1; TIMP 3, tissue inhibitors of metalloproteinase 3; HPSE, heparanase, ND, not determined.

PGC1α transcription coactivators and sirtuins, which are signaling proteins involved in metabolic regulations, are two major families of regulators that function as metabolic controls [687]. PGC1α governs cellular metabolism and mitochondrial biogenesis (namely: glucose uptake, glycolysis, β-oxidation, thermogenesis, mitochondrial respiratory capacity). It is mostly expressed in the brain, skeletal muscles, heart, kidneys, and brown adipose tissue. The capacity of NRF1 to activate its target genes is increased by PGC1α direct interaction with NRF1. PGC1-related coactivator (PRC), another ubiquitously expressed coactivator, similarly improves the ability of NRF1 to activate its targets. Numerous studies have shown how co-expression of PGC1α/β and its downstream targets are involved in the etiology of neurodegenerative diseases [688,689]. For instance, upregulation of

the PGC1α pathway can enhance the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) signaling, plus activate some survival signaling such as the MEK/ERK and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways so to provide neuroprotection from apoptosis in hippocampal neurons after SE [690,691]. On the other hand, downregulating PGC1α by siRNA for *pgc1α* suppresses VEGF/VEGFR2 signaling, as well as pro-survival PI3K/AKT and MEK/ERK pathways that are linked to apoptosis of hippocampal CA3 neurons. These findings would suggest that the PI3K/AKT, MEK/ERK, and VEGF/VEGFR2 signaling pathways, as well as the neuronal survival signaling, are all activated by the PGC1-induced VEGF/VEGFR2 pathway. Consequently, the PGC1/VEGF/VEGFR2 interaction and the subsequent activation of PI3K/AKT and MEK/ERK signaling can be viewed as having an endogenous neuroprotective impact against apoptosis in the hippocampus after SE [692].

Clinical significance of PPARγ agonists

So far, PPARγ agonists have mostly been studied for their possible use in the treatment of type 2 diabetes. The synthetic PPARγ agonists known as TZDs have been demonstrated to improve insulin sensitivity of target tissues and to lower plasma glucose, lipid, and insulin levels [574,693]. In particular, the interaction of TZDs with PPARγ can occur in two ways. First, after forming a heterodimer with RXR, TZDs bind to the PPARγ/RXR complex, resulting in recognition of PPREs in the promoter region of target genes, which are unique DNA response elements. Consequently, PPARγ target genes are eventually transcribed. PPARγ undergoes a conformational change after ligand binding, causing different cofactors to be engaged. Second, PPARγ can restrict gene transcription in a DNA-binding-independent manner by negatively interacting with other signal-transduction pathways, such as the activation of the NF-κB pathway, which prevents gene transcription of pro-inflammatory genes [621]. In 1997, troglitazone was the first TZD approved by the Food and Drug Administration (FDA) to treat type 2 diabetes. However, due to hepatotoxicity, it was recalled as an unsafe drug in 2000 and removed from the market [694]. Two years later, two new TZDs members were approved. Rosiglitazone and pioglitazone are well-established derivatives of TZDs used in diabetes. The anti-diabetic properties of TZDs are thought to be achieved by activating PPARγ in adipose tissue, which allows lipids to be redirected away from muscle and liver, where fat storage creates massive problems [693]. In an animal model of dyslipidemia, PPARγ agonists were found to reduce plasma levels of triglycerides, cholesterol, and unsaturated fatty acids [695].

High interest was also raised in the role of this modulator in cancer treatment. Indeed, phosphatase and tensin homolog (PTEN), a tumor suppressor gene, displays interaction with PPARγ (Table 6). It was found that activation of PPARγ increases PTEN expression in pancreatic cancer cells [663]. In addition, PTEN is activated by TZDs to facilitate their antiproliferative properties [662]. However, the role of PTEN in seizures and epilepsy is still poorly understood. It is suggested that patients with PTEN variants often show an EEG abnormality and epilepsy. For instance, it was reported that about 22 out of 54 patients with PTEN variants enrolled in a multicenter study showed an abnormal EEG and 8 received a diagnosis of epilepsy, especially focal epilepsy (87.5%), with a mean age at seizure

onset of 3.8 years. Moreover, 37.5% of these subjects displayed drug resistant epilepsy [664]. If the development of epilepsy due to the PTEN variant also involves PPARγ was not determined.
Beyond these actions, TDZs were able to reduce blood pressure in diabetic and non-diabetic patients, independently of insulin resistance, and displayed a beneficial impact on cardiovascular risk factors by improving cardiac output and stroke volume [696,697], which indirectly impact on the possibility to develop post-stroke epilepsy. In addition, PPARγ has been recognized to play a key modulatory role in inflammation by preventing the pro-inflammatory cytokine expression and by redirecting immune cell differentiation. PPARγ induces down-regulation of pro-inflammatory genes (Table 6) such as COX-2, metalloproteinase-9 (MMP-9), scavenger receptor A, inducible nitric oxide synthase (iNOS), as well as the production of pro-inflammatory cytokines, chemokines, and interleukins [45,601,602]. Furthermore, PPARγ exhibits neuroprotective activity against oxidative damage, mitochondrial dysfunction, and apoptosis thus rescuing neurons and glial cells. Activation of PPARγ also induces the expression of antioxidant catalase and copper/zinc SOD, two enzymes capable of reducing oxidative stress, and inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [698–700]. PPARγ agonists also prevent oxidative stress by decreasing iNOS activity, blocking NF-κB, and inhibiting TNF-α release or activating nuclear factor (erythroid 2 derived) -like 2 (Nrf2) [701,702]. For instance, eicosanoids, which are pro-inflammatory substances capable of increasing vasodilation derived from polyunsaturated fatty acids, have been shown to reduce their effects in response to PPARγ [567].

PPARγ and its agonists in seizures and epilepsy

Rosiglitazone and pioglitazone are well-established TZDs used in diabetes; however, recent preclinical research studies also suggest that they could be used as an effective epilepsy treatment option. Several studies investigated the effect of rosiglitazone in models of seizures, usually caused by chemical or physical agents, and epilepsy, i.e., spontaneous seizures (Table 7). In an in vitro model of TLE, rosiglitazone could significantly reduce the amplitude and frequency of epileptiform discharges by possibly inhibiting the calcium current of voltage-gated calcium channels (VGCCs) in a PPARγ-dependent manner. Pretreatment with the PPARγ antagonist GW9662 did not inhibit the rosiglitazone impact on discharge frequency, but it inverted the outcome by lowering discharge amplitude. By partially activating PPARγ, rosiglitazone additionally rescued hippocampus slices from NMDA excitotoxicity [703]. Moreover, neuronal loss after pilocarpine-induced SE could be limited by the administration of rosiglitazone, and the elevation of brain-derived neurotrophic factor (BDNF) levels and TrkB signaling were suppressed [567]. Elevation of BDNF levels is neuroprotective but can increase neurogenesis leading to changes in neural circuits in hippocampi exposed to seizures [640,704]. After SE induction, levels of BDNF and TrkB mRNA are up-regulated in the hippocampus, possibly exerting defense mechanisms against neurotoxicity [641]. Importantly, blocking TrkB by its agonist K252a reversed its neuroprotective effects, suggesting that rosiglitazone achieves its neuroprotective role by activation of BDNF/TrkB pathway [567]. Furthermore, pilocarpine-induced seizures were followed by a notable increase in microglia activation, which was effectively counteracted by rosiglitazone administration after SE induction. Rosiglitazone not only suppressed the activation of microglia after SE, but it also inhibited

proinflammatory microglial polarization [705,706]. Microglia serve as a defense player that coordinates immune responses such as phagocytosis, cell proliferation, cell migration, and cytokine production [707]. More research is necessary to understand the molecular mechanism behind the effect of rosiglitazone-dependent suppression of microglia after seizure induction. Another beneficial output of rosiglitazone is its involvement in the up-regulation of glutathione (GHS), a crucial antioxidant helping the detoxification process in astrocytes [708]. Stimulated astrocytes elevate Ca^{2+} influx and therefore release glutamate to enhance synaptic excitatory transmission after SE [709]. Hong et al. reported that rosiglitazone significantly reduced the cognitive impairment caused by SE and potently blocked the activation of astrocyte while balancing GSH homeostasis in the hippocampus following a lithium pilocarpine-induced SE [710]. Importantly, one week after SE, rosiglitazone efficiently diminished neuronal loss in the hippocampus and lowered the formation of reactive oxygen species (ROS) and lipid peroxidation considerably. It was also discovered that rosiglitazone therapy increased antioxidant activity of SOD in combination with decreased HO-1 levels after pilocarpine-induced SE in rats. Co-treatment with the PPARγ antagonist T0070907 could block the aforesaid consequences of rosiglitazone [644]. ROS have been connected to seizure-dependent neurotoxicity and have been implicated in the onset of seizures in pathological situations. The temporal association between free radical generation and epilepsy in several clinical circumstances, as well as the preventive efficiency of antioxidant therapy against cell damage in seizure-induced models are among the evidence. HO-1 is a stress-sensitive protein partially regulated by NF-κB and mitogen-activated protein kinases (MAPK). Either elevated levels of HO-1 in oxidative-stressed tissues may serve to normalize the redox milieu or can otherwise liberate free intracellular iron and carbon monoxide (CO) by heme degradation, which may intensify tissue oxidative stress by increasing oxyradical production inside the mitochondrial compartment [711]. Independently of the presence of a PPARγ agonist, HO-1 was also observed to increase SOD-1 expression in astrocyte mitochondria. For this reason, it has been suggested to be a key endogenous antioxidant defense tool for controlling total ROS generation [712]. With HO-1 being a direct target of PPARγ, and being able to be regulated by rosiglitazone, it could be suggested to be a new target for treatment in combination with TZDs against epilepsy. The ability of rosiglitazone to reduce oxidative stress following pilocarpine-induced SE was also reported by Peng et al. who found that rosiglitazone reversed the SE-induced changes in ROS production and SOD activity [647]. Interestingly, they also demonstrated that rosiglitazone prevents autophagy evoked by SE in an Nrf2-dependent manner. In another study, activation of PPARγ with rosiglitazone in the KA model of SE in rats upregulated the expression of the mitochondrial UCP-2 (Table 6). This protein plays an important role in antioxidant defense by decreasing ROS production. The effect of rosiglitazone on UCP-2 was indeed associated with reduced overproduction of ROS as well as reduced dysfunction of mitochondrial complex I, inhibited translocation of mitochondrial Bax, suppressed cytosolic release of cytochrome, and decreased apoptotic cell death following SE [569].

Furthermore, it was found increased expression of the tripartite motif family 27 (TRIM27) and decreased expression of PPARγ in epilepsy patients. TRIM27 enhances glutamate-induced cytotoxicity in HT22 cells, partly by enhancing glutamate-induced cell apoptosis and pro-inflammatory factor IL-1β release via ubiquitination of PPARγ [630]. Moreover, the neurotoxic

effects of TRIM27 are thought to be decreased by the presence of rosiglitazone. Rosiglitazone suppressed NMDA excitotoxicity in a TLE model, partially by PPARγ activation [703]. Because PPARγ may prevent the transcription of numerous genes that contribute to glutamate-induced neurotoxicity, the suppression of glutamate-induced neurotoxicity in HT22 cells by TRIM27 knockdown may be caused by the inhibition of PPARγ ubiquitination. According to these results, PPARγ agonists reduced neuroinflammation in the mouse hippocampus after convulsions and protected HT-22 cells against oxidative damages caused by glutamate [622,713].

All the aforementioned studies demonstrate that rosiglitazone has neuroprotective properties against SE-induced neuronal damage. However, data on its effect on seizure activity are very limited. Rosiglitazone failed to produce an acute anticonvulsant-like effect in the magnesium deficiency-dependent audiogenic seizure (MDDAs) model in mice [714]. It did not prolong seizure latency, although it was able to attenuate oxidative-stress-induced autophagy by increasing Nrf2 in a rat model of lithium-pilocarpine-induced SE [647]. Moreover, rosiglitazone did not improve the survival rate of mice [705] following pilocarpine-induced SE, even if it was able to reverse microglial polarization in the brain and afford neuroprotection without inducing significant changes in brain inflammation. Interestingly, although in the study by Hong et al. rosiglitazone also did not display anticonvulsant properties during the development of pilocarpine-induced SE in rats, it significantly delayed the development of SRSs demonstrated by EEG recordings [567]. This suggests that rosiglitazone may have an antiepileptic action, due to its ability to suppress SRS in epileptic animals through BDNF/TrkB signaling. By contrast, Boes et al. showed that rosiglitazone did not exert a relevant effect on the development of SRSs and neuronal cell loss following electrically-induced SE in rats, which argues against both the antiepileptic and neuroprotective effects of rosiglitazone. But in this study, rosiglitazone treatment had positive effects on spatial learning performance indicating its possible long-term beneficial disease-modifying effect [715]. Nevertheless, doubts about rosiglitazone's cardiovascular safety have limited the investigations on this drug [716].

Another class member of TZDs is pioglitazone, which displays similar functions as rosiglitazone. In contrast to rosiglitazone, it produced anticonvulsant-like effects in several animal models of seizures and epilepsy (Table 7). In the timed intravenous (i.v.) PTZ seizure threshold test in mice, pioglitazone significantly raised the threshold for clonic seizure after both repeated [627,717] and single administration [718]. Moreover, it prolonged clonic seizure latency in the acute i.p. PTZ-induced seizure model, but it had no effect on tonic seizures [627,717,718]. In the flurothyl-induced seizure test in mice, pioglitazone prolonged latency to generalized tonic-clonic seizure, though it did not modify the features of clonic seizures [719]. Intriguingly, the anticonvulsant-like activity of pioglitazone in the i.v. PTZ and i.p. PTZ-induced seizure tests in female mice was completely abolished by ovariectomy, thus indicating a possible involvement of ovarian hormones in the effects of pioglitazone in female animals. This issue needs further investigation [717]. Another interesting observation is that the anticonvulsant action of pioglitazone in the i.v. PTZ test could be reversed by the iNOS inhibitors aminoguanidine and N-nitroarginine methyl ester (l-NAME), and conversely potentiated by the nitric oxide (NO) precursor l-arginine, suggesting the involvement of the NO pathway [718]. Similar findings were reported by Shafaroodi et al. who concluded that pioglitazone probably exerts its anticonvulsant effects through induction of iNOS. The role of NO

pathway in seizures and epilepsy is a widely recognized phenomenon [627]. For example, in KA-induced SE and the mouse model of PTZ-kindling, increased levels of NO were found, contributing remarkably to hippocampal endoplasmic reticulum stress and oxidative damage [720,721]. In a model of PTZ-induced kindling in mice, pioglitazone could confirm its anticonvulsant efficiency by delaying the seizure onset and suppressing the kindling progression. Furthermore, it could reduce the higher cortical concentration of TNF-α, IL-10, and caspase-3 in the kindling in mice and could decrease the raised level of pro-inflammatory cytokine, IL-10 and caspase-3 activity in acute i.p. PTZ-induced seizures [568]. This phenomenon is likewise observed in genetically epileptic mice. In this study, pioglitazone prolonged the seizure-free period and significantly decreased the convulsion duration. These treated mice were documented to display decreased expression of IL-6 and TNF-α mRNA, implying that PPARγ was able to diminish the neuroinflammatory reaction [631]. Similarly, to rosiglitazone, pioglitazone was reported to provide neuroprotection against neuronal damage caused by seizures. In a model of PTZ-induced SE, pioglitazone prolonged the latency to SE and decreased the loss of neurons. It also prevented the increase of proinflammatory cytokines (IL-1β and IL-6) and the activation of the mTOR pathway [632]. Moreover, pioglitazone significantly reduced seizure activity and decreased neuronal cell damage in the KA model. This effect was accompanied by attenuated activation of astrocytes and microglia as well as by decreased myeloperoxidase and NF-κB expression in glial cells, further indicating that the anti-seizure effects of pioglitazone might be associated with its ability to reduce neuroinflammation [722]. In mice subjected to the pilocarpine model of SE, pioglitazone decreased the seizure severity, neuronal cell loss, and the blood-brain barrier (BBB) damage [723]. A neuroprotective effect of pioglitazone was also observed in rat pups exposed to febrile seizure. It enhanced the seizure latency, and reduced the seizure severity and duration, and also alleviated the cognitive deficits by reducing apoptosis and inflammation [724]. Finally, pioglitazone decreased the seizure frequency and burden in Kv1.1KO mouse – a genetic model of TLE with severe and frequent SRSs [725]. Altogether, the mentioned studies consistently showed an anti-seizure potential for pioglitazone. However, it should be noted that pioglitazone may also act through PPARγ-independent mechanisms. For instance, it may alter neuronal excitability, at least in part, by modulating sodium channels and/or calcium channels, as it was reported that pioglitazone inhibited sodium current (I_{Na}) in mouse hippocampal neurons [723] and L-type VGCCs in vascular smooth muscles [726]. Nevertheless, the possible use of this drug has still to be firmly established as some reports claim that its administration could lead to increased risk of cardiotoxicity or hepatotoxicity or to develop bladder cancer in diabetic patients [727,728]. For instance, in an *in vitro* study using isolated mitochondria from brain and cardiac tissue, pioglitazone at concentrations ranging from 12.5 to 50 µg/ml increased ROS levels, mitochondrion swelling, and release of cytochrome C, indicating neurotoxic and cardiotoxic effects due to impairment of mitochondrial functioning [729]. In a different study using isolated liver mitochondria, three out of six tested TZDs actively inhibited mitochondrial respiration, precisely ciglitazone, troglitazone and darglitazone. Mitochondrial impairment consisting of uncoupled or disrupted respiration was also detected using a combination of the mentioned TZDs [730].

Although TZDs were the most extensively studied PPAR-γ agonists, it is worth mentioning that the novel PPAR-γ agonist N-[9-fluorenylmethoxycarbonyl]-)-L-leucine (FMOC-L-leucine) was shown to produce anti-seizure effect in the MDDAS test, but not in the 6-Hz seizure test in mice. In addition, GW9662 abolished the protective effect of FMOC-L-leucine against audiogenic seizure [714].

Table 7. Investigations performed with PPARγ agonists in animal models of seizures and epilepsy.

Species	Strain	Sex	Age/Weight	Agonist	Treatment	Model	Results	Reference
A. Models of seizures								
Mice	C57Bl/6	M	~7–8 weeks	Pioglitazone	0.1–80 mg/kg (i.p.) 240 min before the test	Flurothyl-induced seizure test	(1) No effect on clonic seizure latency, duration and number; (2) prolonged latency to generalized tonic-clonic seizure; (3) synergistic interaction with KD	[740]
	NMRI	M	Adult	Pioglitazone	10–80 mg/kg (p.o.) 120 or 240 min before the test	i.v. PTZ seizure threshold test	(1) Increased seizure threshold for clonic seizure at 40 and 80 mg/kg (240 min); (2) possible involvement of the NO pathway in the anticonvulsant effect of pioglitazone	[739]
	NMRI	M	23–30 g	Pioglitazone	0.5–20 mg/kg (p.o.) for 10 days	i.v. PTZ seizure threshold test and i.p. PTZ-induced seizure test	(1) Increased seizure threshold for clonic seizure in the i.v. PTZ test at 1 and 5 mg/kg; (2) prolonged clonic seizure latency and no effect on tonic seizure in the i.p. PTZ test; (3) possible involvement of the NO pathway in the anticonvulsant effect of pioglitazone	[648]
	Swiss	M	8 weeks/ 20–25 g	Pioglitazone	10 mg/kg (p.o.) 30 min before the test	i.p. PTZ-induced seizure test	(1) Prolonged seizure latency; (2) no effect on caspase-3 activity; (3) decreased IL-10 and increased TNFα level	[589]
	Swiss	M	8 weeks/ 20–25 g	Pioglitazone	10 mg/kg (p.o.) 30 min before each PTZ injection	PTZ-induced kindling	(1) Suppressed kindling development; (2) decreased caspase-3 activity; (3) reduced neuroinflammation	[589]

Swiss	F	6–8 weeks/ 20–25 g	Pioglitazone	5, 10, and 20 mg/kg (p.o.) for 10 days	i.v. PTZ seizure threshold test and i.p. PTZ-induced seizure test	(1) Increased seizure threshold for clonic seizure in the i.v. PTZ test; (2) prolonged clonic seizure latency and no effect on tonic seizure in the i.p. PTZ test; (3) lack of effect in ovariectomized mice	[738]
Swiss	NP	Adult	Rosiglitazone	up to 100 mg/kg (i.p.) 60 min before the test	MDDASs model	No effect	[735]
Swiss	NP	Adult	FMOC-L-leucine	up to 100 mg/kg (i.p.) 60 min before the test	MDDASs model	Acute anticonvulsant effect	[735]
Swiss	NP	Adult	FMOC-L-leucine	up to 100 mg/kg (i.p.) 15–240 min before the test	6 Hz (at 32 mA) seizure test	No effect	[735]
Wistar	M	Pups (PN7–PN22)	Pioglitazone	5, 10, and 20 mg/kg (i.p.) from PN8 to PN11 and PN13 to PN16	Febrile seizure induced by LPS+KA	(1) Decreased seizure latency, severity and duration; (2) attenuated cognitive impairment; (3) decreased neuronal loss; (4) decreases iNOS level; (5) anti-inflammatory effect	[745]
B. Models of epilepsy							
C57BL/6	M	8–10 weeks	Rosiglitazone	0.1 mg/kg (i.p.) after the termination of SE and then once every 24 h for 72 h	PILO-induced model of SE	(1) No difference in the survival rate and seizure latency when compared to the control group; (2) decreased neuronal loss; (3) reversed microglial polarization; (4) reduced neuroinflammation	[726]
C57BL/6	M	adult	Pioglitazone	10 mg/kg (p.o.) for 14 days before PILO	PILO-induced model of SE	(1) Decreased seizure severity; (2) decreased mortality; (3) decreased neuronal loss and BBB damage	[744]
ICR	M	23–25 g	Pioglitazone	30 mg/kg (p.o.) for 3 days before the test	KA-induced model of SE	(1) Reduced seizure activity; (2) decreased neuronal damage; (3) attenuated activation of astrocytes and microglia; (4) decreased MPO	[133]

C57BL/6							
ICR	M	23–25 g	Pioglitazone	30 mg/kg (p.o.) for 3 days before the test	KA-induced model of SE	(1) Reduced seizure activity; (2) decreased neuronal damage; (3) attenuated activation of astrocytes and microglia; (4) decreased MPO and NF-κB immunoreactivities	[743]
EL	M	5–16 weeks	Pioglitazone	20 mg/kg/day in a rodent chow	Genetic model of epilepsy	(1) Decreased duration of seizures; (2) reduced neuroinflammation	[652]
Kv1.1 KO	NP	~5–6 weeks	Pioglitazone	10 mg/kg (i.p.) for 5 days	Genetic model of epilepsy	(1) Decreased seizure frequency and burden; (2) increased nuclear content of PPARγ2	[746]
Sprague-Dawley	M	12 weeks/ 200–220 g	Rosiglitazone	100 μg/kg (i.c.v.), 25 h before PILO injection and then for 12–24 days in long-term studies	PILO-induced model of SE	(1) No effect on the development and severity of SE in the acute phase; (2) attenuated development of SRSs in the chronic phase; (3) inhibited expression of BDNF and TrkB after SE; (4) decreased neuronal loss	[588]
Sprague-Dawley	M	200–220 g	Rosiglitazone	60 min before both PILO and LiCl injections (the dose and route of administration not provided)	PILO-induced model of SE	(1) No effect on seizure latency; (2) decreased neuronal loss; (3) attenuated oxidative-stress-induced autophagy by regulating Nrf-2	[668]
Sprague-Dawley	M	12 weeks/ 180–220 g	Rosiglitazone	0.1 mg/kg (i.p.) 60 min before PILO and then every 24 h	PILO-induced model of SE	(1) Decreased neuronal loss; (2) antioxidant effect	[665]
Sprague-Dawley	M	12 weeks/ 200–220 g	Rosiglitazone	0.1 mg/kg (i.p.) 24 h before SE and again every 24 h after SE	PILO-induced model of SE	(1) Prevented cognitive impairment; (2) inhibited astrocyte activation; (3) restored GSH homeostasis	[731]
Sprague-Dawley	M	12 weeks/ 200–220 g	Rosiglitazone	0.1 mg/kg (i.p.) 24 h before SE and again every 24 h after SE	PILO-induced model of SE	(1) Decreased neuronal loss; (2) reduced neuroinflammation	[727]

Sprague-Dawley	M	260–300 g	Rosiglitazone	6 nmol (intrahippocampally) 30 min before KA	KA-induced model of SE	(1) Increased UCP2 expression; (2) decreased ROS production; (3) inhibited translocation of Bax; (4) improved dysfunction of mitochondrial complex I; (5) decrease cytosolic release of cytochrome *c*	[590]
Sprague-Dawley	M	200–300 g	Pioglitazone	10 mg/kg (p.o.) for 5 days before the test	PTZ-induced model of SE	(1) Prolonged latency to SE; (2) decreased neuronal loss; (3) reduced neuroinflammation; (4) inhibited activation of the mTOR pathway	[653]
Sprague-Dawley	F	NP	Rosiglitazone	3 mg/kg (i.p.) after SE for 27 days	Electrically-induced model of SE	(1) No effect on the development of SRSs; (2) no effect on neuronal cell loss; (3) attenuated learning impartment	[736]

Abbreviations: F, female; FMOC-L-leucine, (N-[9-fluorenylmethoxycarbonyl]-)-L-leucine; h, hour(s); iNOS, inducible nitric oxide synthase; i.c.v., intracerebroventricular; i.p., intraperitoneal; KA, kainic acid; LPS, lipopolysaccharide; M, male; MDDASs, magnesium deficiency-dependent audiogenic seizure; NP, not provided; PILO, pilocarpine; PN, postnatal day; p.o., per os; PTZ, pentylenetetrazole; ROS, reactive oxygen species; SE, status epilepticus; SRS, spontaneous recurrent seizure; UCP-2, uncoupling protein 2

PPARγ and its agonists at different doses in human and animal models

As presented in Table 7, PPARγ agonists were studied in animal models of seizures or epilepsy at a wide range of doses. Rosiglitazone was administered systemically (i.p.) at 0.1–3 mg/kg, and the dose of 0.1 mg/kg was the most frequently used. In most of the studies, the investigated drug was repeatedly injected. There is also one study in which rosiglitazone was administered acutely at extremely high doses (up to 100 mg/kg). Despite this, it failed to protect against audiogenic seizures [714]. The recommended dose of rosiglitazone in patients with diabetes mellitus type 2 is 4–8 mg/day [731], which corresponds to 0.07–0.13 mg/kg for a person with 60 kg of body weight. Thus, the dose of 0.1 mg/kg used in animal studies falls within the therapeutic range of humans. According to Food and Drug Administration guidelines, a dose translation based on the body surface area (BSA) is much more appropriate than a simple conversion based only on body weight [732]. Although the dose of rosiglitazone used in seizure/epilepsy models (0.1 mg/kg) is lower than the animal equivalent dose calculated on the basis of BSA (0.41-0.82 mg/kg and 0.83-1.64 mg/kg for rats and mice, respectively), it was sufficient to produce neuroprotective effects. However, it should be noticed that rosiglitazone was generally administered i.p. in all the considered animal studies, while in humans it is given orally. This makes the comparison of animal and human doses of rosiglitazone more complex, as the route of administration may affect bioavailability of the drug.

The anti-seizure and/or neuroprotective efficacy of pioglitazone in animal models of seizure and epilepsy was studied at doses ranging from 0.1 to 80 mg/kg, with the average doses of 5–20 mg/kg (see Table 7). Most of the studies were performed in mice and the most common route of administration was oral. The highest dose (80 mg/kg) was used in two studies investigating the acute effects of pioglitazone in the flurothyl- and i.v. PTZ-induced seizure tests. The recommended dose of pioglitazone for diabetes mellitus type 2 is 15-45 mg/day [733]. For a 60 kg human, this translates into a dosage of 0.25-0.75 mg/kg. After BSA-based dose adjustment, the animal equivalent dose of pioglitazone should be around 3.10-9.20 mg/kg for mice and 1.60-4.70 mg/kg for rats, which means that the anti-seizure and neuroprotective effects of pioglitazone in the aforementioned studies were observed at doses similar to or higher than taken by human patients. It is noteworthy that the neuroprotective effects of both rosiglitazone and pioglitazone in animal models of Alzheimer's and Parkinson's disease have been studied at higher doses than in models of seizures or epilepsy [734–736]. Unfortunately, pharmacodynamic/pharmacokinetic studies with TZDs relevant to neurological disorders are missing. The ability of rosiglitazone to cross the BBB in rodents is unknown, while pioglitazone has rather low BBB penetrance [736,737], which suggests that high doses may be necessary to induce some responses. On the other hand, it was suggested that pioglitazone (and possibly other TZDs) may exert some effects with an inverted U-shaped dose-response curve. In a human study, 0.6 mg (but not 2.1, 3.9, and 6.0 mg) of pioglitazone given daily for 14 days increased memory-related hippocampal activity [738]. Likewise, lower doses of pioglitazone (0.04 – 0.16 mg/kg, per os, p.o.) produced larger effects on hippocampal functional connectivity in rats than a higher dose of 0.32 mg/kg [739]. Interestingly, pioglitazone administered for 10 days at doses of 1 and 5 mg/kg (p.o.) significantly increased the seizure threshold in the i.v. PTZ test in mice, while it was ineffective at the lower (0.5 mg/kg) as well as at the higher doses (10 and 20 mg/kg) used [627].

PPAR gamma and ASMs

Currently, valproic acid, carbamazepine, benzodiazepine, lamotrigine, levetiracetam, and topiramate are the most commonly used ASMs [740]. In recent years, alternative medicines such as Epidiolex®, a CBD oral solution, have been found to successfully reduce seizure occurrence in rare pediatric onset refractory epilepsy disorders, such as Dravet syndrome (DS) and Lennox-Gastaut syndrome (LGS). For some ASMs (e.g., valproic acid, carbamazepine, topiramate, and gabapentin), a modulation of PPARγ expression has already been described in different neurological diseases in which these drugs exerted a therapeutic effect, but it still remains to be elucidated this possibility in models of epilepsy [726,741–743]. A possible involvement of PPARγ in the effects of CBD has been described in the systemic KA rat model of TLE [571]. Here, we will focus on valproic acid, phytocannabinoids and their relationship with PPARγ.

PPARγ and valproic acid

Valproic acid, a branched short-chain fatty acid (SCFA) derived from valeric acid, has been used as a monotherapy against absence, partial and generalized tonic-clonic seizures, and juvenile myoclonic epilepsy [744–747]. Its exact molecular pathway is still not fully understood, but it is thought to inhibit voltage-gated sodium channels, facilitate GABAergic transmission, as well as to inhibit GABA degradation and minimize the stimulation of excitatory agents [748]. Its function to inhibit histone deacetylase (HDAC), alter lipid metabolism, boost peroxisomal enzyme activity and to change the ultrastructure of peroxisomes in non-neuronal tissues is thus far well-documented [749–752]. Using primary neuronal cultures, treatment with valproate could change the PPARγ signaling and decreased DNA binding of the receptor by modulating the peroxisome biochemistry [743]. For these reasons, and the fact that PPARγ is involved in lipid homeostasis, a functional interaction between PPARγ and valproate is strongly suggested [743]. In an in vitro model, valproic acid was able to block hemorrhagic shock-induced signaling changes through upstream PTEN signaling through PPARγ activation. Knockdown of PPARγ deleted the favorable effects of valproic acid against signaling alterations and apoptosis [753]. Furthermore, specific polymorphisms of PPARγ and CD36 were shown to be associated with valproate-induced obesity in epileptic patients [638]. Thus, the signaling cascade triggered by the interaction of valproic acid and PPARγ could have a wide range impact on diverse physiological outputs.

PPARγ and plant-based therapeutic compounds

Due to a shorter list of side effects and a higher level of patient acceptance, plant-based therapeutic compounds have been extensively researched. *Aegle marmelos* L. leaf extract (AME) demonstrated a significant increase in onset time and a decrease in seizure duration in PTZ and maximal electroshock mouse models. This effect was proposed to be PPARγ-dependent because of the reverse protective effect of AME when the animals were pretreated with BADGE, a PPARγ inhibitor [754].

For decades, natural compounds derived from *Cannabis sativa* L. have been the primary focus of current research into its use as a broad-spectrum drug. CBD represents a promising approach for treating refractory epilepsy because of its non-psychoactive effect and little side effects. Indeed,

CBD has been shown to modulate several pharmacological targets, such as serotonin 1A receptor, PPARγ and transient receptor potential cation channel subfamily V member 1 channels [755], but it has no psychotropic effects because it cannot activate the cannabinoid receptor type 1 (CB_1) in the central nervous system [756,757].

The anti-inflammatory benefits of cannabinoids in the central nervous system (CNS) could be regulated by PPARγ-induced decrease of lipid peroxidation, MAPK inhibition, and NF-κB downregulation [755]. Although the precise mechanism underlying the interaction between PPARγ and CBD is still unknown, the knowledge that fatty acid amide hydrolase (FAAH) and fatty-acid binding proteins transport CBD within cells while also enhancing PPARγ activity may provide indications for both their synergy and their link to neuroprotective benefits [758,759]. Moreover, FAAH is also able to hydrolyze one of the major endocannabinoids, known as N-arachidonylethanolamide (anandamide, AEA), to arachidonic acid (AA) and ethanolamine. Similarly, another important endocannabinoid, namely 2-arachidonoylglycerol (2-AG), is hydrolyzed to AA and glycerol by monoacylglycerol lipase [428,429]. Interestingly, AA, which is a polyunsaturated omega-6 fatty acid, was shown to control macrophage activation in a PPARγ-dependent manner [760]. The above-mentioned relationships between AA and PPARγ, AA and FAAH and between AEA and 2-AG, plus the partly anticonvulsant properties of AEA and 2-AG, may suggest the possibility of the involvement of PPARγ in their efficacy in diminishing the impact of seizures.

Furthermore, the interaction between PPARγ and CBD has been demonstrated to induce neuroprotective effects in ischemic stroke and neurogenesis in a model of Alzheimer's disease [761–763]. Moreover, CBD has already been shown to modify the immune response by acting via PPARγ and altering NF-κB signaling [755], which is up-regulated in both microglia and astrocytes of patients suffering from Parkinson's disease. In this condition, the activation of PPARγ leads to inhibition of NF-κB signaling and reduces mRNA levels of proinflammatory mediators (i.e., TNF-α, IL-1β, IL-6, and iNOS) [764,765]. The ability of glial cells to promote the inflammatory state by releasing inflammatory mediators during an epileptic seizure suggests a neuroprotective role of CBD, possibly mediated by PPARγ, in epilepsy [766]. Recently, we found that subcutaneous injections of CBD at 120 mg/kg reduced the seizure duration, the number of rats experiencing epileptic seizures, and the total number of seizures in epileptic rats. Moreover, PPARγ immunoreactivity increased in hippocampal and extrahippocampal regions after a repeated administration of CBD, with a remarkable effect observed for the higher dose in the CA3 hippocampal subfield [571].

Like CBD, other minor phytocannabinoids, such as cannabigerol (CBG) and tetrahydrocannabinolic acid (Δ^9-THCA) have been shown to mediate their anti-inflammatory effects through PPARγ [767,768]. Similarly, two derivatives of CBG, known as VCE-003 or VCE-002.3, have also displayed increased affinity for PPARγ, thus maintaining their anti-inflammatory properties, while having little affinity for CB_1 and CB_2 receptors [769]. Furthermore, VCE-003 has been shown to reduce the expression of iNOS in BV2 microglial cells, and this effect can be antagonized by the selective antagonists of PPARγ (GW9662) and CB_2 receptors (AM630) [770]. In an in vivo Parkinson's disease model, the PPARγ antagonist T0070907 was able to block the VCE-003.2-mediated decreases in TNF-α, IL-1β, and iNOS mRNA levels [769]. Also, in this case, the effects of VCE-003 and VCE-003.2

on microglial activation seemed to be related to the activation of PPARγ, which has an important role in regulating the inflammatory response, especially in the central nervous system [771,772].

PPARγ and candidate peptides as targets for the development of anticonvulsant drugs

Several endogenous peptides, such as adrenocorticotropic hormone, angiotensin II, cholecystokinin, cortistatin, dynorphin, galanin, ghrelin, neuropeptide Y, neurotensin, somatostatin, and thyrotropin-releasing hormone, seem to suppress epileptic seizures. At variance, other neuropeptides, including arginine-vasopressin peptide, corticotropin-releasing hormone, enkephalin, β-endorphin, pituitary adenylate cyclase-activating polypeptide, and tachykinins have proconvulsive effects [255,570,773–775]. In this paragraph, we will focus on the ghrelin receptor antagonist EP-80317, which displayed anticonvulsant effects in the 6-Hz corneally stimulated mice through PPARγ involvement [570].

EP-80317 (Haic-D-Mrp-D-Lys-Trp-D-Phe-Lys-NH2) is a hexapeptide with a primary structure similar to that of hexarelin. However, it differs for substitutions in the first and third amino acid residues causing the loss of the growth hormone (GH)-releasing properties possessed by hexarelin [776]. In our studies, we demonstrated that EP-80317 displays anticonvulsant effects in the pilocarpine model and in the repeated 6-Hz corneal stimulation model [255,570,774]. Moreover, we found that GW9662, an inhibitor of PPARγ activity, is able to limit the beneficial effects of EP-80317 in both the animal models. In addition to this, PPARγ immunoreactivity was transiently increased in pyramidal cells and interneurons belonging to parvalbumin and somatostatin-28 subclasses, in coincidence with the anticonvulsant effects of EP-80317 in the repeated 6-Hz corneal stimulation model. Overall, these findings suggested that PPARγ is able to modulate seizures and mediates the anticonvulsant effects of EP-80317 [570].

PPAR gamma and ketogenic diet (KD)

The ketogenic diet (KD) is a high-fat/low-carbohydrate diet applied in refractory epilepsy, predominantly in children with juvenile drug-resistant epilepsy [65]. The combination of high-fat intake and lowering glucose levels is thought to be the key to this diet's effectiveness. There are several studies that report that polyunsaturated fatty acids (PUFAs) and saturated fatty acids display anticonvulsant effects in different mouse and rat models [777–780]. The CNS, excluding adipose tissue, has the highest lipid concentration of any organ in the human body. Lipids make up around 60% of the dry weight of the brain, with PUFAs accounting for about 30% of total lipid content. PUFAs and its eicosanoid derivatives, plus synthetic ligands such as fibrates activate PPARγ and were shown to modulate inflammation [781]. Activation of PPARs increases the transcription of genes involved in fatty acid oxidation by forming heterodimeric transcription-factor complexes with RXR, as stated previously [614,782]. Ingestion of saturated long-chain FAs is the foundation of the basic KD. Ketogenesis happens in the liver when carbohydrates are restricted, hence ketone bodies are released into the blood and cross the BBB. Within one to two days after starting the diet, circulating concentrations of the primary ketone bodies, β-hydroxybutyrate (BHB), acetoacetate (ACA), and acetone, have been demonstrated to considerably increase [783]. Within the first few days to weeks after starting KD, seizure control gradually improves [784]. This is thought to be

caused by the progressive increase in circulating ketone bodies. In addition, preclinical *in vivo* evidence about the anticonvulsant effects of ACA, acetone, L-(+)-β-hydroxybutyrate (L-BHB) and dibenzylamine (DBA) was reported in an audiogenic seizure-susceptible mouse model [785]. But only L-BHB and DBA were able to block NMDA-evoked currents in a concentration and voltage-dependent manner [786]. However, because ketone body levels in the blood do not always correlate with seizure control, it is unclear if these molecules are directly responsible for the reported potential seizure benefits [783,787]. PPARγ2, the splice variant of PPARγ, was implied to be a crucial factor in the mediation of the anticonvulsant effectiveness of KD [725]. Antioxidants like catalase are regulated by PPARγ and are suggested to be a key component in the neuroprotection and anti-epileptic properties of KD [654]. For instance, in in vivo trials with KD-treated rats, as well as in in vitro experiments with hypothesized effectors of various KDs including ketone bodies, and decanoic acid (C10), levels of catalase expression elevated dramatically [652,653]. As a result, investigators revealed that in vivo therapy with KD raises mRNA and protein concentration of catalase in the hippocampus, and that this upregulation demands the presence of PPARγ2. Furthermore, they observed that catalase upregulation promotes the KD associated anticonvulsive impact [654]. Besides, it is hypothesized that the anti-seizure effects of KD mediated by the activation of PPARγ are performed due to the inhibition of KA-induced TNF-α and NF-κB dependent COX-2 expression in the mouse hippocampus after KA-induced seizures [622]. COX-2 is assumed to be involved in neuronal loss in the hippocampus during epileptic episodes [625]. Moreover, these findings reinforce the notion that, in contrast to glial cells, neurons are principally affected by acute inflammatory responses to excitotoxicity [788]. A different research group supported these findings by showing that the KD component C10 activates PPARγ stimulation as well as mitochondrial citrate synthase [652]. Citrate synthase is only found in mitochondria and can be used as a mitochondrial marker [789]. Besides, epileptic patients adopting KD hold increased plasma levels of C10 [790].

Due to the possible involvement of PPARγ in the anti-seizure effects of the KD, the PPARγ agonist pioglitazone was co-administered with the KD to determine whether they could have additive effects and whether their combined administration could allow reduction of the KD ratio. In this regard, interesting results were observed in flurothyl-induced seizures in mice, which did not display a change in clonic seizures in response to solely pioglitazone or solely the KD, but presented a threshold raise to tonic-clonic seizures by co-administration of the KD and pioglitazone [719].

Conclusion

In conclusion, PPARγ represents an interesting therapeutic target with potential disease-modifying outcomes, especially for pharmacoresistant patients with epilepsy, who are also at high risk to develop fatal events such as sudden unexpected death in epilepsy (SUDEP). In particular, the brain of patients displaying refractory epilepsy is reportedly affected by chronic inflammation and mitochondrial dysfunction, which might be related to an abnormal neuronal excitability leading to a remarkable increase in seizure frequency and severity. In this context, the PPARγ activity should be taken into consideration as it displays potent anti-inflammatory effects by inducing MAPK inhibition and NF-κB downregulation, as well as a decreased lipid peroxidation [755,791].

Moreover, PPARγ involvement in neuroinflammation is accompanied by decreased levels of proinflammatory cytokines, such as IL-1β and TNF-α, in the epileptic brain [764,765]. However, PPARγ is not only able to control inflammation, but also to limit oxidative stress and acute brain injury. Moreover, PPARγ promotes tissue repair and fosters long-term functional recovery [792]. Other findings support the possible role of PPARγ as a possible tool for effective disease-modifying interventions, since some PPARγ agonists have been shown to be efficacious in animals displaying epileptic seizures. In preclinical research studies, PPARγ agonists were studied at a wide range of doses. For instance, rosiglitazone was mainly administered systemically (i.p.), and its dose of 0.1 mg/kg commonly used in animal studies falls within the therapeutic range for humans. At this dose, rosiglitazone displayed mainly neuroprotective effects in different seizure/epilepsy models. In regard to pioglitazone, the most common route of administration in mice was oral, and the vast majority of studies regarding the anti-seizure and/or neuroprotective efficacy of pioglitazone in animal models of seizure and epilepsy were done at doses similar to or higher (average doses of 5-20 mg/kg) than taken by patients suffering from diabetes mellitus type 2 [733]. In this regard, pioglitazone repeatedly administered at doses of 1 and 5 mg/kg (p.o.) significantly increased the seizure threshold in the i.v. PTZ test in mice [627]. In addition to this, extensive evidence demonstrates that the PPARγ agonist TZDs rosiglitazone and pioglitazone could have beneficial effects by acting on various molecular pathways.

The availability of FDA-approved agonists of PPARγ might promote the rapid translation of these promising results into clinical trials for epilepsy. Indeed, PPARγ agonists are currently used for clinical treatment of type 2 diabetes. However, the use of these compounds still raises some doubts. For instance, data on the effect of rosiglitazone on seizure activity are limited, and its cardiovascular safety has limited the investigations on its possible use as a drug [716]. At variance, pioglitazone displays anticonvulsant-like effects as well as ameliorative effects on cognitive deficits in animal models of seizures and epilepsy, but it should be clarified whether those effects are PPARγ-dependent or independent mechanisms [724]. In addition to this, the possible use of pioglitazone should be further investigated due to a possible increased risk of developing bladder cancer in diabetic patients [727,728].

Furthermore, the PPARγ activity might be involved in the effects of some ASMs and the KD. Indeed, valproate, a well-known ASM, seems to achieve seizure control by PPARγ-dependent activity [676]. Recently, we have also found that the levels of immunoreactivity for PPARγ were significantly increased within brain regions in a dose-related manner, and that the CA3 hippocampal subfield emerges as the most important target region for the modulation of PPARγ levels by the effective CBD dose. In a KA-model of TLE, this data implies that the anti-seizure effects of CBD may result, at least in part, by the increased PPARγ levels in hippocampal and extrahippocampal regions, and especially in the hippocampal CA3 subfield [571]. Blocking PPARγ also revealed that efficient KD therapy in animal models was only effective in combination with PPARγ stimulation [725]. However, clinical research is still needed to determine this effect in epilepsy patients.

Overall, these data suggest that PPARγ activation in the epileptic brain might have disease modifying effects that lead to long-term benefits in animals. Moreover, these findings highlight the remarkably promising outlook for including PPARγ in the establishment of novel therapeutics

against epilepsy. In this regard, further research should focus on the molecular and cellular pathway of PPARγ (which is not yet decoded) in order to increase the degree of knowledge of the effects induced by PPARγ agonists and other compounds related to PPARγ activation, as well as limiting their side effects[4].

[4] **Conflict of Interest statement**

The authors declare that there are no conflicts of interest.

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Chapter 2
Overview and Aims

Despite the continuous attempt made to discover new ASMs, up to now there are still approximately 30% of patients who are pharmacoresistant to any class of ASMs [366]. For this reason, alternative medicine has received more attention in the last years. In this context, the anticonvulsant activity of CBD in animal models as well as in clinical studies are based on its ability to diminish neuronal excitability. In particular, CBD exerts its functions via three main routes: antagonism at GPR55 receptors, desensitization of TRPV1, and inhibition of adenosine transport [793]. Additional beneficial features of CBD were associated with neuroprotective role in ischemic stroke and enhanced neurogenesis in a model of Alzheimer's disease, which are suggested to be mediated by PPARγ [762,763]. Furthermore, it has been demonstrated that CBD is able to regulate the immune response by modifying NF-κB through PPARγ activation, which is up-regulated in microglia and astrocytes of Parkinson's disease patients [357]. Nevertheless, the exact mechanism of CBD and possible involvement of other receptors, channels and neurotransmitters in an agonist, antagonist or modulatory manner still remain unclear. In numerous rodent models of seizures and epilepsy, CBD was able to reduce seizure severity and duration as well as diminish the susceptibility to develop SE [398,794]. However, how CBD mediates these beneficial effects remains largely unknown.

We have recently observed that PPARγ is involved in the anti-seizure properties of the ghrelin analogue EP-80317, screened in a model of repeated seizure induction [570].

This promising result led to generate the first hypothesis of my thesis, whether the antiseizure effects of CBD are related to the hippocampal levels of PPARγ in an animal model of TLE?

To evaluate this hypothesis, I designed an experiment in which two different doses of CBD (12 or 120 mg/kg) were assessed in epileptic rats previously treated with kainic acid. In these animals, I evaluated the changes in levels of PPARγ immunoreactivity in response to the anti-seizure effects of CBD. Adult male rats were continuously monitored by video-electrocorticography (ECoG) after the KA i.p. injection.

A meta-analysis study of patients with refractory epilepsy treated with CBD-rich extracts from *C. sativa* revealed a safer and more efficient therapeutic outcome than CBD alone. This result can be explained by the synergistic effects of CBD with other phytocannabinoids and terpenes, also called the "entourage effect" [536]. However, further preclinical studies using CBD extracts, as well as clinical control studies need to be carried out to better understand the contribution of these additional substances to the pharmacological effect of the extract.

The aim of the second approach of my thesis was to investigate the *in vivo* anticonvulsant effects of CBD-rich *C. sativa* extracts. In detail, two new preparations of *C. sativa* extracts, differing in terpene concentration, were screened using a modified repeated 6-Hz corneal stimulation test. This model is used to test novel anticonvulsant molecules to overcome the challenge of drug refractoriness. Until now, over 200 terpenes have been identified in cannabis so far, which were shown to possess anti-inflammatory, antioxidant, and anticonvulsant activities [795,796]. For this reason, one decarboxylated *C. sativa* extract containing a high concentration of naturally occurring

terpenes (K2) and one extract with a low concentration of terpenes (K1) were prepared to examine their pharmacological anticonvulsant effect. Adult mice were implanted with an electrode to perform ECoG recording of the induced seizures. First, I evaluated if the two different Cannabis extracts could modify the duration and severity of the induced seizures. Secondly, I characterized the immunoreactivity for the biomarker FosB/ΔFosB, because FosB/ΔFosB markedly increases in the lateral amygdala and hippocampus after seizure induction [253].

Chapter 3

Result 1

Antiseizure Effects of Cannabidiol Leading to Increased Peroxisome Proliferator-Activated Receptor Gamma Levels in the Hippocampal CA3 Subfield of Epileptic Rats[5]

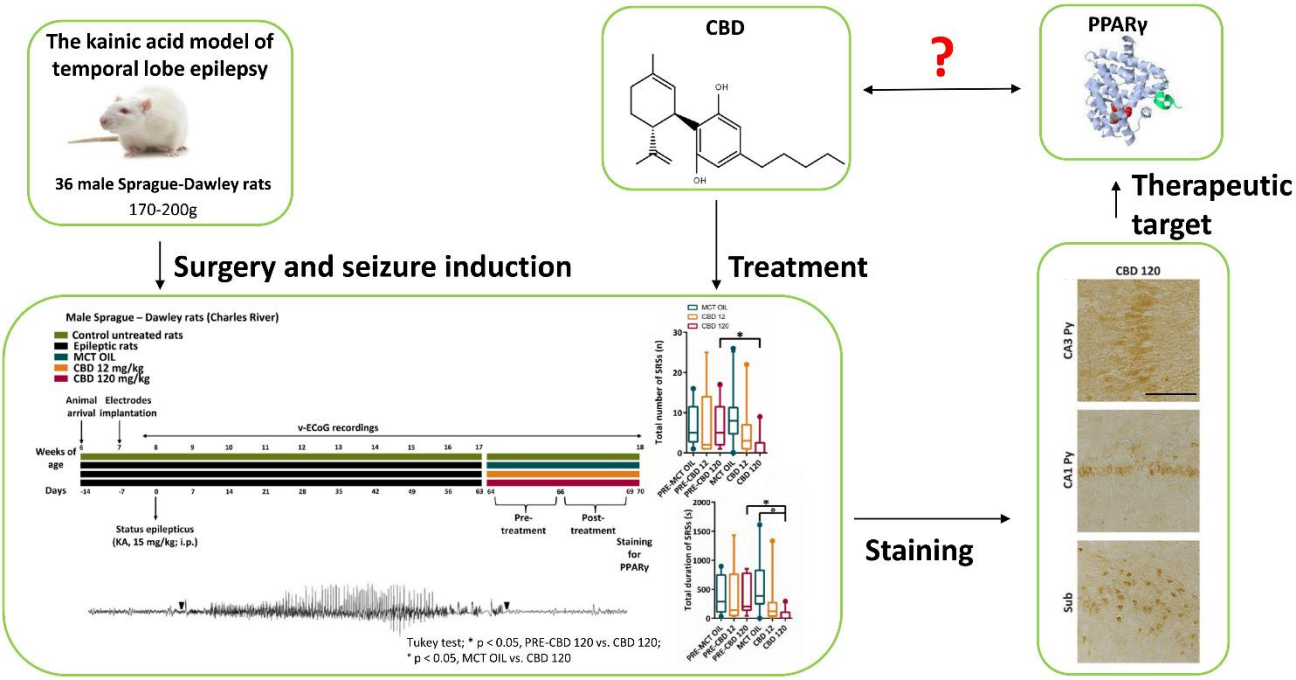

Figure 10. Graphical abstract. Published in *Pharmaceuticals*, 2022 [571].

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Abstract

We evaluated the effects of cannabidiol (CBD) on seizures and peroxisome proliferator- activated receptor gamma (PPARγ) levels in an animal model of temporal lobe epilepsy (TLE). Adult male Sprague-Dawley rats were continuously monitored by video-electrocorticography up to 10 weeks after an intraperitoneal kainic acid (15 mg/kg) injection. Sixty-seven days after the induction of status epilepticus and the appearance of spontaneous recurrent seizures in all rats, CBD was dissolved in medium-chain triglyceride (MCT) oil and administered subcutaneously at 120 mg/kg (n = 10) or 12 mg/kg (n = 10), twice a day for three days. Similarly, the vehicle was administered to ten epileptic rats. Brain levels of PPARγ immunoreactivity were compared to those of six healthy controls. CBD at 120 mg/kg abolished the seizures in 50% of rats ($p = 0.033$ vs. pre-treatment, Fisher's exact test) and reduced total seizure duration ($p < 0.05$, Tukey Test) and occurrence ($p < 0.05$). PPARγ levels increased with CBD in the hippocampal CA1 subfield and subiculum ($p < 0.05$ vs. controls, Holm–Šidák test), but only the highest dose increased the immunoreactivity in the hippocampal CA3 subfield ($p < 0.001$), perirhinal cortex, and amygdala ($p < 0.05$). Overall, these results suggest that the anti-seizure effects of CBD are associated with upregulation of PPARγ in the hippocampal CA3 region.

Keywords: cannabinoids; cannabidiol; drug-resistant epilepsy; kainic acid; nuclear receptors; peroxisome proliferator-activated receptor gamma; temporal lobe epilepsy

Introduction

Epilepsy is a chronic disorder displaying a high impact on psychological, social, and economic conditions of patients and their caregivers, besides the problems of the disease itself [563]. Its prevalence of 0.5% to 1.0% within the world's population ranks epilepsy on the fourth place of neurological diseases after migraine-headache, stroke, and dementia [16]. Among others, temporal lobe epilepsy (TLE) is the most common form of epilepsy in adult patients [45]. The administration of anti-seizure medications (ASMs) results in seizure freedom in about 65% of patients [366]. However, the remaining patients continue to present spontaneous recurrent seizures (SRSs). For this reason, the use of alternative drugs as new treatments is in the focus of the latest research.

Cannabidiol (CBD) and tetrahydrocannabinol (Δ^9-THC) are the two major active substances found in the female flowers of Cannabis sativa L. Due to the lack of psychotropic properties of CBD, which are the principal drawbacks of Δ^9-THC, its application is declared to be more secure and effective in reducing seizures. Therefore, in the last five decades, CBD has gained considerable interest as an add-on medication for drug-resistant epilepsy. In 2018, CBD was approved by the Food and Drug Administration (FDA) for the treatment of severe forms of infantile epilepsy, such as Lennox–Gastaut and Dravet syndromes [403,538].

The medicinal value of CBD has been recognized for many years [357], nevertheless, significant progress in establishing the clinical protocol to use CBD as an add-on ASM has only been set in the recent period. Although the exact mechanism behind the functioning of CBD is not fully confirmed yet, there are several targets exhibiting promising results in their mutual dependence and interaction [761,797,798]. For example, CBD has been shown to act as a reuptake inhibitor of the

endocannabinoid anandamide, changing the excitatory and inhibitory dynamics of synapses [435]. In addition to this, it has been demonstrated that the PI3K/mTOR pathway, which is linked to numerous neurological disorders, including epilepsy, is involved in the anticonvulsant effects of CBD [799]. Moreover, CBD inhibits adenosine transport type 1 equilibrative nucleoside transporter (ENT-1) activity and lowers neuronal excitability by functionally antagonizing G-protein-coupled receptor 55 (GPR55) and desensitizing transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors [491].

Another beneficial interaction was found between peroxisome proliferator-activated receptor gamma (PPARγ) and CBD, which was associated with neuroprotective properties in ischemic stroke and with stimulated neurogenesis in a model of Alzheimer's disease [761–763]. CBD has already been shown to regulate immune responses by modulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, a pathway activated in both reactive microglia and astrocytes and hypothesized to contribute to neurodegenerative diseases such as Parkinson's disease. Notably, PPARγ activation inhibits NF-κB signaling and lowers the mRNA levels of proinflammatory mediators including tumor necrosis factor alpha, interleukin 6 (IL-6), and inducible nitric oxide synthase [764,765]. The ability of glial cells to promote the inflammatory state by producing inflammatory mediators during an epileptic seizure emphasizes the neuroprotective role of CBD, possibly mediated by PPARγ, in epilepsy [766]. However, the fact that CBD presents agonist, antagonist and other modulatory actions on diverse receptors and channels, complicates the determination of its exact pathway [797].

In preclinical studies, it was suggested that CBD attenuates seizures, improves survival, and behavioral comorbidities of Dravet syndrome in mice [466,800]. Similarly, CBD exerts anticonvulsant activities in a wide variety of acute seizure models, such as the audiogenic seizure, maximal electroshock, and 6 Hz-seizure models [794,801], or other models in which seizures are induced by isoniazid, cocaine, or gamma-aminobutyric acid type A ($GABA_A$) receptor antagonists [802–804]. Additionally, CBD seems to display anti-ictal activities in rodents when administered prior to the pilocarpine or penicillin-induced seizures [805,806]. It can also decrease the percentage of rats developing *status epilepticus* (SE) after the intrahippocampal administration of pilocarpine [398]. Then, antiepileptogenic effects of CBD have been reported in a rat model of pentylenetetrazole (PTZ)-induced kindling [807], whereas anticonvulsant effects against the motor manifestations of a focal epilepsy have been described in cobalt-treated rats [808]. Furthermore, chronic CBD administration was shown to ameliorate both reference and working memory errors after the onset of SRSs, in a model of TLE [806].

Considering the above-mentioned data, it could be possible that the antiepileptic effects of CBD depend, at least in part, on unconventional targets [761], such as PPARγ. In this regard, we recently found that PPARγ is involved in the anti-seizure properties of EP-80317, a ghrelin analogue tested in a model of repeated seizure induction [570]. Thus, to evaluate this hypothesis, we designed an experiment in which two different doses of CBD (12 or 120 mg/kg) [794,809–811] were tested in epileptic rats previously treated with kainic acid (KA) [177,812]. In these animals, we evaluated the changes in levels of PPARγ immunoreactivity in response to the anti-seizure effects of CBD.

Results

Different Responses to Treatment

Thirty epileptic rats were obtained by injecting KA in 32 animals (mortality, approximately 7%), as illustrated in Figure 11, and monitoring the epileptic activity by continuous video-electrocorticographic (v-ECoG) recordings until treatment with CBD or its vehicle. Further details about the experimental design are available in the method section.

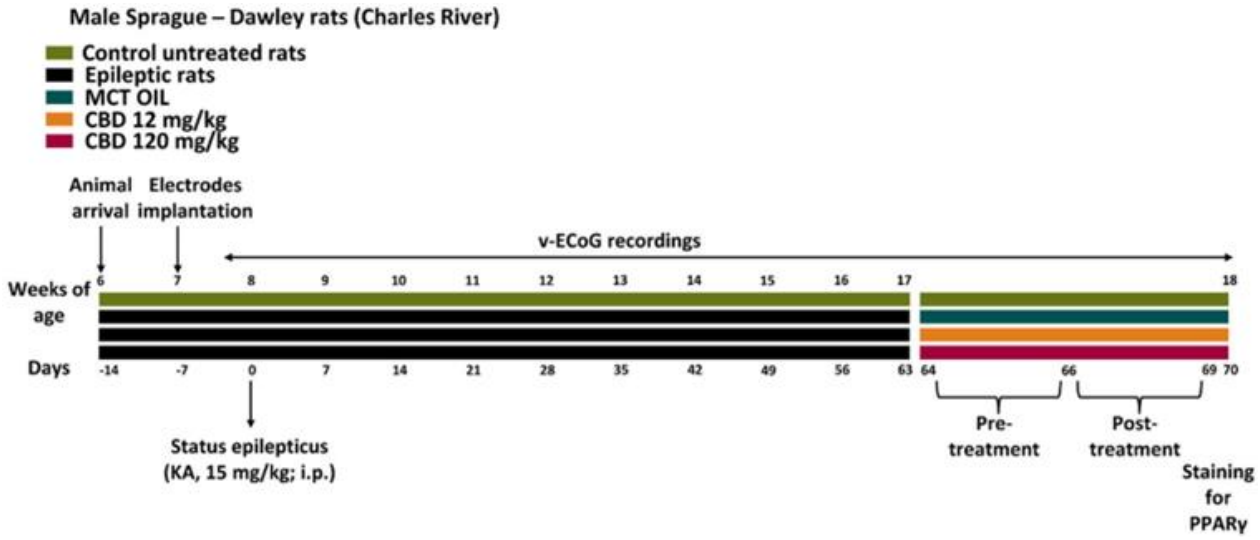

Figure 11. Experimental design. Thirty-eight male Sprague-Dawley rats were purchased at 6 weeks of age, and cortical electrodes were implanted 1 week later for video-electrocorticographic (v-ECoG) recordings of recurrent seizures. Status epilepticus (SE) was induced with an intraperitoneal (i.p.) administration of kainic acid (KA; 15 mg/kg) at 8 weeks of age (n = 2 rats died during the SE). Ten epileptic rats per group were treated with cannabidiol (CBD) at 12 mg/kg, 120 mg/kg, or with medium-chain triglyceride (MCT) oil. The remaining animals (n = 6) were used as control, non-treated rats.

During the treatment with CBD (120 mg/kg dissolved in medium-chain triglyceride (MCT) oil, injected subcutaneously, s.c.), 50% of the rats presented with a complete response, consisting of the suppression of SRSs during the overall period of treatment. The remaining rats experienced an incomplete response to CBD, as the seizure suppression was not complete but only reduced to 63%. By comparing the occurrence of SRSs under the administration of a high dose of CBD with that observed in the pre-treatment period (during which all the rats displayed at least one SRS) of the same animals, we observed a significant difference ($p = 0.033$, Fisher's exact test), suggesting that the high dose of CBD efficaciously suppressed ictogenesis. In comparison to the pre-treatment period, the treatment with a low dose of CBD or MCT oil did not affect the number of rats that displayed seizures, as only 2 out of 10 rats did not present SRSs after the treatment with CBD at 12 mg/kg, and only 1 out of 10 rats did not develop SRSs during the administration of MCT oil.

Characterization of the Duration of SRSs

By visual inspection, SRSs recorded in CBD-treated rats (120 mg/kg), which did not completely respond to CBD administration, differed in duration when compared to the SRSs observed in the same group of rats during the days preceding the treatment, and from those characterized in epileptic rats treated with 12 mg/kg of CBD or MCT in the same period (Figure 12).

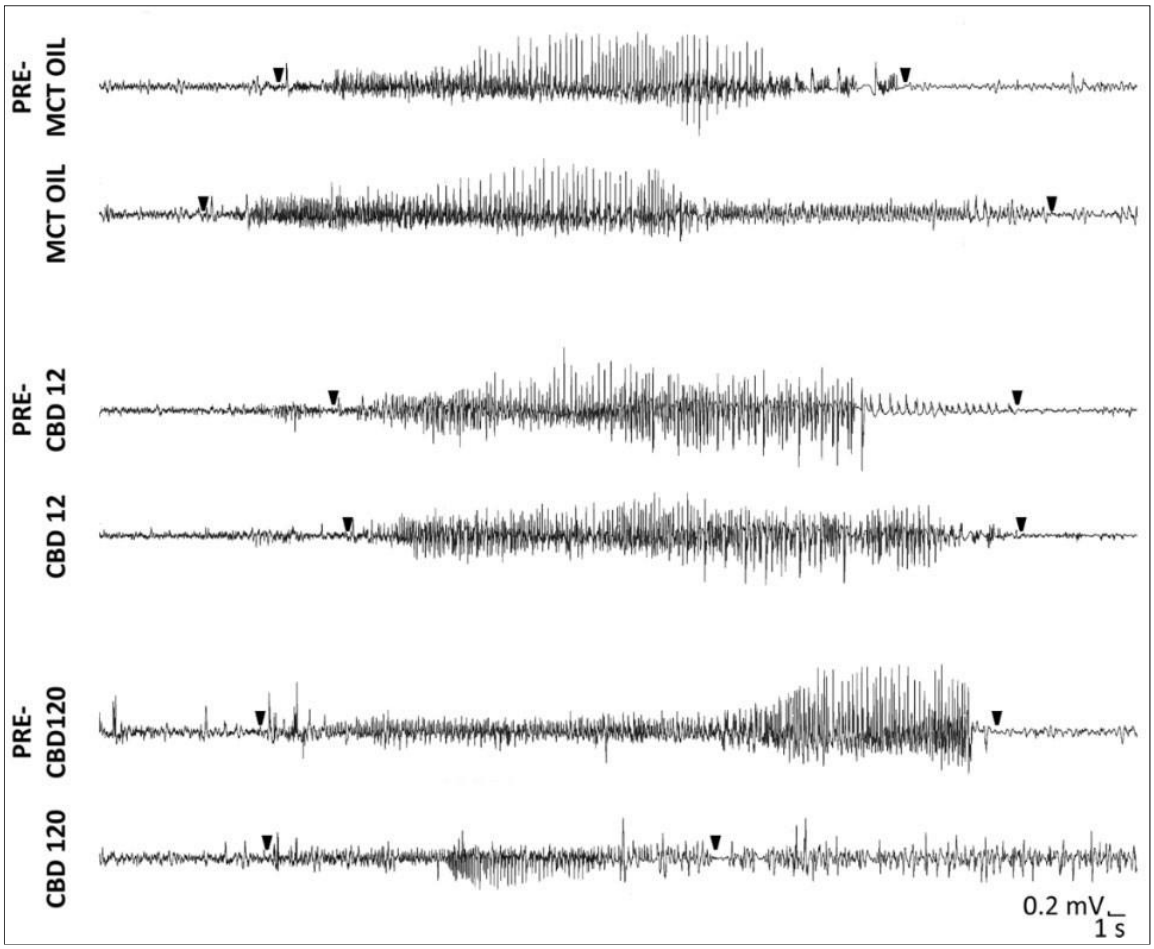

Figure 12. Spontaneous recurrent seizures (SRSs) after induction of status epilepticus in epileptic rats treated with cannabidiol (CBD) at 12 mg/kg, 120 mg/kg, or with medium-chain triglyceride (MCT) oil. SRSs were recorded in rats during the treatment with CBD or MCT oil, but also 3 days before (PRE-MCT OIL, PRE-CBD12, and PRE-CBD120) the treatment. Notably, SRSs during the high concentrations of CBD belong to incomplete responders. Arrowheads represent the onset and termination of SRSs.

The changes in the duration of SRSs with different treatment conditions were evaluated through the Friedman repeated measures analysis of variance (ANOVA) on Ranks. This analysis revealed that differences in the median duration of total SRSs were significantly different across treatment groups ($p = 0.026$). The Tukey test showed that the treatment with high dose of CBD induced a significant decrease in the total duration of all SRSs ($p < 0.05$), in comparison to the pre-treatment period. In the same period, a significant difference was also observed between the treatment with a high dose of CBD and MCT oil ($p < 0.05$). Interestingly, these changes were not significantly different when stages (ST.) 0–3 and ST. 4–5 SRSs were considered separately (Figure 13A–C).

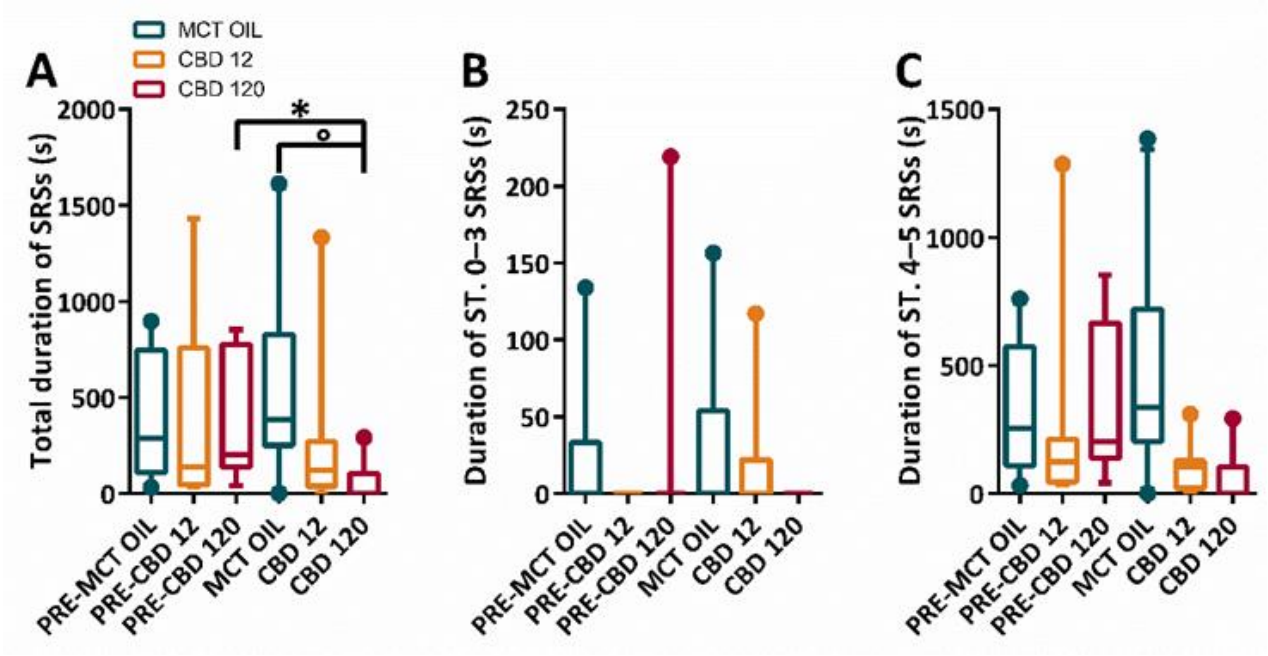

Figure 13. Duration of spontaneous recurrent seizures (SRSs) in epileptic rats treated with cannabidiol (CBD) at 12 mg/kg, 120 mg/kg or with medium-chain triglyceride (MCT) oil. Rats (n = 30) were treated with CBD 12 mg/kg (n = 10, 2 of which did not display SRSs and were attributed a 0 value), CBD 120 mg/kg (n = 10, 5 of which did not display SRSs and were attributed a 0 value), or MCT oil (n = 10, 1 of which did not display SRSs and were attributed a 0 value). In (A), CBD at 120 mg/kg significantly decreased (Tukey test; * $p < 0.05$, PRE-CBD 120 vs. CBD 120; ◦ $p < 0.05$, MCT OIL vs. CBD 120) the total number of SRSs. The high dose of CBD displayed no significant effects when stages (ST.) 0–3 (B) and ST. 4–5 (C) SRSs were considered separately.

Characterization of the Total Number of SRSs

In Table 8, the total number of SRSs with different treatment conditions was reported.

Table 8. Median and interquartile range values of the number of spontaneous recurrent seizures (SRSs) in the various treatment groups with cannabidiol (CBD) at 12 mg/kg, 120 mg/kg, or with medium-chain triglyceride (MCT) oil, subdivided by stages (ST.), or considered altogether. Ten animals per group were included, except where a single outlier per group was identified and removed.

Total Number of SRSs			
Group	**Pre-Treatment**	**Treatment**	**Outlier Removal**
MCT OIL	5.00 (2.75–11.50)	8.00 (4.75–11.25)	no
CBD 12	2.00 (1.00–14.00)	3.00 (1.00–7.00)	yes
CBD 120	5.00 (2.00–11.50)	0.00 (0.00–2.50)	yes
Number of ST. 0–3 SRSs			
MCT OIL	0.00 (0.00–2.25)	0.00 (0.00–2.25)	no
CBD 12	0.00 (0.00–0.00)	0.00 (0.00–1.00)	yes
CBD 120	0.00 (0.00–0.00)	0.00 (0.00–1.00)	yes
Number of ST. 4–5 SRSs			
MCT OIL	4.00 (2.75–7.00)	6.50 (3.75–11.25)	no
CBD 12	2.00 (1.00–13.00)	3.00 (1.00–4.50)	yes
CBD 120	5.00 (2.00–11.50)	0.00 (0.00–2.50)	yes

Then, the changes in the occurrence of SRSs were evaluated through the Friedman repeated measures ANOVA on Ranks. This analysis revealed that differences in the median number of total SRSs were significantly different across treatment groups ($p = 0.035$). The Tukey test (Figure 14A–C) showed that the treatment with high dose of CBD induced a significant decrease in the total number of all SRSs ($p < 0.05$), in comparison to the pre-treatment period. Interestingly, the effects of CBD on the median number of SRSs were limited to tonic-clonic SRSs ($p < 0.05$).

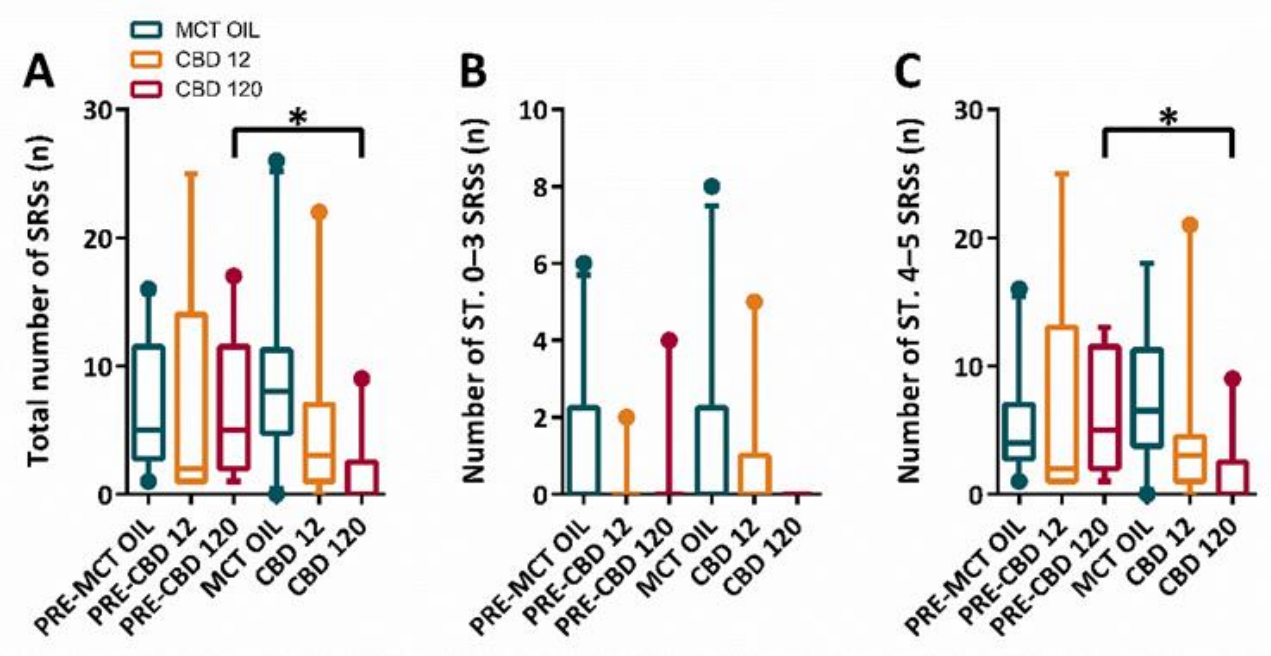

Figure 14. Total number of spontaneous recurrent seizures (SRSs) in epileptic rats treated with cannabidiol (CBD) at 12 mg/kg, 120 mg/kg, or with medium-chain triglyceride (MCT) oil. Rats (n = 30) were treated with CBD 12 mg/kg (n = 10, 2 of which did not display SRSs and were attributed a 0 value), CBD 120 mg/kg (n = 10, 5 of which did not display SRSs and were attributed a 0 value), or MCT oil (n = 10, 1 of which did not display SRSs and were attributed a 0 value). In (A), CBD at 120 mg/kg significantly decreased (Tukey test; * $p < 0.05$, PRE-CBD 120 vs. CBD 120) the total number of SRSs. The high dose of CBD displayed no significant effects on stages (ST.) 0–3 SRSs (B), but only on ST. 4–5 SRSs (C).

PPAR-γ Immunoreactivity

We evaluated the effects of treatment with CBD or MCT oil on PPARγ immunoreactivity (Figure 15A–D) in the Cornu Ammonis 3 stratum pyramidalis (CA3 Py), Cornu Ammonis 1 stratum pyramidalis (CA1 Py), and subiculum (Sub). Note that six healthy control rats and six rats per treatment group were randomly chosen for the immunohistochemical staining, whereas 4 out of 10 epileptic rats per treatment group were monitored by video- electrocorticography for an additional week to assess possible changes in seizure frequency after treatment. However, no remarkable changes in seizure frequency were visually observed in the week following the treatment.

In comparison to controls, the treatment with CBD at 120 mg/kg significantly increased PPARγ levels in CA3 Py (0.166 ± 0.009 vs. 0.251 ± 0.014, $p < 0.001$; Holm–Šidák test), CA1 Py (0.225 ± 0.009 vs. 0.331 ± 0.035, $p = 0.01$), and Sub (0.238 ± 0.014 vs. 0.307 ± 0.018, $p = 0.024$). Furthermore, PPARγ levels in CA3 Py were significantly increased after the treatment with a high dose of CBD, in comparison to the groups treated with CBD at 12 mg/kg (0.198 ± 0.008 vs. 0.251 ± 0.014, $p = 0.013$) or MCT (0.200 ± 0.009 vs. 0.251 ± 0.014, $p = 0.014$). In comparison to controls, PPARγ levels were

significantly higher in CA1 Py (0.225 ± 0.009 vs. 0.305 ± 0.013, p = 0.04) and Sub (0.238 ± 0.014 vs. 0.299 ± 0.019, p = 0.045) after the treatment with CBD at 12 mg/kg.

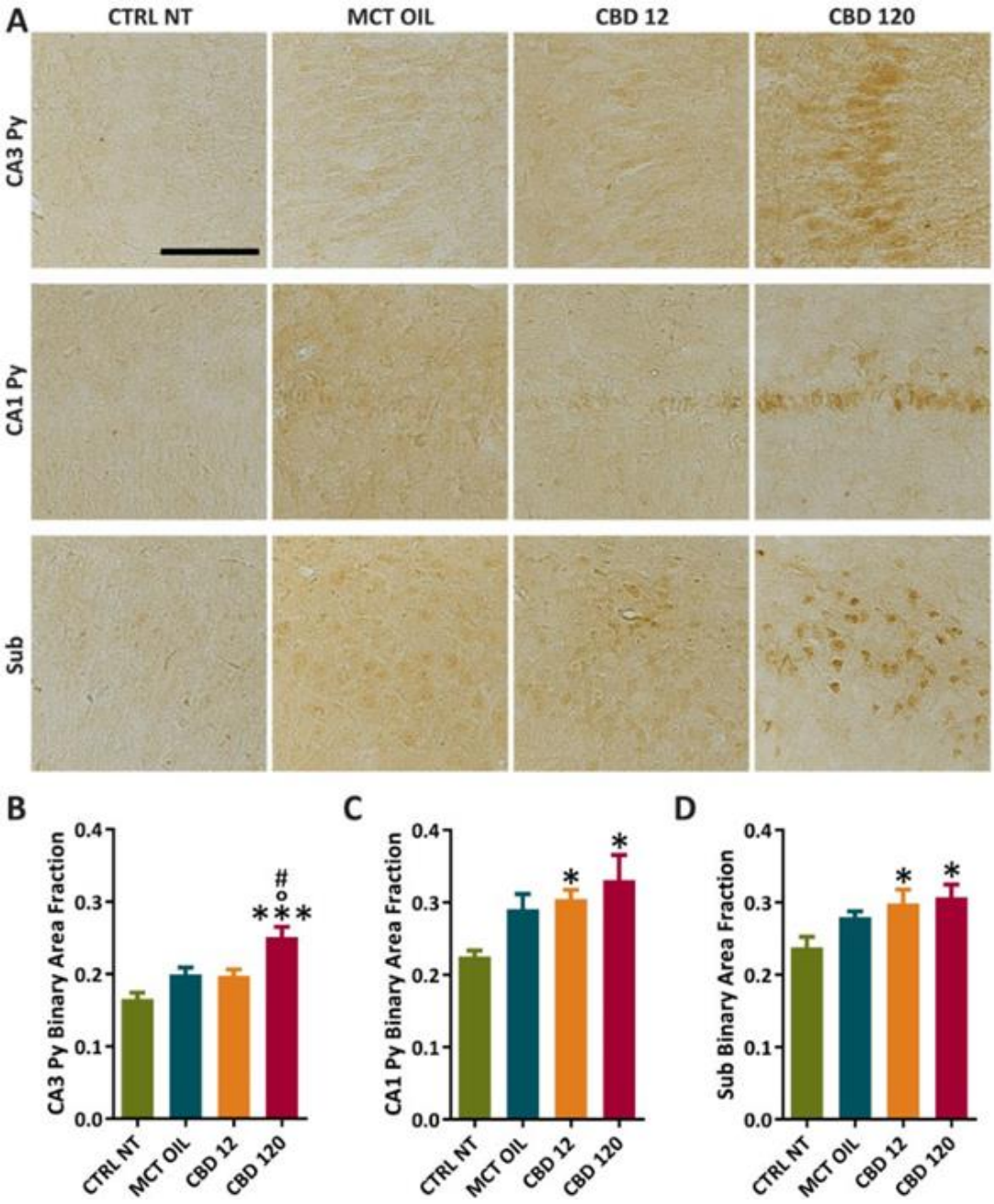

Figure 15. PPARγ immunoreactivity in hippocampal regions of controls and rats treated with cannabidiol (CBD) or with medium-chain triglyceride (MCT) oil. Non-treated controls (CTRL NT, n = 6) and epileptic animals (A) treated with CBD at high dose (CBD 120, n = 6), CBD at low dose (CBD 12, n = 6), or MCT oil (MCT OIL, n = 6) were used to determine the levels of PPARγ in CA3 Py (B), CA1 Py (C), and Sub (D). * $p < 0.05$, *** $p < 0.001$, CBD 12 or CBD120 vs. CTRL NT; ◦ $p < 0.05$, CBD 120 vs. MCT OIL; # $p < 0.05$, CBD 120 vs. CBD 12; Holm-Šidák test. CTRL NT, non-treated control; CBD, cannabidiol. CA3 Py, Stratum pyramidalis of Cornu Ammonis 3; CA1 Py, Stratum pyramidalis of Cornu Ammonis 1; Sub, Subiculum. Scale: 0.1 mm.

Furthermore, we assessed the effects of treatment with CBD or MCT oil on PPARγ immunoreactivity (Figure 16A–C) in the lateral amygdala (La) and perirhinal cortex (PRh). In comparison to controls, the treatment with CBD at 120 mg/kg significantly increased PPARγ levels in La (0.225 ± 0.015 vs. 0.312 ± 0.030, p = 0.012) and PRh (0.215 ± 0.011 vs. 0.269 ± 0.012, p = 0.045).

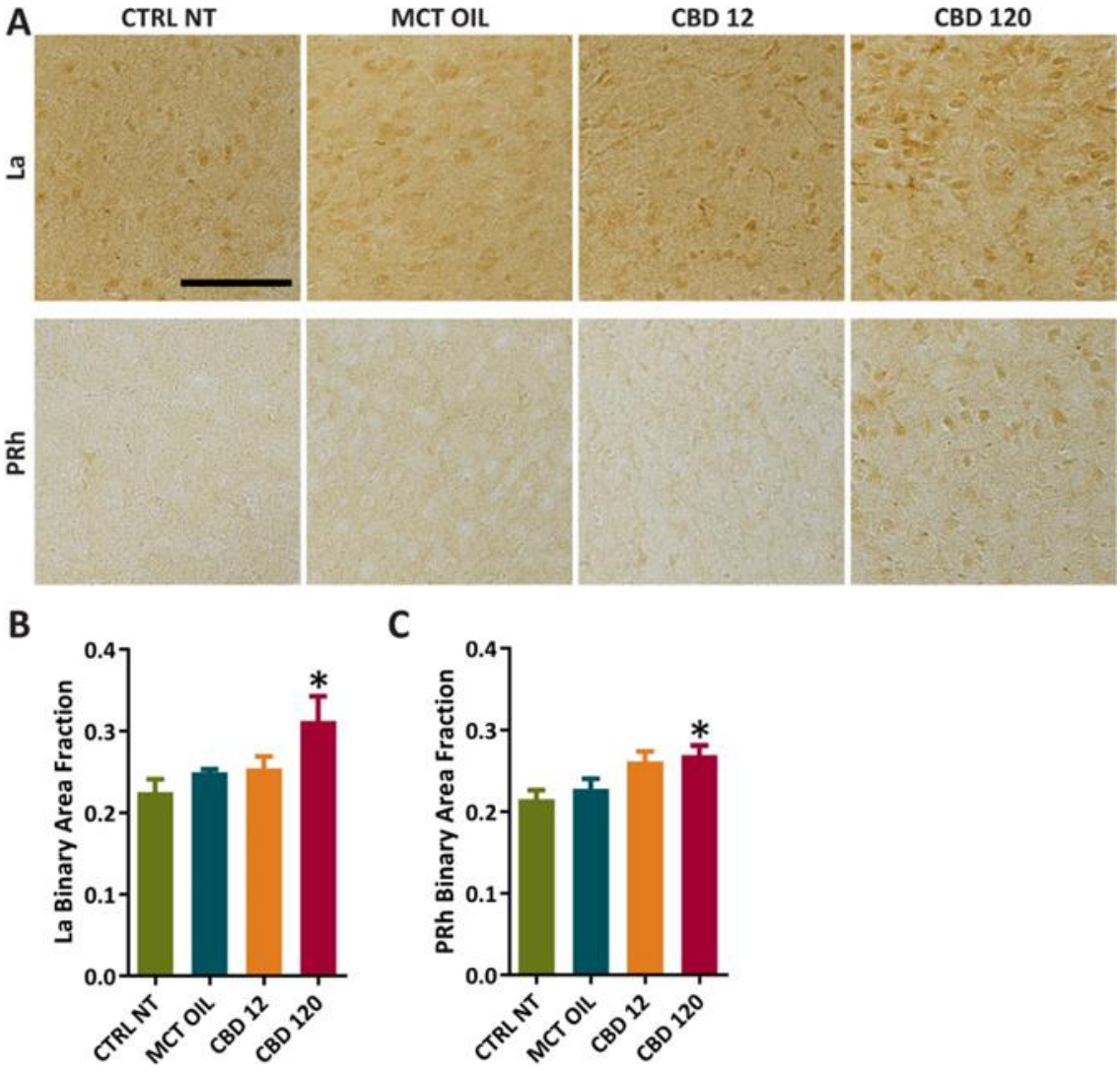

Figure 16. PPARγ immunoreactivity in extrahippocampal regions of controls and rats treated with cannabidiol (CBD) or with medium-chain triglyceride (MCT) oil. Non-treated controls (CTRL NT, n = 6) and epileptic animals (A) treated with CBD at high dose (CBD 120, n = 6), CBD at low dose (CBD 12, n = 6), or MCT oil (MCT OIL, n = 6) were used to determine the levels of PPARγ in lateral amygdala (La) (B), and perirhinal cortex (PRh) (C). * $p < 0.05$, CBD 120 vs. CTRL NT; Holm-Šidák
test. Scale: 0.1 mm.

Discussion

The present study aimed to evaluate the efficacy of CBD in an animal model of TLE and to analyze the modulation of PPARγ immunoreactivity by CBD. Particularly, the subcutaneous injections of

CBD at 120 mg/kg reduced: (i) the seizure duration; (ii) the number of rats experiencing SRSs; (iii) the total number of SRSs in epileptic rats. Moreover, PPARγ immunoreactivity was increased in hippocampal and extrahippocampal regions after the repeated administration of CBD, with a remarkable effect of the higher dose in the CA3 hippocampal subfield.

A compulsory step to assess the efficacy of CBD in controlling SRSs was to define its therapeutic dose. To this aim, we adopted the intraperitoneal (i.p.) KA model [177,812], and CBD was administered at 12 or 120 mg/kg. In our experiment, only the highest dose displayed a therapeutic effect in epileptic rats. Thus, this finding confirms the results of other models. For instance, the i.p. injection of CBD at 25 mg/kg did not change the seizure thresholds in the maximal electroshock seizure threshold test and the 6-Hz-induced seizure test, whereas its injection at higher doses was significantly more powerful in counteracting seizures [794]. Indeed, only CBD at 100 mg/kg significantly potentiated the anticonvulsant effect of topiramate, oxcarbazepine, and pregabalin in the maximal electroshock seizure threshold test. Similarly, only CBD at 50 and 100 mg/kg enhanced the activity of tiagabine and gabapentin in the 6-Hz seizure test [794]. This agrees with recent data, which demonstrated that the administration of CBD at 25 mg/kg by gavage was ineffective at reducing seizures induced by the repeated 6-Hz corneal stimulation test in mice. However, a more complete whole-plant extract (i.e., oil enriched with terpenes) was more effective and reduced the therapeutic dose [801].

An important issue to consider when adopting CBD to control SRSs might be that the mechanisms underlying the anticonvulsant activity of CBD are not completely defined [761,797]. CBD could mediate the hyperpolarization of presynaptic membrane and reduce the neuronal hyperactivity through an indirect action on CB_1 receptors, as it seemed to act as a reuptake inhibitor of the endocannabinoid anandamide [759]. However, the involvement of additional targets could further explain the anti-seizure properties of CBD in our animal model of TLE. For instance, CBD induced neuroprotection in ischemic stroke though PPARγ involvement [762], which seemed to be also related to the anti-seizure properties of the ghrelin receptor antagonist EP-80317 [570], and of the ketogenic diet [654]. Moreover, CBD might exert anti-inflammatory effects by acting both on the peripheral system and central nervous system. More precisely, CBD seemed to reduce peripheral inflammation by acting at TRPV1, cannabinoid receptor 2 (CB_2), and GPR55 receptors. These interactions were, in turn, involved in the downregulation of enzymes related to the production of prostaglandins, reactive oxygen species, and cytokines. At variance, the anti-inflammatory effects of CBD in the central nervous system seemed to involve the inhibition of the mitogen-activated protein kinase pathway and the downregulation of NF-κB, together with PPARγ-mediated reduction of lipid peroxidation [357]. In this regard, the possible involvement of PPARγ in the neuroprotective and anti-inflammatory effects of CBD could be further supported by the fact that also the PPARγ-agonist pioglitazone was shown to reduce neuronal loss and decrease the levels of IL-1β and IL-6 in the hippocampus of a rat model of PTZ induced SE [632]. Additionally, pioglitazone was shown to attenuate pilocarpine-induced seizure severity, neuronal loss, blood-brain-barrier impairment, and sodium currents in hippocampal neurons. Neuronal excitability and excitotoxicity, in contrast, were exacerbated by PPARγ inhibition [723].

In general, the complete understanding of the signaling pathways involved in the beneficial effects of CBD could help in defining the efficacy and safety of CBD as an add-on anticonvulsant therapy in different populations of epileptic patients [775], so not only in those populations in which CBD was already shown to display beneficial effects (e.g., patients with treatment-resistant Lennox–Gastaut syndrome and Dravet syndrome) [538,813]. In our experiment, data suggested that the levels of immunoreactivity for PPARγ were significantly increased within brain regions in a dose-related manner, and the CA3 hippocampal subfield emerged as the most important target region for the modulation of PPARγ levels by the effective CBD dose. Overall, these results might contribute to explaining, at least in part, the anti-seizure properties of CBD in our animal model of TLE.

Materials and Methods

Animals

The study protocol was authorized by the Italian Ministry of Health (729/2021-PR), after approval by the university Animal Welfare Body. All experiments were performed in agreement with the European Directive 2010/63/EU and the consequent Italian act (DM 26/2014). Thirty-eight adult male Sprague-Dawley rats (Charles River, Calco, Italy), with initial weights of 175-200 g, were housed in a specific pathogen-free facility in a controlled environment and with ad libitum access to water and food. All efforts were made to refine procedures and protect the animals' welfare.

Experimental Design

SE was induced by an i.p. injection of KA (15 mg/kg; Sigma-Aldrich, Milan, Italy). At the end of SE, softened rat chow was administered to minimize discomfort. The mortality rate in our systemic KA rat model was similar to that previously determined [35]. In ten epileptic rats per group, both CBD (12 or 120 mg/kg; provided by Farmabios, Gropello Cairoli, Italy) and MCT oil (1 mL/kg; USP pharmaceutical grade MCT Lean; MCT Foods, Glencoe, IL, USA) were administered s.c. twice a day for three days, starting from 67 days after SE onset. Particularly, the subcutaneous administration was preferred because substances might be absorbed at a slower rate compared with other parenteral routes, providing a sustained effect [814]. Moreover, the injection was performed twice a day to mimic the administration of Epidiolex® in patients affected by drug-resistant SRSs [5]. For immunohistochemical analyses, six rats per treatment group (n = 18) and six untreated controls were considered. Specifically, the control rats were used to determine the basal levels of PPARγ.

Electrode Implantation and Video-Electrocorticography

Guiding holes were drilled and electrodes (stainless steel Ø = 1 mm; PlasticsOne, Roanoke, VA, USA) were implanted in frontal (bregma 0 mm, 3.5 mm lateral from midline) and occipital cortices (bregma -6.5 mm, 3.5 mm lateral from midline) of both hemispheres. One additional electrode was implanted below the lambda on the midline in all rats and used as a reference. During electrode implantation, volatile isoflurane was used to induce a deep anesthesia, assessed by deep breath, loss of tail and eye reflexes. Gel containing 2.5 g lidocaine chloride, 0.5 g neomycin sulfate, and 0.025 g fluocinolone acetonide (Neuflan® gel; Molteni Farmaceutici, Scandicci, Italy) was applied at the end of the surgical procedure to reduce acute pain and risk of infection. All animals were

monitored until complete recovery from anesthesia and housed in single cages with no grids or environmental enrichments to avoid risk of headset loss.
The EcoG data were recorded via cable connection between headset and preamplifiers. Electrical activity was digitally filtered (0.3 Hz high-pass, 500 Hz low-pass), and acquired at 1 kHz per channel. All data was stored on a personal computer after the mathematical subtraction of traces of recording electrodes from trace of reference electrode by using a PowerLab8/30 amplifier connected to 4 BioAmp preamplifiers (ADInstruments; Dunedin, Otago, New Zealand). Videos were digitally captured through a camera connected to the dedicated computer and synchronized to the ECoG traces through LabChart 8 PRO internal trigger.

Behavioral and v-ECoG Analysis

ECoG traces were digitally filtered offline (band-pass: high 50 Hz, low 1 Hz) and manually analyzed using LabChart 8 PRO software (AD Instruments, Sydney, Australia) by expert raters. SE was defined as the period of time in which rats either did not recover normal behavior between a seizure and the other, or in which they displayed continuous shaking for more than 5 min. The end of SE was defined by a progressive reduction in frequency of the continuous electrographic spikes, accompanied by the recovery of normal behavior. In our animal model, SE was allowed to self-terminate, and the mean duration was about 10 h [812].
Seizures were defined as ECoG segments with a minimum duration of 10 s, continuous synchronous high-frequency activity, and an amplitude of at least twice the previous baseline. The mean duration of SRSs per day was equal to 0 s when the animal did not display SRSs for at least 24 h. Seizures and their durations were determined in the ECoG traces, then investigated for correlated behavior by using the synchronized video recordings. In particular, all seizures were scored as ST. 0 (or subclinical) if a clear epileptiform ECoG signal was observed without corresponding evident behavior in the video; ST. 1–2 in the presence of absence-like immobility, “wet-dog shakes”, facial automatisms, and head nodding; ST. 3, when presenting with forelimb clonus and lordosis posture; ST. 4, corresponding to generalized seizures and rearing; ST. 5, when seizures consisted of rearing with loss of posture and/or wild running, followed by generalized convulsions.

Immunohistochemistry

Rats deeply anesthetized with isoflurane were transcardially perfused with phosphate buffered saline (PBS, pH 7.4) followed by Zamboni’s fixative (pH 6.9), 12 h after the last subcutaneous injection of CBD or MCT oil. Brains were post-fixed at 4 °C in the same fixative for 24 h, cryoprotected in 15 and 30% sucrose solutions, and stored at 80 °C until used. Horizontal sections of 50 µm were cut using a freezing sliding microtome (Leica SM2000 R; Leica, Nussloch, Germany). Brain sections were washed in Tris-buffered saline (TBS) and incubated in 3% H_2O_2 in TBS (30 min). After washing, sections were blocked 1 h in TBS containing 2% bovine serum albumin, 0.3% Triton X-100 (Tx) and 5% normal goat serum. Brain sections were then placed at 4 °C with a polyclonal rabbit anti-PPARγ (Ab209350; Abcam, Cambridge, UK, dilution 1:2000, 48 h). Following another washing step, sections were incubated for 1 h with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:200), and later with the avidin- biotin-peroxidase

complex (Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA). The staining was performed in 0.05% 3,3-diaminobenzidine tetrahydrochloride for 5 min (DAB, Sigma–Aldrich, Milan, Italy) and developed by adding 0.03% H_2O_2. In the end, sections were washed again in TBS, mounted on gelatin-coated slides and cover slipped with Eukitt (Eukitt R, O. Kindler GmbH & Co., Freiburg, Germany).

Image Analysis

The levels of immunoreactivity for PPARγ were in the CA3 Py, CA1 Py, Sub, La, and in the PRh. The staining was performed as previously detailed [570]. Immunostained sections from -8.04 mm to -5.04 mm Bregma level were evaluated with a Nikon Eclipse CiL (Nikon Instruments) at 20X, and images were digitally captured by a Nikon DS-Fi3 digital camera. PPARγ immunoreactivity was measured as a binary area fraction after discrimination from background staining. Particularly, the binary area fraction (binary area/measured area) was analyzed using NIS-Elements software. Particularly, the binary area represented the sum of areas of all binary objects, whereas the measured area was the region of interest (ROI) within the measurement frame. In all sections, the ROI was manually traced.

Statistical Analysis

For all groups, we compared the number of rats with SRSs before and during the treatment period by using the Fisher's exact test. Data on the total number and duration of SRSs were analyzed by Friedman repeated measures ANOVA on Ranks, followed by the Tukey test. PPARγ levels were compared using one-way ANOVA, followed by Holm–Šidák test. One outlier per data set was identified using the Grubbs' test and removed. Statistical analyses were performed by using SigmaPlot 13 (Systat Software; San Jose, CA, USA). The results are shown as medians and interquartile ranges (seizures) or mean ± standard error of the mean (SEM) (PPARγ) and considered significant at $p < 0.05$.

Conclusions

The present work was aimed at evaluating the effects of CBD on SRSs and PPARγ levels in an animal model of TLE.

The results obtained on the systemic KA rat model suggested that the anti-seizure effects of CBD might lead, at least in part, to increased PPARγ levels in hippocampal and extrahippocampal regions, and especially in the hippocampal CA3 subfield.

A possible limitation suggested in this study is the lack of definitive proof of the involvement of PPARγ in the anti-seizure effects of CBD. In the future, we will assess whether PPARγ antagonists block the anti-seizure effects of CBD[6].

[6] **Author Contributions**: Conceptualization, A.-M.C., G.C. and G.B.; methodology, A.-M.C., F.R., L.S. and D.I.; validation, A.-M.C. and G.B.; formal analysis, A.-M.C., F.R., L.S. and D.I.; investigation, A.-M.C., G.C. and G.B.; resources, G.C. and G.B.; data curation, A.-M.C. and G.B.; writing—original draft preparation, A.-M.C. and G.B.; writing—review and editing A.-M.C., F.R., L.S., D.I., G.C. and G.B.;

visualization, A.-M.C.; supervision, G.C. and G.B.; project administration, G.C. and G.B.; funding acquisition, G.C. and G.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study protocol was authorized by the Italian Ministry of Health (729/2021-PR), after approval by the university Animal Welfare Body. All experiments were performed in agreement with the European Directive 2010/63/EU and the consequent Italian act (DM 26/2014).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data is contained within article.

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Conflicts of Interest: The authors declare no conflict of interest.

Chapter 4

Result 2

Antiseizure Seizure Effects of Fully Characterized Non-Psychoactive *Cannabis Sativa* L. Extracts in the Repeated 6-Hz Corneal Stimulation Test[7]

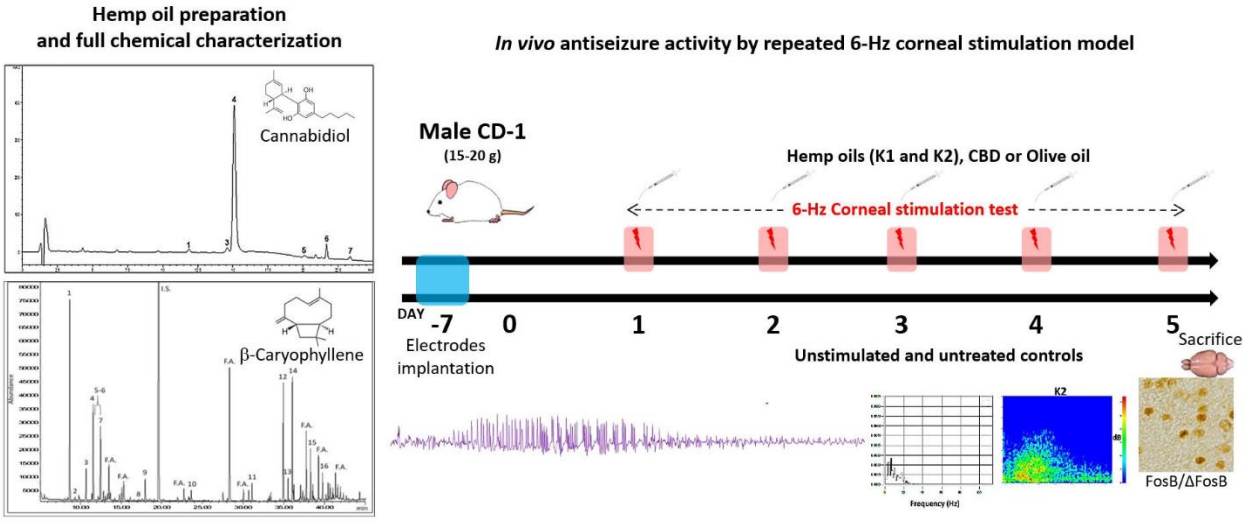

Figure 17. Graphical abstract. Published in *Pharmaceuticals*, 2021 [801].

Abstract

Compounds present in *Cannabis sativa* L. preparations have recently attracted much attention in the treatment of drug-resistant epilepsy. Here, we screened two olive oil extracts from a non-psychoactive *C. sativa* variety, fully characterized by high-performance liquid chromatography and gas chromatography. Particularly, hemp oils with different concentrations of terpenes were administered at the same dose of cannabidiol (25 mg/kg/day orally), 1 h before the 6-Hz

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corneal stimulation test (44 mA). Mice were stimulated once a day for 5 days and evaluated by video- electrocorticographic recordings and behavioral analysis. Neuronal activation was assessed by FosB/ΔFosB immunoreactivity. Both oils significantly reduced the percentage of mice experiencing convulsive seizures in comparison to olive oil-treated mice ($p < 0.050$; Fisher's exact test), but only the oil enriched with terpenes (K2) significantly accelerated full recovery from the seizure. These effects occurred in the presence of reduced power of delta rhythm, and, instead, increased power of theta rhythm, along with a lower FosB/ΔFosB expression in the subiculum ($p < 0.050$; Duncan's method). The overall findings suggest that both cannabinoids and terpenes in oil extracts should be considered as potential therapeutic agents against epileptic seizures and epilepsy.

Keywords: *Cannabis sativa* L.; hemp; cannabidiol; terpenes; epilepsy; 6-Hz corneal stimulation; antiseizure activity; power band spectrum; FosB/ΔFosB

Introduction:

Epilepsy is characterized by the recurrence of spontaneous seizures, and represents one of the most common neurological diseases [14]. Notably, its prevalence rate of 0.5% to 1.0% within the global population ranks epilepsy in fifth place within neurological diseases, after stroke, migraine, dementia, and meningitis [16,17]. Moreover, epilepsy is accompanied by a remarkable psychological, social, and economic burden for patients and their caregivers, which complicates the scenario of this disease [563]. Despite the efforts made to develop new anti-seizure medications (ASMs) for the treatment of epilepsy, for which several clinical trials have been performed, one third of patients still display drug-refractory epilepsy [366]. In this context, the beneficial effects of the *Cannabis sativa* L. plant in the treatment of seizures have been known since ancient times. The active ingredients of this plant are mainly Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), the latter considered much safer and more effective in treating seizures, because it is devoid of the adverse psychotropic effects which represent the main limitation of Δ^9-THC. Although the therapeutic potential of CBD in seizure disorders has been known for many years, it is only in the last decade that major progress has been made in characterizing its preclinical and clinical properties as a new ASM [815]. While the precise mechanism of action of CBD in humans remains unknown, there are several plausible targets engaged by CBD. The preclinical evidence strongly implicates three main molecular targets in CBD anticonvulsive properties [793]. In particular, CBD reduces neuronal excitability through functional antagonism of GPR55 receptors, desensitization of TRPV1 receptors, and inhibition of adenosine transport [793], even if other receptors and channels possibly involved in its activity cannot be excluded [797]. A pharmaceutical-grade formulation of purified CBD, derived from *C. sativa*, was evaluated in several randomized placebo-controlled adjunctive-therapy trials, which resulted in its regulatory approval for the treatment of seizures associated with Dravet syndrome, Lennox–Gastaut syndrome, and tuberous sclerosis complex [815]. Interpretation of the results of these trials was complicated by the occurrence of an interaction with clobazam in CBD-treated patients [815].

In a very recent study, cannabigerolic acid (CBGA), which is the biosynthetic precursor of all cannabinoids, was found to be more potent than CBD in reducing seizures triggered by a febrile event in a mouse model of Dravet syndrome [816]. Higher doses of CBGA also had pro-convulsant effects on other seizure types, highlighting a limitation of this cannabis constituent in comparison with CBD [816].

Cannabidiolic acid (CBDA), the direct precursor of CBD which is spontaneously generated under the action of heat and light, showed anticonvulsant activity in an acute rat model of seizures [817]. However, the chemical instability of CBDA poses challenges for its potential clinical utility [817]. All these issues would need to be overcome before another cannabinoid supersedes CBD as a new anticonvulsant drug.

A meta-analysis paper described the results of observational clinical studies on the treatment of refractory epilepsy with CBD-based products. CBD-rich extracts from *C. sativa* seem to present a better therapeutic profile than purified CBD, at least in this population of patients with refractory epilepsy [818]. This difference is probably due to synergistic effects of CBD with other compounds, in particular with terpenes, in the so-called entourage effect, but this remains to be confirmed [818]. The use of hemp extracts could offer advantages in comparison with pure CBD, in view of the above-mentioned entourage effect, due to volatile compounds, as described for both mood disorders and pain [819,820]. The bottleneck of this approach is related to the required standardization of the plant material, as well as the extraction method, in order to ensure not only the efficacy and the reproducibility of the pharmacological action, but also its safety. Indeed, it is well documented that there is a high degree of variability in the composition of medical cannabis oils on the basis of the extraction method [821].

To further evaluate the potential use of non-psychotropic *C. sativa* extracts that are highly rich in CBD as a new therapeutic treatment for resistant epilepsy, we tested CBD and *C. sativa* extracts in the 6-Hz corneal stimulation mouse model [250,255,570,822,823]. Specifically, we aimed to test the in vivo anti-seizure effect of olive oil extracts from hemp inflorescences, which were fully characterized for their content of both cannabinoids and terpenes. Even though it was challenging to study the chemical composition of plant extracts, in view of their complex chemical composition, the full characterization of both cannabinoids and terpenes was made possible and reliable thanks to specific and fully validated analytical methods based on high-performance liquid chromatography (HPLC) and gas chromatography (GC) [824–828]. The influence of the compounds found in the extracts, specifically terpenes in combination with cannabinoids, on the experimental results obtained after the administration of the oil extracts and pure CBD in mice is illustrated in detail.

Results

Qualitative and Quantitative Analysis of Cannabinoids in Hemp Oils

The identification of the secondary metabolites in hemp oils was carried out by ultra- high-performance liquid chromatography (UHPLC), coupled with high-resolution mass spectrometry (HRMS). In order to verify the presence of additional cannabinoids in hemp, for which analytical standards were not available, a target metabolomic analysis was employed.

This was performed using a compiled list of 213 cannabinoids, on the basis of those described in the literature [829], and additional potential cannabinoids that were added according to the biosynthesis and decomposition pathways previously described [829–831]. The MS and MS/MS data for the compound identified in hemp oils are shown in Table 9.

Table 9. Chemical formula, MS, and MS/MS data of the compounds identified in hemp oils both in the positive and negative ion mode by UHPLC-HRMS.

Compound	Formula	MS (*m/z*)	MS/MS (*m/z*)
Positive ion mode			
Cannflavin B	$C_{21}H_{20}O_6$	369.1333	315.2315
Dehydrocannabifuran-C5 (DCBF)	$C_{21}H_{24}O_2$	309.1775	239.8728
Epoxycannabigerol	$C_{21}H_{32}O_3$	333.2424	315.2319
Cannabinodiol (CBND)	$C_{21}H_{26}O_2$	311.2006	293.1898
Cannabidivarin (CBDV)	$C_{19}H_{26}O_2$	287.0060	277.2160
Hydroxycannabidiol (OH-CBD)	$C_{21}H_{30}O_3$	331.2268	313.2160
Cannabidiol-C4 (CBD-C4)	$C_{20}H_{28}O_2$	301.2153	245.1530
Cannflavin A	$C_{26}H_{28}O_6$	437.1959	359.2211
Cannabigerol (CBG)	$C_{21}H_{32}O_2$	317.2475	245.1547
Cannabidiol (CBD)	$C_{21}H_{30}O_2$	315.2319	245.1547
Cannabidiol monomethyl ether-C5 (CBDM)	$C_{22}H_{32}O_2$	329.2474	313.1800
Bisnor-cannabielsoin-C1 (CBEO)	$C_{17}H_{22}O_3$	275.1635	207.1013
Cannabinol (CBN)	$C_{21}H_{26}O_2$	311.2006	293.1898
Δ^9-Tetrahydrocannabinol (Δ^9-THC)	$C_{21}H_{30}O_2$	315.2321	245.1547
Cannabichromene (CBC)	$C_{21}H_{30}O_2$	315.2321	245.1547
Negative ion mode			
Hydroxycannabidiolic acid (OH-CBDA)	$C_{22}H_{30}O_5$	373.2028	345.2076
Cannabidiolic acid-C4 (CBDA-C4)	$C_{21}H_{28}O_4$	343.1932	325.1845
Cannabidiolic acid (CBDA)	$C_{22}H_{30}O_4$	357.2021	313.2176
Cannabigerolic acid (CBGA)	$C_{22}H_{32}O_4$	359.2228	331.2285
Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA)	$C_{22}H_{30}O_4$	357.2021	313.2177
Cannabichromenic acid (CBCA)	$C_{22}H_{30}O_4$	357.2021	313.2177

Cannabinoic acids showed a higher intensity of signals in the negative ion mode, as opposed to the neutral compounds which ionized better in the positive ion mode. A totalof 15 neutral cannabinoids were identified in the positive ion mode, while 6 cannabinoic acids were identified in the negative ion mode. Cannflavins A and B, which are isoprenic flavones present in minor amounts in hemp, were detected in the positive ion mode [825].It should be pointed

out that the qualitative profile was the same for the two hemp oils, due to the application of the same extraction procedure for this class of compounds.

Among all the identified cannabinoids, the most representative ones were further quantified by HPLC-UV. Figure 18 shows a representative HPLC-UV chromatogram of K2 hemp oil, acquired at 210 nm. The most abundant peak is that of CBD, at a retention time of 15.4 min.

Table 10 shows the quantitative results obtained from the HPLC-UV analysis of cannabinoids both in K1 and K2 oils. The data revealed that the decarboxylation process had successfully occurred for both the oils. As a matter of fact, Δ^9-THCA, CBDA, and CBGA were under the limit of quantification (LOQ) in both cases. The content of neutral cannabinoids in K1 and K2 oils was of the same order of magnitude.

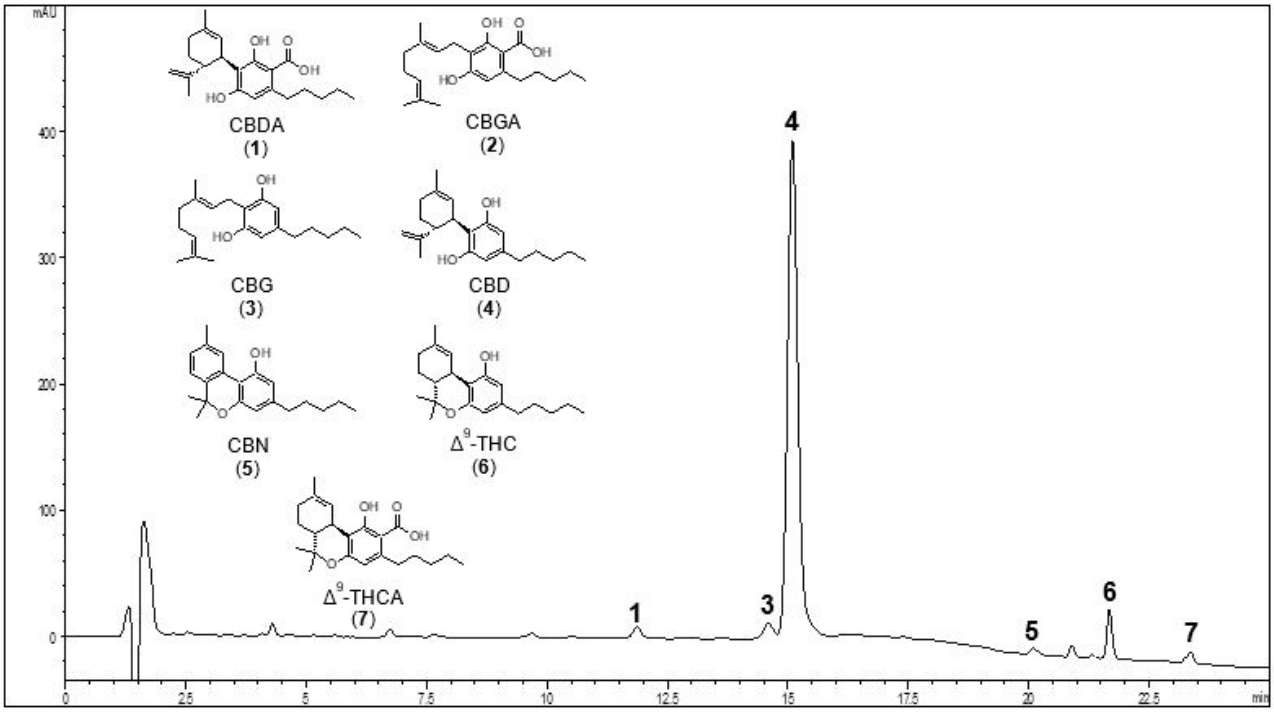

Figure 18. Representative HPLC-UV chromatogram of hemp oil 2 (K2) recorded at 210 nm. The peaks of the main neutral and acid cannabinoids are shown. For peak identification, see Table 10. The chemical structures of the compounds selected for quantitative analysis are shown in the slot on the left.

Table 10. Quantification of cannabinoids in hemp oils using HPLC-UV. Data expressed as mg/mL ± SD.

Peak Number	t_R (min)	Compound	Hemp Oil 1 (K1)	Hemp Oil 2 (K2)
1	11.6	CBDA	0.34 ± 0.01	<LOQ
2	13.4	CBGA	<LOQ	<LOQ
3	14.9	CBG	0.18 ± 0.03	0.20 ± 0.10
4	15.4	CBD	4.78 ± 0.23	4.24 ± 0.05
5	19.8	CBN	0.14 ± 0.03	0.14 ± 0.01
6	21.6	Δ^9-THC	0.42 ± 0.02	0.29 ± 0.01
7	23.6	Δ^9-THCA	<LOQ	<LOQ

LOQ= Limit of quantification

GC-MS Analysis of Terpenes in Olive Oil Extracts

Hemp volatile compounds in the oils were analyzed by GC-MS and identified according to their MS spectra, which were compared with available data in the MS spectral library.

As shown in Figure 19 and Table 11, the GC analysis allowed us to identify 16 compounds in samples K1 and K2.

The two samples displayed the same qualitative profile, since these compounds were identified in both oils. Peaks from 1 to 11 in Figure 19 were identified as monoterpenes,while peaks eluted later consisted of sesquiterpenes and caryophyllene oxide, which is a sesquiterpenoid. Among the components identified, the most representative terpenes were α-pinene and β-caryophyllene. The terpenes found in these hemp oils were the same as those previously described for dried hemp inflorescences and essential oil [825,826].

As to the quantitative analysis, the most representative terpenes in the oils, on the basis of the full-scan GC-MS data, were quantified in the SIM mode, using calibration curves built with an appropriate I.S. Quantitative data for samples K1 and K2 are shown in Table 12.

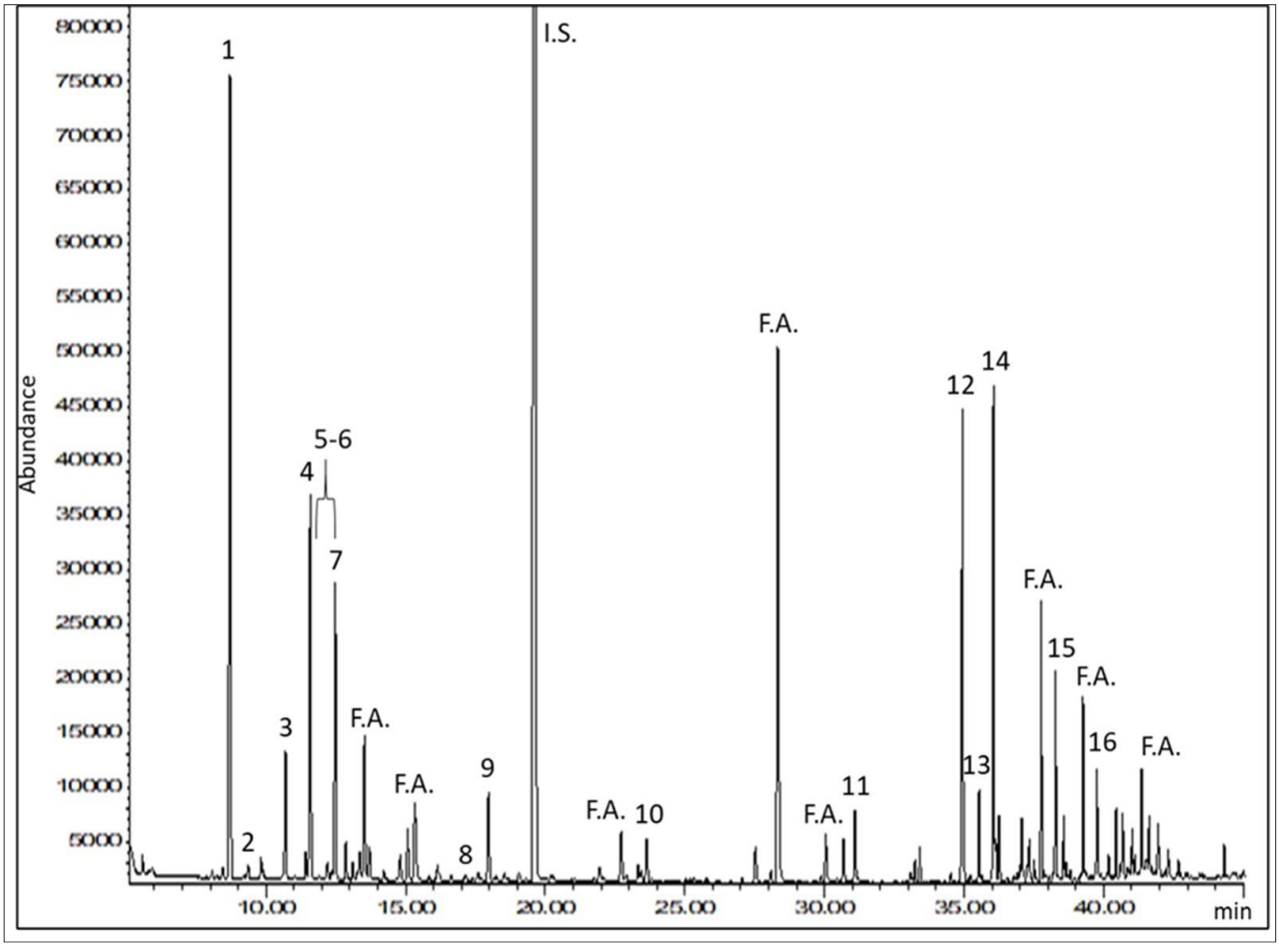

Figure 19. Representative GC-MS selected ion monitoring (SIM) chromatogram of hemp oil 2 (K2), obtained by monitoring the ion at 93 *m/z*. It is possible to see the peak of the internal standard (I.S.). Fatty acids (F.A.) from olive oil, used as the extraction solvent, eluted among the terpenes of interest. For peak identification, see Table 11.

Table 11. Retention time, molecular formula, and molecular weight of the compounds identified in hemp oils by GC-MS.

Peak Number	t_R (min)	Compound	Formula	MW
1	8.6	α-Pinene	$C_{10}H_{16}$	136.23
2	9.3	Camphene	$C_{10}H_{16}$	136.23
3	10.7	β-Pinene	$C_{10}H_{16}$	136.23
4	11.5	β-Myrcene	$C_{10}H_{16}$	136.23
5	12.2	α-Phellandrene	$C_{10}H_{16}$	136.23
6	12.4	Δ3-Carene	$C_{10}H_{16}$	136.23
7	12.5	Limonene	$C_{10}H_{16}$	136.23
8	17.0	α-Terpinolene	$C_{10}H_{16}$	136.23
9	17.9	Linalool	$C_{10}H_{18}O$	154.25
10	23.3	p-Cymen-8-ol	$C_{10}H_{14}O$	150.22
11	33.4	Ylangene	$C_{15}H_2$	204.35
12	34.9	β-Caryophyllene	$C_{15}H_{24}$	204.35
13	35.5	α-Bergamotene	$C_{15}H_{24}$	204.35
14	36.0	α-Humulene	$C_{15}H_{24}$	204.35
15	38.6	*trans*-Nerolidol	$C_{15}H_{26}O$	222.37
16	39.8	Caryophyllene oxid	$C_{15}H_{24}O$	220.35

The terpene content of sample K1 was found to be very low, having only α-pinene above the LOQ. On the other hand, K2 showed consistently higher terpene concentrations. In particular, α-pinene represented the most abundant compound among monoterpenes in K2 oil, with a concentration of 11.65 µg/mL, followed by limonene, β-myrcene, and linalool. As for the sesquiterpenes, β-caryophyllene was the most abundant compound, with a concentration of 26.61 µg/mL in K2 oil, followed by α-humulene and caryophyllene oxide.

Table 12. Quantification of terpenes in hemp oils using GC-MS. Data expressed as μg/mL ± SD.

Compound	Hemp Oil (K1)	Hemp Oil 2 (K2)
α-Pinene	3.48 ± 0.18	11.65 ± 0.98
β-Pinene	<LOQ	<LOQ
β-Myrcene	<LOQ	7.29 ± 0.19
Limonene	<LOQ	8.99 ± 0.46
Linalool	<LOD	5.29 ± 0.24
β-Caryophyllene	<LOQ	26.61 ± 3.00
α-Humulene	<LOQ	9.74 ± 1.08
trans-Nerolidol	<LOQ	4.15 ± 0.77
Caryophyllene oxide	<LOQ	8.25 ± 1.55

LOQ = limit of quantification. LOD = limit of detection.

Characterization of the Seizures Induced by 6-Hz Corneal Stimulations

Daily seizures were induced in mice by electroshocks delivered to both eyes for 5 consecutive sessions with intervals of 24 h. After the last session of 6-Hz corneal stimulation, seizures induced in K1- and K2-treated mice seemed to partially differ from those displayed by mice treated with a low dose of CBD (25 mg/kg) or olive oil (Figure 20A). This observation was also supported by considering the power spectral density referred to the first 10 s after the 6-Hz stimulation (Figure 20B). Moreover, the hypothesis was also in agreement with the power attenuation maps related to the electrocorticographic (ECoG) traces (Figure 20C).

Different Responses to Treatment

The repeated 6-Hz test induced progressive changes in the seizure severity, and percentage of mice developing generalized tonic–clonic convulsions, with and without loss of posture (Figure 21A–E). In comparison to the olive oil-treated group, the changes were not significantly different in CBD-, K1-, and K2-treated mice up to the fourth session of the 6-Hz test. After 5 sessions of 6-Hz corneal stimulation, 7 out of 8 mice treated with olive oil (87.5%) and 5 out of 8 mice treated with a low dose of CBD (62.5%) developed generalized tonic–clonic convulsions, with and without loss of posture. At variance, only 1 out of 8 mice treated with a low dose of K1 (12.5%) and 2 out of 8 mice treated with a low dose of K2 (25.0%) developed generalized tonic–clonic convulsions, with and without loss of posture. By comparing the percentage of olive oil-treated mice developing stage 4–5 seizures to those observed in the other groups, we found a significant difference in comparison to the K1- ($p = 0.010$; Fisher's exact test) and K2-treated groups ($p = 0.041$). Instead, no differences were reported between the mice treated with olive oil and CBD, when CBD (25 mg/kg/day orally) was administered at the same dose of K1 and K2.

Characterization of the Duration of Ictal ECoG Recordings and Time to Recover from Seizures

The duration of the ictal ECoG recordings and the time to recover from seizures were analyzed by two-way (treatment time) repeated measures analysis of variance (ANOVA). This analysis showed no statistically significant effect of both treatment ($F(3,112) = 0.832$, $p = 0.488$) and time for the duration of the ictal ECoG recordings ($F(4,112) = 0.723$, $p = 0.578$). Similarly, there was no statistically significant interaction between treatment and time for the duration of the ictal ECoG recordings in mice ($F(12,112) = 1.684$, $p = 0.080$), indicating that all treatments did not modify the duration of the ictal ECoG (Figure 22A). Furthermore, the analysis showed no statistically significant effect of treatment ($F(3,107) = 0.932$, $p = 0.438$), nor time, for the duration of recovery from seizures ($F(4,107) = 0.0877$, $p = 0.986$). However, there was a statistically significant interaction between treatment and time ($F(12,107) = 1.876$, $p = 0.045$). Precisely, time to recover from seizures was shorter in K1-treated mice compared to CBD and K2 ($p < 0.050$; Duncan's method), after the first session of 6-Hz corneal stimulation. K1 seemed to display a beneficial effect only in the first, compared to the third, session of stimulation ($p < 0.050$). At variance, K2 displayed beneficial effects on recovery only after 3 sessions of 6-Hz corneal stimulations ($p < 0.050$) (Figure 22).

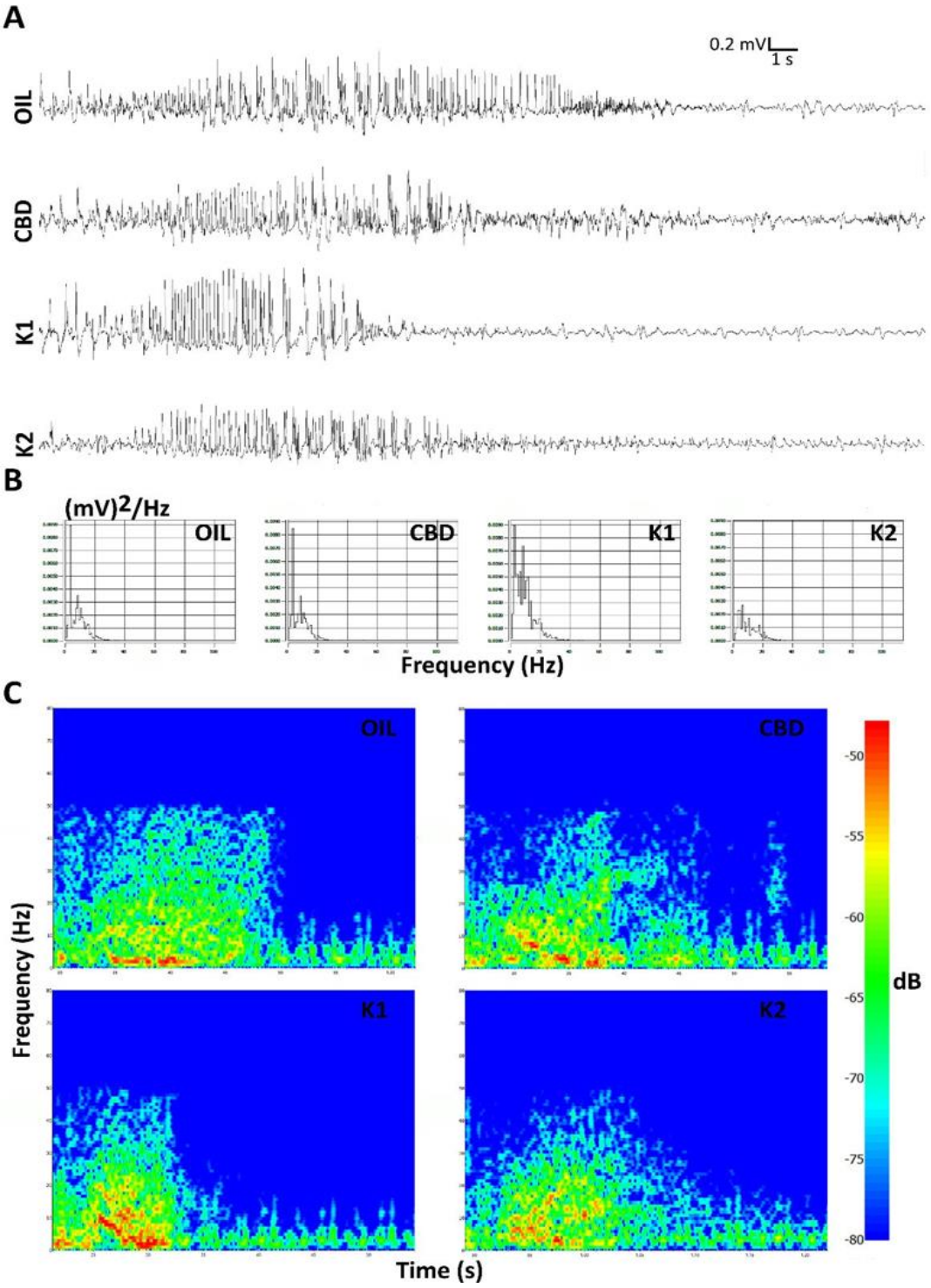

Figure 20. Seizures induced after the last session of 6-Hz corneal stimulation are illustrated. Seizures induced in K1- and K2-treated mice seemed to partially differ from those displayed by mice treated with either CBD alone or olive oil in the last session of the 6-Hz test. In (A), electrocorticographic (ECoG) traces are reported for all treatment groups. The power spectral density and power attenuation maps are represented, respectively, in (B, C). Scale: 0.2 mV/s. OIL, olive oil; CBD, cannabidiol; K1, hemp oil 1; K2, hemp oil 2.

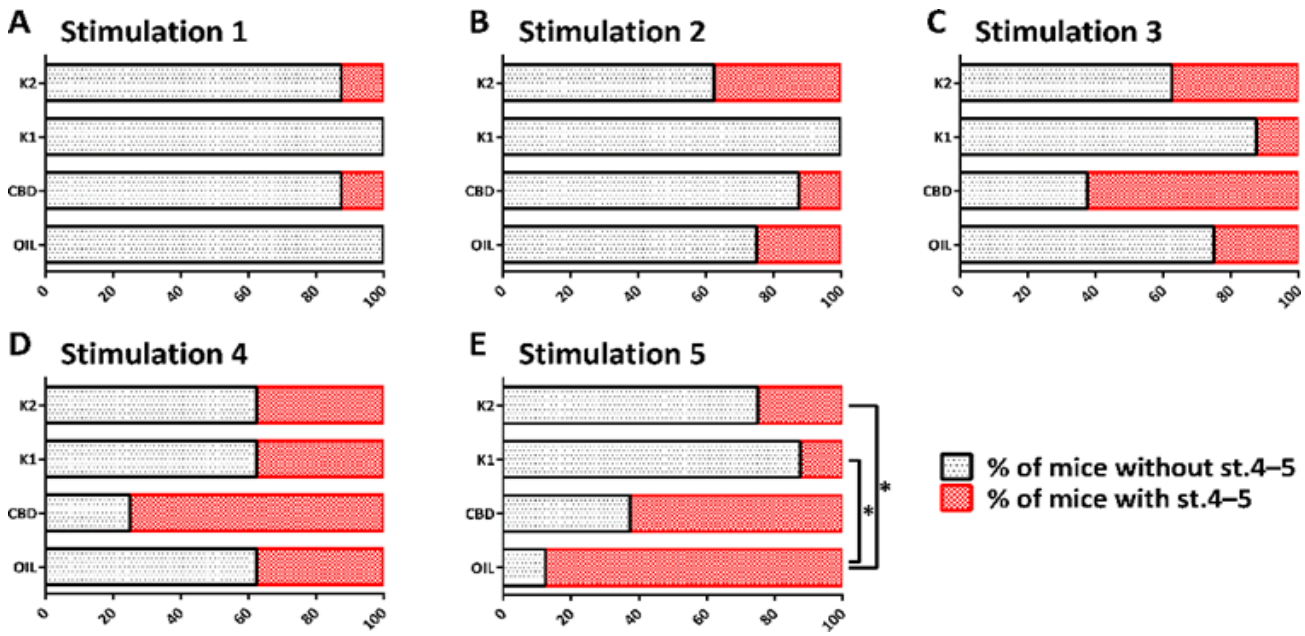

Figure 21. Percentage of mice developing (in red), or not developing (in black), generalized tonic– clonic convulsions, with (stage 5, st. 5) and without loss of posture (st. 4), after each session of 6-Hz corneal stimulation. The repeated 6-Hz test induced progressive changes in the seizure severity (A–E) and, at the end, the percentage of K1- and K2-treated mice developing st. 4–5 seizures was significantly reduced in comparison to olive oil-treated mice. * $p < 0.050$; Fisher's exact test. OIL, olive oil; CBD, cannabidiol; K1, hemp oil 1; K2, hemp oil 2.

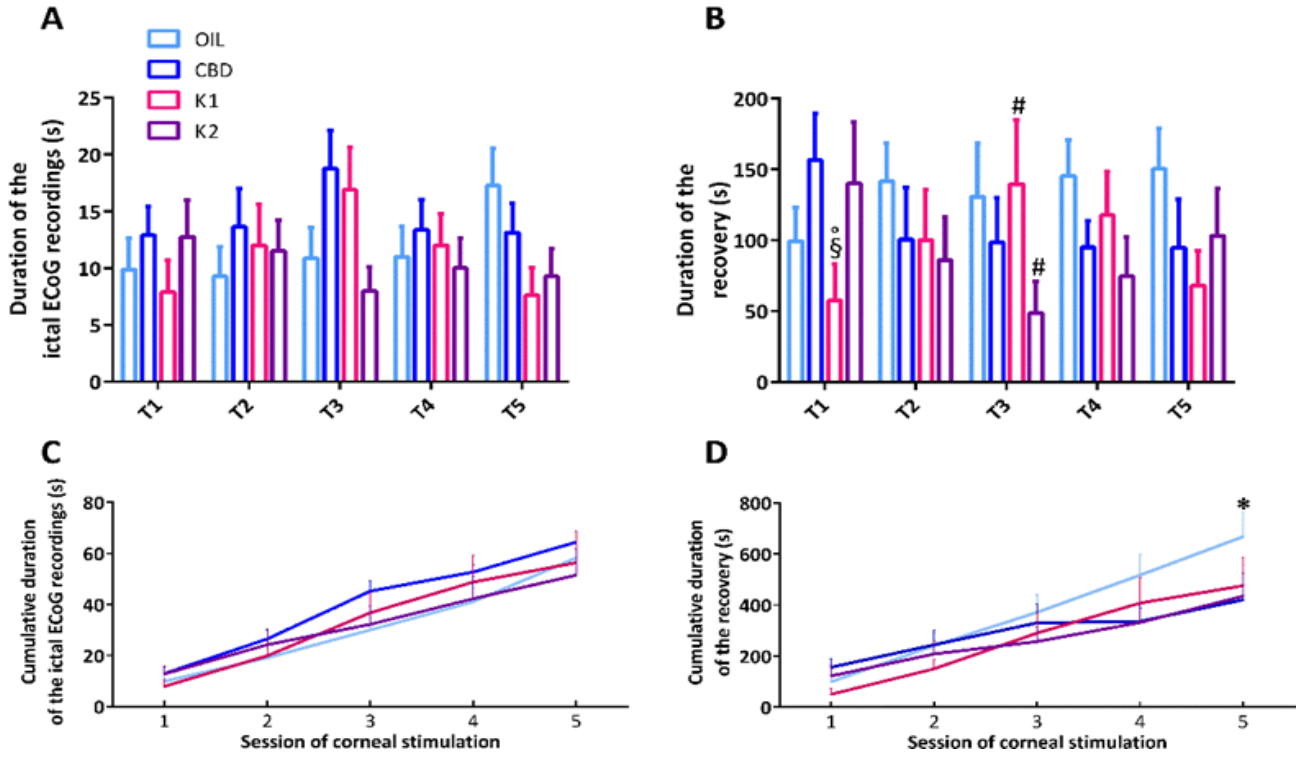

Figure 22. Duration of ictal electrocorticographic (ECoG) recordings, time to recover from seizures, and the cumulative effect of the treatments. All mice (n = 32) were treated with CBD (n = 8), K1 (n = 8), K2 (n = 8), or olive oil (n = 8). In (A), no treatment had beneficial effects on the total duration of seizures after the 6-Hz corneal stimulations. In (B), K1 and K2 had occasional beneficial effects on time to recover from seizures. In (C), no treatment had beneficial effects on the cumulative duration of the ictal ECoG recordings, but this cumulative duration significantly increased from the first to last session of stimulation in all groups. In (D), the cumulative duration of recovery significantly increased from the first to last session of stimulation in all groups, but only K2 had a significant cumulative effect on the time to recover from seizures. * $p < 0.050$ K2 vs. OIL; § $p < 0.050$ K1 vs. CBD; ◦ $p < 0.050$ K1 vs. K2; # $p < 0.050$ vs. session 1; Duncan's method. T1–T5, from the first to the fifth session of corneal stimulation; OIL, olive oil; CBD, cannabidiol; K1, hemp oil 1; K2, hemp oil 2.

To evaluate the overall duration of seizures induced by the various sessions of 6-Hz corneal stimulation, we also analyzed the cumulative duration of ictal ECoG recordings and recovery by summing up the respective time intervals. A two-way repeated measures ANOVA showed no statistically significant effect of treatment ($F(3,110) = 0.515$, $p = 0.676$), nor an interaction between treatment and time ($F(12,110) = 1.069$, $p = 0.393$). As expected, a significant effect of time ($F(4,110) = 122.213$, $p < 0.001$) was observed for the cumulative duration of the ictal ECoG recordings (Figure 22C). Then, the analysis showed no statistically significant effect of treatment ($F(3,108) = 0.738$, $p = 0.538$) and a predictable significant effect of time for the cumulative duration of recovery ($F(4,108) = 84.926$, $p < 0.001$). Interestingly, there was a statistically significant interaction between treatment and time for the cumulative duration of recovery ($F(12,108) = 2.737$, $p = 0.003$), which was reduced by K2, compared to olive oil ($p < 0.050$), after the last 6-Hz test (Figure 22D).

Analysis of the Power Band Spectrum

The power band spectrum, expressed in percentage, was analyzed by a two-way ANOVA. In particular, the percentage of delta power showed a significant effect of treatment ($F(3,108) = 8.971$, $p < 0.001$), but no significant effect of time for delta power ($F(4,108) = 0.748$, $p = 0.562$). There were no statistically significant interactions between treatment and time for delta power in mice ($F(12,108) = 0.630$, $p = 0.812$). Delta power was not statistically different between groups until the third session of 6-Hz corneal stimulation, but it was significantly decreased in all groups in comparison to olive oil-treated mice in the fourth session of 6-Hz corneal stimulation ($p < 0.050$; Duncan's method). In the last 6-Hz test, delta power remained significantly lower only in K2-treated mice in comparison to oil- and CBD-treated mice (Figure 23A).

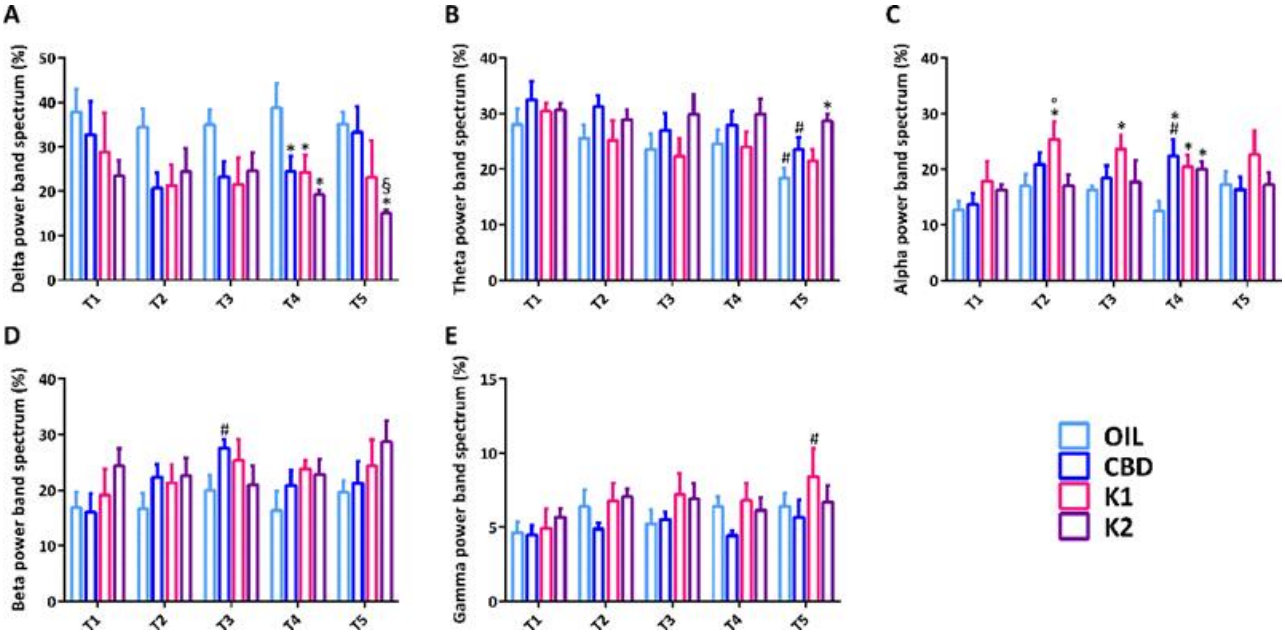

Figure 23. Power band spectrum analysis after each session of the 6-Hz test. All mice (n = 32) were treated with CBD (n = 8), K1 (n = 8), K2 (n = 8), or olive oil (n = 8). A relative indication of distribution of power over the frequency, expressed in percentage, was determined in seizures induced by the 6-Hz corneal stimulation (A–E). * $p < 0.050$ vs. OIL; § $p < 0.050$ vs. CBD; ◦ $p < 0.050$ vs. K2; # $p < 0.050$ vs. first session of the same group, with the same treatment; Duncan's method. T1–T5, from the first to the fifth session of corneal stimulation; OIL, olive oil; CBD, cannabidiol; K1, hemp oil 1; K2, hemp oil 2.

The percentage of theta power showed a significant effect of treatment ($F(3,109) = 5.381$, $p = 0.002$) and time for theta power ($F(4,109) = 3.996$, $p = 0.005$). However, there was no statistically significant interaction between treatment and time ($F(12,109) = 0.410$, $p = 0.957$). In comparison to the first 6-Hz test, the percentage of theta power was significantly reduced in olive oil- and CBD-treated mice in the last session of 6-Hz stimulation ($p < 0.050$). At variance, theta power was significantly higher in the K2-treated mice in comparison to the oil-treated mice, in the same session of 6-Hz stimulation ($p < 0.050$) (Figure 23B).

Similarly, the percentage of alpha power showed a significant effect of treatment ($F(3,109) = 6.781$, $p < 0.001$) and time ($F(4,109) = 2.473$, $p = 0.049$). There was no significant interaction between treatment and time ($F(12,109) = 0.934$, $p = 0.561$). In comparison to the olive oil-treated mice, alpha power was significantly higher in the K1-treated mice from the second to the fourth sessions of 6-Hz stimulation ($p < 0.050$). In the second 6-Hz test, alpha power was also markedly higher in the K1-treated mice in comparison to the K2-treated mice ($p < 0.050$). Alpha power was also significantly increased in CBD- and K2-treated groups, in comparison to the olive oil-treated group, in the fourth session of 6-Hz corneal stimulation ($p < 0.050$) (Figure 23C).

The percentage of beta power showed a significant effect of treatment ($F(3,108) = 3.511$, $p = 0.018$) but not time ($F(4,108) = 1.388$, $p = 0.243$). Moreover, there was no significant interaction between treatment and time for beta power ($F(12,108) = 0.663$, $p = 0.783$). In comparison to the first 6-Hz test, beta power was significantly higher in the CBD-treated mice in the third session of 6-Hz corneal stimulation ($p < 0.050$) (Figure 23D).
Furthermore, the percentage of gamma power showed a significant effect of treatment ($F(3,107) = 3.939$, $p = 0.010$) but not time ($F(4,107) = 2.137$, $p = 0.081$). Also, there was no significant interaction between treatment and time for gamma power ($F(12,107) = 0.368$, $p = 0.972$). In comparison to the first 6-Hz test, gamma power was significantly higher in the K1-treated mice in the last session of 6-Hz corneal stimulation ($p < 0.050$) (Figure 23E).

FosB/ΔFosB Immunoreactivity

We evaluated the effects of repeated exposure to 6-Hz corneal stimulation (44 mA) on FosB/ΔFosB immunoreactivity in the stratum pyramidalis of the cornu ammonis 1 (CA1) region (Figure 24A), and in the subiculum (Figure 24B). FosB/ΔFosB levels were barely detectable in all sampled regions of unstimulated control mice. In comparison to the non-stimulated control group, neuronal expression was increased in all groups. These changes were significant in the Sub ($p < 0.050$; Duncan's method), but not in CA1. Interestingly, a prominent increase in FosB/ΔFosB levels was observed in the Sub of oil-, CBD-, and K1-treated mice in comparison to the non-stimulated control group. On the other hand, treatment with K2 prevented a significant increase in FosB/ΔFosB levels in the Sub.
We tested the anti-seizure activity of two different hemp oils, which were compared to CBD at a concentration similar to that found in the extracts. The results of our investigation suggest that both extracts exerted an anti-seizure activity, and they were more efficient than CBD when tested as the only administered anticonvulsant. Additionally, the extract enriched with terpenes was advantageous in affording a faster recovery from seizures. This effect was observed in the presence of preserved theta activity in the ECoG and restrained FosB/ΔFosB expression in the subiculum. These findings confirm the validity of hemp oils to address seizures, and further suggest a CBD-independent role of terpenes as an ASM [832–834].
As for the influence of the chemical composition of hemp oils on bioactivity data, the difference in the content of volatile compounds between hemp oils K1 and K2 could be responsible for the results obtained in the 6-Hz seizure test, there being neutral cannabinoids present in a very similar amount in both the samples. In particular, the quantitatively higher content of terpenes in K2 may act in a synergistic way with cannabinoids to enhance the anticonvulsant effect of K2 compared with K1 and with pure CBD. A similar entourage effect between cannabinoids and terpenes was previously described for mood disorder, as well as for pain [819,820]. Furthermore, some representative terpenes of K2, such as β-caryophyllene (a CB_2 receptors agonist), β-myrcene and limonene have displayed anticonvulsant activity against seizures induced by pentylenetetrazol [835,836]. Moreover, linalool has been able to act as an anticonvulsant in quinolinic acid-induced seizures *in vivo* [837] and to alleviate maximal electroshock-induced seizures in mice [838].

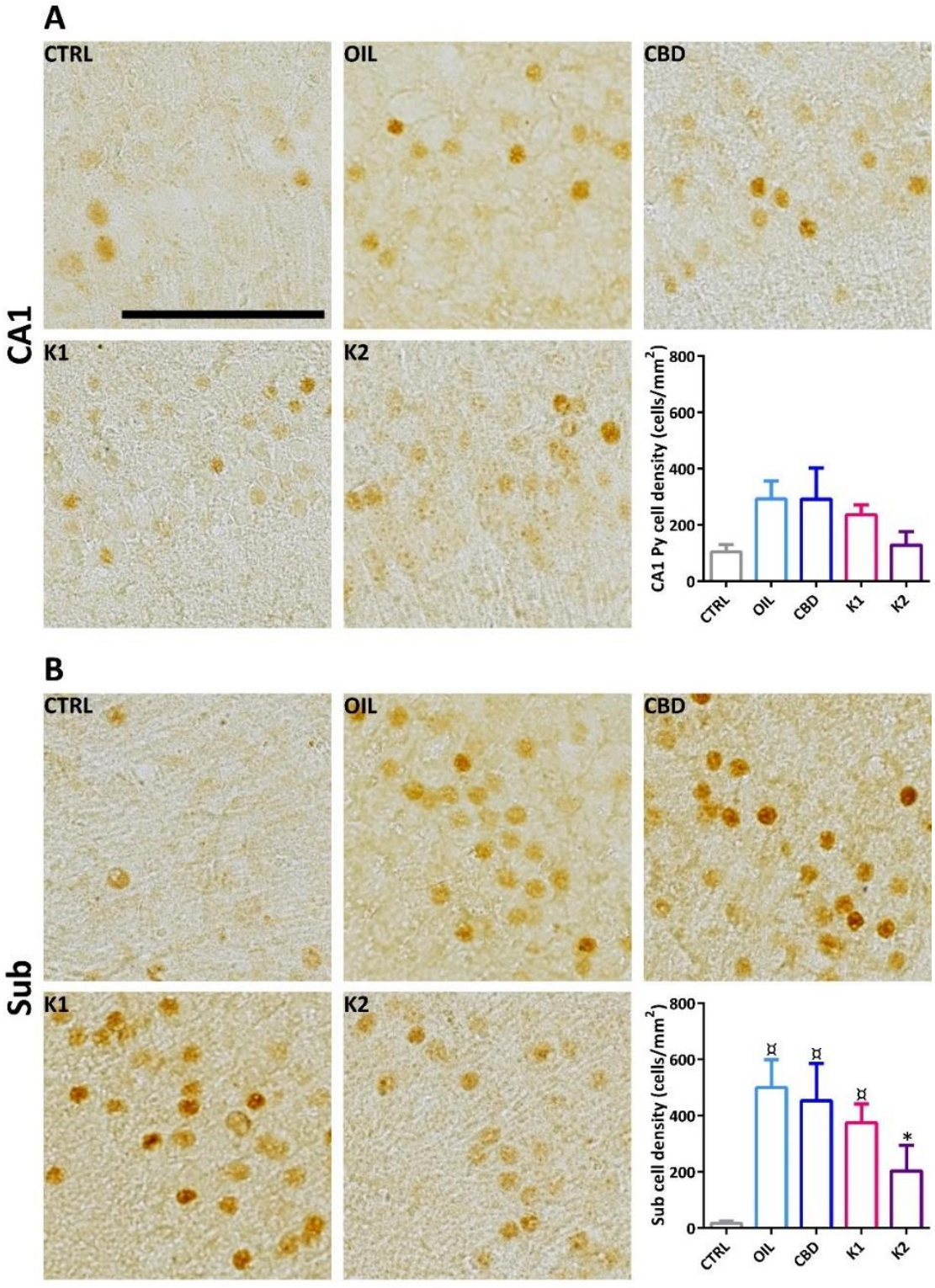

Figure 24. FosB/ΔFosB immunoreactivity in the hippocampal CA1 region and in the subiculum (Sub) of mice treated with CBD (n = 6), K1 (n = 6), K2 (n = 6), or olive oil (n = 6), and exposed to different sessions of 6-Hz corneal stimulation. FosB/ΔFosB immunoreactivity was also illustrated in a representative unstimulated control (Ctrl, n = 6). Particularly, FosB/ΔFosB immunoreactivity in the CA1 was reported

in (A), whereas that of the Sub, in (B). $p < 0.050$ vs. CTRL; * $p < 0.050$ vs. OIL; Duncan's method. Scale: 0.1 mm. CTRL, control; OIL, olive oil; CBD, cannabidiol; K1, hemp oil 1; K2, hemp oil 2.

Discussion

Although both K1 and K2 reduced the occurrence of convulsive seizures, K2 appeared to promote a prompt recovery from seizures, therefore suggesting a possible advantage. Interestingly, this effect was observed in the presence of better-preserved theta activity in the ECoG, which was kept at the same level in all sessions of 6-Hz corneal stimulation, whereas it declined in the other treatment groups. We already observed changes in the power of the theta band in other experiments, which suggested a role for this frequency in seizure characteristics [177,812]. Specifically, during *status epilepticus* evoked by kainic acid in rats, a drop in theta power preceded the appearance of generalized tonic–clonic seizures, the most severe epileptic activity observed in this model [812]. However, in the present experiment, theta power seemed to be more related to the faster recovery from seizures observed in the K2-treated group, in which the involvement of reduced activation of subicular neurons could also be hypothesized. The subiculum is a region of the hippocampal formation able to promote seizure activity, and it is also involved in refractoriness to some ASMs [839–841]. Thus, the observed effect of K2 on the subiculum in the 6-Hz model of corneal stimulation, which was suggested as a model of resistance to ASMs, encourages further studies to assess the possible efficacy of this hemp oil in addressing difficult to treat seizures.

Other effects of treatments on the power spectrum of the ECoG were also observed. Most of them were apparently not related to the anticonvulsant effects of the treatment, with the exception of delta power. In addition, this spectrum band was kept at the same level in the K2 group until the last session of 6-Hz corneal stimulation. In a previous experiment, we reported that the power of the delta band in postictal depression was significantly related to the remarkable increase in seizure occurrence found in epileptic rats after the withdrawal of levetiracetam, previously administered for a week as an ASM [177]. For this reason, the changes in the power band observed in the other treatment groups could have contributed to the seizure characteristics found in the present experiment.

It is important to highlight that the chronic administration of CBD alone might markedly induce anticonvulsant and antiepileptogenic effects [842]. However, the crucial role of the volatile component of K2 in the overall activity of the extract, together with the presence of CBD, could be supported by the fact that CBD alone, at a low dose, seemed not to be effective [794]. Indeed, CBD injected intraperitoneally at a dose of 25 mg/kg did not significantly modify the seizure thresholds in the maximal electroshock seizure threshold test and the 6-Hz-induced seizure test in mice. At variance, when injected at higher doses of 50 and 100 mg/kg, it was significantly powerful [794]. Thus, our approach, using a dose below the threshold of CBD, definitely unmasked the anticonvulsant properties of the other compounds present in hemp oils.

Materials and Methods

Chemical and Solvents

Stock methanolic solutions (1.0 mg/mL) of cannabidiol (CBD), cannabigerol (CBG) and cannabinol (CBN), as well as stock acetonitrile solutions (1.0 mg/mL) of cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA), were purchased from Cerilliant Corporation (Round Rock, TX, USA).

Stock 2-propanol solutions (1.0 mg/mL) of α-pinene, β-pinene, β-myrcene, limonene, linalool, β-caryophyllene, α-humulene, caryophyllene oxide and trans-nerolidol were obtained from Agilent Technologies (Santa Clara, CA, USA).

Acetonitrile (ACN), formic acid (HCOOH), *n*-hexane, methanol (MeOH), 2-propanol and methyl-octanoate were purchased from Sigma Aldrich s.r.l. (Milan, Italy). Water (H_2O) was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

Extra virgin olive oil was of European Pharmacopoeia grade. CBD powder (> 99%) and hemp female inflorescences (Kompolti variety), listed within the EU database of admitted agricultural species, were purchased from Materia Medica Processing (Bolzano, Italy).

Animals

All experiments were performed according to the Italian Act (DM 26/2014) and the European Directive 2010/63/EU. Thirty-eight male CD-1 mice (Charles River, Calco, Italy), with initial weights of 15–20 g, were housed in a specific pathogen-free facility with a controlled environment and ad libitum access to water and food. All efforts were made to refine procedures and protect the animals' welfare.

Preparation of the Olive Oil Extracts

Hemp inflorescences were crushed before the decarboxylation step and extraction with olive oil. Decarboxylation allows for more standardized extracts, avoiding the spontaneous conversion of cannabinoic acids into their neutral counterparts during storage of the extract [821].

All hemp oils were prepared using a drug–solvent ratio of 1:10 (w/v), as previously described [821]. To prepare hemp oil 1 (K1), not preserving volatile components, 5 g of dried plant material were decarboxylated in a beaker, placed into an oven at 110 °C for 15 min, and then at 120 °C for 60 min. The plant material was extracted with 50 mL of olive oil with a turbo-emulsifier for 10 min, keeping the temperature below 40 °C. This operation was repeated three times. The third time, an additional 4 min of sonication were applied. The combined oil extracts were finally filtered to remove the solid residue.

To prepare hemp oil 2 (K2), 5 g of dried plant material were decarboxylated in an oven at 115 °C for 1.30 h in a sealed flask, in order to retain the volatile compounds inside. The plant material was then extracted with 50 mL of olive oil directly in the flask, following the same extraction procedure described for K1.

CBD oil was prepared by dissolving a weighted amount of pure compound (225 mg) in 50 mL of olive oil. Hemp oils were diluted to 1:50 and 1:5 with 2-propanol prior to the injection into the HPLC and GC systems, respectively.

UHPLC-HRMS Analysis

Qualitative analysis of hemp oil extracts was performed by means of UHPLC-HRMS. The analyses were performed on a Thermo Scientific (Waltham, MA, USA) UHPLC Ultimate 3000, equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment, and a Q-Exactive Orbitrap mass spectrometer, with a heated electro-spray ionization (HESI) source. An Ascentis Express C_{18} column (150 mm x 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used.

With regard to olive oil hemp extracts, a ternary A/B/C multistep gradient (solvent A: 0.1% HCOOH in H_2O, solvent B: 0.1% HCOOH in ACN, and solvent C: MeOH) was used. Solvent C was kept constant at 5% throughout the run. On the basis of previous work [829], the multistep gradient program was established as follows: 0–2 min from 50 to 67% B, which was kept for 4 min; 6–10 min from 67 to 90% B, which was kept for 4 min; and 14–15 min from 90 to 50% B, which was kept for 5 min, for re-equilibration of the system prior to the next injection. A flow rate of 0.3 mL/min was used. The column temperature was 30 °C. The injection volume was 1.0 µL.

MS acquisition was carried out with a heated electro-spray ionization (ESI) source operated both in the positive and negative ion modes. As for the MS detector, the source parameters were set as follows: sheath gas (N_2) 40, auxiliary gas (N_2) 30, auxiliary gas temperature 290 °C, and electrospray voltage 3.5 kV (+) and 3.2 kV (-). The analyses were acquired in the full mass data-dependent (FM-dd-MS2) mode at a resolving power of 35.000 full width at half maximum (FWHM). The other mass analyzer parameters were set as follows: scan range *m/z* 150–2000, automatic grain control (AGC) target 1 x 10^6 ions in the Orbitrap analyzer, ion injection time 100 ms, and isolation window for the filtration of the precursor ions *m/z* 1.0. The fragmentation of precursors ions was performed at 20, 30, and 50 as normalized collision energies (NCE).

HPLC-UV Analysis

HPLC-UV analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II system, consisting of a quaternary pump, a manual injector, and a UV variable wavelength detector. Chromatograms were recorded by using an Agilent OpenLab ChemStation (Rev. C.01.10). An Ascentis Express C_{18} column (150 mm x 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used, with a mobile phase composed of 0.1% HCOOH in both (A) H_2O and (B) ACN. The gradient elution was as follows: 0–13 min 60% B, 13–17 min from 60 to 80% B, 17–22 min from 80 to 90% B, which was kept for 8 min. The post-running time was 15 min. The flow rate was 0.4 mL/min. The sample injection volume was 3 µL. Chromatograms were acquired at 210 nm (for decarboxylated cannabinoids) and at 220 nm (for cannabinoic acids). Calibration curves for target compounds were constructed at five

calibration levels in the range of 2.5–100 µg/mL, by plotting the peak areas of the analytes vs. their concentration. Δ^9-THC and Δ^9-THCA were quantified on the basis of the calibration curves built for CBD and CBDA. Two injections were performed for each sample. The value corresponding to the limit of quantification (LOQ) was 2.5 µg/mL for each cannabinoid, while the limit of detection (LOD) was 0.8 µg/mL.

GC-MS Analysis

GC-MS analysis was carried out on a 7890B gas chromatograph coupled with a 5977B GC/MSD mass spectrometer (Agilent Technologies, Germany). Compounds were separated on an Agilent Technologies HP-5 cross-linked poly-5% diphenyl-95% dimethyl polysiloxane (30 m x 0.32 mm i.d., 0.25 µm film thickness) capillary column. The column temperature was initially set at 45 °C, then increased at a rate of 2 °C/min up to 100 °C, then raised to 250 °C at a rate of 5 °C/min, and again raised up to 280 °C at a rate of 11 °C/min, and finally held for 15 min. The injection volume was 1.0 µL, with a split ratio of 1:15. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injector, transfer line and ion-source temperature were 250, 280 and 230 °C, respectively. Mass spectra were acquired with an electronic ionization (EI) source at 70 eV, operating in the full-scan acquisition mode in the range of 40–400 *m/z*. MS spectra of eluting peaks were compared with those reported in the NIST mass spectral database (version 1.4)

The identification of terpenes was performed by the comparison of the experimental linear retention index (LRI) values obtained for the chromatographic peaks with those reported in the literature for the same plant material [826]. In addition, a mass spectra database search for the compounds present in the chromatogram, acquired in the full-scan mode, was performed.

For quantitative analysis, the selected ion monitoring (SIM) mode was applied to monitor the most intense ion at 93 *m/z* for all the terpenes of interest, and the ion at 74 *m/z* for methyl-octanoate used as the I.S. Calibration curves for target compounds were constructed at five concentration levels in the range of 1–50 µg/mL, by plotting the peak areas of the analytes vs. their concentration. The concentration of the I.S. was 25 µg/mL. Two injections were performed for each level. The concentration value corresponding to the LOQ was 1.0 µg/mL for limonene, linalool, β-caryophyllene, trans-nerolidol and caryophyllene oxide, and 0.5 µg/mL for α-pinene, β-pinene, β-myrcene and α-humulene. The concentration value corresponding to the LOD was 0.3 µg/mL for limonene, linalool, β-caryophyllene, trans-nerolidol and caryophyllene oxide, and 0.2 µg/mL for α-pinene, β-pinene, β-myrcene and α-humulene.

Experimental Design

Thirty-eight mice were implanted with electrodes and randomly divided into 5 groups: (i) 6 control mice were neither treated nor stimulated, (ii) 8 mice received olive oil alone, (iii) 8 mice received CBD (25 mg/kg/day orally) dissolved in olive oil, (iv) 8 mice received K1 (25 mg/kg/day orally) dissolved in olive oil, and (v) 8 mice received K2 (25 mg/kg/day orally) dissolved in olive

oil. Except for the control group, the remaining mice were stimulated once and allowed to recover for 24 h before being stimulated for up to 5 sessions.
All 6-Hz stimulated mice (n = 32) were considered for behavior and video-ECoG recordings. At variance, a total of 6 animals per group (n = 30) were considered for immunohistochemical staining. In this case, the control mice were used to determine basal levels of the investigated markers, because they were handled and exposed to the same procedure as the others.

Electrodes Implantation and v-ECoG Recordings

Before electrode implantation, anesthesia was induced with volatile isoflurane (4% induction and 1–2% maintenance), and deep anesthesia was assessed by deep breathing and loss of tail and eye reflexes. Accordingly, the skin was shaved, disinfected with povidone-iodine 10% (Betadine® skin solution; Meda Pharma, Milano, Italy), cut, and opened to expose the skull.
Guiding holes were drilled and epidural electrodes (stainless steel Ø D 1 mm; PlasticsOne, Roanoke, VA, USA) were implanted in the frontal (bregma 0 mm, 3 mm lateral from midline) and occipital cortices (-bregma 3.5 mm, 3 mm lateral from midline) of both hemispheres. One electrode was implanted below lambda on the midline in all mice and used as a reference.
All electrodes were connected through steel wire to terminal gold pins (Bilaney Consultant GmbH, Düsseldorf, Germany) inserted in a plastic pedestal (PlasticsOne) cemented on the heads. After surgery, gel containing 2.5 g lidocaine chloride, 0.5 g neomycin sulfate, and 0.025 g fluocinolone acetonide (Neuflan® gel; Molteni Farmaceutici, Scandicci, FI, Italy) was applied to minimize acute pain and risk of infection. All mice were monitored until complete recovery from anesthesia and housed in single cages without grids or environmental enrichments to reduce the risk of headset loss.
The ECoG was recorded during all sections of corneal stimulation via cable connection between the headset and preamplifiers. Electrical activity was digitally filtered (0.3 Hz high-pass, 500 Hz low-pass), acquired at 1 kHz per channel, and stored on a personal computer after the mathematical subtraction of traces of recording electrodes from traces of reference electrodes, using a PowerLab8/30 amplifier connected to 4 BioAmp preamplifiers (AD Instruments; Dunedin, Otago, New Zealand). Videos were digitally captured through a camera connected to the computer and synchronized to the ECoG traces through a LabChart 8 PRO internal trigger.

Corneal Stimulation Protocol

Corneal stimulation was performed once a day for 5 days and was preceded by the application of a topical eye anesthetic (0.4% oxybuprocaine hydrochloride eye drops, Novesin, Novartis, Switzerland) 1–2 min before. The injection of CBD, K1, K2, or olive oil was performed by oral gavage 1 h before each session of corneal stimulation.
Particularly, the stimulation (fixed current intensity of 44 mA, pulse width of 0.2 ms, duration of 3 s, frequency of 6-Hz) was delivered via corneal electrodes connected to a stimulator (ECT Unit 5780; Ugo Basile, Comerio, Italy).

Behavioral and ECoG Analysis

Seizures were defined as ECoG segments with a minimum duration of 5 s, continuous synchronous high-frequency activity, and an amplitude of at least twice the previous baseline. Notably, ECoG traces were digitally filtered offline (band-pass: high 50 Hz, low 1 Hz) and manually analyzed, using LabChart 8 PRO software (AD Instruments), by expert raters. The LabChart 8 PRO software was also used to observe power attenuation during the seizures induced by the last session of 6-Hz corneal stimulation. Particularly, the power attenuation gave a spectrum with a wide range of powers and was expressed in logarithmic unit decibels (dB), relative to the reference power (attenuation = 10 log (power/reference power)).

Seizures were further analyzed using EDFbrowser (1st order Butterworth high-pass filter: 1 Hz; powerline interference removal: 50 Hz) [843] to understand whether significant changes in the cortical power band spectrum occurred during the induced seizures [177,812]. The power spectral density of the corresponding signal was shown as $(\mu V)^2$/FFT-resolution. Moreover, a relative indication of the distribution of power over the frequency regions, ranging from 1 to 100 Hz, expressed in percentage (%) and recorded from the frontal electrodes, was determined. Especially, power band spectrum analysis was expressed in percentage, and included delta (δ, 0–4 Hz), theta (θ, 4–8 Hz), alpha (α, 8–12 Hz), beta (β, 12–24 Hz) and gamma (γ, 24–100 Hz) frequencies in 10-s epochs on a continuous ECoG.

Seizure severity was ranked according to the following scores: (i) stunned posture and eye blinking; (ii) head nodding, Straub tail and chewing; (iii) unilateral or alternating forelimb clonus; (iv) generalized tonic–clonic convulsions without loss of posture and rearing; (v) generalized tonic–clonic convulsions with loss of posture. Seizure severity and duration were first recorded through direct observation, and then reanalyzed on video recordings by an investigator unaware of the stimulation session. The time to recover from seizures was calculated from the end of the ECoG seizure to the reappearance of normal exploratory behavior.

Immunohistochemistry

One hour after the last 6-Hz test, mice were deeply anesthetized with isoflurane for about 10 s and perfused transcardially with phosphate buffered saline (PBS, pH 7.4), followed by Zamboni's fixative (pH 6.9). Brains were removed and postfixed at 4 °C in the same fixative for 24 h [255,570]. Tissues were transferred to 15% and 30% sucrose solutions, and then horizontal sections (50 µm thickness) were cut using a freezing-sliding microtome (Leica SM2000 R; Leica, Nussloch, Germany).

Sections were washed three times in PBS, treated with 3% H_2O_2 in PBS (10 min), and blocked for 1 h in PBS containing 5% normal goat serum and 0.1% Triton X-100. The sections were subsequently incubated overnight at 4 °C with rabbit polyclonal anti-FosB/ΔFosB (H-75, sc-7203, Santa Cruz Biotechnology, CA, USA; 1:250). The next day, the sections were incubated for 1 h with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:200). After washing, the brain sections were incubated for 1 h with the avidin-biotin-peroxidase complex (Elite ABC Kit; Vector Laboratories). The immunostaining was

developed in 0.05% 3,3-diaminobenzidine tetrahydrochloride for 5 min (Sigma-Aldrich, Milan, Italy) by adding 0.03% H_2O_2.

Image Analysis

Immunostained sections from -8.04 mm to -5.04 mm Bregma level were evaluated with a Nikon Eclipse CiL (Nikon Instruments) at 20, and for each area of interest (cornu ammonis 1 stratum pyramidalis—CA1 Py; Subiculum—Sub) images were digitally captured by a Nikon DS-Fi3 digital camera. The cellular density (n/mm^2) of FosB/ΔFosB immunoreactive cells was quantified and analyzed using the ImageJ and NIS-Elements software. Particularly, FosB/ΔFosB-positive cells were counted by using ImageJ, whereas the region of interest (ROI) was manually traced in all analyzed sections by using NIS- Elements software.

Statistics

Fisher's exact test was used to compare the number of animals experiencing convulsive seizures, with and without loss of posture, in the various treatment groups. The duration of the ictal ECoG recordings, and the time needed to recover from seizures, were analyzed by two-way (treatment time) repeated measures ANOVA, followed by the Duncan's method for post-hoc comparisons. Furthermore, the power band spectrum, expressed in percentage, was analyzed by two-way ANOVA, followed by Duncan's method. Then, FosB/ΔFosB positivity values were compared using one-way ANOVA, followed by Duncan's method. Statistical analyses were carried out using Sigmaplot 11 (Systat Software, San Jose, CA, USA). Data were represented as means, with standard error of the mean (SEM).

Conclusions

The present work was aimed at the preparation and chemical characterization of hemp oils, and at the evaluation of their in vivo antiepileptic effects in the 6-Hz corneal stimulation model. Olive oil formulations of medical *C. sativa*, currently used in patients, and oral administration, represent an easy route for animal testing as well.

The extraction procedure used in this work allowed us to obtain standardized hemp oils, as regards their cannabinoid content. The novelty of the present study is represented by the complete qualitative and quantitative characterization of terpenes in the extracts, with particular interest in the K2 hemp oil, which was prepared to preserve volatile compounds. The results obtained on the 6-Hz corneal stimulation model suggested a role of terpenic components in enhancing the antiepileptic activity of cannabinoids in K2 oil, compared to K1 and pure CBD, even if other minor compounds in the extracts can contribute to the overall activity.

Future work should be focused on the assessment of the bioactivity of K2 oil, as well as its main compounds, such as CBD and β-caryophyllene, on additional in vivo models of drug-resistant seizures, including Dravet and Lennox–Gastaut syndromes, although the high number of potentially active compounds in hemp extracts could complicate further studies on the

mechanism of their anti-seizure activity. The possible involvement of CB_2 receptors in the observed anti-seizure effect should also be evaluated[8].

[8] **Author Contributions**: Conceptualization, F.P. and G.B.; methodology, A.-M.C. and V.B.; software, L.S. and L.A.; validation, F.P. and G.B.; formal analysis, A.-M.C., L.S., L.A. and V.B.; investigation, L.S. and L.A.; resources, F.P. and G.B.; data curation, A.-M.C., L.S., L.A. and V.B.; writing—original draft preparation, A.-M.C., L.S. and L.A.; writing—review and editing, F.P. and G.B.; visualization, A.-M.C. and V.B.; supervision, F.P. and G.B.; project administration, F.P. and G.B.; funding acquisition, F.P. and G.B. All authors have read and agreed to the published version of the manuscript.

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Chapter 5

Summary, conclusion, and future perspectives

Summary

Epilepsy is a chronic neurological disorder displaying a world prevalence between 0.5% - 1.0% [16,17]. This disorder is placed fourth in importance among other neurological disorders, following migraine, stroke, and dementia. Among other forms of epilepsy, TLE is the most common type in adult patients and is associated with brain injuries especially in the hippocampus, cognitive impairment and pharmacoresistance [42]. Just about 65% of patients, who are treated with ASMs, experience seizure freedom, the rest remains caught in the consequences and impact of the disease [52]. Additionally, ASMs might diminish the seizures, in patients who are sensitive to them, but do not resolve the underlying mechanism of the disease and cause important side effects [844].

In this respect, the ultimate goal of pharmacological research is in developing a more potent, effective and safer medication. Therefore, researchers are going back to using compounds found in nature as a possible cure for epilepsy. In particular, in this thesis two different doses of CBD were used in the chronic KA model, which mimics the effects of TLE in patients with regard to SRSs occurrence and pharmacoresistance. Furthermore, this work focused on investigating derivatives found in the *Cannabis sativa* L. plant in models of seizure and epilepsy. Cannabis extracts enriched with terpenes were used in a modified repeated 6-Hz corneal stimulation test, which has been developed to screen anti-seizure compounds. The original protocol was changed to enable the evaluation of potential changes in seizure phenotype and the responsiveness to anti-seizure treatments in relation to an increased seizure exposure.

The introduction provides a general overview of epilepsy itself, including the classification and types of seizures and epilepsy, as well as discusses different hypotheses of the mechanisms of drug-refractoriness. Most importantly, the challenge of developing new ASMs and possibly new potent compounds such as CBD-rich extracts and single cannabis compounds and their interaction with PPARγ are shown in the first part of the thesis.

In the third and fourth chapters, the following questions are addressed: 1. Are the anti-seizure effects of CBD in a model of TLE mediated by PPARγ? 2. Are there changes in the expression of PPARγ specific to certain hippocampal regions in epileptic rats? 3. Can CBD-rich Cannabis extracts be considered as a potential medication in pharmacoresistant epilepsy? How important are terpenes in treating seizures and epilepsy? 4. Are there suitable markers for the identification of neurological changes after the repeated 6-Hz corneal stimulation and of the aggravation of seizures?

In the fifth and last chapters, using the findings of chapter 3 and 4, an overall conclusion of these questions is drawn, and future perspectives proposed.

Are the anti-seizure effects of CBD in a model of TLE mediated by PPARγ?

CBD is a well-described cannabinoid exhibiting curative effects in systemic and neurological disorders, evaluated in animal models, and more importantly, in clinical studies [806,845,846]. Especially in epilepsy, CBD is marketed as an add-on therapy to other ASMs, demonstrating promising anticonvulsant activity and has already been administered successfully in patients suffering from drug-resistant epilepsy [402,538,813].

In our chronic model of TLE by injecting KA, CBD induced a significant decrease in the total duration of all SRSs compared to the pre-treatment period. In the same period, a significant difference was also seen between CBD-treated rats and the control group treated with MCT oil. In detail, our investigation demonstrated that CBD at 120 mg/kg successfully eliminated seizure in 50% of the rats and diminished total seizure duration and occurrence. Interestingly, these changes were not significantly different when non-convulsive and convulsive stages SRSs were considered separately. Immunohistochemical staining revealed an increased PPARγ level in hippocampal and extrahippocampal regions after the repeated administration of CBD at a low (12 mg/kg) and high doses (120 mg/kg).

CBD exerts its anticonvulsant activity by modulating different targets, including GPCRs and ion channels. Notably, CBD lowers neuronal excitability by functionally antagonizing GPR55 receptors and blocking the opening of Nav1.1 to Nav1.7. A number of other effector molecules may also be affected by CBD and involved in its activity such as PPARγ [761,797,847]. In this regard, it has been observed that PPARγ is involved in the anticonvulsant effects of EP-80317, a ghrelin analogue tested in a model of repeated 6-Hz corneal stimulation test. Indeed, blocking PPARγ with its inhibitor GW9662 was able to counteract the anti-seizure activity of EP-80317 [570]. Additionally, it has been proposed that PPARγ is essential for the anticonvulsant actions of the KD in addition to the anti-seizure properties of the non-selective cannabinoid agonists WIN 55,212-2 [622,848].

Even though the precise mechanism underlying the interaction between PPARγ and CBD is still unknown, the fact that FAAH and FABPs transport CBD within cells while also increasing PPARγ activity may give clues to both their synergy and their connection to neuroprotective advantages [758,759]. For instance, it was found that a CBD-PPARγ-interaction causes neuroprotective effects in ischemic stroke and neurogenesis in an Alzheimer's disease model [762,763]. Furthermore, CBD has already been demonstrated to affect PPARγ-mediated NF-κB signaling, which is up-regulated in both microglia and astrocytes of Parkinson's disease patients [357]. In this regard, PPARγ activity inhibits NF-κB signaling and lowers the amounts of mRNA for proinflammatory mediators, such as TNF-α and IL-1β [764,765]. Hence, the PPARγ-mediated reduction of lipid peroxidation, NF-κB downregulation and the suppression of the MAPK pathway appeared to be associated with the anti-inflammatory actions of CBD [357]. Moreover, pioglitazone, a PPARγ agonist, was able to suppress neuronal loss and reduce IL-1β and IL-6 levels in the hippocampus of PTZ-induced SE in rats [632]. Pioglitazone has additionally been demonstrated to reduce the severity of seizures induced by pilocarpine, as well as neuronal loss, BBB damage, and sodium currents in hippocampal neurons, while PPARγ inhibition, increased neuronal excitability and excitotoxicity [723]. For these reasons, a beneficial interaction between CBD and PPARγ is assumed, with its major neuroprotective role

played after seizure appearance, as well as in epileptogenesis. We hypothesize that the anticonvulsant effects of CBD in a chronic model of TLE are, at least in part, mediated by PPARγ activity.

Are there changes in the expression of PPARγ specific to certain hippocampal regions in epileptic rats?

PPARγ is detectable in numerous regions of the CNS, including the frontal cortex, nucleus accumbens, amygdala, hypothalamus, ventral tegmental area, thalamic paraventricular, nucleus reuniens, substantia nigra, thalamus, granular layers of the cerebellum and hippocampus. These brain regions are known to play an important role in a number of neuroanatomical alterations that occur during epileptic seizures [601]. Especially the hippocampus seems to be markedly affected by seizures and epilepsy, resulting e.g. in volume change, loss of neurons, impaired function of neuronal networks and sprouting [45,849,850]. Previously, the ability of EP-80317 to enhance PPARγ expression in hippocampal pyramidal neurons as well as in different interneuron subpopulations, was described in a mouse model of 6-Hz corneal stimulation [570].
Our work confirmed these findings, showing that both low and high dose of CBD was able to increase immunoreactivity for PPARγ in CA1 and subiculum, whereas just the high dose of CBD significantly increased PPARγ levels in CA3, lateral amygdala and perirhinal cortex in comparison to the MCT-oil treated control group. Additionally, PPARγ levels in CA3 were significantly increased in rats treated with the high dose of CBD, compared to the low-dose of CBD and MCT-oil treated animals. These outcomes highlight that the most significant target area for the regulation of PPARγ by high CBD is the CA3 hippocampus subfield, which supports that the anti-seizure effects of CBD might be mediated by an upregulation of PPARγ in this area.

Can CBD-rich Cannabis extracts be considered as a potential medication in pharmacoresistant epilepsy? How important are terpenes in treating seizures and epilepsy?

Most approaches to developing new medications aim to find an effective single compound. The same thought applies to find a suitable ASM established from derivatives derived from the *Cannabis sativa* L. plant. So far, there are numerous studies about the anti-seizure effects of CBD in animal models and clinical studies. In these reports, CBD was able to diminish seizure occurrence, as well as reduce the seizure severity and to display a beneficial effect on the epileptogenesis [815,851–853]. Until now, about 550 different chemical compounds have been identified in *C. sativa* from which at least 110 are phytocannabinoids [854,855]. The most well-known and studied phytocannabinoid is THC, which was demonstrated to have curative effects in diverse diseases, including epilepsy, but due to its psychoactive activity it is a non-suitable medication for adults and infants suffering from epilepsy [856,857]. Besides, it was reported that CBGA, the biosynthetic precursor to all cannabinoids is more effective than CBD in reducing seizures triggered by a febrile-

like event in a Dravet syndrome mouse model. In contrast to CBD, higher doses of CBGA also showed pro-convulsant effects on other seizure types, revealing a drawback of this cannabis compound [816]. Moreover, CBDA exhibited anticonvulsant properties in the rat MES test. The chemical instability of CBDA, however, gives rise to a number of difficulties for its prospective clinical application [817]. These challenges would need to be addressed before another single cannabis compound replaces CBD as a novel ASM. Taking into account that many other phytocannabinoids show anticonvulsant activity, but are as a single compound not as effective as CBD [457,492], lately, researchers focused on the potency of their synergistic effects in seizure control of Cannabis extracts. The outcome of a meta-analysis on observational clinical studies on the use of CBD-based medicines to treat patients with DRE were recently published. At least in the group of patients with refractory epilepsy, CBD-rich extracts were shown to have a superior therapeutic function than pure CBD alone. From the two third of patients positively responding to either CBD or CBD-rich extracts, 71% of extract-receivers were reported to have better results than patients treated with purified CBD alone (46%) [536].

Our preclinical *in vivo* study, using a modified 6-Hz corneal stimulation test, supports these results. Two different hemp oils were used in this approach: K1 with a low concentration of terpenes and K2 with a high concentration of terpenes. We found that just after the fifth stimulation, 87.5% of mice treated with olive oil developed generalized tonic–clonic convulsions, with and without loss of posture, whereas in mice treated with CBD only 62.5% showed convulsions. Noteworthy, only 12.5% of mice treated with K1 and 25.0% of mice treated with K2 developed generalized tonic–clonic convulsions, with and without loss of posture. No statistical significance was detected about the duration of ictal ECoG recordings in the different groups, indicating that all treatments did not modify the duration of the ictal ECoG. However, after the last stimulation, K2 was able to significantly reduce the cumulative duration of the recovery from seizures compared to olive oil [801].

Apart from cannabinoids, terpenes are another abundant class of compounds present in cannabis, which are essential oil components and are responsible for the aroma of the plant [858,859]. In numerous animal studies, terpenes, such as β-caryophyllene and limonene have been described as anticonvulsant agents against PTZ-induced seizures [835,836]. In addition, positive effects on oxidative stress and neuroinflammation caused by seizures were described [834]. Moreover, terpenes are thought to be essential compounds to exert the full activity of cannabinoid extracts due to their synergistic effects [520]. The “entourage effect” was first postulated by Mechoulam and Ben-Shabat in 1998 and suggests that the overall impact is greater than the sum of its constituent parts [860]. Consequently, cannabinoids are currently not only tested for their single molecule effect but are also studied in combination with terpenes and other phytocannabinoids to identify their full therapeutic potential. In this regard, the enhancement of pain and mood disorders could be associated with the entourage effect between terpenes and cannabinoids [819,820]. Consistent with these studies, our data support the hypothesis that cannabis extracts enriched with terpenes (K2) are beneficial in providing a faster recovery from seizures compared to extracts with a low terpene-concentration and CBD alone, emphasizing the significance of terpenes. In our work, we do not assess potential adverse effects of cannabis extracts. However, data from a clinical meta-

analysis suggested appetite alteration, sleepiness, gastrointestinal disturbances, weight changes, fatigue, and nausea as the most frequent, but mild side effects [818]. Regarding using cannabinoids alone, pure CBD tends to lower the occurrence of unwanted events 5-10 times when administered as an add-on medication to other ASMs [861]. Interestingly, the placebo effect, normally ranging from 15-25%, can be as high as 40% in patients using cannabis compounds, especially in children [538,804,813]. This may indicate that claimed therapeutic responses are significantly impacted by people's beliefs about the current trendy treatments employing cannabis.

Are there suitable markers for the identification of neurological changes after the repeated 6-Hz corneal stimulation and of the aggravation of seizures?

To identify new effective ASMs, precise and reliable biomarkers for epilepsy and epileptogenesis would be needed. There are already numerous well-explored biomarkers which change their expression after seizure appearance or in the time course of the disease tested *in vivo* in rodents and humans (Table 5) [270,281,282]. For instance, FosB/ΔFosB antigens are known to be rapidly, but transiently induced in seizure and epilepsy animal models [862,863]. FosB/ΔFosB immunoreactivity was reported to be elevated after the induction of SE, as well as reappeared in the hippocampus after the reinduction of SE in epileptic rats [305,864]. Additionally, FosB/ΔFosB isoforms act as indicators of changes in neuronal activity, and have been described to be directly involved in epileptogenesis since their deletion was associated with adult-onset spontaneous epilepsy [304,306]. Furthermore, there is relevant documentation that FosB isoforms are responsible for the upregulation of the NR1 subunit of the NMDA receptor in cortical pyramidal neurons after repeated electroconvulsive seizures [865]. Activity and expression of NMDA receptors is described to be altered in epilepsy, as well as antagonism at these receptors has anti-seizure effects [866]. An opposite trend was shown in a previous study, in which FosB/ΔFosB immunoreactivity was initially increased in the lateral amygdala and hippocampus after induced seizures, which, however, returned to control levels after subsequent stimulations [253].

In our study, using a modified repeated 6-Hz corneal stimulation mouse model, FosB/ΔFosB levels were barely detectable in all hippocampal regions of unstimulated control mice. In contrast, neuronal expression was increased in olive oil, CBD, K1 and K2-treate mice after corneal stimulation. These changes were statistically significant in the subiculum but not in CA1. Interestingly, oil-, CBD-, and K1-treated and stimulated mice had significantly higher levels of FosB/ΔFosB than unstimulated mice. On the contrary, treatment with K2 prevented a significant increase in FosB/ΔFosB levels in the subiculum. Determining the immunoreactivity after repeated stimulation, when mice displayed the most severe stage of our modified Racine scale, as tonic-clonic convulsions with and without the loss of posture, suggests FosB/ΔFosB as a suitable marker in animal models of epilepsy for seizure aggravation.

In contrast to previously published results, that observed after several stimulations a return to control levels of FosB/ΔFosB [253], our findings in animals exposed to several seizures, showed an increase in immunoreactivity. This phenomenon might be caused by its nuclear accumulation due

to its unusually long half-life. This hypothesis is consistent with the finding that epigenetic suppression of the hippocampal calcium-binding protein calbindin-D28k, which is regulated by ΔFosB, persists even during seizure-free periods [867]. The immunohistochemical staining further aligns the significance of the entourage effect of cannabinoids and terpenes in counteracting neuronal modifications induced by seizures.

Conclusion and future perspectives

With the present work, I give insight into new possible ASMs for patients suffering from drug-resistant epilepsy. The efficacy of ASMs might be challenged by different factors, including alterations of efflux transporters across the BBB, desensitization of target ion channels and receptors or variations in genes encoding enzymes that display key functions in the pharmacodynamics and -kinetics of ASMs and/or genes associated with the epileptic phenotype [77,103,110]. Other mechanisms, such as epilepsy-associated structural alterations (neurodegeneration and axonal sprouting) and gliosis, may contribute to the formation of an abnormal neural network and the access of drugs to epileptic foci, thus reducing the efficacy of ASMs [76,145].

The goal of my study was to assess the *in vivo* anti-seizure effects of cannabis extracts in a modified model of repeated 6-Hz corneal stimulation in mice. Furthermore, this work aimed at evaluating the *in vivo* antiepileptic activity of CBD on SRSs and PPARγ levels in a chronic animal model of TLE, induced by KA. In this regard, the results of the 6-Hz corneal stimulation screening test indicated that terpenes are important components in improving the anti-seizure effects of K2, in comparison to K1 and pure CBD. Additionally, K2 was able to prevent an increase of FosB/ΔFosB levels in the subiculum suggesting that K2 can reduce overexcitation in the hippocampus. Moreover, data from the systemic KA rat model propose that the antiepileptic actions of CBD might partially be mediated by elevated PPARγ levels in the hippocampal subfield CA3, suggesting an involvement of PPARγ in the anti-seizure effects of CBD.

Future work should be focused on the assessment of the efficacy of cannabis extracts in the KA model as well as in *in vivo* models of DRE in genetically modified animals. The bioactivity and evaluation of single terpenes, such as β-caryophyllene could be addressed, as well as the involvement of CB_2 in the described anti-seizure effects. Moreover, further research is necessary to elucidate the molecular targets of diverse cannabinoids and the underlying mechanism of their synergistic effects with terpenes. Additionally, it could be of interest to explore whether the neurons expressing FosB/ΔFosB potentially constitute a subgroup of cells that is particularly susceptible to seizures.

Further investigations in antagonizing PPARγ might allow more precise indication about its role in CBD-dependent anti-seizure mechanisms. To overcome the challenge of drug-refractoriness, more research needs to be performed in evaluating the BBB permeability for *C. sativa* molecules and extracts. A possible solution might be the use of biodegradable nanoparticles. Their ability to reduce

side effects, as well as their protection of the chemical structure of the compound allows a more adequate and efficient targeting and penetration to the site of action [868].

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