CHALLENGES AND CONUNDRUMS IN CANNABINOID-BASED TREATMENTS FOR EPILEPSY SYNDROMES AND ASSOCIATED NEUROBEHAVIORAL COMORBIDITIES

EDITED BY: Dolores E. López García, Ricardo Gómez-Nieto and Norberto Garcia-Cairasco

PUBLISHED IN: Frontiers in Behavioral Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-966-2
DOI 10.3389/978-2-88971-966-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

CHALLENGES AND CONUNDRUMS IN CANNABINOID-BASED TREATMENTS FOR EPILEPSY SYNDROMES AND ASSOCIATED NEUROBEHAVIORAL COMORBIDITIES

Topic Editors:
Dolores E. López García, University of Salamanca, Spain
Ricardo Gómez-Nieto, University of Salamanca, Spain
Norberto Garcia-Cairasco, University of São Paulo, Brazil

Citation: García, D. E. L., Gómez-Nieto, R., Garcia-Cairasco, N., eds. (2021). Challenges and Conundrums in Cannabinoid-Based Treatments for Epilepsy Syndromes and Associated Neurobehavioral Comorbidities. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-966-2

Table of Contents

frontiers in Behavioral Neuroscience

EDITORIAL
published: 11 November 2021
doi: 10.3389/fnbeh.2021.781852

Editorial: Challenges and Conundrums in Cannabinoid-Based Treatments for Epilepsy Syndromes and Associated Neurobehavioral Comorbidities

Ricardo Gómez-Nieto [1,2,3*†], *Dolores E. López* [1,2,3*†] *and Norberto Garcia-Cairasco* [4,5*†]

[1] *Institute of Neuroscience of Castilla y León, University of Salamanca, Salamanca, Spain,* [2] *Institute for Biomedical Research of Salamanca (IBSAL), Salamanca, Spain,* [3] *Department of Cell Biology and Pathology, Faculty of Medicine, University of Salamanca, Salamanca, Spain,* [4] *Neurophysiology and Experimental Neuroethology Laboratory, Physiology Department, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil,* [5] *Department of Neuroscience and Behavioral Sciences, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil*

Keywords: antiseizure medications, cannabidiol, cannabinoids, comorbidities, epilepsy, experimental-animal models, clinical epilepsy, seizure

OPEN ACCESS

Edited and reviewed by:
Emilio Russo,
University of Catanzaro, Italy

***Correspondence:**
Ricardo Gómez-Nieto
richard@usal.es
Dolores E. López
lopezde@usal.es
Norberto Garcia-Cairasco
ngcairas@usp.br

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *23 September 2021*
Accepted: *19 October 2021*
Published: *11 November 2021*

Citation:
Gómez-Nieto R, López DE and Garcia-Cairasco N (2021) Editorial: Challenges and Conundrums in Cannabinoid-Based Treatments for Epilepsy Syndromes and Associated Neurobehavioral Comorbidities. Front. Behav. Neurosci. 15:781852. doi: 10.3389/fnbeh.2021.781852

Editorial on the Research Topic

Challenges and Conundrums in Cannabinoid-Based Treatments for Epilepsy Syndromes and Associated Neurobehavioral Comorbidities

Epilepsy is the one of most common neurological disorder that affects people of all ages. This devastating disease presents a very different etiology and a wide variety of symptoms. Although the hallmark of those symptoms is the recurrent and unprovoked seizures, epilepsy should be considered a spectrum disorder that can lead to many physiological disturbances accompanied by a great diversity of behavioral manifestations. Thus, epilepsy syndromes include not only seizures, but also several comorbid conditions related to behavioral and psychiatric disorders such as cognitive impairments, depression, and anxiety. Despite the availability of numerous and different antiseizure treatments, many fail to manage the uncontrolled electrical activity in the epileptic brain. This becomes a major medical problem, the so-called pharmacoresistant or refractory epilepsy, as it is often a chronic and lifelong condition for many patients. Uncontrolled seizures are linked in both cause and consequence to significant risk of severe brain injuries with irreversible modifications of cerebral organization, which can result in poorly controlled seizures, despite ongoing antiseizure medications, and can even cause death. Pharmacoresistant epilepsy further leads to debilitating psychopathic consequences and many antiseizure substances are found to have a role in exacerbating physical and/or psychiatric symptoms. Thus, it is not uncommon for epileptic patients with pharmacoresistance to also experience several episodes of significant depression and/or anxiety. Bearing in mind that the major goal of epilepsy therapy is for patients to be free of seizures and adverse effects, a reinforced understanding of pharmacoresistance is considered a hot spot in epilepsy research. The availability of newer anticonvulsant components and the development of a promising pipeline of future antiseizure medications is becoming a reality. In this regard, and despite the cannabis usage in ancient times and further criminalization, it is striking the strong interest developed in recent years for the use of cannabis-derived compounds to treat epilepsy syndromes and associated neurobehavioral comorbidities. Among all the phytocannabinoid present in *Cannabis sp*., it is particularly noteworthy the cannabidiol (CBD)

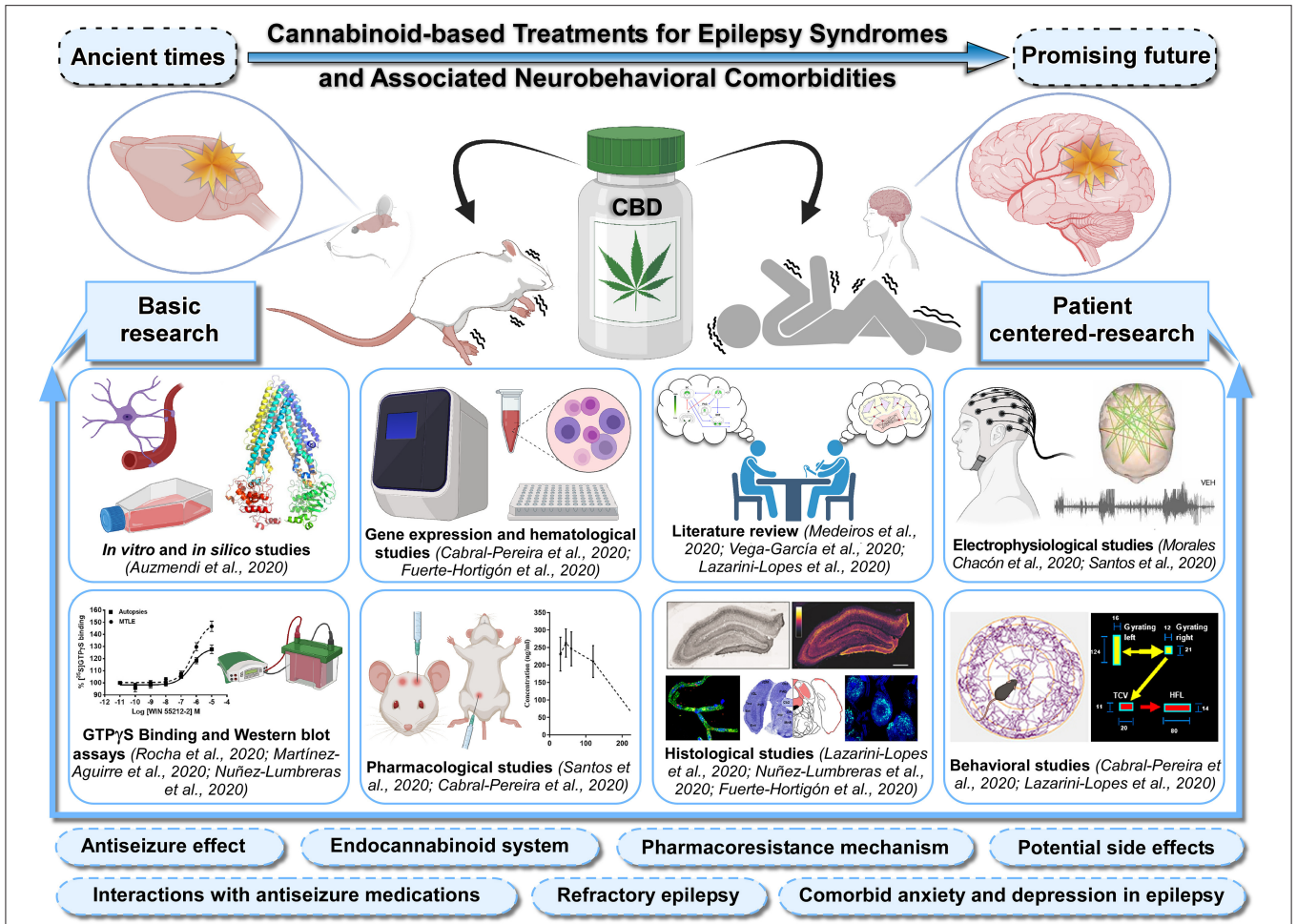


FIGURE 1 | Sketch of the Research Topic's main issues. Advances in the knowledge of cannabinoid-based treatments for epilepsy syndromes and associated neurobehavioral comorbidities should be fostered by greater integration between basic and patient-centered research. This Special Research Topic brings together scientific studies in experimental models and patients with epilepsy using a broad range of cutting-edge techniques, including molecular, histological, electrophysiological, pharmacological, and behavioral approaches as well as *in silico* and *in vitro* methodologies. In addition, it also contains integrative literature reviews that generate new frameworks and perspectives on the topic. This Topic collectively provides a comprehensive insight into the anticonvulsant effects of cannabidiol (CBD), role of the endocannabinoid system, pharmacoresistance mechanisms, potential interactions with classic antiseizure medications, possible side effects, with a view to tackle the use of cannabinoids in refractory epilepsy as well as comorbid anxiety and depression in epilepsy. Articles shown in the figure were grouped by methods and experimental approaches. *Figure created with Canvas Software and icons based on Biorender.com*.

that interacts with therapeutic targets in body and brain and exerts a well-defined anticonvulsant profile, without adverse psychoactive effects and abuse liability. The cannabinoid system is overwhelming as is the complex association of many ligands, various types of receptors, multi-signal pathways as well as different ion channels, and furthermore, not all cannabinoids act through the endocannabinoid system. Although previous studies have found that cannabinoids improve seizure control and have benefits on neurobehavioral function, unresolved questions and controversy persist.

This Research Topic aimed to highlight basic and patient-centered research focused on the cannabinoid-based treatments for epilepsy syndromes and associated neurobehavioral comorbidities (**Figure 1**). This Topic has gathered twelve articles, including three reviews, one brief research report, and eight original research contributions from high-profile scientists in the field. Readers will find high-level information on the multidisciplinary and cutting-edge techniques, including molecular, histological, electrophysiological, pharmacological, and behavioral approaches as well as *in silico* and *in vitro* methodologies. This article collection is meant to provide a comprehensive insight into the therapeutic use of cannabinoids, including the anticonvulsant effects, the involvement of the endocannabinoid system, pharmacoresistance mechanisms, potential interactions with classic antiseizure medications and possible side effects, making a wide variety of key information available to clarify the relevance of cannabinoid-based treatments in refractory epilepsy as well as comorbid anxiety- and depression-like behavior (**Figure 1**). The first article of this Topic (Auzmendi et al.) reported inhibitory effects of CBD on the

active efflux of P-glycoprotein-dependent Rhodamine-123 by using cultures of rat astrocytes and vascular endothelial cells subjected to hypoxia. The outcome of this *in vitro* approach contributes to elucidate how overexpression of P-glycoprotein at the blood-brain barrier level can limit the access of antiseizure medications to the brain parenchyma. Additionally, the authors performed *in silico* studies to predict a possible direct interaction between P-glycoprotein and CBD as a substrate/competitive inhibitor, which supported the use of CBD as an adjuvant therapy in refractory epilepsy. In this context, three original research articles deal with the CBD's mechanism of action and the role of endocannabinoid system in patients with drug-resistant mesial temporal lobe epilepsy (DR-MTLE) and comorbid mood disorders. First, Rocha et al. evaluated the tissue levels of endocannabinoids and the cell signaling transduction after the activation of cannabinoid-receptor-1 in the hippocampus (epileptogenic area) and the temporal neocortex (seizure propagation area) of patients with DR-MTLE, with and without anxiety and depression. The results indicated that enhanced endocannabinoid neurotransmission is involved in the absence of comorbid mood disorders. Consistently, Martínez-Aguirre et al. focused on evaluating the interaction between CBD and 5-hydroxytryptamine-1A receptors in cell membranes obtained from the hippocampus and temporal neocortex of patients with DR-MTLE. The radioligand displacement and GTPγS-binding assays showed that CBD interacts with human 5-hydroxytryptamine-1A receptors, acting as an inverse agonist that might modify neuronal excitation and epileptic seizures in DR-MTLE patients. Complementing these studies, Nuñez-Lumbreras et al. assessed the protein expression levels and Gαi/o protein-induced activation by cannabinoid-receptors-1 and 2 in the brain microvascular endothelial cells of patients with DR-MTLE. The results obtained from the immunofluorescence, Western blot and GTPγS-binding approaches were compared with those from autopsies of non-epileptic patients and correlated with the clinical data. This study revealed differences in the protein expression of cannabinoid-receptors between the hippocampus and the temporal neocortex, suggesting that cannabinoid-receptors with high efficiency represent an important therapeutic target for maintaining the integrity of the blood-brain barrier in DR-MTLE patients. The research article by Morales-Chacón et al. further deals with the impact of CBD on the brain function of patients with pharmacoresistant epileptic encephalopathy who received pharmaceutical-grade CBD (Epidiolex®) as adjunctive antiseizure therapy. The authors used functional connectivity and network topology derived from electroencephalograms, and interestingly found that CBD treatment was related to inhibition of the transition of the interictal to the ictal state as well as the improvement of electroencephalogram organization and brain function. They advocated for using this network analysis approach on electroencephalographic signals to assess the effects of CBD in clinical practice. Seizure disorders are common during childhood and the immature brain is highly susceptible to developing neuronal hyperexcitability under different pathological conditions. In this Topic, the review by Vega-García et al. compiles crucial information from pre-clinical and clinical studies that examine the effects of cannabinoids on epileptogenesis in early life. The authors spotlight the beneficial therapeutic effects of cannabinoids and pointed out methodological limitations such as the small sample size and the short follow-up period in pediatric studies. The efficacy and safety of CBD treatment in pediatric epilepsy was also discussed. In a brief research report, Santos et al. examined the effects of systemic administration and intracerebral microinfusion of several cannabinoid-receptor agonists in two rodent models of epilepsy, the genetically epilepsy-prone rats (GEPR-3) and the *Area Tempestas* model. By assessing and comparing seizure scores and electroencephalograms in baseline, vehicle-treated and treatment conditions of both experimental models, this study add to our understanding of potential sites of action of cannabinoids in the context of putative antiseizure treatment. Since there are strong indications that the endocannabinoid system modulates epileptic seizures by regulating neuronal excitability, it is vital to investigate the expression of cannabinoid receptors in pre-clinical animal models of epilepsy. In this respect, this topic contains two original research articles that determined the protein expression of cannabinoid-receptors-1 in the brain of two well-established genetically audiogenic rodent strains, in which the generalized tonic–clonic seizures are triggered by intense sound stimulation. Lazarini-Lopes et al. used a detailed anatomical analysis to assess the effects of acute and chronic audiogenic seizures on cannabinoid-receptor-1 expression in the hippocampus and amygdala of the Wistar Audiogenic Rat (WAR). Similarly, Fuerte-Hortigón et al. used immunohistochemistry and gene expression analysis to study the differential distribution of the cannabinoid-receptor-1 in the brain of the genetically audiogenic seizure-prone hamster (GASH/Sal). These complementary studies shed light on the relationship between the cannabinoid-receptor-1 and seizure susceptibility in both genetic model of audiogenic seizures, setting it up as a potent regulator of neuronal excitability. In this context, readers might wonder about the anticonvulsant effects of cannabinoids and their synergistic interactions with conventional antiseizure agents in audiogenic seizure models. The review article by Lazarini-Lopes et al. provides an overview on the pharmacological modulation of the endocannabinoid system in audiogenic seizure susceptibility as well as the effects of *Cannabis*-derived compounds, with special attention to CBD. The authors pointed out that the assessment of cannabinoids in epilepsy related comorbidities is an under-explored research field and should be further investigated. To delve deeper into these issues, the article by Cabral-Pereira et al. examined the behavioral and molecular effects of acute and chronic administrations of CBD and valproate on the GASH/Sal audiogenic seizures, as well as the coadministration of both drugs. It was found that CBD slightly attenuated seizure behaviors without adverse effects, and the combination of both drugs did not alter the therapeutic outcome of the valproate monotherapy, which helps prevent the animals from getting convulsions. The effects on the gene expression of protein channel and receptors targeted by the CBD were further explored in the epileptogenic focus. The review article by Medeiros et al. explores the endocannabinoid system as a possible pharmacological landmark for mimicking a form of

"on-demand" desynchronization analogous to those proposed by deep brain stimulation in the treatment of epilepsy. The review also discusses the evidence supporting the role of the endocannabinoid system in modulating the synchronization and/or coupling of distinct local neural circuitry, which presents implications on the physiological setting of proper sensory-motor integration.

To overcome the challenges and conundrums in the cannabinoid-based treatments for epilepsy syndromes at the present time, the investigators worldwide are tirelessly designing and conducting experiments. This Research Topic represents an excellent example of this collaborative effort and commitment. The Topic Editors hope that these thoughtful papers would benefit those researchers to advance in this exciting research field, inspiring novel bench-to-bedside ventures.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by the research grant from the Instituto de Salud Carlos III (ISCIII), co-financed with European Union FEDER funds (#PI19/01364, PIs: DL and RG-N), the São Paulo Research Foundation (FAPESP grants: 14/50891-1, 19/05957-8, 19/00849-2, and 2019/02787-4, PI: NG-C), the National Council for Scientific and Technological Development (CNPq grants: 465458/2014-9 and 305883/2014-3, PI: NG-C), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES—Finance code 001, PI: NG-C), and CAPES-Print (Process no. 88887.370299/2019-00, PI: NG-C), as well as the SPRINT-São Paulo Researchers in International Collaboration program from the USAL/FAPESP (#2019/16574-2, PIs: NG-C and DL).

ACKNOWLEDGMENTS

The Guest Editors were sincerely grateful to all contributors who joined this Special Research Topic with commitment and diligence, devoting their time to make these dedicated collections of articles as comprehensive and advance as possible. We would also like to thank the Editorial Office staff of Frontiers in Behavioral Neuroscience, particularly the Editor-in-Chief Professor Nuno Sousa, for their continued support.

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 17 March 2020
doi: 10.3389/fnbeh.2020.00032

Cannabidiol (CBD) Inhibited Rhodamine-123 Efflux in Cultured Vascular Endothelial Cells and Astrocytes Under Hypoxic Conditions

*Jerónimo Auzmendi[1,2], Pablo Palestro[3], Agustín Blachman[4], Luciana Gavernet[3], Amalia Merelli[1], Alan Talevi[3], Graciela Cristina Calabrese[4], Alberto Javier Ramos[2,5] and Alberto Lazarowski[1]**

[1] Instituto de Fisiopatología y Bioquímica Clínica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [3] Laboratorio de Investigaciones Bioactivas y Desarrollo, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad de La Plata, La Plata, Argentina, [4] Cátedra de Biología Celular y Molecular, Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [5] Laboratorio de Neuropatología Molecular, Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis," Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

OPEN ACCESS

Edited by:
Norberto Garcia-Cairasco, University of São Paulo, Brazil

Reviewed by:
Luiz E. Mello, Federal University of São Paulo, Brazil
Mustapha Muzaimi, Universiti Sains Malaysia Health Campus, Malaysia

***Correspondence:**
Alberto Lazarowski
nadiatom@ffyb.uba.ar

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 16 October 2019
Accepted: 17 February 2020
Published: 17 March 2020

Citation:
Auzmendi J, Palestro P, Blachman A, Gavernet L, Merelli A, Talevi A, Calabrese GC, Ramos AJ and Lazarowski A (2020) Cannabidiol (CBD) Inhibited Rhodamine-123 Efflux in Cultured Vascular Endothelial Cells and Astrocytes Under Hypoxic Conditions. Front. Behav. Neurosci. 14:32. doi: 10.3389/fnbeh.2020.00032

Despite the constant development of new antiepileptic drugs (AEDs), more than 30% of patients develop refractory epilepsy (RE) characterized by a multidrug-resistant (MDR) phenotype. The "transporters hypothesis" indicates that the mechanism of this MDR phenotype is the overexpression of ABC transporters such as P-glycoprotein (P-gp) in the neurovascular unit cells, limiting access of the AEDs to the brain. Recent clinical trials and basic studies have shown encouraging results for the use of cannabinoids in RE, although its mechanisms of action are still not fully understood. Here, we have employed astrocytes and vascular endothelial cell cultures subjected to hypoxia, to test the effect of cannabidiol (CBD) on the P-gp-dependent Rhodamine-123 (Rho-123) efflux. Results show that during hypoxia, intracellular Rho-123 accumulation after CBD treatment is similar to that induced by the P-gp inhibitor Tariquidar (Tq). Noteworthy, this inhibition is like that registered in non-hypoxia conditions. Additionally, docking studies predicted that CBD could behave as a P-gp substrate by the interaction with several residues in the α-helix of the P-gp transmembrane domain. Overall, these findings suggest a direct effect of CBD on the Rho-123 P-gp-dependent efflux activity, which might explain why the CBD add-on treatment regimen in RE patients results in a significant reduction in seizure frequency.

Keywords: cannabidiol, P-glycoprotein, hypoxia, endothelial cells, astrocytes

INTRODUCTION

Refractory epilepsies (REs) are characterized by high recurrence of seizures that cannot be controlled by at least two well-tolerated antiepileptic drugs (AEDs) appropriate for the particular epilepsy type (Kwan and Brodie, 2010). Whereas the continuous development of new AEDs has offered important improvements regarding treatment adherence, pharmacokinetics, tolerability,

and efficacy in certain particular epilepsy types (Löscher et al., 2013; Hanaya and Arita, 2016; Talevi, 2016), similar percentage of non-responder patients remains the same as that observed for the AEDs since two centuries ago (Kwan and Brodie, 2000). Several hypotheses to explain this common multidrug-resistant (MDR) phenotype in RE have been proposed. Among them, the "transporter hypothesis" and the "pharmacokinetics hypothesis" suggest that ABC transporters could play a central role in RE (Leandro et al., 2019). Both hypotheses could explain the first pioneering description of a MDR phenotype in an RE with persistent low levels of AEDs in plasma associated with P-glycoprotein (P-gp) overexpression (Tishler et al., 1995; Lazarowski et al., 1999, 2004a). P-gp, so far the most studied of these transporters, is encoded by ABCB1 gene. P-gp expression is regulated by hypoxia, inflammation, and stress-related transcriptional factors such as HIF-1α, NFκB, STAT3, or PXR (Ho and Piquette-Miller, 2006; Jain et al., 2014; Zhang Z.-L. et al., 2018). Moreover, upregulation of P-gp was reported in a wide spectrum of experimental models and clinical studies of RE (Lazarowski et al., 2004b, 2007b; Hartz et al., 2017; Deng et al., 2018; Weidner et al., 2018). During hypoxia, P-gp was overexpressed in different tissues, including brain and heart (Lazarowski et al., 2007a; Aviles-Reyes et al., 2010; Merelli et al., 2011a,b). Remarkably, this overexpression was also registered in brain and heart after repetitive seizures and status epilepticus, showing a hypoxic–ischemic scenario (Auzmendi et al., 2014, 2018).

Some alternative therapies are eligible, before the highly invasive surgical removal of the epileptic focus, to improve life quality of patients with RE. Vagal nerve stimulation or ketogenic diet has been applied with variable outcomes (Johnson and Wilson, 2018; Liu et al., 2018; D'Andrea Meira et al., 2019; Dibué-Adjei et al., 2019; Hwang et al., 2019). More recently, the use of different Cannabis compounds to treat seizures has emerged as a potential therapeutic opportunity. Particularly, Cannabis compounds are employed to control the so-called catastrophic epilepsies in children (Maa and Figi, 2014). Several reports and reviews have highlighted the benefits of a rational and selective use of these compounds for the treatment of RE (Filloux, 2015; Rosenberg et al., 2015; Friedman and Devinsky, 2016; Chen et al., 2018). Among the wide spectrum of Cannabis compounds, cannabidiol (CBD) has been suggested as a relevant candidate for RE control because of its no psychoactive effect unlike those produced by Δ^9-tetrahydrocannabinol (Tasker et al., 2015). Recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have recently approved oral CBD as an add-on treatment for Dravet and Lennox–Gastaut syndromes (Devinsky et al., 2019).

Cannabis compounds act through specific endocannabinoid receptors (CB1, CB2) (du Plessis et al., 2015; Basavarajappa et al., 2017). In the central nervous system (CNS), the CB1 receptor is widely expressed in neurons of hippocampus and neocortex, while glial cells express both CB1 and CB2 (Scotter et al., 2010; Maccarrone et al., 2011). Interestingly, the stimulation of the endocannabinoid system plays an important role in a variety of physiological functions and conditions such as neuronal development and plasticity, food intake, energy balance, and cell apoptosis (Marzo et al., 2004; Moreno et al., 2005; Pacher et al., 2006). More recently, it has also been proposed that CBD action involves non-canonical targets such as the transient receptor potential (TRP) channel (Hassan et al., 2014; Iannotti et al., 2014), the adenosine receptor A2A (A2AAR) (Ohta and Sitkovsky, 2001), and the orphan receptor (GPR55) (Chiurchiù et al., 2015) in addition to its known activity on CB1. Furthermore, CBD has also been reported to elicit anti-inflammatory effects (Booz, 2011; Burstein, 2015) that may decrease the glial response after seizures. Besides, CBD activates the microglia promoting their phagocytic activity (Hassan et al., 2014). To date, few studies have assayed CBD and other Cannabis compounds as blockers, substrates, or modulators of the expression of P-gp and other ABC transporters (Holland et al., 2006, 2009; Zhu, 2006; Feinshtein et al., 2013; Brzozowska et al., 2016). Despite the fact that CB1 activation by seizure-induced release of endocannabinoids could play a neuroprotective role (Alger, 2004), its effects on the MDR phenotype in RE remain to be disclosed. Inhibition of P-gp and other ABC transporters could facilitate the normal action of AEDs, bringing new opportunities to improve seizure control with classical AEDs in patients with RE (Höcht et al., 2007; Feinshtein et al., 2013; Hisham et al., 2018; Xie et al., 2018; Zhang H.-L. et al., 2018). Consequently, these transporters emerge as potential new pharmacological targets in RE (Robey et al., 2008).

The aim of this study was to evaluate the possible inhibitory effect of CBD on the active efflux of the fluorescent P-gp-substrate Rhodamine-123 (Rho-123) by *in vitro* studies. Taking into account all above described, P-gp overexpression at the blood–brain barrier (BBB) level can limit the access of AEDs to the brain parenchyma. Cultures of both members of neurovascular unit (NVU) astrocytes and vascular endothelial cells were analyzed in hypoxia condition. CBD inhibitory effect on P-gp activity was tested through Rho-123 efflux assay, comparing it with that of the highly specific P-gp inhibitor Tariquidar (Tq). Additionally, *in silico* studies were performed to explore and predict a possible direct interaction between P-gp and CBD as a substrate/competitive inhibitor.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animals and their care were conducted in accordance with our institutional guidelines, which comply with the NIH guidelines for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience, and were approved by the CICUAL committee (0092357/2019) of the School of Medicine of the University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Reagents

Cell culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA, United States). Fetal calf serum

(FCS) was purchased from Natocor (Córdoba, Argentina). Poly-L-lysine, Rhodamine 123, third-generation P-gp inhibitor Tq, and other chemicals were obtained from Sigma–Aldrich (United States). CBD was obtained from Enecta.

Glial Culture

Primary cortical glial cell cultures were obtained from nine 3–5 postnatal day Wistar rats as described previously (Merelli et al., 2019). Briefly, rats were decapitated, and brains were surgically removed from the skull under sterile condition. Using a fine tip tweezer, meninges were eliminated, and brain cortices were removed and mechanically disrupted within Dulbecco's modified Eagle medium (DMEM). After several centrifugations, dissociated glial cells were resuspended in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 μg/ml penicillin–streptomycin. Then, 1.5×10^4 cells/ml glial cells were plated in 96-multi-well plate, incubated in supplemented DMEM at 37°C, 5% CO_2. The medium was exchanged every 48 h until the cells reached 70–80% confluence.

Vascular Endothelial Cell Culture

Cells derived from polyoma middle T-transformed murine heart endothelium (H5V) (Calabrese et al., 2011) were grown in DMEM supplemented with 10% FBS, streptomycin (100 mg/ml), and penicillin (100 UI/ml) in 5% CO_2 atmosphere, at 37°C. The medium was exchanged every 48 h until the cells reached 70–80% confluence.

Chemical Hypoxia Induction

Glial and endothelial cells (1.5×10^6 cells/ml) were depleted of serum for 4 h before incubating with or without 0.3 mM $CoCl_2$ in medium supplemented with 0.5% FBS for 6 h (final volume 5 ml). **Figure 1** summarizes the experimental scheme and the molecular mechanisms of chemical hypoxia induction.

Rho-123 Efflux Assay

This approach to test the P-gp activity was performed as described previously (Merelli et al., 2019). Briefly, following the chemical hypoxia induction, glial and endothelial cells were incubated in the presence of increasing concentration CBD for 30 min [5, 50, or 100 μM, prepared from a stock solution of 0.1 mM in dimethyl sulfoxide (DMSO)].Then, Rho-123 (1 μM in DMEM) was added for 5 min. Controls were carried out in the absence of CBD or with Tq (5 μM) used as a specific blocker of P-gp. All treatments were performed in triplicate. **Figure 1** summarizes the experimental scheme and the molecular mechanisms of Rho-123 efflux studies.

Computational Studies

In previous investigations, a human P-gp homology model was constructed based on the structure encoded as 3G61 in Protein Data Bank, which showed the highest sequence coverage (92%) and percent of identity (82%) among other alternatives. Comparison of several docking software and conditions (see next paragraph) allowed us to conclude that Autodock Vina

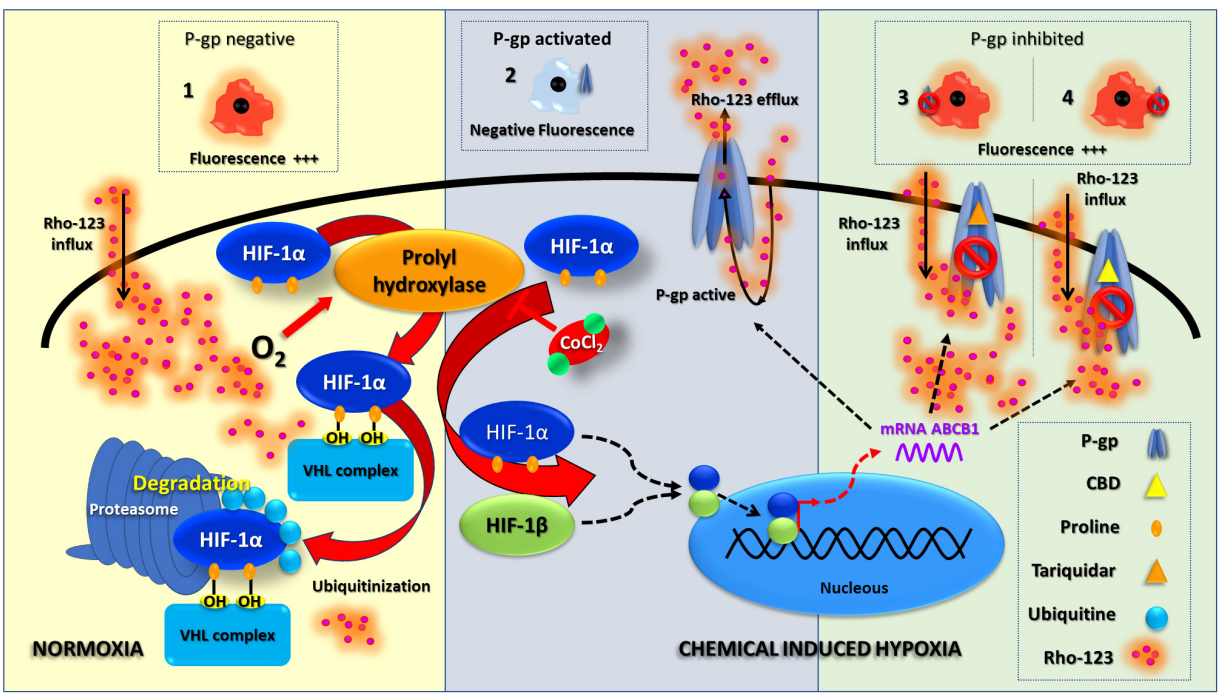


FIGURE 1 | Chemical hypoxia induction. This scheme summarizes the HIF-1 molecular pathway under normoxia and chemical-induced hypoxia; this also shows an interpretation of Rho-123 efflux assay results. Under normoxia, P-gp is absent and a negative result of Rho-123 efflux assay is shown as an increased fluorescence (1). Since the chemical hypoxia is induced, a stabilization of HIF-1 leads to a P-gp overexpression; thus, a positive result of Rho-123 efflux assay is related to a decreased fluorescence (2). The inhibition of P-gp activity (with TQ or CBD—3 and 4, respectively) correlates with an increase in fluorescence.

flexible docking was the best choice among the tested options. As further validation, the model was subsequently used to find new anticonvulsant compounds that do not interact with P-gp and might then constitute potential therapeutic options to treat refractory patients with high expression levels of such transporter (Jäger et al., 1997; Semenza, 2009; Parasrampuria and Mehvar, 2010).

To select the best docking protocol, we run different simulations (by changing the docking software and/or conditions) to predict the interactions with P-gp of a dataset of compounds. The dataset comprised small molecules with known interaction with the transporter (from now on, binders) and structures with no interaction with the protein (non-binders). Using the docking score as discriminating variable, we analyzed the capacity of different docking protocols to classify them correctly; that is, the non-binders should have higher docking score than binders. We quantified this information through the area under the receiver operating characteristic (ROC) curves (Palestro et al., 2018). This metric also allowed us to decide the threshold score that can be used to discern between the classes. Three docking software were tested: Glide (version 5.7, Schrodinger Suite 2011), Autodock4.2, and Autodock Vina (The Scripps Research Institute[1]) (Trott and Olson, 2010). The "docking active site" was defined through a $24 \times 24 \times 24$ Å^3 grid, centered on the relative position of the ligand in the crystallographic structure of mouse P-gp (PDB code 3G61). Such region comprises the entire transmembrane region, since the binding subsites for P-gp substrates and inhibitors reside in this area (Aller et al., 2009).

In terms of flexibility of the target, we run both rigid and flexible docking simulations. The flexibility of the target was considered by allowing two different sets of amino acids to move in the simulations. In one system, we set the binding site residues Phe-335, Phe-343, Phe-728, Phe-732, and Phe-978 as flexible (model A), whereas in the other simulation, we selected as flexible Tyr-307, Tyr-953, Phe-343, and Phe-978 (model B). The selection of the mobile residues in model A was founded on the analysis of the amino acids that interact with the ligands in the experimental mouse complexes (PDB codes 3G60 and 3G61). For model B, we analyzed first the conformation of the flexible residues in model A after the docking simulations. We found that Phe-343 and Phe-978 showed different conformations depending on the ligand, whereas Phe335, Phe732, and Phe728 adopted almost the same conformation in all simulations. Then, we choose as flexible residues Phe-343, Phe-978, and other amino acids that interact with the ligands according to the docking results with model A.

Our results pointed to Autodock Vina as the best solution. We computed 20 docking runs for each compound using the default parameters for the rest of the variables, and model B as target. This system was also able to reproduce the binding mode of the inhibitor co-crystallized in the mouse experimental structure (ligand named QZ59, PBD code 3G60). A more detailed explanation about the selection and validation of the docking protocol is given in the original research previously published (Palestro et al., 2014). This same procedure has been used here to predict the interaction between CBD and P-gp.

Quantification and Statistical Analysis

Microscopic images were taken using an Olympus IX-81 microscope equipped with a DP71 camera (Olympus, Japan). Morphometrical and densitometric analyses were performed with ImageJ (NIH) and statistical analysis was done with GraphPad Prism software. Glial morphology was evaluated in a central field of 15 hypoxic and 15 non-hypoxic wells.

The fluorescence intensity was evaluated as a parameter of Rho-123 retention in cell cultures. After checking the normal distribution of the data, differences were analyzed by one-way ANOVA and Student Newman Keuls post-test comparison or by two-tailed Student *t*-test.

RESULTS

Effect of CBD on Glial and Endothelial Cells

In vitro Studies

Initially, we tested the effect of different concentrations of CBD (5, 50, and 100 μM) on glial cells under hypoxia conditions. Microglia and astrocytes were identified based on morphological clues established by Giulian and Baker (1986) and Gebicke-Haerter et al. (1989). While the astrocytes grow at the bottom of the plate with a large soma and long processes, the microglial cells grow on top of them, characterized by a small soma and few prolongations (**Figure 2**). No changes in the number of glial cells were registered under hypoxia, along the experiments (control: microglia/field = 128.6 ± 6.05 and astrocytes/field = 82.40 ± 4.675; vs hypoxia treatment: microglia/field = 116.8 ± 7.123 and astrocytes/field = 77.00 ± 1.51). Besides, the cell ratio between astrocytes and microglia remained constant during experiments (control = 0.6436 ± 0.031 vs hypoxia = 0.6724 ± 0.054; $p = 0.2982$).

On the other hand, when Rho-123 efflux was assayed under hypoxic conditions, astrocytes showed a decreased Rho-123 retention, while in microglia, it remained unchanged. In this context, Tq treatment recovered the Rho-123 retention in astrocytes from mixed glial cultures (**Figure 3**). Noteworthy, CBD produced a similar retention of Rho-123 to that achieved by Tq. Moreover, astrocytes exposed to hypoxia and subsequent CBD treatment recovered Rho-123 retention in a concentration-dependent manner (5–100 μM) (**Figure 3**).

When Rho-123 retention was evaluated on H5V endothelial cell cultures exposed to hypoxia, a similarly low level of the fluorescent compound retention was observed in both control and hypoxic conditions (**Figure 4A**). Nevertheless, when H5V cells, under hypoxia, were exposed to CBD 5, 50, or 100 μM, fluorescence was increased in a concentration-dependent manner (**Figure 4B**). Additionally, when H5V cells, in normoxia, were exposed to CBD 100 μM, Rho-123 retention was also increased (**Figure 4C**).

In silico Studies

Since CBD blocked the active Rho-123 efflux, and this activity is a known property of P-gp, we performed *in silico* studies to test the possible interaction between CBD and P-gp. In our docking

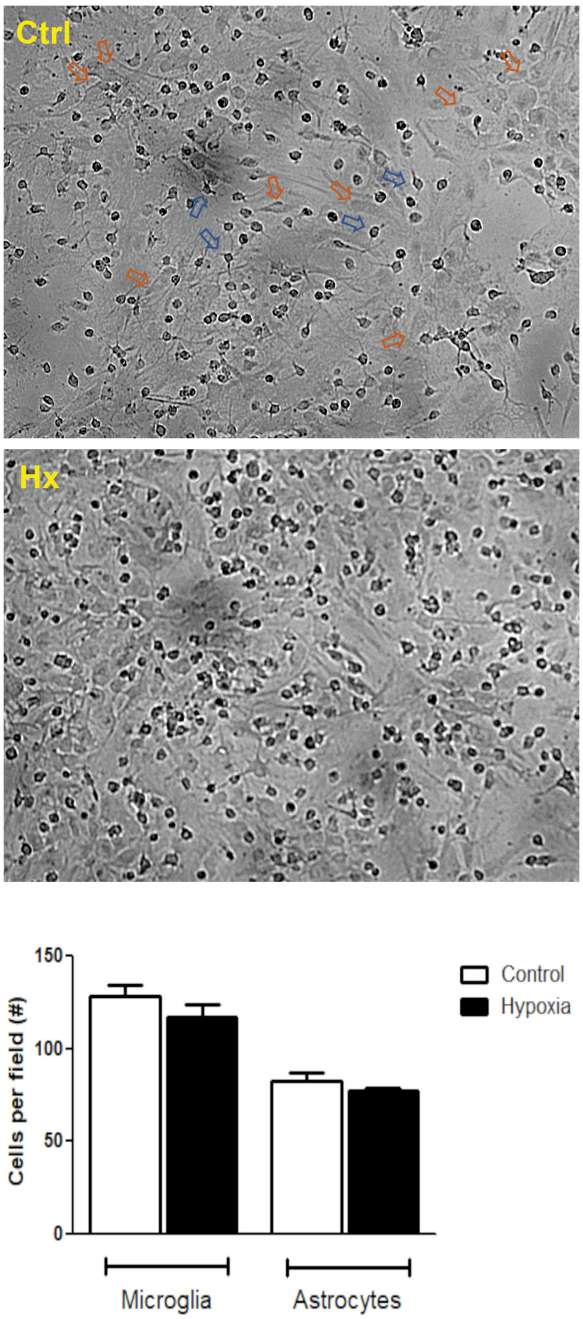


FIGURE 2 | Hypoxia effect on glial mixed culture. Representative phase contrast images showing glial mixed culture. Morphological differences between microglia and astrocytes are indicated by blue or orange arrows, respectively. Glial mixed cultures exposed to control conditions (DMEM, upper image) or chemical $CoCl_2$-induced hypoxia (bottom image). Images were acquired at total magnification of 40×.

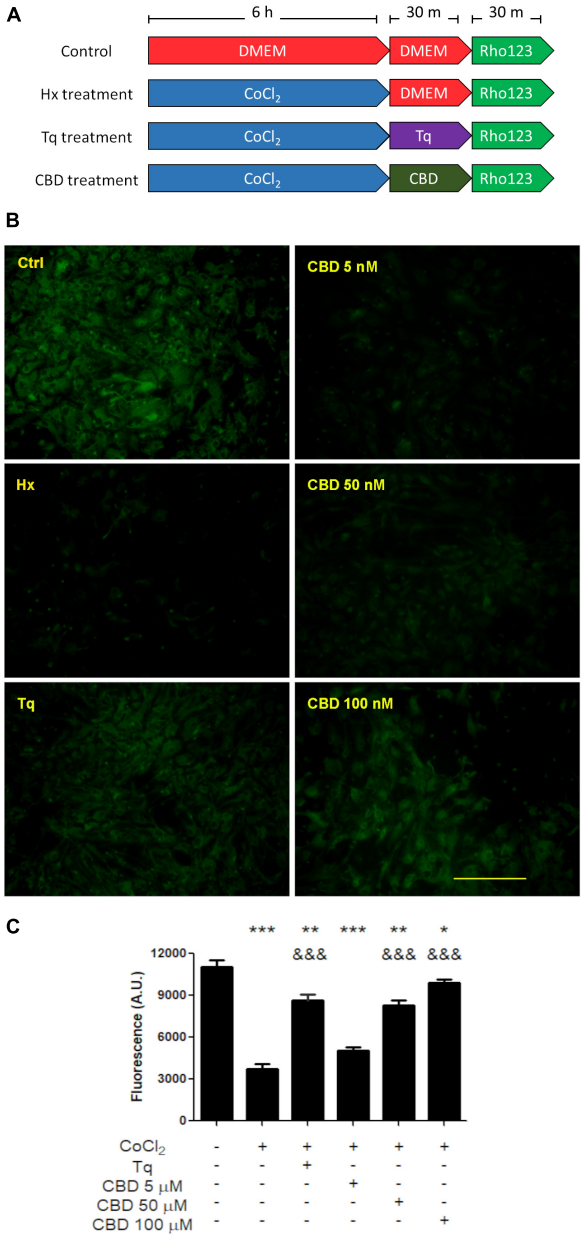


FIGURE 3 | Effect of CBD on glial cells. **(A)** Schematic representation of the treatments and temporal profile applied on mixed glial cell cultures. **(B)** Representative images of the Rho-123 retention by glial culture after each treatment. **(C)** Quantification of the Rho-123 retention as fluorescence intensity for each treatment. At least 20–30 astrocytes were measured in a central field per well, but microglia were not included. Bars represent the mean ± SEM. The differences were analyzed by one-way ANOVA ($p < 0.001$) and Bonferroni post-test. Comparisons with the control were represented by an asterisk (*) while (&) represents comparisons against hypoxia treatment.

experiment, the lowest energy pose displayed an estimated binding energy of -9.0 kcal/mol. These data suggest that CBD may act as a P-gp substrate (thus behaving as a competitive inhibitor). **Figure 5** shows the homology model of human P-gp and the predicted binding mode of CBD to the docking binding site.

DISCUSSION

Refractory epilepsies have been associated with ABC transporter overexpression (Löscher and Potschka, 2002; Lazarowski et al., 2007b; Löscher et al., 2011), in direct relation with the recently

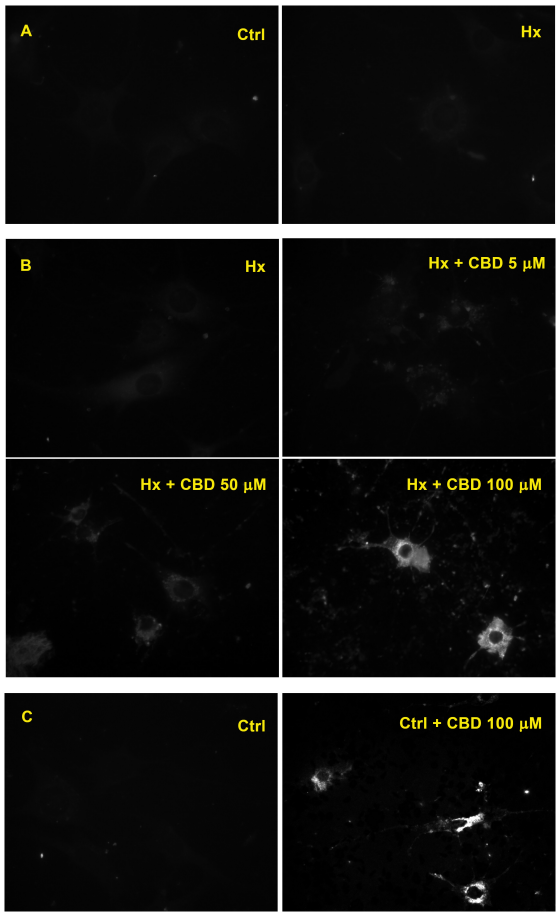


FIGURE 4 | Effect of CBD on endothelial cells. **(A)** Fluorescence microscopy of H5V cells. **(B)** H5V cells exposed to hypoxia in the presence of CBD (5, 50, or 100 μM). **(C)** H5V cells, in normoxia, exposed to CBD (100 μM). All images were obtained with a magnification of 60×.

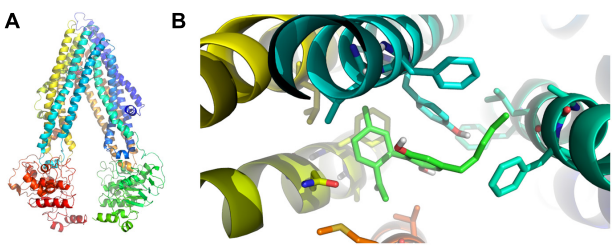


FIGURE 5 | *In silico* binding between P-gp and CBD. **(A)** Homology model of human P-gp. **(B)** Predicted binding mode between CBD (light green) and P-gp amino acid residues in the binding foci. The aromatic residues shown in the figure correspond to Phe 728 and Tyr 307 (both in light blue), the latter of which seems to be involved in the most relevant specific interaction through pi stacking.

proposed pharmacokinetic hypothesis (Tang et al., 2017; Leandro et al., 2019). In this scenario, ABC transporter overexpression at the NVU level could be responsible for sub-therapeutic levels of AEDs at the brain parenchyma (**Figure 6A**).

Our present study demonstrates that CBD inhibits the active efflux of Rho-123, a recognized P-gp substrate, in two prominent members of the NVU such as astrocytes and vascular endothelial cells. Furthermore, these results were more evident under chemically induced hypoxia, a condition in which P-gp is induced to be overexpressed *in vivo*, in vascular endothelial cells, astrocytes, and neurons (Lazarowski et al., 2007a; Merelli et al., 2011a). As shown in **Figure 1**, $CoCl_2$ inhibits the prolyl hydroxylase, a HEM enzyme that is regulated by oxygen concentration. Inhibition of prolyl hydroxylase allows the stabilization of HIF-1α with its concomitant gene expression to hypoxic response. Furthermore, 50 and 100 μM of CBD recovered a similar amount of Rho-123 intracellular retention than the specific P-gp inhibitor Tq, suggesting that these concentrations of CBD produce an inhibitory effect on ABC transporter efflux activity, particularly on P-gp, as demonstrated on trophoblast cell line, mouse embryonic fibroblast, Caco-2, and LLC-PK1/MDR1 cells (Holland et al., 2006; Zhu, 2006; Feinshtein et al., 2013).

Our docking analysis suggests that CBD could act as a competitive inhibitor of P-gp-mediated transport. Also, we observed that CBD inhibited the Rho-123 efflux of non-hypoxic H5V cells, a type of vascular endothelial cells that normally express ABC transporters (**Figure 4**). These data clearly are in accord with the "transporters hypothesis," where MDR phenotype is related with P-gp overexpression at this type cell level.

From these observations, several aspects should be considered. Despite the fact that different new AEDs have been developed, some of them involving novel modes of action, 30% of epileptic patients continue to have seizures, being carriers of the so-called MDR epilepsy phenotype. The "transporter hypothesis" suggests that brain overexpression of several ABC transporters is responsible for the MDR phenotype in patients with RE. As previously reported by our group and other authors, brain overexpression of P-gp appears as one of the more common mechanisms related with RE carriers of this MDR phenotype. Several reports have also described the potential effects of P-gp inhibitors as adjuvant therapy next to AEDs, to achieve better control of seizures in different cases of drug-resistant epilepsies (Lazarowski et al., 2007b; Robey et al., 2008).

Interestingly, for several thousand years, humanity has given medicinal use to *Cannabis sativa* (Marijuana), even for the treatment of epileptic patients. In addition to their behavioral and psychotropic effects, cannabinoids have complex pharmacological effects by binding to two specific plasma membrane G protein-coupled receptors: the CB1 receptor expressed mainly in the brain and in some peripheral tissues and the CB2 receptor expressed mainly outside of the CNS have been described (Pertwee and Ross, 2002). Several experimental studies have documented that the endocannabinoid system is strongly activated by seizures, and the upregulation of CB1R activity has antiseizure effects; furthermore, depending on the type of pharmacological action on CB1R, it can both increase and suppress the seizure-like discharges in hippocampus (Marsicano et al., 2003; Deshpande et al., 2007a,b).

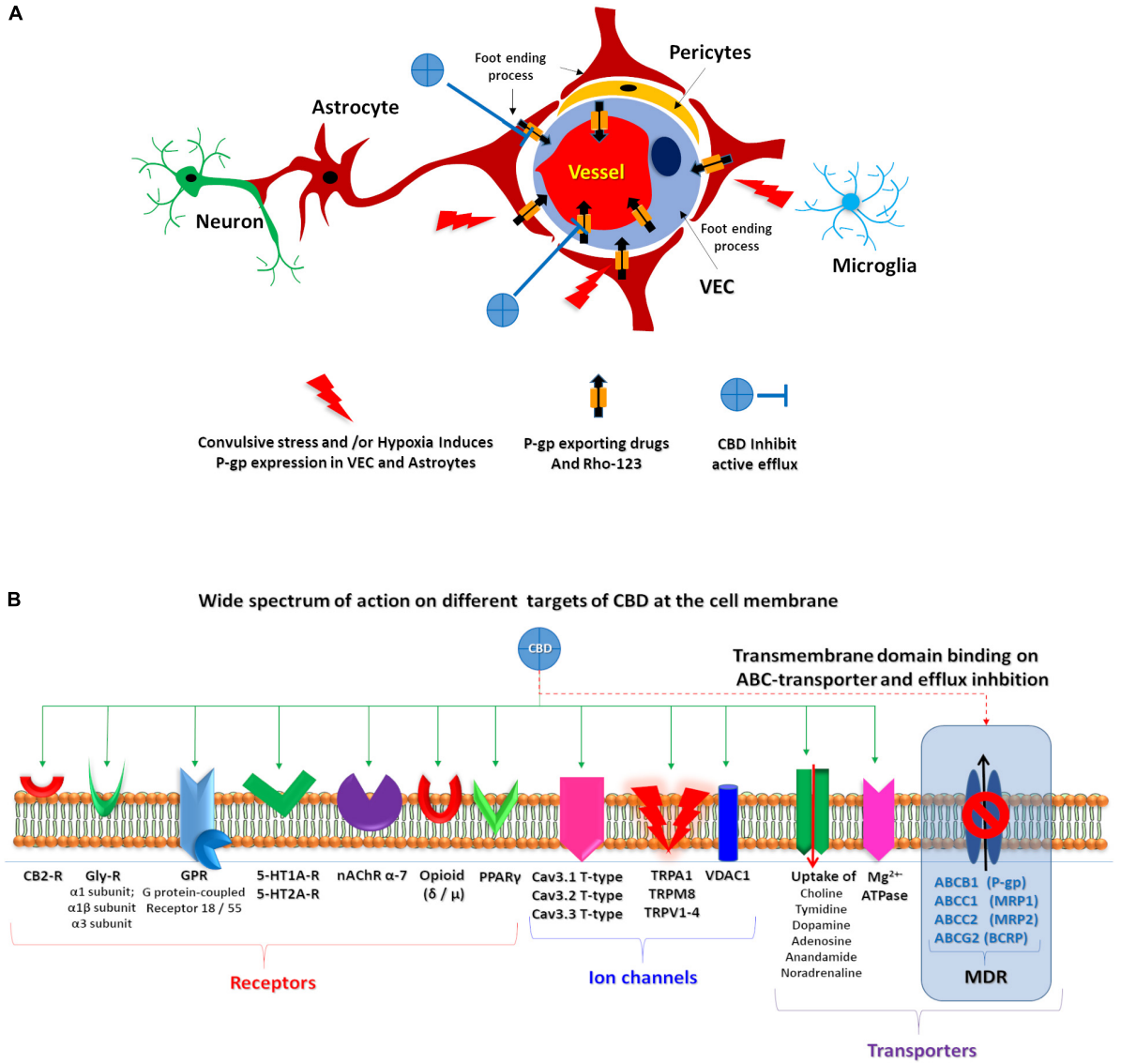


FIGURE 6 | CBD proposed targets. **(A)** Schematic representation of the proposed CBD action on endothelial cells and astrocytes after convulsive stress and or hypoxia. **(B)** Representation of wide spectrum of CBD target on a membrane cell. Green solid arrows identified several described CBD target. Red dashed arrow highlights the proposed CBD action on ABC transporters.

The antiepileptic effects of some cannabinoids were revised, showing that they can act on multiple targets (Friedman and Devinsky, 2016). Because CBD lacks THC psychotropic action, it could be an excellent alternative candidate to treat patients with RE (Cilio et al., 2014); in fact, oral CBD has recently been approved as an add-on treatment of severe drug-resistant epilepsies, such as Dravet and Lennox–Gastaut syndromes (Devinsky et al., 2019). However, due to its complex pharmacology, its specific mechanism(s) of action in epilepsy is (are) yet to be described. Whereas the wide spectrum of known CBD targets are not directly related to the MDR phenotype observed in RE (**Figure 6**), the multi-target nature of CBD could explain its effects on RE, as multi-target drugs are being actively explored as potential treatment of complex disorders, in line with a network pharmacology/systems biology perspective (Margineanu, 2014, 2016).

In previous experimental studies, our group has provided *in vivo* evidence that focal brain injection of $CoCl_2$ (1 mM) induces P-gp expression surrounding the lesion site in neurons, astrocytic end-foot, and their related endothelial cells on blood vessels, and that higher $CoCl_2$ doses (200 mM) resulted in additional P-gp immunostaining of the whole astrocytic and neuronal soma (Lazarowski et al., 2007a). Nuclear translocation of hypoxia-inducible factor 1-alpha (HIF-1α) was also observed in this experimental paradigm (Caltana et al., 2009) along with erythropoietin receptor (Epo-R) and P-gp co-expression in

neurons, astrocytes, and vascular endothelial cells. Interestingly, both Epo-R and P-gp expression are inducible by HIF-1α (Comerford et al., 2002; Semenza, 2009). On the other hand, we also demonstrated that repetitive seizures and/or status epilepticus induce high expression of P-gp in neurons, astrocytes, and vessels, associated with the MDR phenotype (Lazarowski et al., 2004b; Höcht et al., 2007; Auzmendi et al., 2013; Merelli et al., 2019). Furthermore, repetitive seizures and/or status epilepticus also activate HIF-1α and induce P-gp overexpression in heart, which appears to be associated with heart failure and sudden unexpected death in epilepsy (SUDEP) (Auzmendi et al., 2018).

In our current study, a clear inhibition by CBD of Rho-123 efflux was observed in hypoxic astrocytes and vascular endothelial cells. It is known that Rho-123 is a substrate of P-gp; however, Rho-110, a metabolic product of Rho-123, can also be transported by other ABC transporters as MRP2 is also expressed at the BBB (Jäger et al., 1997; Semenza, 2009; Parasrampuria and Mehvar, 2010). According to docking studies presented here, the estimated binding energy of CBD to P-gp suggests that it may act as a weak substrate for the transporter. This observation is in line with the high CBD concentration needed (50–100 μM) to produce a significant retention of Rho-123 compared to a potent TQ specific blocker (5 μM). Previous reports using P-gp knockout mice showed that the lack of P-gp does not limit the brain uptake of CBD in healthy mice (Brzozowska et al., 2016). These reports do not necessarily imply that CBD does not interact with P-gp, especially in the context of high-expression levels of the transporter, as those observed in RE patients (Löscher et al., 2011). Consistent with these evidences, Holland et al. reported that low CBD concentration does not improve uptake of the Rho-123 in CEM/VLB100 cells expressing high levels of P-gp. In contrast, high levels of CBD (10 μM) sensitize such cells to vinblastine, another known P-gp substrate (Holland et al., 2006).

It is known that CBD is a multi-target compound, acting on ionic channels, neurotransmitter receptors, and other transmembrane transporters, with different effects in each of them acting such as activator, modulator, agonist, antagonist, etc. Additionally, not only is CBD metabolized by the enzymatic cytochrome system, but it can also inhibit some of these enzymes, inducing a slowdown in the metabolism of more common AEDs, which is an alternative way of affecting their pharmacokinetics (Rocha et al., 2020). Therefore, CBD effects on ABC transporters are only one among multiple mechanisms through which this drug could have an impact on the MDR phenotype in RE patients.

CONCLUSION

Our results indicate that, in addition to the various effects previously described by CBD, this drug can also inhibit the active efflux of Rho-123, a known P-gp substrate, in two types of cells of the NVU, in a similar (though less potent) manner to TQ. Consistently, our *in silico* study indicates that CBD may bind the transmembrane domain of P-gp, possibly acting as a competitive inhibitor. It remains to be studied whether CBD may also impair P-gp-mediated transport in a non-competitive manner. CBD could thus be used as an adjuvant therapy to reverse the MDR phenotype as observed in patients with RE, which could explain its recent approval as an add-on therapy to treat severe refractory childhood epilepsies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by CICUAL.

AUTHOR CONTRIBUTIONS

JA, AM, AT, AR, GC, and AL: conceptualization and investigation. JA, PP, AB, and LG: methodology. JA, PP, AB, LG, and AT: software and data curation. JA, PP, LG, AT, GC, and AL: formal analysis. JA, AM, AT, GC, and AL: resources. JA, AM, AT, and AL: writing – original draft preparation, review, and editing. JA and AL: supervision and project administration.

FUNDING

This work was supported by the following grants: PICT 2014-2178 (JA), UBACyT-20720170200024BA (AM), UBACYT 20020150100003BA (AR and AL), UBACYT 20020170100128BA (GC), UNLP X83 (PP, LG, and AT), and PICT 2016-0165 (PP, LG, and AT).

ACKNOWLEDGMENTS

Special thanks to Enecta for the CBD provision.

REFERENCES

Alger, B. E. (2004). Endocannabinoids and their implications for epilepsy. *Epilepsy Curr.* 4, 169–173. doi: 10.1111/j.1535-7597.2004.04501.x

Aller, S. G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., et al. (2009). Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* 323, 1718–1722. doi: 10.1126/science.1168750

Auzmendi, J., Buchholz, B., Salguero, J., Cañellas, C., Kelly, J., Men, P., et al. (2018). Pilocarpine-induced status epilepticus is associated with P-glycoprotein

induction in cardiomyocytes. Electrocardiographic changes, and sudden death. *Pharmaceuticals (Basel)* 11:21. doi: 10.3390/ph11010021

Auzmendi, J. A., Merelli, A. M., Girardi, E. S., Orozco Suáre, S., Rocha Arrieta, L. L., Lazarowski, A. J., et al. (2014). Progressive heart P-glycoprotein (P-gp) overexpression after experimental repetitive seizures (ERS) associated with fatal status epilepticus (FSE). Is it related with SUDEP? *Smart Sci. Technol. Mol. Cell. Epilepsy* 1, 1–9.

Auzmendi, J. A., Orozco-Suárez, S., Bañuelos-Cabrera, I., González-Trujano, M. E., Calixto, González E, Rocha, L., et al. (2013). P-glycoprotein contributes to cell membrane depolarization of hippocampus and neocortex in a model of repetitive seizures induced by pentylenetetrazole in rats. *Curr. Pharm. Des.* 19, 6732–6738. doi: 10.2174/13816128113193800006

Aviles-Reyes, R. X., Angelo, M. F., Villarreal, A., Rios, H., Lazarowski, A., and Ramos, A. J. (2010). Intermittent hypoxia during sleep induces reactive gliosis and limited neuronal death in rats: implications for sleep apnea. *J. Neurochem.* 112, 854–869. doi: 10.1111/j.1471-4159.2009.06535.x

Basavarajappa, B. S., Shivakumar, M., Joshi, V., and Subbanna, S. (2017). Endocannabinoid system in neurodegenerative disorders. *J. Neurochem.* 142, 624–648. doi: 10.1111/jnc.14098

Booz, G. W. (2011). Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. *Free Radic. Biol. Med.* 51, 1054–1061. doi: 10.1016/j.freeradbiomed.2011.01.007

Brzozowska, N., Li, K. M., Wang, X. S., Booth, J., Stuart, J., McGregor, I. S., et al. (2016). ABC transporters P-gp and Bcrp do not limit the brain uptake of the novel antipsychotic and anticonvulsant drug cannabidiol in mice. *PeerJ* 4:e2081. doi: 10.7717/peerj.2081

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg. Med. Chem.* 23, 1377–1385. doi: 10.1016/j.bmc.2015.01.059

Calabrese, G. C., Gazzaniga, S., Oberkersch, R., and Wainstok, R. (2011). Decorin and biglycan expression: its relation with endothelial heterogeneity. *Histol. Histopathol.* 26, 481–490. doi: 10.14670/HH-26.481

Caltana, L., Merelli, A., Lazarowski, A., and Brusco, A. (2009). Neuronal and glial alterations due to focal cortical hypoxia induced by direct cobalt chloride (CoCl2) brain injection. *Neurotox. Res.* 15, 348–358. doi: 10.1007/s12640-009-9038-9

Chen, K. A., Farrar, M., Cardamone, M., Gill, D., Smith, R., Cowell, C. T., et al. (2018). Cannabidiol for treating drug-resistant epilepsy in children: the New South Wales experience. *Med. J. Aust.* 209, 217–221. doi: 10.5694/mja18.00023

Chiurchiù, V., Lanuti, M., De, Bardi M, Battistini, L., and Maccarrone, M. (2015). The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells. *Int. Immunol.* 27, 153–160. doi: 10.1093/intimm/dxu097

Cilio, M. R., Thiele, E. A., and Devinsky, O. (2014). The case for assessing cannabidiol in epilepsy. *Epilepsia* 55, 787–790. doi: 10.1111/epi.12635

Comerford, K. M., Wallace, T. J., Karhausen, J., Louis, N. A., Montalto, M. C., and Colgan, S. P. (2002). Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res.* 62, 3387–3394.

D'Andrea Meira, I, Romão, T. T., Pires do Prado, H. J., Krüger, L. T., Pires, M. E. P., and da, Conceição P. O. (2019). Ketogenic diet and epilepsy: what we know so far. *Front. Neurosci.* 13:5. doi: 10.3389/fnins.2019.00005

Deng, X., Xie, Y., and Chen, Y. (2018). Effect of neuroinflammation on ABC transporters: possible contribution to refractory epilepsy. *CNS Neurol. Disord. Drug Targets* 17, 728–735. doi: 10.2174/1871527317666180828121820

Deshpande, L. S., Blair, R. E., Ziobro, J. M., Sombati, S., Martin, B. R., and DeLorenzo, R. J. (2007a). Endocannabinoids block status epilepticus in cultured hippocampal neurons. *Eur. J. Pharmacol.* 558, 52–59. doi: 10.1016/j.ejphar.2006.11.030

Deshpande, L. S., Sombati, S., Blair, R. E., Carter, D. S., Martin, B. R., and DeLorenzo, R. J. (2007b). Cannabinoid CB1 receptor antagonists cause status epilepticus-like activity in the hippocampal neuronal culture model of acquired epilepsy. *Neurosci. Lett.* 411, 11–16. doi: 10.1016/j.neulet.2006.09.046

Devinsky, O., Nabbout, R., Miller, I., Laux, L., Zolnowska, M., Wright, S., et al. (2019). Long-term cannabidiol treatment in patients with Dravet syndrome: an open-label extension trial. *Epilepsia* 60, 294–302. doi: 10.1111/epi.14628

Dibué-Adjei, M., Brigo, F., Yamamoto, T., Vonck, K., and Trinka, E. (2019). Vagus nerve stimulation in refractory and super-refractory status epilepticus – a systematic review. *Brain Stimul.* 12, 1101–1110. doi: 10.1016/j.brs.2019.05.011

du Plessis, S. S., Agarwal, A., and Syriac, A. (2015). Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility. *J. Assist. Reprod. Genet.* 32, 1575–1588. doi: 10.1007/s10815-015-0553-8

Feinshtein, V., Erez, O., Ben-Zvi, Z., Erez, N., Eshkoli, T., Sheizaf, B., et al. (2013). Cannabidiol changes P-gp and BCRP expression in trophoblast cell lines. *PeerJ* 1:e153. doi: 10.7717/peerj.153

Filloux, F. M. (2015). Cannabinoids for pediatric epilepsy? Up in smoke or real science? *Transl. Pediatr.* 4, 271–282. doi: 10.3978/j.issn.2224-4336.2015.10.03

Friedman, D., and Devinsky, O. (2016). Cannabinoids in the treatment of epilepsy. *N. Engl. J. Med.* 374, 94–95. doi: 10.1056/nejmc1512758

Gebicke-Haerter, P. J., Bauer, J., Schobert, A., and Northoff, H. (1989). Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. *J. Neurosci.* 9, 183–194. doi: 10.1523/jneurosci.09-01-00183.1989

Giulian, D., and Baker, T. J. (1986). Characterization of ameboid microglia isolated from developing mammalian brain. *J. Neurosci.* 6, 2163–2178. doi: 10.1523/jneurosci.06-08-02163.1986

Hanaya, R., and Arita, K. (2016). The new antiepileptic drugs: their neuropharmacology and clinical indications. *Neurol. Med. Chir. (Tokyo)* 56, 205–220. doi: 10.2176/nmc.ra.2015-0344

Hartz, A. M., Pekcec, A., Soldner, E. L., Zhong, Y., Schlichtiger, J., and Bauer, B. (2017). P-gp protein expression and transport activity in rodent seizure models and human epilepsy. *Mol. Pharm.* 14, 999–1011. doi: 10.1021/acs.molpharmaceut.6b00770

Hassan, S., Eldeeb, K., Millns, P. J., Bennett, A. J., Alexander, S. P., and Kendall, D. A. (2014). Cannabidiol enhances microglial phagocytosis via transient receptor potential (TRP) channel activation. *Br. J. Pharmacol.* 171, 2426–2439. doi: 10.1111/bph.12615

Hisham, M., Lakshmikanthcharan, S., Chaitanya Juluri, S. K., and Nandakumar, S. M. (2018). Verapamil as an adjuvant treatment for drug-resistant epilepsy. *Indian J. Crit. Care Med.* 22, 680–682. doi: 10.4103/ijccm.IJCCM_250_18

Ho, E. A., and Piquette-Miller, M. (2006). Regulation of multidrug resistance by pro-inflammatory cytokines. *Curr. Cancer Drug Targets* 6, 295–311. doi: 10.2174/156800906777441753

Höcht, C., Lazarowski, A., Gonzalez, N. N., Auzmendi, J., Opezzo, J. A., Bramuglia, G. F., et al. (2007). Nimodipine restores the altered hippocampal phenytoin pharmacokinetics in a refractory epileptic model. *Neurosci. Lett.* 413, 168–172. doi: 10.1016/j.neulet.2006.11.075

Holland, M. L., Lau, D. T. T., Allen, J. D., and Arnold, J. C. (2009). The multidrug transporter ABCG2 (BCRP) is inhibited by plant-derived cannabinoids. *Br. J. Pharmacol.* 152, 815–824. doi: 10.1038/sj.bjp.0707467

Holland, M. L., Panetta, J. A., Hoskins, J. M., Bebawy, M., Roufogalis, B. D., Allen, J. D., et al. (2006). The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochem. Pharmacol.* 71, 1146–1154. doi: 10.1016/j.bcp.2005.12.033

Hwang, S. T., Stevens, S. J., Fu, A. X., and Proteasa, S. V. (2019). Intractable generalized epilepsy: therapeutic approaches. *Curr. Neurol. Neurosci. Rep.* 19:16. doi: 10.1007/s11910-019-0933-z

Iannotti, F. A., Hill, C. L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., et al. (2014). Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem. Neurosci.* 5, 1131–1141. doi: 10.1021/cn5000524

Jäger, W., Winter, O., Halper, B., Salamon, A., Sartori, M., Gajdzik, L., et al. (1997). Modulation of liver canalicular transport processes by the tyrosine-kinase inhibitor genistein: implications of genistein metabolism in the rat. *Hepatology* 26, 1467–1476. doi: 10.1002/hep.510260613

Jain, S., Rathod, V., Prajapati, R., Nandekar, P. P., and Sangamwar, A. T. (2014). Pregnane X receptor and P-glycoprotein: a connexion for Alzheimer's disease management. *Mol. Divers.* 18, 895–909. doi: 10.1007/s11030-014-9550-6

Johnson, R. L., and Wilson, C. G. (2018). A review of vagus nerve stimulation as a therapeutic intervention. *J. Inflamm. Res.* 11, 203–213. doi: 10.2147/JIR.S163248

Kwan, P., and Brodie, M. J. (2000). Early Identification of refractory epilepsy. *N. Engl. J. Med.* 342, 314–319. doi: 10.1056/nejm200002033420503

Kwan, P., and Brodie, M. J. (2010). Definition of refractory epilepsy: defining the indefinable? *Lancet Neurol.* 9, 27–29. doi: 10.1016/s1474-4422(09)70304-7

Lazarowski, A., Caltana, L., Merelli, A., Rubio, M. D., Ramos, A. J., and Brusco, A. (2007a). Neuronal mdr-1 gene expression after experimental focal hypoxia: a new obstacle for neuroprotection? *J. Neurol. Sci.* 258, 84–92. doi: 10.1016/j.jns.2007.03.004

Lazarowski, A., Czornyj, L., Lubienieki, F., Girardi, E., Vazquez, S., and D'Giano, C. (2007b). ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. *Epilepsia* 48(Suppl. 5), 140–149. doi: 10.1111/j.1528-1167.2007.01302.x

Lazarowski, A., Lubieniecki, F., Camarero, S., Pomata, H., Bartuluchi, M., Sevlever, G., et al. (2004a). Multidrug resistance proteins in tuberous sclerosis and refractory epilepsy. *Pediatr. Neurol.* 30, 102–106. doi: 10.1016/s0887-8994(03)00407-7

Lazarowski, A., Ramos, A. J., García-Rivello, H., Brusco, A., and Girardi, E. (2004b). Neuronal and glial expression of the multidrug resistance gene product in an experimental epilepsy model. *Cell. Mol. Neurobiol.* 24, 77–85. doi: 10.1023/b:cemn.0000012726.43842.d2

Lazarowski, A., Sevlever, G., Taratuto, A., Massaro, M., and Rabinowicz, A. (1999). Tuberous sclerosis associated with MDR1 gene expression and drug-resistant epilepsy. *Pediatr. Neurol.* 21, 731–734. doi: 10.1016/s0887-8994(99)00074-0

Leandro, K., Bicker, J., Alves, G., Falcão, A., and Fortuna, A. (2019). ABC transporters in drug-resistant epilepsy: mechanisms of upregulation and therapeutic approaches. *Pharmacol. Res.* 144, 357–376. doi: 10.1016/j.phrs.2019.04.031

Liu, H., Yang, Y., Wang, Y., Tang, H., Zhang, F., Zhang, Y., et al. (2018). Ketogenic diet for treatment of intractable epilepsy in adults: a meta-analysis of observational studies. *Epilepsia Open* 3, 9–17. doi: 10.1002/epi4.12098

Löscher, W., Klitgaard, H., Twyman, R. E., and Schmidt, D. (2013). New avenues for anti-epileptic drug discovery and development. *Nat. Rev. Drug Discov.* 12, 757–776. doi: 10.1038/nrd4126

Löscher, W., Luna-Tortós, C., Römermann, K., and Fedrowitz, M. (2011). Do ATP-binding cassette transporters cause pharmacoresistance in epilepsy? Problems and approaches in determining which antiepileptic drugs are affected. *Curr. Pharm. Des.* 17, 2808–2828. doi: 10.2174/138161211797440212

Löscher, W., and Potschka, H. (2002). Role of multidrug transporters in pharmacoresistance to antiepileptic drugs. *J. Pharmacol. Exp. Ther.* 301, 7–14. doi: 10.1124/jpet.301.1.7

Maa, E., and Figi, P. (2014). The case for medical marijuana in epilepsy. *Epilepsia* 55, 783–786. doi: 10.1111/epi.12610

Maccarrone, M., Bernardi, G., Agrò, A. F., and Centonze, D. (2011). Cannabinoid receptor signalling in neurodegenerative diseases: a potential role for membrane fluidity disturbance. *Br. J. Pharmacol.* 163, 1379–1390. doi: 10.1111/j.1476-5381.2011.01277.x

Margineanu, D. G. (2014). Systems biology, complexity, and the impact on antiepileptic drug discovery. *Epilepsy Behav.* 38, 131–142. doi: 10.1016/j.yebeh.2013.08.029

Margineanu, D. G. (2016). Neuropharmacology beyond reductionism – a likely prospect. *BioSystems* 141, 1–9. doi: 10.1016/j.biosystems.2015.11.010

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208

Marzo, V., Di, Bifulco, M., Petrocellis, L., and De Petrocellis, L. (2004). The endocannabinoid system and its therapeutic exploitation. *Nat. Rev. Drug Discov.* 3, 771–784. doi: 10.1038/nrd1495

Merelli, A., Caltana, L., Girimonti, P., Ramos, A. J., Lazarowski, A., and Brusco, A. (2011a). Recovery of motor spontaneous activity after intranasal delivery of human recombinant erythropoietin in a focal brain hypoxia model induced by CoCl2 in rats. *Neurotox. Res.* 20, 182–192. doi: 10.1007/s12640-010-9233-8

Merelli, A., Caltana, L., Lazarowski, A., and Brusco, A. (2011b). Experimental evidence of the potential use of erythropoietin by intranasal administration as a neuroprotective agent in cerebral hypoxia. *Drug Metabol. Drug Interact.* 26, 65–69. doi: 10.1515/DMDI.2011.007

Merelli, A., Ramos, A. J., Lazarowski, A., and Auzmendi, J. (2019). Convulsive stress mimics brain hypoxia and promotes the P-glycoprotein (P-gp) and erythropoietin receptor overexpression. Recombinant human erythropoietin effect on P-gp activity. *Front. Neurosci.* 13:750. doi: 10.3389/fnins.2019.00750

Moreno, M., Lopez-Moreno, J. A., Rodríguez de Fonseca, F., and Navarro, M. (2005). Behavioural effects of quinpirole following withdrawal of chronic treatment with the CB1 agonist, HU-210, in rats. *Behav. Pharmacol.* 16, 441–446. doi: 10.1097/00008877-200509000-00017

Ohta, A., and Sitkovsky, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414, 916–920. doi: 10.1038/414916a

Pacher, P., Bátkai, S., and Kunos, G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* 58, 389–462. doi: 10.1124/pr.58.3.2

Palestro, P. H., Enrique, N., Goicoechea, S., Villalba, M. L., Sabatier, L. L., Martin, P., et al. (2018). Searching for new leads to treat epilepsy: target-based virtual screening for the discovery of anticonvulsant agents. *J. Chem. Inf. Model.* 58, 1331–1342. doi: 10.1021/acs.jcim.7b00721

Palestro, P. H., Gavernet, L., Estiu, G. L., and Bruno Blanch, L. E. (2014). Docking applied to the prediction of the affinity of compounds to p-glycoprotein. *Biomed Res. Int.* 2014:358425. doi: 10.1155/2014/358425

Parasrampuria, R., and Mehvar, R. (2010). Effects of P-glycoprotein and Mrp2 inhibitors on the hepatobiliary disposition of rhodamine 123 and its glucuronidated metabolite in isolated perfused rat livers. *J. Pharm. Sci.* 99, 455–466. doi: 10.1002/jps.21831

Pertwee, R. G., and Ross, R. A. (2002). Cannabinoid receptors and their ligands. *Prostaglandins Leukot. Essent. Fatty Acids* 66, 101–121.

Robey, R. W., Lazarowski, A., and Bates, S. E. (2008). P-glycoprotein–a clinical target in drug-refractory epilepsy? *Mol. Pharmacol.* 73, 1343–1346. doi: 10.1124/mol.108.046680

Rocha, L., Frías-Soria, C. L., Ortiz, J. G., Auzmendi, J., and Lazarowski, A. (2020). Is cannabidiol a drug acting on unconventional targets to control drug−resistant epilepsy? *Epilepsia Open* 5, 36–49. doi: 10.1002/epi4.12376

Rosenberg, E. C., Tsien, R. W., Whalley, B. J., and Devinsky, O. (2015). Cannabinoids and epilepsy. *Neurotherapeutics* 12, 747–768. doi: 10.1007/s13311-015-0375-5

Scotter, E. L., Abood, M. E., and Glass, M. (2010). The endocannabinoid system as a target for the treatment of neurodegenerative disease. *Br. J. Pharmacol.* 160, 480–498. doi: 10.1111/j.1476-5381.2010.00735.x

Semenza, G. L. (2009). Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology* 24, 97–106. doi: 10.1152/physiol.00045.2008

Talevi, A. (2016). Computational approaches for innovative antiepileptic drug discovery. *Expert Opin. Drug Discov.* 11, 1001–1016. doi: 10.1080/17460441.2016.1216965

Tang, F., Hartz, A. M. S., and Bauer, B. (2017). Drug-resistant epilepsy: multiple hypotheses, few answers. *Front. Neurol.* 8:301. doi: 10.3389/fneur.2017.00301

Tasker, J. G., Chen, C., Fisher, M. O., Fu, X., Rainville, J. R., and Weiss, G. L. (2015). Endocannabinoid regulation of neuroendocrine systems. *Int. Rev. Neurobiol.* 125, 163–201. doi: 10.1016/bs.irn.2015.09.003

Tishler, D. M., Weinberg, K. I., Hinton, D. R., Barbaro, N., Annett, G. M., and Raffel, C. (1995). MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* 36, 1–6. doi: 10.1111/j.1528-1157.1995.tb01657.x

Trott, O., and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi: 10.1002/jcc.21334

Weidner, L. D., Kannan, P., Mitsios, N., Kang, S. J., Hall, M. D., Theodore, W. H., et al. (2018). The expression of inflammatory markers and their potential influence on efflux transporters in drug-resistant mesial temporal lobe epilepsy tissue. *Epilepsia* 59, 1507–1517. doi: 10.1111/epi.14505

Xie, Y., Shao, Y., Deng, X., Wang, M., and Chen, Y. (2018). MicroRNA-298 reverses multidrug resistance to antiepileptic drugs by suppressing MDR1/P-gp expression in vitro. *Front. Neurosci.* 12:602. doi: 10.3389/fnins.2018.00602

Zhang, H.-L., Lin, Y.-H., Qu, Y., and Chen, Q. (2018). The effect of miR-146a gene silencing on drug-resistance and expression of protein of P-gp and MRP1 in epilepsy. *Eur. Rev. Med. Pharmacol. Sci.* 22, 2372–2379. doi: 10.26355/eurrev_201804_14829

Zhang, Z.-L., Jiang, Q.-C., and Wang, S.-R. (2018). Schisandrin A reverses doxorubicin-resistant human breast cancer cell line by the inhibition of P65 and Stat3 phosphorylation. *Breast Cancer* 25, 233–242. doi: 10.1007/s12282-017-0821-9

Zhu, H.-J. (2006). Characterization of P-glycoprotein inhibition by major cannabinoids from marijuana. *J. Pharmacol. Exp. Ther.* 317, 850–857. doi: 10.1124/jpet.105.098541

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 06 May 2020
doi: 10.3389/fnbeh.2020.00052

Endocannabinoid System and Cannabinoid 1 Receptors in Patients With Pharmacoresistant Temporal Lobe Epilepsy and Comorbid Mood Disorders

Luisa Rocha[1], Resat Cinar[2], Rosalinda Guevara-Guzmán[3], Mario Alonso-Vanegas[4], Daniel San-Juan[4], Iris Martínez-Juárez[4], José Luis Castañeda-Cabral[1] and Francia Carmona-Cruz[1]*

[1] Department of Pharmacobiology, Center of Research and Advanced Studies, Mexico City, Mexico, [2] Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD, United States, [3] Department of Physiology, School of Medicine, National Autonomous University of Mexico, Mexico, [4] National Institute of Neurology and Neurosurgery "Manuel Velasco Suarez", Mexico City, Mexico

OPEN ACCESS

Edited by:
Norberto Garcia-Cairasco,
University of São Paulo, Brazil

Reviewed by:
Salim Yalcin Inan,
Meram Faculty of Medicine, Turkey
Tao Tan,
University at Buffalo, United States

***Correspondence:**
Luisa Rocha
lrocha@cinvestav.mx

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 20 November 2019
Accepted: 20 March 2020
Published: 06 May 2020

Citation:
Rocha L, Cinar R, Guevara-Guzmán R, Alonso-Vanegas M, San-Juan D, Martínez-Juárez I, Castañeda-Cabral JL and Carmona-Cruz F (2020) Endocannabinoid System and Cannabinoid 1 Receptors in Patients With Pharmacoresistant Temporal Lobe Epilepsy and Comorbid Mood Disorders. Front. Behav. Neurosci. 14:52. doi: 10.3389/fnbeh.2020.00052

Experimental evidence points out that the activation of the endocannabinoid system induces neuroprotective effects and reduces mood disorders. In the hippocampus of patients with mesial temporal lobe epilepsy (MTLE), studies indicated augmented cannabinoid 1 receptor (CB_1R) binding, in spite of its low mRNA and protein expressions. Although this situation suggests an enhanced CB_1R-induced neurotransmission in patients with MTLE, especially those with pharmacoresistant seizures, which present important neuronal damage and high comorbid mood disorders. The present study focused to investigate the status of CB_1R and the endocannabinoid system by obtaining CB_1R-induced G-protein signaling efficacy and measuring the tissue levels of endocannabinoids in the hippocampus and the temporal neocortex of patients with pharmacoresistant MTLE. Furthermore, the obtained results were correlated with comorbid anxiety and depression. The experiments revealed that patients with MTLE present increased CB_1R-induced G-protein signaling efficacy (Emax) as well as an augmented tissue content of anandamide and oleoylethanolamine and low 2-arachidonoylglycerol. Some of these changes were more evident in patients with MTLE without mood disorders. The current findings indicate that pharmacoresistant MTLE is associated with increased CB_1R-induced transductional mechanisms as well as augmented tissue content of specific endocannabinoids in the hippocampus and the temporal neocortex. The enhanced endocannabinoid neurotransmission may be involved in the absence of comorbid mood disorders in some patients with MTLE.

Keywords: mesial temporal lobe epilepsy, anandamide, oleoylethanolamine, 2-arachidonoylglycerol, cannabinoid 1 receptor, anxiety, depression

INTRODUCTION

Endocannabinoids are synthesized "on demand" as a consequence of enhanced neuronal depolarization and elevated intracellular calcium levels (Stella et al., 1997). According to this notion, it is expected that the augmented neuronal depolarization produced during a seizure activity may result in the activation of the endocannabinoid system. This notion is supported by experimental evidence indicating that the tissue levels of endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) were augmented subsequent to the induction of acute seizures and induce neuroprotective effects through the activation of cannabinoid 1 receptors (CB_1Rs) (Marsicano et al., 2003; Wallace et al., 2003). However, drug-naive patients with temporal lobe epilepsy (TLE), a disorder associated with enhanced glutamatergic neurotransmission during ictal and interictal periods (During and Spencer, 1993), present reduced AEA levels in CSF, whereas the 2-AG levels are not modified (Romigi et al., 2010).

Concerning endocannabinoid receptors, studies revealed reduced mRNA and protein expression of CB_1Rs in the hippocampus of patients with pharmacoresistant TLE, especially in glutamatergic axon terminals (Ludányi et al., 2008). In contrast, positron emission tomography (PET) imaging experiments using [^{18}F]MK-9470 indicate that patients with mesial TLE (MTLE) present augmented CB_1R availability in the temporal lobe ipsilateral to the epileptic focus (Goffin et al., 2011). These studies indicate that, in spite of their low mRNA and protein expression, CB_1R binding is enhanced in the brain of patients with MTLE. This situation leads to propose an enhanced CB_1R-induced neurotransmission with subsequent inhibitory and neuroprotective effects in brains with epilepsy. However, this notion is not supported by the progressive and severe hippocampal damage found in patients with pharmacoresistant MTLE that suggests a hypoactive endocannabinoid neurotransmission (Nearing et al., 2007).

A deficient endocannabinoid neurotransmission is also associated with anxiety and depression (Boorman et al., 2016; Korem et al., 2016; Kranaster et al., 2017), whereas the augmentation of endocannabinoids is related with antidepressant effects (Bortolato et al., 2007). Considering that pharmacoresistant MTLE frequently coexists with anxiety and depression (Nogueira et al., 2017), it is possible to suggest that the hypoactivity of the endocannabinoid system may play a significant role in their comorbidity. However, no evidence exists to support this notion.

The present study focused to establish that MTLE is associated with alterations in the endocannabinoid system that facilitate the seizure activity and the comorbid anxiety and depression. Experiments were designed to evaluate the tissue content of endocannabinoids and the transductional mechanisms subsequent to the activation of CB_1Rs in the hippocampus and the temporal neocortex of patients with pharmacoresistant MTLE, with and without anxiety and depression (A/D).

MATERIALS AND METHODS

Patients and Tissue Collection

Hippocampus and temporal neocortex tissues were obtained from 49 patients (29 females and 20 males) with pharmacoresistant MTLE. Every patient underwent an extensive presurgical evaluation, including video electroencephalogram (EEG), magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT) within the Epilepsy Surgery Program of the National Institute of Neurology and Neurosurgery (Instituto Nacional de Neurologia y Neurocirugia "Manuel Velasco Suarez," Mexico). Scalp EEG played an important role in lateralizing and focalizing interictal epileptiform activity. Video-EEG was performed, and at least two complex partial seizures were recorded in each patient. Since we could not perform ictal SPECT in each patient, interictal SPECT offered valuable information regarding the hypoperfusion area (Tae et al., 2005; Huberfeld et al., 2006). MRI performed with either a 1.5- or 3-T machine demonstrated mesial sclerosis and the reduced volume of the temporal pole area of epileptic patients with MTLE, but no significant changes in T2–T3 gyri from all epileptic patients. In addition, MRI findings showed a clear matching with the EEG recordings. Patients with focal cortical dysplasia or neocortical temporal lobe epilepsy were specifically excluded from the study.

During the neurological evaluation, the prevalence of depression and anxiety disorders was established using the Structured Clinical Interview for DSM-IV Axis I (First et al., 1999). A Spanish version of the Hospital Anxiety and Depression Scale (HADS), previously validated in a Spanish population (Herrero et al., 2003; Gómez-Arias et al., 2012), was applied to all patients to identify symptoms of anxiety and/or depression. The HADS scale considers symptoms over the previous week and is not affected by coexisting general medical conditions. Patients with other psychiatric or somatic disturbances interfering with mood disorders, such as addiction, were excluded from the present study. This study was approved by the scientific committees of the institutions involved in the present research, and informed consent was obtained from every patient.

The patients had "en block" anterior lobectomy, ipsilateral to the epileptic focus, at least 48 h after the last seizure. Intraoperative electrocorticography was performed with grids of 4×8 electrodes (Ad-Tech, Racine, WI, United States) in order to identify spiking neocortex. Samples from both the epileptic hippocampus and the spiking T2–T3 gyri (from 2.5 to 5 cm posterior to the temporal pole) were obtained in every patient. Tissue was collected immediately upon resection, quickly frozen in pulverized dry ice, and stored at $-70°C$.

It is known that endocannabinoid AEA and another *N*-acyl ethanolamine oleoylethanolamide (OEA) present a progressive accumulation after death, a condition that correlates with the postmortem interval (Patel et al., 2005; Schmid et al., 2005). Then, biopsies from the hippocampus and the temporal neocortex obtained from seven patients (three males and four females) who had a cerebral lesion without epilepsy (four with tumor and three with vascular malformation) were used as control

condition for endocannabinoid tissue content. These patients had to have a surgical resection of a portion of these brain areas. As control condition for the binding experiments, we used autopsy samples obtained from 11 subjects (seven males and four females) who died by vehicular accident (n = 6), cardiac arrest (n = 4), pneumonia (n = 1), and without history of neurological disease. These autopsy samples were obtained with a postmortem interval of 14.8 ± 0.9 h and immediately stored at −70°C. The fragments from the neocortex included gray matter only.

Evaluation of Endocannabinoid Tissue Content

Endocannabinoids AEA, 2-AG, and OEA were quantified in the brain by liquid chromatography/tandem mass spectrometry as previously described (Cinar et al., 2014). Briefly, brain tissue weighing 100–150 mg was homogenized in 0.5 ml of ice-cold methanol/Tris buffer (50 mM, pH 8.0), 1:1, containing 11.2 ng [$^2H_4$]AEA as internal standard. The homogenates were extracted three times with $CHCl_3$: MeOH (2:1, vol/vol), dried under nitrogen flow, and reconstituted with MeOH after precipitating proteins with ice-cold acetone. The mass spectrometer was set for electrospray ionization operated in positive ion mode. The levels of each compound were analyzed by multiple reactions monitoring. The molecular ion and fragment for each compound were measured as follows: m/z 348.3→62.1 for AEA, m/z 352.3→66.1 for [$^2H_4$]AEA, m/z 326.3→62.1 for OEA, and m/z 379.3→91.1 for 2−AG. The analytes were quantified using MassHunter Workstation LC/QQQ Acquisition and MassHunter Workstation Quantitative Analysis software (Agilent Technologies). The amount of AEA, 2-AG, and OEA in the samples was determined against standard curves. Values are expressed as fmol/mg (AEA and OEA) or pmol/mg (2-AG), respectively.

Analysis of Gi/o Protein Activation by CB1Rs

Membrane Preparations

Crude membrane fraction from human temporal neocortex and hippocampus was prepared according to the method previously described (Benyhe et al., 1997). Briefly, samples (50–100 mg) were homogenized on ice in centrifugation buffer (50 mM Tris HCl, 1 mM EGTA, and 3 mM $MgCl_2$; pH 7.4) using a Teflon glass homogenizer. The homogenate was centrifuged at 20,000 × g for 45 min at 4°C, and the resulting pellet was resuspended in assay buffer (50 mM Tris HCl, 9 mM $MgCl_2$, 0.2 mM EGTA, and 150 mM NaCl; pH 7.4). The centrifugation step was repeated. The final pellet was resuspended in assay buffer and homogenized. Protein levels were determined by the method of Lowry (Lowry et al., 1951). The sample was diluted to a concentration of 2 μg/ml with assay buffer and stored at −70°C until use in the binding assays.

[^{35}S]GTPγS Binding Assay

Receptor-mediated Gi/o protein activation was measured as described previously (Cinar et al., 2008) with slight modifications. Cell membrane fractions (≈10 μg of protein/sample) were incubated at 30°C for 60 min in assay buffer containing 0.1% fatty acid-free bovine serum albumin with GDP (100 μM), [^{35}S]GTPγS (0.05 nM), and increasing concentrations (10^{-9} to 10^{-5} M) of WIN 55212-2 in assay tubes with a final volume of 1 ml. Total binding was measured in the absence of the tested compound. Non-specific binding was determined in the presence of 100 μM unlabeled GTPγS and subtracted from the total binding to calculate the specific binding. The reaction was initiated with incubation at 30°C for 60 min and terminated by the addition of ice-cold wash buffer (50 mM Tris HCl and 5 mM $MgCl_2$; pH 7.4), followed by rapid filtration under vacuum through Whatman GF/B glass-fiber filters. The filters were washed three times with ice-cold wash buffer using Brandel M48 Cell Harvester and then dried, and bound radioactivity was detected in Sigma-Fluor™ Scintillation Cocktail (Sigma) with Beckman LS6000-SC liquid scintillation counter. Stimulation was established as percent of the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity). [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times. Data were subjected to non-linear regression analysis of concentration effect curves performed by Prism (GraphPad Software, Inc.) to determine the potency (EC_{50}) and the maximal stimulation (Emax) values.

Data Analysis

The results were examined statistically by one-way ANOVA and a *post hoc* Tukey test to determine significant differences. Pearson correlation calculations were carried out to identify the influence of clinical factors (age of patients, age at seizure onset, epilepsy duration, and seizure frequency) on the results obtained. Data were expressed as mean ± SME.

RESULTS

Clinical Data

Patients with MTLE without A/D (n = 25) had the following clinical data (mean ± SE): age of subjects, 30.7 ± 1.8 years; age at seizure onset, 10.5 ± 2 years; epilepsy duration, 19.2 ± 2.2 years; and frequency of seizures, 8.5 ± 1.5 per month. Patients with MTLE and comorbid A/D (n = 24) presented similar clinical data (age of subjects, 37.9 ± 1.7 years; age at seizure onset, 10.9 ± 1.4 years; and frequency of seizures, 13.8 ± 4.2 per month) when compared with patients without A/D, except that they presented a longer epilepsy duration (27.1 ± 2.1 years, $p < 0.02$). The age of the patients with cerebral lesion without epilepsy and the autopsy subjects was not significantly different from that of the patients with MTLE (35.2 ± 7.7 and 42.9 ± 6.1 years, respectively; $p > 0.05$).

Endocannabinoids in the Hippocampus and the Temporal Neocortex of Patients With Pharmacoresistant Mesial Temporal Lobe Epilepsy

The tissue levels of endocannabinoids and OEA in the hippocampus and the temporal neocortex of control

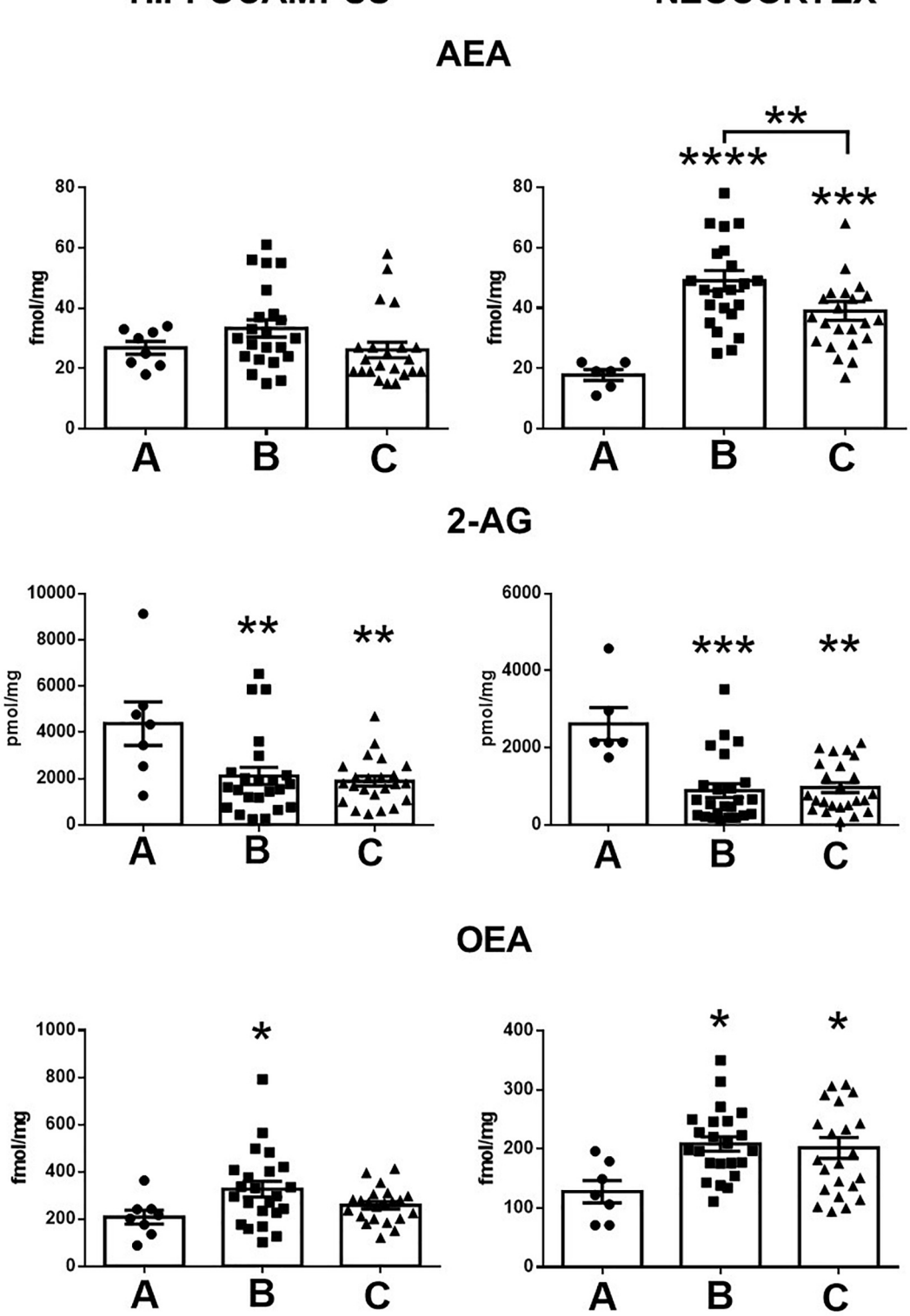


FIGURE 1 | Tissue content of anandamide (AEA), 2-arachidonoylglycerol (2-AG), and oleoylethanolamine (OEA) in the hippocampus and the temporal neocortex of autopsies **(A)** and patients with mesial temporal lobe epilepsy without **(B)** and with comorbid anxiety and depression **(C)**. Values are expressed as mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

subjects showed the following values: AEA, 26.8 ± 2.1 and 17.8 ± 1.8 fmol/mg, respectively; 2-AG, 4378 ± 941 and 2620 ± 423 pmol/mg, respectively; and OEA, 208.9 ± 29 and 127 ± 18 fmol/mg, respectively.

When compared with the control conditions, the hippocampus of patients with MTLE without mood disorders showed a high tissue content of OEA (57%, $p < 0.04$) and low 2-AG tissue levels (51%, $p < 0.005$) (**Figure 1**). The temporal neocortex of these patients presented a high tissue content of AEA and OEA (175%, $p < 0.001$ and 63%, $p < 0.02$, respectively) and a low tissue content of 2-AG (65%, $p < 0.0001$).

In contrast with the control condition, the hippocampus of patients with MTLE plus A/D presented low tissue levels of 2-AG (53%, $p < 0.002$). In the temporal neocortex, experiments revealed a high tissue content of AEA and of OEA (118%, $p < 0.008$ and 58%, $p < 0.04$, respectively) and a low tissue content of 2-AG (62%, $p < 0.0001$).

In contrast to patients with A/D, the tissue levels of AEA in the temporal neocortex of patients with MTLE without comorbid alterations were significantly higher (25%, $p < 0.02$). No further significant differences were found between both groups of patients. In addition, statistical analysis did not reveal significant correlations between the tissue content of endocannabinoids and the clinical factors (**Table 1**).

Gi/o Protein Activation by CB_1Rs in Patients With Mesial Temporal Lobe Epilepsy

In autopsy samples, the binding assay in the presence of WIN 55212-2 revealed a maximal incorporation of [^{35}S]GTPγS (Emax) of 25.5 and 29.6% (hippocampus and neocortex, respectively), with EC_{50} values of 704 ± 127 and 378 ± 64 nM (hippocampus and temporal neocortex, respectively). When compared with the autopsy samples, the tissue from patients with MTLE showed higher Emax values in both the hippocampus (14%, $p < 0.01$) and the temporal neocortex (17%, $p < 0.01$), whereas the EC_{50} values were similar (511 ± 110 and 637 ± 80 nM, respectively) (**Figure 2**).

A comparison of Emax and EC_{50} values according to the presence or the absence of comorbid A/D maintained these significant differences with the autopsies. However, this analysis revealed a higher Emax value in the temporal neocortex of patients with MTLE without A/D. No significant differences were detected between both groups of patients with MTLE (**Figure 3**). In addition, no significant correlations were detected between the Emax values and the clinical factors (**Table 1**).

These results indicate that WIN 55212-2, an agonist for CB_1R and CB_2R (Eissenstat et al., 1995), induces a higher Gi/o protein activation in the tissue obtained from patients with MTLE. In order to identify the contribution of CB_1R and/or CB_2R in this effect, Emax values were obtained in the presence of AM251 and AM630 (antagonists of CB_1R and CB_2R, respectively) at 100 μM. The results obtained revealed that AM251 attenuated the WIN 55212-2-induced efficacy (Emax) in both the hippocampus and the temporal neocortex of patients with MTLE. This effect was not evident with AM630 (**Figure 2**). These findings support that the higher Gi/o protein activation induced by WIN 55212-2 in the tissue of patients with MTLE was mediated by CB_1Rs.

DISCUSSION

The present study revealed a higher CB_1R-induced Gi/o protein activation and significant changes in the tissue content of

TABLE 1 | Correlations between clinical data and parameters evaluated in hippocampus and temporal neocortex of patients with mesial temporal lobe epilepsy with and without mood disorders.

Parameters	Patients	Brain area	Age of patient	Seizure onset age	Duration of epilepsy	Frequency of seizures
AEA	**A/D**	**Hipp**	−0.1500	0.1356	−0.2140	−0.0404
		Cx	−0.1104	0.1527	−0.1988	−0.0009
	No A/D	**Hipp**	−0.0375	0.1084	−0.2451	−0.1648
		Cx	−0.0560	0.1175	−0.2515	−0.1510
2-AG	**A/D**	**Hipp**	0.3291	−0.1964	0.4300	−0.0041
		Cx	0.3405	−0.0656	0.3212	−0.2224
	No A/D	**Hipp**	−0.0819	0.0909	−0.1177	−0.1361
		Cx	0.2408	−0.1382	0.3074	−0.0203
OEA	**A/D**	**Hipp**	−0.1032	0.0891	−0.1426	−0.0823
		Cx	−0.0595	0.0931	−0.1181	0.0135
	No A/D	**Hipp**	−0.0661	−0.0222	−0.0961	−0.0664
		Cx	−0.0544	0.0300	−0.1926	−0.0635
Emax CB_1Rs	**A/D**	**Hipp**	0.3659	0.0233	0.2367	0.0191
		Cx	0.0305	0.5300	−0.4265	0.0474
	No A/D	**Hipp**	−0.4980	−0.3488	−0.0406	−0.0726
		Cx	−0.0362	0.4122	−0.5234	−0.2319

Values represent the Pearson Correlation Coefficients. A/D, patients with mesial temporal lobe epilepsy with anxiety and/or depression; AEA, anandamide; CB_1Rs, cannabinoid 1 receptor; Cx, neocortex; Emax, G-protein signaling efficacy; Hipp, hippocampus; No A/D, patients with mesial temporal lobe epilepsy without anxiety and/or depression; OEA, oleoylethanolamine; 2-AG, 2-arachidonoylglycerol.

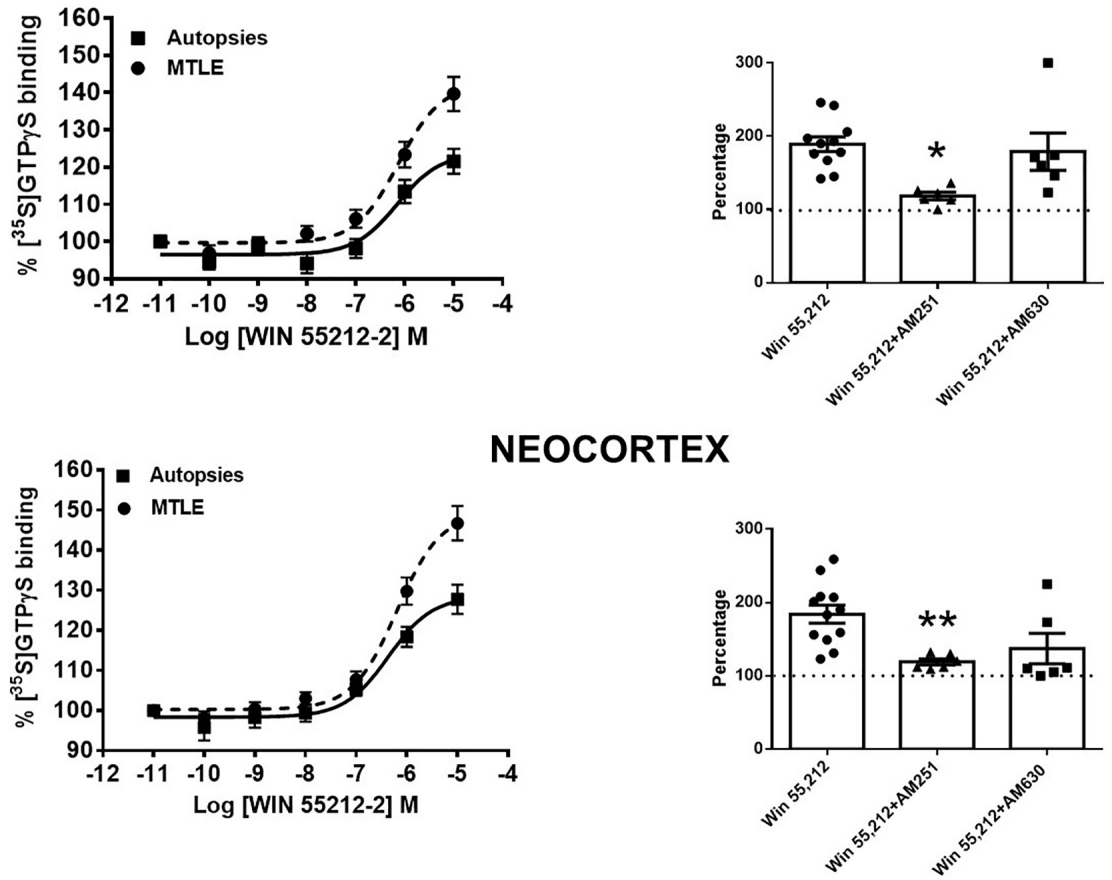


FIGURE 2 | Left side: specific [^{35}S]GTPγS binding to cell membranes obtained from the hippocampus and the temporal neocortex of autopsies and patients with mesial temporal lobe epilepsy (MTLE) as a function of increasing concentrations of WIN 55212-2. Each point represents the mean ± SME of the individual percentage stimulation over the basal values. The absolute basal values from patients with MTLE were similar to those from the autopsy samples. Notice that in patients with MTLE, the [^{35}S]GTPγS binding stimulation by WIN 55212-2 was higher with respect to autopsies. Right side: representation of the maximal stimulation (Emax) values induced by WIN 55212-2 alone and in the presence of an antagonist of CB1Rs (AM251) or CB2Rs (AM630) in the hippocampus and the temporal neocortex of patients with MTLE. Notice that AM251 avoided the augmentation of Emax in both brain areas. Values are expressed as mean ± SE of the individual percentage stimulation over basal values (dotted lines). *$p < 0.05$; **$p < 0.01$.

AEA, OEA, and 2-AG in the epileptic hippocampus and the temporal neocortex of patients with pharmacoresistant MTLE. Some of these changes were more evident in patients without comorbid A/D.

CB_1Rs are involved in the modulation of glutamatergic and GABAergic transmission in the hippocampus and the neocortex (Hoffman et al., 2003; Domenici et al., 2006; Kawamura et al., 2006; Hill et al., 2007). The effects of CB_1Rs depend on their location, i.e., increased CB_1R signaling on glutamatergic terminals induces inhibition and neuroprotective effects, while those located on GABAergic terminals induce excitatory effects (Chiarlone et al., 2014; Guggenhuber et al., 2015). Studies support that the endocannabinoid system induces protective effects in several neurological disorders (Kaur et al., 2016). In neuropathic pain, CB_1Rs along with endocannabinoids are augmented, a situation explained as a compensatory condition (Mitrirattanakul et al., 2006).

In the present study, we found that WIN 55,212-2, a potent agonist for both CB_1R and CB_2R (Eissenstat et al., 1995), induced an overactivation of Gi/o proteins in both the hippocampus and the temporal neocortex of patients with pharmacoresistant MTLE. According to the obtained results, it is possible that the WIN 55,212-2-induced overactivation of Gi/o protein was mediated by CB_1R activation. The increased CB_1R-induced Gi/o protein activation found in the present study correlates with the high CB_1R binding detected by PET in the temporal lobe of patients with MTLE (Goffin et al., 2011). This suggests an increase of neurotransmission mediated by CB_1Rs in these brain areas, in spite of the lower mRNA and protein expressions of CB_1R (Ludányi et al., 2008).

The functional consequence of the increased CB_1Rs-induced Gi/o protein activation in the present study is that endocannabinoid neurotransmission is augmented at brain areas involved in MTLE. This notion was supported by previous

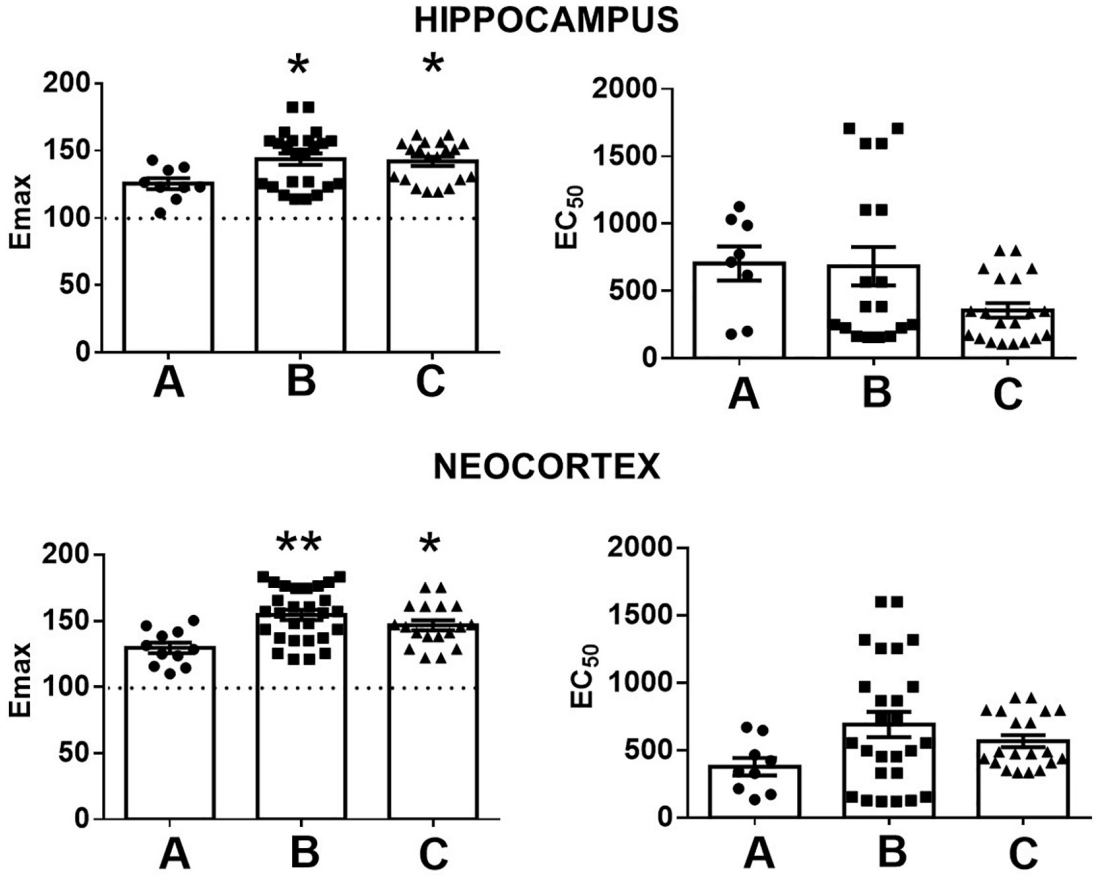


FIGURE 3 | Representation of the maximal stimulation (Emax) and potency (EC_{50}) values induced by WIN 55212-2 in the hippocampus and the temporal neocortex of autopsies **(A)** and patients with mesial temporal lobe epilepsy without **(B)** and with comorbid anxiety and depression **(C)**. Values are expressed as mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$.

studies in which cannabinoid agonists were more effective in suppressing recurrent excitation in the dentate gyrus of animals with augmented expression of CB_1Rs subsequent to epileptic activity rather than in the controls (Bhaskaran and Smith, 2010).

Epilepsy induces a significant reorganization of the CB_1Rs (Falenski et al., 2007). In the hippocampus of patients with pharmacoresistant MTLE, there is a reduction in the number of excitatory synapses, an effect associated with a low expression of CB_1Rs (Ludányi et al., 2008), whereas the inhibitory presynaptic terminals present a high expression of CB_1Rs (Maglóczky et al., 2010). In addition, the hippocampus of patients with MTLE presents reactive astrogliosis, a condition in which endocannabinoid neurotransmission can augment the glutamate release and then promote the seizure activity (Navarrete and Araque, 2010; Coiret et al., 2012). In the neocortex of patients with pharmacoresistant epilepsy, the activation of CB_1Rs inhibits $GABA_A$ receptor-mediated synaptic transmission (Kovacs et al., 2012). Then, it is possible to suggest that the increased CB_1R-induced Gi/o protein activation found in the hippocampus and the temporal neocortex of patients with MTLE reduces the seizure threshold and induces proconvulsant effects.

The blockage or genetic disruption of CB_1Rs induces depression and anxiogenic effects (Navarro et al., 1997; Haller et al., 2004; Christensen et al., 2007; Mikics et al., 2009). An important finding from the present study was that the enhanced CB_1R-induced Gi/o protein activation was similar in patients with MTLE with and without comorbid A/D. These results support that the augmentation in CB_1R-induced transductional mechanisms in the hippocampus and the temporal neocortex of patients with MTLE is not involved in comorbid A/D. However, additional studies are necessary to support this hypothesis.

Concerning endocannabinoid tissue levels, the results of the present study revealed that patients with MTLE present opposite changes, i.e., high levels of AEA and OEA and decreased levels of 2-AG. This condition was more evident in the temporal neocortex. Changes in the opposite direction have been found in the brain areas of patients with schizophrenia (Muguruza et al., 2013). They can be explained as a consequence of the distinct metabolism and the catabolism of each endocannabinoid (Di Marzo and Maccarrone, 2008). In addition, studies support that the augmentation of AEA reduces the levels, metabolism, and effects of 2-AG, an effect mediated by TRPV1 channels (Maccarrone et al., 2008).

AEA and 2-AG are endogenous ligands for CB_1Rs (Hillard, 2000) with anticonvulsant and neuroprotective effects (Wallace et al., 2002; Marsicano et al., 2003; Vilela et al., 2013; Mounsey et al., 2015; Sugaya et al., 2016). The low tissue levels of 2-AG detected in the hippocampus and the temporal neocortex of patients with MTLE can be the consequence of the low expression of the enzyme responsible for its synthesis (diacylglycerol lipase) as found in these subjects (Ludányi et al., 2008). Regarding the AEA, a previous study indicated that the hippocampus of patients with MTLE do not present alterations in the tissue content of this endocannabinoid, a condition associated with the absence of changes in its synthesis and metabolism (Ludányi et al., 2008). The present study supports the absence of alterations in the tissue levels of AEA in the epileptic hippocampus. In contrast, our results revealed an increase of AEA tissue content in the temporal neocortex, a condition that can be associated to the excessive glutamatergic neurotransmission related with epileptic activity (During and Spencer, 1993; Hansen et al., 2001).

It is important to notice that the changes in OEA tissue levels found in the present study were similar to those detected for AEA. This finding can be explained because OEA is a structural analog of AEA. Their synthesis and degradation are controlled by the same enzymes such as *N*-acyl phosphatidylethanoplamine specific phospholipase D and fatty acid amide hydrolase, respectively. It is known that OEA induces anti-inflammatory, neuroprotective, and anti-depressant effects (Antón et al., 2017). Then, the increased tissue content of OEA in both the hippocampus and the temporal neocortex of patients with pharmacoresistant MTLE may represent a mechanism to reduce cell damage.

Experimental evidence indicates that enhanced levels of endocannabinoids induce antidepressive and anxiolytic effects (Kathuria et al., 2003; Hill and Gorzalka, 2005; Yu et al., 2015; Brellenthin et al., 2017; Kranaster et al., 2017; Meyer et al., 2019). Subjects with major depression show low serum levels of endocannabinoids (Hill et al., 2008, 2009), whereas impaired 2-AG signaling in the hippocampus facilitates an anxiety-like behavior (Guggenhuber et al., 2015). In contrast, other studies suggest that the increased endocannabinoid neurotransmission is associated with mood disorders (Vinod et al., 2005). Our results revealed that patients with MTLE without A/D present higher tissue content of OEA in the hippocampus, whereas AEA is augmented in the temporal neocortex, when compared with patients with MTLE and comorbid A/D. The high tissue content of OEA in the hippocampus is in agreement with a previous study indicating that its oral administration induces antidepressant effects associated with the regulation of brain-derived neurotrophic factor levels in the hippocampus (Jin et al., 2015). On the other hand, decreased levels of AEA are associated with depression (Vinod et al., 2012) and anxiogenic effects (Rubino et al., 2008), whereas its augmentation reverses depressive-like responses through the activation of CB_1Rs (de Morais et al., 2016). It is possible that the higher levels of AEA in the temporal neocortex and OEA in the hippocampus of patients with MTLE avoid the comorbid A/D. Additional studies are essential to support this notion.

An important limitation of the present study is the lack of correlation between the results obtained and the conditions that may modify the endocannabinoid system in both autopsies and patients with MTLE. Concerning this issue, it is known that the endocannabinoid system is modified by antiseizure drugs, diets rich in fats and sugars, weight changes, herbal remedies, chronic stress, exercise, and cannabis consumption, among other conditions (McPartland et al., 2014). Unfortunately, these conditions are not considered as criteria for diagnosing and they are not rigorously investigated in autopsies and patients with epilepsy. Further clinical trials are essential to determine if the changes found in the present study are mediated by conditions different from epileptic activity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the National Institute of Neurology and Neurosurgery. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LR designed the study and organized the manuscript. RC carried out the experiments to determine the tissue levels of endocannabinoids. RG-G obtained and evaluated the autopsy samples. MA-V did the neurosurgery of patients. DS-J and IM-J identified and evaluated the patients with epilepsy. JC-C participated in the analysis of the results and organization of the manuscript. FC-C carried out the binding experiments.

FUNDING

This study was supported by the National Council of Science and Technology (CONACyT) (grants A3-S-26782 and 261481). Additional support was given through the postdoctoral fellowship (no. 710924) to JC-C and by the intramural funds of the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health.

ACKNOWLEDGMENTS

We thank Ms. Judith Harvey-White (NIAAA, NIH) for her technical assistance with the LC-MS/MS experiments.

REFERENCES

Antón, M., Alén, F., Gómez de Heras, R., Serrano, A., Pavón, F. J., Leza, J. C., et al. (2017). Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration. *Addict. Biol.* 22, 724–741. doi: 10.1111/adb.12365

Benyhe, S., Farkas, J., Tóth, G., and Wollemann, M. (1997). Met5-enkephalin-Arg6-Phe7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in rat brain. *J. Neurosci. Res.* 48, 249–258. doi: 10.1002/(sici)1097-4547(19970501)48:3<249::aid-jnr7>3.0.co;2-f

Bhaskaran, M. D., and Smith, B. N. (2010). Effects of TRPV1 activation on synaptic excitation in the dentate gyrus of a mouse model of temporal lobe epilepsy. *Exp. Neurol.* 223, 529–536. doi: 10.1016/j.expneurol.2010.01.021

Boorman, E., Zajkowska, Z., Ahmed, R., Pariante, C. M., and Zunszain, P. A. (2016). Crosstalk between endocannabinoid and immune systems: a potential dysregulation in depression? *Psychopharmacology* 233, 1591–1604. doi: 10.1007/s00213-015-4105-9

Bortolato, M., Mangieri, R. A., Fu, J., Kim, J. H., Arguello, O., Duranti, A., et al. (2007). Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biol. Psychiatry* 62, 1103–1110. doi: 10.1016/j.biopsych.2006.12.001

Brellenthin, A. G., Crombie, K. M., Hillard, C. J., and Koltyn, K. F. (2017). Endocannabinoid and mood responses to exercise in adults with varying activity levels. *Med. Sci. Sports Exerc.* 49, 1688–1696. doi: 10.1249/MSS.0000000000001276

Chiarlone, A., Bellocchio, L., Blázquez, C., Resel, E., Soria-Gómez, E., Cannich, A., et al. (2014). A restricted population of CB1 cannabinoid receptors with neuroprotective activity. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8257–8262. doi: 10.1073/pnas.1400988111

Christensen, R., Kristensen, P. K., Bartels, E. M., Bliddal, H., and Astrup, A. (2007). Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. *Lancet* 370, 1706–1713. doi: 10.1016/s0140-6736(07)61721-8

Cinar, R., Freund, T. F., Katona, I., Mackie, K., and Szucs, M. (2008). Reciprocal inhibition of G-protein signaling is induced by CB(1) cannabinoid and GABA(B) receptor interactions in rat hippocampal membranes. *Neurochem. Int.* 52, 1402–1409. doi: 10.1016/j.neuint.2008.02.005

Cinar, R., Godlewski, G., Liu, J., Tam, J., Jourdan, T., Mukhopadhyay, B., et al. (2014). Hepatic CB1 receptors mediate diet-induced insulin resistance by increasing *de novo* synthesis of long chain ceramides. *Hepatology* 59, 143–153. doi: 10.1002/hep.26606

Coiret, G., Ster, J., Grewe, B., Wendling, F., Helmchen, F., Gerber, U., et al. (2012). Neuron to astrocyte communication via cannabinoid receptors is necessary for sustained epileptiform activity in rat hippocampus. *PLoS One* 7:e37320. doi: 10.1371/journal.pone.0037320

de Morais, H., de Souza, C. P., da Silva, L. M., Ferreira, D. M., Baggio, C. H., Vanvossen, A. C., et al. (2016). Anandamide reverses depressive-like behavior, neurochemical abnormalities and oxidative-stress parameters in streptozotocin-diabetic rats: role of CB1 receptors. *Eur. Neuropsychopharmacol.* 26, 1590–1600. doi: 10.1016/j.euroneuro.2016.08.007

Di Marzo, V., and Maccarrone, M. (2008). FAAH and anandamide: is 2-AG really the odd one out? *Trends Pharmacol. Sci.* 29, 229–233. doi: 10.1016/j.tips.2008.03.001

Domenici, M. R., Azad, S. C., Marsicano, G., Schierloh, A., Wotjak, C. T., Dodt, H. U., et al. (2006). Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission. *J. Neurosci.* 26, 5794–5799. doi: 10.1523/JNEUROSCI.0372-06.2006

During, M. J., and Spencer, D. D. (1993). Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet* 341, 1607–1610. doi: 10.1016/0140-6736(93)90754-5

Eissenstat, M. A., Bell, M. R., D'Ambra, T. E., Alexander, E. J., Daum, S. J., Ackerman, J. H., et al. (1995). Aminoalkylindoles: structure-activity relationships of novel cannabinoid mimetics. *J. Med. Chem.* 38, 3094–3105. doi: 10.1021/jm00016a013

Falenski, K. W., Blair, R. E., Sim-Selley, L. J., Martin, B. R., and DeLorenzo, R. J. (2007). Status epilepticus causes a long-lasting redistribution of hippocampal cannabinoid type 1 receptor expression and function in the rat pilocarpine model of acquired epilepsy. *Neuroscience* 146, 1232–1244. doi: 10.1016/j.neuroscience.2007.01.065

First, M. B., Pincus, H. A., and Frances, A. (1999). Another perspective on "putting DSM-IV in perspective". *Am. J. Psychiatry* 156, 499–500. doi: 10.1176/ajp.156.3.499a

Goffin, K., Van Paesschen, W., and Van Laere, K. (2011). In vivo activation of endocannabinoid system in temporal lobe epilepsy with hippocampal sclerosis. *Brain* 134, 1033–1040. doi: 10.1093/brain/awq385

Gómez-Arias, B., Crail-Meléndez, D., López-Zapata, R., and Martínez-Juárez, I. E. (2012). Severity of anxiety and depression are related to a higher perception of adverse effects of antiepileptic drugs. *Seizure* 21, 588–594. doi: 10.1016/j.seizure.2012.06.003

Guggenhuber, S., Romo-Parra, H., Bindila, L., Leschik, J., Lomazzo, E., Remmers, F., et al. (2015). Impaired 2-AG signaling in hippocampal glutamatergic neurons: aggravation of anxiety-like behavior and unaltered seizure susceptibility. *Int. J. Neuropsychopharmacol.* 19:yv091. doi: 10.1093/ijnp/pyv091

Haller, J., Varga, B., Ledent, C., and Freund, T. F. (2004). CB1 cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB1-specific agents. *Behav. Pharmacol.* 15, 299–304. doi: 10.1097/01.fbp.0000135704.56422.40

Hansen, H. H., Schmid, P. C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., et al. (2001). Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo neurodegeneration. *J. Neurochem.* 78, 1415–1427. doi: 10.1046/j.1471-4159.2001.00542.x

Herrero, M. J., Blanch, J., Peri, J. M., De Pablo, J., Pintor, L., and Bulbena, A. (2003). A validation study of the hospital anxiety and depression scale (HADS) in a Spanish population. *Gen. Hosp. Psychiatry* 25, 277–283. doi: 10.1016/s0163-8343(03)00043-4

Hill, E. L., Gallopin, T., Férézou, I., Cauli, B., Rossier, J., Schweitzer, P., et al. (2007). Functional CB1 receptors are broadly expressed in neocortical GABAergic and glutamatergic neurons. *J. Neurophysiol.* 97, 2580–2589. doi: 10.1152/jn.00603.2006

Hill, M. N., Carrier, E. J., McLaughlin, R. J., Morrish, A. C., Meier, S. E., Hillard, C. J., et al. (2008). Regional alterations in the endocannabinoid system in an animal model of depression: effects of concurrent antidepressant treatment. *J. Neurochem.* 106, 2322–2336. doi: 10.1111/j.1471-4159.2008.05567.x

Hill, M. N., and Gorzalka, B. B. (2005). Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test. *Eur. Neuropsychopharmacol.* 15, 593–599. doi: 10.1016/j.euroneuro.2005.03.003

Hill, M. N., Miller, G. E., Carrier, E. J., Gorzalka, B. B., and Hillard, C. J. (2009). Circulating endocannabinoids and N-acylethanolamines are differentially regulated in major depression and following exposure to social stress. *Psychoneuroendocrinology* 34, 1257–1262. doi: 10.1016/j.psyneuen.2009.03.013

Hillard, C. J. (2000). Biochemistry and pharmacology of the endocannabinoids arachidonylethanolamide and 2-arachidonylglycerol. *Prostaglandins Other Lipid Mediat.* 61, 3–18. doi: 10.1016/s0090-6980(00)00051-4

Hoffman, A. F., Riegel, A. C., and Lupica, C. R. (2003). Functional localization of cannabinoid receptors and endogenous cannabinoid production in distinct neuron populations of the hippocampus. *Eur. J. Neurosci.* 18, 524–534. doi: 10.1046/j.1460-9568.2003.02773.x

Huberfeld, G., Habert, M. O., Clemenceau, S., Maksud, P., Baulac, M., and Adam, C. (2006). Ictal brain hyperperfusion contralateral to seizure onset: the SPECT mirror image. *Epilepsia* 47, 123–133. doi: 10.1111/j.1528-1167.2006.00378.x

Jin, P., Yu, H. L., Tian-Lan, Zhang, F., and Quan, Z. S. (2015). Antidepressant-like effects of oleoylethanolamide in a mouse model of chronic unpredictable mild stress. *Pharmacol. Biochem. Behav.* 133, 146–154. doi: 10.1016/j.pbb.2015.04.001

Kathuria, S., Gaetani, S., Fegley, D., Valiño, F., Duranti, A., and Tontini, A. (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nat. Med.* 9, 76–81. doi: 10.1038/nm803

Kaur, R., Ambwani, S. R., and Singh, S. (2016). Endocannabinoid system: a multi-facet therapeutic target. *Curr. Clin. Pharmacol.* 11, 110–117. doi: 10.2174/1574884711666160418105339

Kawamura, Y., Fukaya, M., Maejima, T., Yoshida, T., Miura, E., Watanabe, M., et al. (2006). The CB1 cannabinoid receptor is the major cannabinoid receptor

at excitatory presynaptic sites in the hippocampus and cerebellum. *J. Neurosci.* 26, 2991–3001. doi: 10.1523/JNEUROSCI.4872-05.2006

Korem, N., Zer-Aviv, T. M., Ganon-Elazar, E., Abush, H., and Akirav, I. (2016). Targeting the endocannabinoid system to treat anxiety-related disorders. *J. Basic Clin. Physiol. Pharmacol.* 27, 193–202. doi: 10.1515/jbcpp-2015-0058

Kovacs, F. E., Knop, T., Urbanski, M. J., Freiman, I., Freiman, T. M., Feuerstein, T. J., et al. (2012). Exogenous and endogenous cannabinoids suppress inhibitory neurotransmission in the human neocortex. *Neuropsychopharmacology* 37, 1104–1114. doi: 10.1038/npp.2011.262

Kranaster, L., Hoyer, C., Aksay, S. S., Bumb, J. M., Leweke, F. M., Janke, C., et al. (2017). Electroconvulsive therapy enhances endocannabinoids in the cerebrospinal fluid of patients with major depression: a preliminary prospective study. *Eur. Arch. Psychiatry Clin. Neurosci.* 267, 781–786. doi: 10.1007/s00406-017-0789-7

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.

Ludányi, A., Eross, L., Czirják, S., Vajda, J., Halász, P., Watanabe, M., et al. (2008). Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. *J. Neurosci.* 28, 2976–2990. doi: 10.1523/JNEUROSCI.4465-07.2008

Maccarrone, M., Rossi, S., Bari, M., De Chiara, V., Fezza, F., Musella, A., et al. (2008). Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. *Nat. Neurosci.* 11, 152–159. doi: 10.1038/nn2042

Maglóczky, Z., Tóth, K., Karlócai, R., Nagy, S., Eross, L., Czirják, S., et al. (2010). Dynamic changes of CB1-receptor expression in hippocampi of epileptic mice and humans. *Epilepsia* 51, 115–120. doi: 10.1111/j.1528-1167.2010.02624.x

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208

McPartland, J. M., Guy, G. W., and Di Marzo, V. (2014). Care and feeding of the endocannabinoid system: a systematic review of potential clinical interventions that upregulate the endocannabinoid system. *PLoS One* 9:e89566. doi: 10.1371/journal.pone.0089566

Meyer, J. D., Crombie, K. M., Cook, D. B., Hillard, C. J., and Koltyn, K. F. (2019). Serum endocannabinoid and mood changes after exercise in major depressive disorder. *Med. Sci. Sports Exerc.* 51, 1909–1917. doi: 10.1249/MSS.0000000000002006

Mikics, E., Vas, J., Aliczki, M., Halasz, J., and Haller, J. (2009). Interactions between the anxiogenic effects of CB1 gene disruption and 5-HT3 neurotransmission. *Behav. Pharmacol.* 20, 265–272. doi: 10.1097/FBP.0b013e32832c70b1

Mitrirattanakul, S., Ramakul, N., Guerrero, A. V., Matsuka, Y., Ono, T., Iwase, H., et al. (2006). Site-specific increases in peripheral cannabinoid receptors and their endogenous ligands in a model of neuropathic pain. *Pain* 1268, 102–114. doi: 10.1016/j.pain.2006.06.016

Mounsey, R. B., Mustafa, S., Robinson, L., Ross, R. A., Riedel, G., Pertwee, R. G., et al. (2015). Increasing levels of the endocannabinoid 2-AG is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Exp. Neurol.* 273, 36–44. doi: 10.1016/j.expneurol.2015.07.024

Muguruza, C., Lehtonen, M., Aaltonen, N., Morentin, B., Meana, J. J., and Callado, L. F. (2013). Quantification of endocannabinoids in postmortem brain of schizophrenic subjects. *Schizophr. Res.* 148, 145–150. doi: 10.1016/j.schres.2013.06.013

Navarrete, M., and Araque, A. (2010). Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron* 68, 113–126. doi: 10.1016/j.neuron.2010.08.043

Navarro, M., Hernández, E., Muñoz, R. M., Del Arco, I., Villanua, M. A., Carrera, M. R., et al. (1997). Acute administration of the CB1 cannabinoid receptor antagonist SR 141716A induces anxiety-like responses in the rat. *Neuroreport* 8, 491–496. doi: 10.1097/00001756-199701200-00023

Nearing, K., Madhavan, D., and Devinsky, O. (2007). Temporal lobe epilepsy: a progressive disorder? *Rev. Neurol. Dis.* 4, 122–127.

Nogueira, M. H., Yasuda, C. L., Coan, A. C., Kanner, A. M., and Cendes, F. (2017). Concurrent mood and anxiety disorders are associated with pharmacoresistant seizures in patients with MTLE. *Epilepsia* 58, 1268–1276. doi: 10.1111/epi.13781

Patel, S., Carrier, E. J., Ho, W. S., Rademacher, D. J., Cunningham, S., Reddy, D. S., et al. (2005). The postmortal accumulation of brain N-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *J. Lipid Res.* 46, 342–349. doi: 10.1194/jlr.M400377-JLR200

Romigi, A., Bari, M., Placidi, F., Marciani, M. G., Malaponti, M., Torelli, F., et al. (2010). Cerebrospinal fluid levels of the endocannabinoid anandamide are reduced in patients with untreated newly diagnosed temporal lobe epilepsy. *Epilepsia* 51, 768–772. doi: 10.1111/j.1528-1167.2009.02334.x

Rubino, T., Realini, N., Castiglioni, C., Guidali, C., Viganó, D., Marras, E., et al. (2008). Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb. Cortex* 18, 1292–1301. doi: 10.1093/cercor/bhm161

Schmid, P. C., Krebsbach, R. J., Perry, S. R., Dettmer, T. M., Maasson, J. L., and Schmid, H. H. (2005). Occurrence and postmortem generation of anandamide and other long-chain N-acylethanolamines in mammalian brain. *FEBS Lett.* 375, 143–147. doi: 10.1016/0014-5793(95)01194-j

Stella, N., Schweitzer, P., and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388, 773–778. doi: 10.1038/42015

Sugaya, Y., Yamazaki, M., Uchigashima, M., Kobayashi, K., Watanabe, M., Sakimura, K., et al. (2016). Crucial roles of the endocannabinoid 2-arachidonoylglycerol in the suppression of epileptic seizures. *Cell Rep.* 16, 1405–1415. doi: 10.1016/j.celrep.2016.06.083

Tae, W. S., Joo, E. Y., Kim, J. H., Han, S. J., Suh, Y. L., Kim, B. T., et al. (2005). Cerebral perfusion changes in mesial temporal lobe epilepsy: SPM analysis of ictal and interictal SPECT. *Neuroimage* 24, 101–110. doi: 10.1016/j.neuroimage.2004.08.005

Vilela, L. R., Medeiros, D. C., Rezende, G. H., de Oliveira, A. C., Moraes, M. F., and Moreira, F. A. (2013). Effects of cannabinoids and endocannabinoid hydrolysis inhibition on pentylenetetrazole-induced seizure and electroencephalographic activity in rats. *Epilepsy Res.* 104, 195–202. doi: 10.1016/j.eplepsyres.2012.11.006

Vinod, K. Y., Arango, V., Xie, S., Kassir, S. A., Mann, J. J., Cooper, T. B., et al. (2005). Elevated levels of endocannabinoids and CB1 receptor-mediated G-protein signaling in the prefrontal cortex of alcoholic suicide victims. *Biol. Psychiatry* 57, 480–486. doi: 10.1016/j.biopsych.2004.11.033

Vinod, K. Y., Xie, S., Psychoyos, D., Hungund, B. L., Cooper, T. B., and Tejani-Butt, S. M. (2012). Dysfunction in fatty acid amide hydrolase is associated with depressive-like behavior in Wistar Kyoto rats. *PLoS One* 7:e36743. doi: 10.1371/journal.pone.0036743

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and DeLorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920

Wallace, M. J., Martin, B. R., and DeLorenzo, R. J. (2002). Evidence for a physiological role of endocannabinoids in the modulation of seizure threshold and severity. *Eur. J. Pharmacol.* 452, 295–301. doi: 10.1016/s0014-2999(02)02331-2

Yu, H. L., Sun, L. P., Li, M. M., and Quan, Z. S. (2015). Involvement of norepinephrine and serotonin system in antidepressant-like effects of oleoylethanolamide in the mice models of behavior despair. *Neurosci. Lett.* 593, 24–28. doi: 10.1016/j.neulet.2015.03.019

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

frontiers in Behavioral Neuroscience

REVIEW
published: 13 November 2020
doi: 10.3389/fnbeh.2020.603245

The Endocannabinoid System Activation as a Neural Network Desynchronizing Mediator for Seizure Suppression

*Daniel de Castro Medeiros[1], Vinícius Rosa Cota[2], Antonio Carlos P. Oliveira[3], Fabricio A. Moreira[3] and Márcio Flávio Dutra Moraes[1,4]**

[1] Núcleo de Neurociências, Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [2] Laboratório Interdisciplinar de Neuroengenharia e Neurociências, Departamento de Engenharia Elétrica, Universidade Federal de São João Del-Rei, São João Del-Rei, Brazil, [3] Departamento de Farmacologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [4] Centro de Tecnologia e Pesquisa em Magneto Ressonância, Programa de Pós-Graduação em Engenharia Elétrica, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

OPEN ACCESS

Edited by:
Dolores E. López García, University of Salamanca, Spain

Reviewed by:
Carlos De Cabo, Complejo Hospitalario Universitario de Albacete, Spain
Salim Yalcin Inan, Meram Faculty of Medicine, Turkey

***Correspondence:**
Márcio Flávio Dutra Moraes
mfdm@ufmg.br;
mfdm@icb.ufmg.br

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *05 September 2020*
Accepted: *20 October 2020*
Published: *13 November 2020*

Citation:
Medeiros DC, Cota VR, Oliveira ACP, Moreira FA and Moraes MFD (2020) The Endocannabinoid System Activation as a Neural Network Desynchronizing Mediator for Seizure Suppression. Front. Behav. Neurosci. 14:603245. doi: 10.3389/fnbeh.2020.603245

The understanding that hyper-excitability and hyper-synchronism in epilepsy are indissociably bound by a cause-consequence relation has only recently been challenged. Thus, therapeutic strategies for seizure suppression have often aimed at inhibiting excitatory circuits and/or activating inhibitory ones. However, new approaches that aim to desynchronize networks or compromise abnormal coupling between adjacent neural circuitry have been proven effective, even at the cost of enhancing local neuronal activation. Although most of these novel perspectives targeting circuitry desynchronization and network coupling have been implemented by non-pharmacological devices, we argue that there may be endogenous neurochemical systems that act primarily in the desynchronization component of network behavior rather than dampening excitability of individual neurons. This review explores the endocannabinoid system as one such possible pharmacological landmark for mimicking a form of "on-demand" desynchronization analogous to those proposed by deep brain stimulation in the treatment of epilepsy. This essay discusses the evidence supporting the role of the endocannabinoid system in modulating the synchronization and/or coupling of distinct local neural circuitry; which presents obvious implications on the physiological setting of proper sensory-motor integration. Accordingly, the process of ictogenesis involves pathological circuit coupling that could be avoided, or at least have its spread throughout the containment of other areas, if such endogenous mechanisms of control could be activated or potentiated by pharmacological intervention. In addition, we will discuss evidence that supports not only a weaker role played on neuronal excitability but the potential of the endocannabinoid system strengthening its modulatory effect, only when circuitry coupling surpasses a level of activation.

Keywords: epilepsy, cannabinoid system, neural synchrony oscillations, deep brain electrical stimulation, network decoupling, pharmacological treatment

INTRODUCTION

Epilepsy is a severe brain disorder intimately associated with excessive neural excitability and synchrony whose treatment is still limited to a few pharmacological and non-pharmacological approaches (ketogenic diets, surgery; Devinsky et al., 2018; Loscher, 2020). The antiepileptic drugs primarily aim to reduce epileptic seizure occurrence by restraining the neuronal activity (reducing the excitatory or increasing the inhibitory transmission); thus, somehow, considering that network desynchronization would follow as a natural consequence of diminished excitability. Nevertheless, even with the substantial pharmacological arsenal available, drug treatment is still insufficient to ameliorate the symptoms and the course of the disease in some patients (refractory epilepsies; Perucca and Gilliam, 2012). Thus, new paradigms and strategies should be considered when approaching the neurobiology of epilepsy and developing new therapeutic interventions. This review will focus on the hypothesis that the endocannabinoid system can mediate epileptic seizure suppression by desynchronizing the neural networks rather than acting only at the excitation/inhibition balance.

Cannabinoids and the Endocannabinoid System

The use of the herb *Cannabis sativa* ("marijuana") for the treatment of epilepsy has been suggested for centuries (Zuardi, 2006). However, its clinical application was limited by its psychotropic effects, abuse liability, and the fact that its chemical composition remained unidentified until recently. Only in the second half of the twentieth century were its constituents, termed phytocannabinoids, finally characterized. Cannabis's primary active substance is delta-9-tetrahydrocannabinol (THC; Mechoulam, 1970). However, various other compounds are interesting from a pharmacological standpoint, including cannabidiol (CBD), delta-9-tetrahydrocannabivarin, cannabidivarin, among others (Hill et al., 2013; Patra et al., 2019).

The chemical characterization of cannabis and THC isolation and synthesis has made it possible to obtain numerous synthetic derivatives (i.e., synthetic cannabinoids). The pharmacological studies with phytocannabinoids and synthetic cannabinoids finally led to the identification of their mechanisms of action and to the description of a new signaling mechanism in the brain, the endocannabinoid system (Pertwee et al., 2010; **Figure 1**). The endocannabinoid system comprises the Gi-coupled cannabinoid receptors CB_1 and CB_2 (the molecular targets of THC), the endogenous ligands (endocannabinoids) arachidonoyl ethanolamide (AEA, also anandamide) and 2-arachidonoylglycerol (2-AG), and the enzymes responsible for their metabolism. Endocannabinoids are proposed to function as a retrograde neurotransmission system, being produced from lipid membranes in postsynaptic neurons. Their actions are terminated after they are removed from the synaptic cleft by a membrane transporter and hydrolyzed in the intracellular medium (Pertwee et al., 2010; Mechoulam et al., 2014). The main enzymes responsible for metabolizing anandamide and 2-AG are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. Other enzymes also contribute to the biotransformation of endocannabinoids in the brain, such as cyclo-oxygenase 2 (COX-2), and alpha/beta-Hydrolase domain containing 6 (ABHD6). Additional receptors have also been described for endocannabinoids, among them the transient receptor potential vanilloid-1 channel (TRPV1), which can be activated by endogenous anandamide (Mechoulam et al., 2014).

In terms of clinical applications, THC and synthetic cannabinoids are of limited use as they can induce psychosis, abuse liability, amnesia, hyperphagia, and motor impairment. In fact, direct CB_1 receptor agonists may even induce or aggravate epileptic seizures, depending on the dose (Asth et al., 2019). CBD, on the other hand, has been demonstrated to be efficacious in reducing epileptic seizures in both experimental and clinical settings (Billakota et al., 2019). Contrary to THC, CBD does not act as a CB_1 receptor agonist; instead, its antiepileptic activity seems to occur by inhibiting anandamide reuptake and hydrolysis, increasing the brain levels of this endocannabinoid and thereby activating CB_1 receptor signaling (Vilela et al., 2013). Accordingly, CBD antiepileptic effects depend on the PI3K/mTOR intracellular pathway, a signal transduction mechanism coupled to the CB_1 receptor (Gobira et al., 2015; Lima et al., 2020). Other phytocannabinoids have been reported as potential antiepileptic drugs, among them, cannabidivarin, and delta-9-cannabivarin (Hill et al., 2010, 2013).

Cannabinoid on-Demand and Circuit Breaker Functions

The detailed understanding of the physiological aspects of the endocannabinoid system has offered new pharmacological possibilities beyond the phytocannabinoids. Evidence converging from various experimental approaches suggest that endocannabinoid synthesis can be triggered by post-synaptic neurons in response to calcium influx after excessive glutamate release and neuronal excitability. Once in the synaptic cleft, they bind to presynaptic CB_1 receptors whose activation restrains hyperexcitability and attenuate neurotransmitter release, therefore working as a negative feedback mechanism modulating synaptic transmission (Maejima et al., 2001; Wilson and Nicoll, 2001). Remarkably, seizure-inducing substances increase anandamide levels in the hippocampus and have their effect magnified by CB_1 receptor blockade (Marsicano et al., 2003; Wallace et al., 2003). Based on these observations, endocannabinoids have been proposed to function as an on-demand mechanism protecting the brain against hyperexcitability and activity-dependent excitotoxicity (Marsicano et al., 2003). The molecular mechanisms at the synaptic levels possibly entail presynaptic glutamate release followed by calcium-triggered endocannabinoid synthesis and release from the post-synaptic terminal; endocannabinoids activate presynaptic CB_1 receptor, which activates a Gi-protein and triggers an intracellular cascade whose consequence is a reduction in calcium influx and glutamate release. Altogether, this mechanism would work as a synaptic circuit breaker (Katona and Freund, 2008; Katona, 2015; Soltesz et al., 2015).

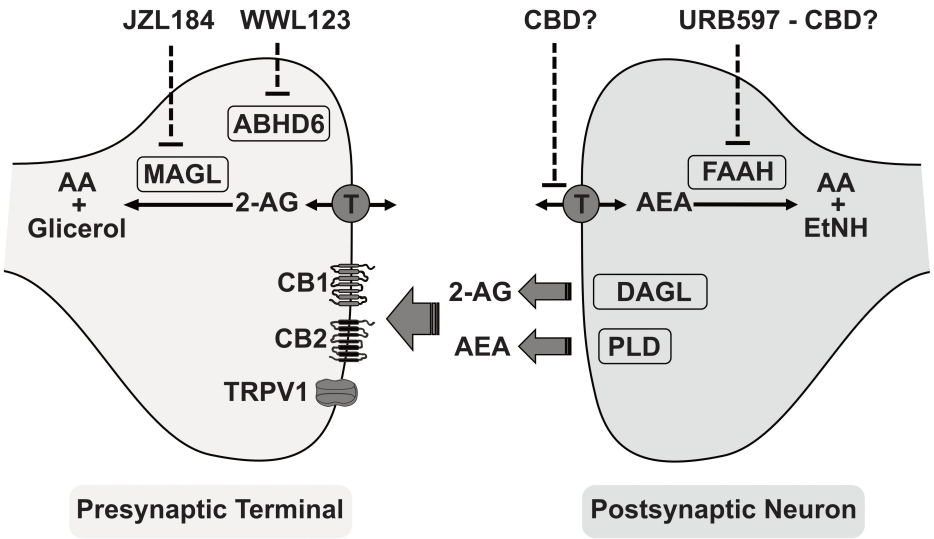


FIGURE 1 | A simplified view of the endocannabinoid system and its main components. Endocannabinoids: Arachidonoylethanolamide (AEA, anandamide) and 2-arachidonoylglicerol (2-AG). Synthesizing enzymes: Diacylglycerol lipase (DAGL) and phospholipase-D (PLD). Membrane transporter (T). Cannabidiol (CBD) as inhibitor of anandamide reuptake and hydrolysis. Hydrolyzing enzymes: Monoacylglycerol lipase (MAGL), alpha/beta-Hydrolase domain containing 6 (ABHD6), and fatty acid amide hydrolase (FAAH). Enzymes inhibitors: JZL184 irreversible inhibitor for MAGL, URB597 relatively selective inhibitor of FAAH, WWL123 inhibitor of ABHD6. Receptors: cannabinoid type-1 (CB1), cannabinoid type-2 (CB2), and transient receptor potential vanilloid-1 (TRPV1). AEA metabolites: arachidonic acid (AA) and ethanolamide (EtNH). 2-AG metabolites: arachidonic acid (AA) and glicerol.

These unique characteristics point to the endocannabinoid system as an attractive target for pharmacological intervention for the treatment of epilepsies. Theoretically, the selective inhibition of endocannabinoid-hydrolyzing enzymes could work with anatomical and temporal resolution, restraining synaptic activity only under circumstances in which excessive activity (excitotoxicity) would occur. In line with this hypothesis, synthetic compounds that inhibit the enzymes responsible for degrading anandamide and 2-AG yields favorable results in experimental models of seizure and epilepsy. Concerning anandamide hydrolysis, the selective FAAH inhibitor AM374 inhibits kainic acid-induced seizure and neurotoxicity (Karanian et al., 2007). Moreover, the FAAH inhibitor URB597 increases the threshold of pentylenetetrazole-induced behavioral and electroencephalographic seizures (Vilela et al., 2013). As for the 2-AG-related enzymes, ABHD-6 inhibition also reduces PTZ-induced seizures (Naydenov et al., 2014), whereas MAGL inhibition delays the consequences of kindling induced by electrical stimulation (ES) of the amygdala (von Rüden et al., 2015). Importantly, endocannabinoid hydrolysis inhibitors tend to have a safer pharmacological profile as compared to direct CB_1 agonists, as they seem less prone to induce psychosis, motor impairment, and addiction, which can be attributed to the on-demand functioning of endocannabinoids (Asth et al., 2019).

Therefore, the endocannabinoid receptors and molecules deal with the brain hyperexcitability, one crucial aspect of epilepsy, by modulating the synaptic transmission in a neural activity-dependent manner. Nevertheless, recent studies have demonstrated that the cannabinoid system also modifies the neural synchrony, in some cases with a marginal effect on overall excitability, which is intimately associated with complex brain functions (e.g., sensation, perception, and cognition) and neurological disorders as schizophrenia, Alzheimer's disease, and epilepsy (Uhlhaas and Singer, 2006).

Epilepsy as a Network Dysfunction and Hypersynchronous Disease

Although synchronization and hypersynchronization are largely used to describe neural phenomena in general and epilepsy in particular, these terms are somehow loosely defined in the literature. In a system containing multiple oscillating subsystems such as the brain, synchronism can be described as a driving influence of an oscillator toward another one (Jensen and Colgin, 2007). This means that objective dynamical descriptors (e.g., amplitude, phase, and frequency, etc.) will display a mathematical relation of the kind $y = f(x)$ between oscillations if they are synchronized. Given the timescale of neural events of interest (in the order of milliseconds), electrographic recordings such as scalp electroencephalogram (EEG) in humans or intracranial local field potentials (LFP) in experimental animals are the main choice for objectively assessing neurodynamical synchronism underlying brain function and disease. Myriad approaches have been used to perform such investigation of EEG and LFP signals, ranging from assessment of occurrence and temporal coincidence of meaningful electrographic signatures by visual inspection to advanced computerized mathematical analyses such as cross-correlation, coherence, partial directed coherence, Granger causality, mutual information, phase lock value, and cross-frequency phase-amplitude coupling (CFC; Quian Quiroga et al., 2002; Kreuz et al., 2007).

In this perspective, while normal levels of synchronism between neural structures underlie brain function (Schnitzler and Gross, 2005; Womelsdorf et al., 2007), aberrations lead to dysfunction (Uhlhaas and Singer, 2006). For instance, it is now well-established that consolidation of declarative memory largely relies on triple phase-amplitude coupling between cortico-cortical slow oscillations, thalamocortical spindles, and hippocampal ripples across the sleep-wake cycle (Klinzing et al., 2019). In contrast, epilepsy is understood as a disease of hypersynchronization; a rationale supported by an ever-increasing number of experimental observations. Starting from the occurrence of highly-synchronous paroxysms such as epileptiform polyspikes (Wu et al., 2013), hypersynchronization can also be evidenced by the temporal and spatial pattern of spread of aberrant activity across nodes of ictogenic networks involving the hippocampus, amygdala, and parahippocampal areas in Temporal Lobe Epilepsy (TLE) and also midbrain and hindbrain structures in generalized tonic-clonic seizures (de Curtis and Avanzini, 2001; Avoli et al., 2002; de Guzman et al., 2004; Moraes et al., 2005a; Bertram, 2013). In the same vein, modifications in the expression of electrographic activity induced by manipulations of neural circuitry (lesions and transections) are additional proof of network synchronization underlying epileptic phenomena (Imamura et al., 2001; Moraes et al., 2005b). Finally, increases in phase-amplitude CFC between different pairs of band frequencies (Nariai et al., 2011; Guirgis et al., 2013; Edakawa et al., 2016) and *in silico* findings from non-linear dynamics analysis (Kalitzin et al., 2019) further corroborate this view. It is important to highlight, though, that hypersynchronization is not ubiquitous during ictogenesis, and there has also been evidence of desynchronization, at least in specific areas, frequencies, and time points of the process (Netoff and Schiff, 2002; Jiruska et al., 2013).

Not only epileptic phenomena have been quantitatively studied by this measure, but also novel therapeutic interventions (pharmacological or not) are screened according to their effects on synchronization levels of brain signals. For instance, different modalities of Deep Brain Stimulation (DBS) have been found, among other effects, to suppress aberrant oscillations while inducing beneficial rhythms (Udupa and Chen, 2015). In fact, the DBS delivered by a responsive neurostimulation system (RNS^{TM} System, NeuroPace, Inc.) to patients with epilepsy acutely suppressed gamma frequency (35–100 Hz) phase-locking (Sohal and Sun, 2011). Using eigenvalue dynamics computed over cross-correlation matrices, Schindler and colleagues (2007) have also found that EEG synchronization levels depend on parameter settings of low-frequency stimulation of the seizure onset zone in humans (Schindler et al., 2007).

Of particular interest here, some ES approaches have been tailored to specifically tackle synchronization as a means to treat epilepsy in further corroboration of the notion of anticonvulsant effects of desynchronization. A non-standard form of low-frequency stimulation (four pulses per second in average) with randomized intervals between pulses, termed non-periodic stimulation (NPS) and devised by our group, has been shown to effectively suppress acute seizures induced by PTZ (Cota et al., 2009) and in chronic seizures induced in the late phase of the pilocarpine model of TLE (de Oliveira et al., 2014). Electrographically, NPS has been shown to rectify spectral signatures (de Souza Silva et al., 2019) and possibly to decrease the duration of epileptiform activity, the number and the frequency of epileptiform spikes (de Oliveira et al., 2019). An approach very similar to NPS termed Temporally Irregular DBS (TiDBS) has been used to effectively impair epileptogenesis induced by amygdalar kindling, shortening daily afterdischarge duration, and interfering with propagation patterns of epileptiform activity (Santos-Valencia et al., 2019). Other forms of desynchronizing ES have been used to suppress seizures or decrease cortical excitability, with correlated electrographical findings (Quinkert et al., 2010; Wyckhuys et al., 2010). In fact, the temporal pattern of ES is now considered to have a central role in the modulation of neuronal activity (Zheng et al., 2020) and to suppress aberrant synchronization in epilepsy and many other neurological disorders (Grill, 2018).

From this set of findings, one can conclude that assessing complex epileptic phenomena in the network level alongside its emerging properties such as synchronization (Garcia-Cairasco, 2009) may represent not only a fruitful approach to understand the pathophysiology of epilepsy, but also to develop novel treatment (pharmacological or not) in an engineered and thus efficacious way (Sunderam et al., 2010). This is exactly the venue this review explores, associating the on-demand endocannabinoid system and pharmacological targets to its ability to modulate coupling among distinct network oscillators without necessarily dampening individual neuronal activity itself. This framework is further explored in the following sections.

Endocannabinoid System Diminishes the Neural Organization

Fluctuations in the electrical field potential (LFP) are permanently present at the cerebral extracellular medium, reflecting the alternating pockets of higher/lower recruitment probability of localized population of neurons (Buzsáki et al., 2012). The pace activity offers a temporal-organized framework for neural communication (Fries, 2005; Buzsáki, 2010), and both local and distant neuronal ensembles (task-demanding cells that fire in a constricted window) transiently synchronize the oscillatory activity during information processing (O'Keefe and Recce, 1993; Varela et al., 2001; Bosman et al., 2012). Disturbance in the fine-tuning of the network time-coupling [mainly regulated by inhibitory synapses (Buzsáki and Chrobak, 1995; Whittington et al., 2000)] is associated with cognitive disorders and neurological pathologies (Uhlhaas and Singer, 2012). Important to note that the cannabinoid receptors are the most abundant G protein-coupled receptor in the brain and present at GABAergic and glutamatergic axon terminals (Devane et al., 1988), but up to ten times more prevalent at the former (Kawamura et al., 2006). Hence, alterations at the cannabinoid system may potentially perturb the neural connections and, consequently, the coupling and generation of oscillatory patterns related to physiological functions.

The LFP brain oscillations range from very slow (<0.01 Hz) to ultrafast frequencies (200–600 Hz), and distinct band rhythms

become prominent when cerebral structures engage in specific tasks (Buzsáki and Draguhn, 2004). Perceptual functions are closely related to gamma oscillations (30–80 Hz) and involve timed interaction of distributed neural groups (Fries, 2005, 2009). Even though gamma rhythm typically emerges from local networks, its remote synchronization can be performed by long-distant neurons and by the interaction with slower frequencies that modulate activity over extensive spatial regions (Buzsáki et al., 2013), i.e., phase coding. Gamma synchrony is considered an essential mechanism for binding sensory features in sparse structures, an element present in consciousness, and modifications in this rhythm may underline perceptual disturbances seen in psychosis (Fries, 2005, 2009; Uhlhaas and Singer, 2006; Wang, 2010). Schizophrenic subjects present deficits in the perceptual organization, correlated with the reduction of gamma power and synchrony over distributed areas (Uhlhaas et al., 2006). Similarly, healthy humans administered with CB1R agonist (THC) exhibit psychosis-relevant effects associated with gamma oscillation disorder (coherence reduction during auditory evoked response test; Cortes-Briones et al., 2015). *In vitro* and *in vivo* animal investigations additionally demonstrated that CB1-agonist disturbs gamma rhythm in limbic system areas (reduction synchrony and power, respectively) and impair auditory processing (sensory gating), acting mainly in GABAergic synapses (Hájos et al., 2000, Hajós et al., 2008). Nonetheless, the disturbance induced by cannabinoids expands beyond gamma and perceptual functions, also affecting low frequency generation, phase-coding and other cognitive faculties.

Compared with faster frequencies, slow oscillatory rhythms are associated with a more extensive brain volume alteration, longer time-window discharging probability and, the integration of a significant higher number of neurons (von Stein and Sarnthein, 2000; Quilichini et al., 2010; Buzsáki and Wang, 2012). Of particular interest to phenomena involving the function/dysfunction of the hippocampus (i.e., memory/TLE, respectively), the slow frequency theta rhythm (4–12 Hz) is related to the temporal organization of faster frequencies (e.g., gamma-band by cross-frequency coupling) and the coordination of local and distant unit-firing (Mizuseki et al., 2009; Quilichini et al., 2010; Lisman and Jensen, 2013). Human and animal studies have shown the effect of potentiating the cannabinoid system in theta oscillations disruption and memory impairment. Morrison et al. demonstrated that healthy patients administered with CB1 agonist (THC) performed poorly at working-memory tests and presented a reduction of theta power and coherence in frontal lobe electrodes. Importantly, the disrupting of network dynamics, revealed by coherence diminishing, correlated with positive psychotic symptoms (Morrison et al., 2011). CB1R activation (CP55940-potent agonist) also disturbed the synchrony of the rats' neural oscillations at the medial prefrontal cortex (mPFC) and hippocampus during end-to-end T-maze spatial working memory task. In addition, animals presented power reduction in the gamma-band at the mPFC and in the theta-band at the hippocampus, decreasing the theta coherence between hippocampus-mPFC. This work also showed a substantial compromise of the prefrontal unit phase-locking activity to the hippocampus theta rhythm, which correlates to reduced cognitive performance (Kucewicz et al., 2011). Robe et al. also demonstrated that the CB1 agonist CP55940 decreased hippocampal theta power (in freely moving rats) associated with memory impairment. Interestingly, the activation of CB1R occasioned a severe disruption of cell assembly time coordination. However, there was no spatial remap of the place cells and only a marginal reduction in fire rate and no correlation with LFP power change. The authors argued that the CB1 dyssynchrony-effect might be the origin of theta power reduction, since the excitatory/inhibitory firing rate balance was minimally affected (Robbe et al., 2006; Robbe and Buzsáki, 2009). If this claim is valid, the CB1-induced disruption of the time organization may play a far more important role at the network synchrony than on overall network excitability – which would explain behavioral disturbances and cognitive impairment. Additionally, it could contribute to the LFP power decrease seen in several structures, as described previously.

Despite the rhythm disordering in healthy subjects, the CB activation may be beneficial to neural networks prone to develop hypersynchronous state. The synchrony reduction caused by CBR1 could balance the abnormal coupling among microcircuits present in epileptic brains, decreasing the pathological oscillatory attractor-effect, and, consequently, the occurrence of the seizures. Important to note, the endocannabinoid system offers an "on-demand" approach, with a major effect in excessively active neural ensembles [higher coupling probability (Kudela et al., 2003)], which is quite suitable for long-term treatments.

Cannabinoid Activity on Inhibitory Cells as a Mechanism for Neural Network Desynchrony and Seizure Suppression

Although pertaining to the same neurochemical system, targeting directly CB1 receptors or the anabolic/catabolic pathways of endocannabinoid metabolism are fundamentally very different approaches. The most noticeable particularity of this specific neurochemical system, as shown in previous sections of this review, is its ability to promote homeostatic modulation of synaptic activity by targeting the presynaptic neuron, through a feedback mechanism. And, such backward modulation is mostly triggered by "abnormal" or excessive postsynaptic activation, that, consequently, promotes increased endocannabinoid release aimed at presynaptic receptors (**Figure 2**). It should be clear that such "on-demand" recruitment of synaptic homeostatic modulation would be lost if pharmacological agents were to target the receptors directly [for review see Katona and Freund (2008)].

The quasi-specific co-expression of CB1 receptors in CCK GABAergic interneurons may help explain the endocannabinoid system's prominent role in regulating coupling-strength between neuronal oscillators rather than on the hippocampal network excitability itself (Katona et al., 1999). Evidence shows that parvalbumin-positive GABAergic interneurons (PV^+) are fast-spiking, create time-delimited pockets of oscillations – sometimes referred to as the hippocampal "clocks," but are also involved in very strong lateral inhibition modulation of similar feedforward/feedback microcircuit motifs within the hippocampus. Altogether, PV^+ seems to promote an efficient

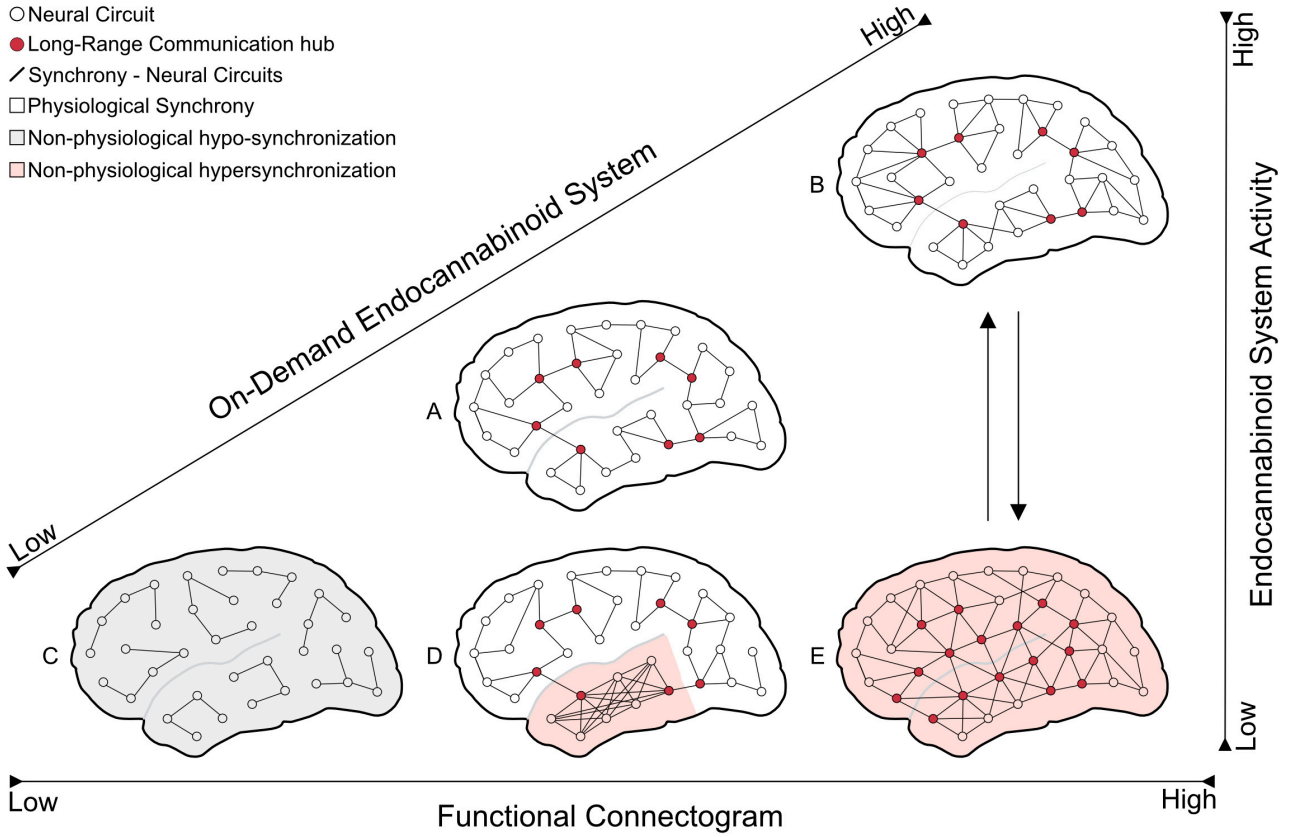


FIGURE 2 | The schematic model depicts the endocannabinoid system's hypothetical effect on network synchrony at different functional connectogram levels. The on-demand endocannabinoid system activity would suppress the pathological excessive neural synchrony (seizure) and maintain the brain in physiological conditions even in functional connectogram fluctuations. **(A)** brain in the physiological resting state; **(B)** brain in the physiological state of a hard mental workload (increasing of neural network synchronization compared with A); **(C)** brain at anesthetized/comatose state (severe decrease in circuits communication – hypo-synchronization); **(D)** disordered/excessive neural synchrony at a distinct brain area (focal seizure – hypersynchronization); and **(E)** extensive and unspecific pathological synchronization (generalized seizure – hypersynchronization).

mechanism of pattern separation with these circuit motifs that are consistent with engram formation and discrimination associated with different memory traces (Espinoza et al., 2018). On the other hand, the role of CCK positive interneurons seems much less specific – as well as less known. The CCK^{+} are slower firing interneurons (Klausberger et al., 2005), with a lesser strict set of connectivity rules within the hippocampus and have been suggested to modulate much more complex behavior traits (e.g., mood regulation), that are certainly dependent on the temporal and spatial organization of multiple engrams (Freund, 2003). Thus, the endocannabinoid system may play an important part in how spatial pockets of hippocampal microcircuit patterns interact with each other in time, a hypothesis that can extend for other neural areas (Iball and Ali, 2011). In fact, if such a claim were true, one would expect the pharmacological manipulation of the cannabinoidergic system to affect slower oscillations, associated with the temporal arrangement of hippocampal microcircuit motifs (Robbe et al., 2006; Robbe and Buzsáki, 2009), to a much greater degree than the faster oscillators (associated to a more local or specific circuit motifs – e.g., fast gamma oscillations).

The loss or "silencing" of GABAergic interneurons are known to play an important role in TLE (McNamara, 1994; Zhang and Buckmaster, 2009). In fact, it has been suggested that the circuit rearrangement promotes the sustained epileptiform activity by compromising inhibitory feedback/feedforward microcircuits in hippocampal networks (Paz and Huguenard, 2015). Even under physiological conditions, considering the untampered hippocampal circuitry, rhythm generation is known to be highly dependent on GABAergic interneurons (Cobb et al., 1995; Buzsáki and Wang, 2012). The Medial Septal (MS) neurons projecting to hippocampal GABAergic interneurons and its ability to coordinate the firing patterns of specific circuit motifs generating GAMA activity has been proven essential to produce theta wave oscillations (Dragoi et al., 1999; Hangya et al., 2009). In addition, the temporal and spatial organization of multiple engrams has a strict phase correlation with the overall theta oscillation, rather than with fixed time delays between the local oscillators themselves (Petersen and Buzsáki, 2020). Thus, if one interprets the ictogenic process as several microcircuits being coupled together, throughout massive amounts of neural tissue, with complete disregard to

a specific patterns associated to a memory trace, or traces presented in sequence; the pathophysiological counterpart would be that the system responsible for circuit discrimination and organization must have been compromised. Indeed, there is a selective loss of CCK^+ interneurons in TLE (Wyeth et al., 2010), theta oscillations are much more compromised than gamma oscillations (Inostroza et al., 2013), MS GABAergic interneurons project to CCK^+ interneurons (although not exclusively; Freund and Antal, 1988; Unal et al., 2015), and, as mentioned before, express endocannabinoid receptors. Altogether, CCK^+ interneurons seem to play an important role in synchronizing and differentiating the microcircuits composed of localized groups of hippocampal pyramidal cells and, when compromised, unleash PV + interneurons to synchronize the entire network.

Therefore, some of the same mechanisms associated with the behavioral manifestations after the recreational use of *Cannabis sativa*, might explain its success in treating patients with epilepsy. The same synchrony disturbance, or disorganization of microcircuit synchronous recruitment, that would make a subject under the influence to express disconnected phrases and ideas, would be very beneficial to disrupt an "abnormal attractor" coupling a massive group of pyramidal cell discharges that exist in epilepsy. In point of fact, it would be even better if such a disruption would occur only when absolutely needed, i.e., "on-demand." That is obviously the case of asynchronous electrical stimulation triggered by abnormal ictal activity and (closed-loop), and, as suggested by this review, the potentiation of the endocannabinoid system could render the same effect as an independent pharmacological treatment.

CONCLUSION

The NPS – DBS and the endocannabinoid pharmacological therapeutic approaches are obviously quite different treatment strategies for epilepsy, with no evidence in the literature of reciprocal modulation. Nevertheless, as proposed by this review, both strategies may share the common goal of focusing on desynchronizing network activity without necessarily affecting excitation/inhibition balance (Medeiros and Moraes, 2014). It is quite important to clarify the fact that the strategies are not mutually exclusive and may very well have a synergetic effect if considered as a form of polytherapy. In addition, NPS – DBS could also benefit from exploratory probing stimulation, to test for abnormal network coupling, conferring an "on-demand" characteristic to its presentation (Medeiros et al., 2014). Altogether, both strategies would be complementary in the sense that on-demand-DBS would have a much faster action, with a narrower time-window constant, while endocannabinoid targeting would present long-term background action on network hypersynchronization.

More research and experimental data are needed in order to determine if DBS therapy (time-fixed pulses or NPS) predominantly has its effect by modulating or recruiting the endocannabinoid system; which is, at this time, speculative and solely based on the possible coincidental mechanisms of both therapeutical approaches. Aside from this potential caveat, a hypothetical rationale is that particular DBS patterns (high-frequency stimulation – over 50 Hz, or short NPS interpulse lengths – below 20 ms) could recruit the on-demand release of endogenous CBR1 ligands triggered by the increase in abnormal neural activity/connectivity. Thus, the DBS would directly modulate the brain's dynamic functional connectogram by recruiting a built-in on-demand "circuit-breaker system" (i.e., the endocannabinoid system), consequently disrupting neural network abnormal synchronization. According to this proposal, the DBS and cannabinoid system would have a bidirectional collaborative effect on seizure suppression, substantially enhancing the common outcomes.

AUTHOR CONTRIBUTIONS

DM, FM, and MM conceived the presented idea and supervised the project. DM, VC, FM, and MM wrote the manuscript. AO provided critical feedback and helped shape the manuscript. All authors reviewed, edited, and approved the manuscript.

FUNDING

The authors thank the funding agencies that supported this work: FAPEMIG (CBB-APQ-02290-13, CBB-APQ-03261-16, and APQ 02485-15), CNPq (307354/2017-2), and CAPES (PROCAD 88881.068460/2014-01 and BEX 5826/15-2). FM and MM were supported by Research Productivity Fellowship from CNPq.

ACKNOWLEDGMENTS

In memory of Professor Elisado Carlini (1930–2020), one of the founders of cannabinoid research in Brazil.

REFERENCES

Asth, L., Iglesias, L. P., De Oliveira, A. C., Moraes, M. F. D., and Moreira, F. A. (2019). Exploiting cannabinoid and vanilloid mechanisms for epilepsy treatment. *Epilepsy Behav*. doi: 10.1016/j.yebeh.2019.106832 [Epub ahead of print].

Avoli, M., D'Antuono, M., Louvel, J., Köhling, R., Biagini, G., Pumain, R., et al. (2002). Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system *in vitro*. *Prog. Neurobiol.* 68, 167–207. doi: 10.1016/s0301-0082(02)00077-1

Bertram, E. H. (2013). Neuronal circuits in epilepsy: Do they matter? *Exp. Neurol.* 244, 67–74. doi: 10.1016/j.expneurol.2012.01.028

Billakota, S., Devinsky, O., and Marsh, E. (2019). Cannabinoid therapy in epilepsy. *Curr. Opin. Neurol.* 32, 220–226. doi: 10.1097/wco.0000000000000660

Bosman, C. A., Schoffelen, J.-M., Brunet, N., Oostenveld, R., Bastos, A. M., Womelsdorf, T., et al. (2012). Attentional stimulus selection through selective synchronization between monkey visual areas. *Neuron* 75, 875–888. doi: 10.1016/j.neuron.2012.06.037

Buzsáki, G. (2010). Neural syntax: cell assemblies, synapsembles, and readers. *Neuron* 68, 362–385. doi: 10.1016/j.neuron.2010.09.023

Buzsáki, G., Anastassiou, C. A., and Koch, C. (2012). The origin of extracellular fields and currents — EEG, ECoG, LFP and spikes. *Nat. Rev. Neurosci.* 13, 407–420. doi: 10.1038/nrn3241

Buzsáki, G., and Chrobak, J. J. (1995). Temporal structure in spatially organized neuronal ensembles: a role for interneuronal networks. *Curr. Opin. Neurobiol.* 5, 504–510. doi: 10.1016/0959-4388(95)80012-3

Buzsáki, G., and Draguhn, A. (2004). Neuronal oscillations in cortical networks. *Science* 304, 1926–1929. doi: 10.1126/science.1099745

Buzsáki, G., Logothetis, N., and Singer, W. (2013). Scaling brain size, keeping timing: evolutionary preservation of brain rhythms. *Neuron* 80, 751–764. doi: 10.1016/j.neuron.2013.10.002

Buzsáki, G., and Wang, X.-J. (2012). Mechanisms of gamma oscillations. *Annu. Rev. Neurosci.* 35, 203–225. doi: 10.1146/annurev-neuro-062111-150444

Cobb, S. R., Buhl, E. H., Halasy, K., Paulsen, O., and Somogyi, P. (1995). Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature* 378, 75–78. doi: 10.1038/378075a0

Cortes-Briones, J., Skosnik, P. D., Mathalon, D., Cahill, J., Pittman, B., Williams, A., et al. (2015). Δ9-THC disrupts gamma (γ)-band neural oscillations in humans. *Neuropsychopharmacology* 40, 2124–2134. doi: 10.1038/npp.2015.53

Cota, V. R., de Castro Medeiros, D., da PáscoaVilela, M. R. S., Doretto, M. C., and Moraes, M. F. D. (2009). Distinct patterns of electrical stimulation of the basolateral amygdala influence pentylenetetrazole seizure outcome. *Epilepsy Behav.* 14(Suppl. 1), 26–31. doi: 10.1016/j.yebeh.2008.09.006

de Curtis, M., and Avanzini, G. (2001). Interictal spikes in focal epileptogenesis. *Prog. Neurobiol.* 63, 541–567. doi: 10.1016/s0301-0082(00)00026-5

de Guzman, P., D'Antuono, M., and Avoli, M. (2004). Initiation of electrographic seizures by neuronal networks in entorhinal and perirhinal cortices *in vitro*. *Neuroscience* 123, 875–886. doi: 10.1016/j.neuroscience.2003.11.013

de Oliveira, J. C., Drabowski, B. M. B., Rodrigues, S. M. A. F., Maciel, R. M., Moraes, M. F. D., and Cota, V. R. (2019). Seizure suppression by asynchronous non-periodic electrical stimulation of the amygdala is partially mediated by indirect desynchronization from nucleus accumbens. *Epilepsy Res.* 154, 107–115. doi: 10.1016/j.eplepsyres.2019.05.009

de Oliveira, J. C., Medeiros, D., de, C., de Souza, E., Rezende, G. H., Moraes, M. F. D., et al. (2014). Temporally unstructured electrical stimulation to the amygdala suppresses behavioral chronic seizures of the pilocarpine animal model. *Epilepsy Behav.* 36, 159–164. doi: 10.1016/j.yebeh.2014.05.005

Devane, W. A., Dysarz, F. A. III, Johnson, M. R., Melvin, L. S., and Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* 34, 605–613.

Devinsky, O., Vezzani, A., O'Brien, T. J., Jette, N., Scheffer, I. E., de Curtis, M., et al. (2018). Epilepsy. *Nat. Rev. Dis. Primers* 4:18024.

Dragoi, G., Carpi, D., Recce, M., Csicsvari, J., and Buzsáki, G. (1999). Interactions between hippocampus and medial septum during sharp waves and theta oscillation in the behaving rat. *J. Neurosci.* 19, 6191–6199. doi: 10.1523/jneurosci.19-14-06191.1999

Edakawa, K., Yanagisawa, T., Kishima, H., Fukuma, R., Oshino, S., Khoo, H. M., et al. (2016). Detection of epileptic seizures using phase–amplitude coupling in intracranial electroencephalography. *Sci. Rep.* 6:25422. doi: 10.1038/srep25422

Espinoza, C., Guzman, S. J., Zhang, X., and Jonas, P. (2018). Parvalbumin interneurons obey unique connectivity rules and establish a powerful lateral-inhibition microcircuit in dentate gyrus. *Nat. Commun.* 9:4605.

Freund, T. F. (2003). Interneuron diversity series: rhythm and mood in perisomatic inhibition. *Trends Neurosci.* 26, 489–495. doi: 10.1016/s0166-2236(03)00227-3

Freund, T. F., and Antal, M. (1988). GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature* 336, 170–173. doi: 10.1038/336170a0

Fries, P. (2005). A mechanism for cognitive dynamics: neuronal communication through neuronal coherence. *Trends Cogn. Sci.* 9, 474–480. doi: 10.1016/j.tics.2005.08.011

Fries, P. (2009). Neuronal gamma-band synchronization as a fundamental process in cortical computation. *Annu. Rev. Neurosci.* 32, 209–224. doi: 10.1146/annurev.neuro.051508.135603

Garcia-Cairasco, N. (2009). Puzzling challenges in contemporary neuroscience: insights from complexity and emergence in epileptogenic circuits. *Epilepsy Behav.* 14(Suppl. 1), 54–63. doi: 10.1016/j.yebeh.2008.09.010

Gobira, P. H., Vilela, L. R., Gonçalves, B. D. C., Santos, R. P. M., de Oliveira, A. C., Vieira, L. B., et al. (2015). Cannabidiol, a Cannabis sativa constituent, inhibits cocaine-induced seizures in mice: possible role of the mTOR pathway and reduction in glutamate release. *Neurotoxicology* 50, 116–121. doi: 10.1016/j.neuro.2015.08.007

Grill, W. M. (2018). Temporal pattern of electrical stimulation is a new dimension of therapeutic innovation. *Curr. Opin. Biomed. Eng.* 8, 1–6. doi: 10.1016/j.cobme.2018.08.007

Guirgis, M., Chinvarun, Y., Carlen, P. L., and Bardakjian, B. L. (2013). The role of delta-modulated high frequency oscillations in seizure state classification. *Annu. Int. Conf. IEEE Eng. Med. Biol. Soc.* 2013, 6595–6598.

Hajós, M., Hoffmann, W. E., and Kocsis, B. (2008). Activation of cannabinoid-1 receptors disrupts sensory gating and neuronal oscillation: relevance to schizophrenia. *Biol. Psychiatry* 63, 1075–1083. doi: 10.1016/j.biopsych.2007.12.005

Hájos, N., Katona, I., Naiem, S. S., MacKie, K., Ledent, C., Mody, I., et al. (2000). Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur. J. Neurosci.* 12, 3239–3249. doi: 10.1046/j.1460-9568.2000.00217.x

Hangya, B., Borhegyi, Z., Szilágyi, N., Freund, T. F., and Varga, V. (2009). GABAergic neurons of the medial septum lead the hippocampal network during theta activity. *J. Neurosci.* 29:8094. doi: 10.1523/jneurosci.5665-08.2009

Hill, A. J., Weston, S. E., Jones, N. A., Smith, I., Bevan, S. A., Williamson, E. M., et al. (2010). Δ9-Tetrahydrocannabivarin suppresses *in vitro* epileptiform and *in vivo* seizure activity in adult rats. *Epilepsia* 51, 1522–1532. doi: 10.1111/j.1528-1167.2010.02523.x

Hill, T. D., Cascio, M. G., Romano, B., Duncan, M., Pertwee, R. G., Williams, C. M., et al. (2013). Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. *Br. J. Pharmacol.* 170, 679–692. doi: 10.1111/bph.12321

Iball, J., and Ali, A. B. (2011). Endocannabinoid release modulates electrical coupling between CCK cells connected via chemical and electrical synapses in CA1. *Front. Neural Circuits* 5:17. doi: 10.3389/fncir.2011.00017

Imamura, S., Tanaka, S., Akaike, K., Tojo, H., Takigawa, M., and Kuratsu, J. (2001). Hippocampal transection attenuates kainic acid-induced amygdalar seizures in rats. *Brain Res.* 897, 93–103. doi: 10.1016/s0006-8993(01)02098-4

Inostroza, M., Brotons-Mas, J. R., Laurent, F., Cid, E., and de la Prida, L. M. (2013). Specific impairment of "what-where-when" episodic-like memory in experimental models of temporal lobe epilepsy. *J. Neurosci* 33, 17749–17762. doi: 10.1523/jneurosci.0957-13.2013

Jensen, O., and Colgin, L. L. (2007). Cross-frequency coupling between neuronal oscillations. *Trends Cogn. Sci.* 11, 267–269. doi: 10.1016/j.tics.2007.05.003

Jiruska, P., de Curtis, M., Jefferys, J. G. R., Schevon, C. A., Schiff, S. J., and Schindler, K. (2013). Synchronization and desynchronization in epilepsy: controversies and hypotheses. *J. Physiol.* 591, 787–797. doi: 10.1113/jphysiol.2012.239590

Kalitzin, S., Petkov, G., Suffczynski, P., Grigorovsky, V., Bardakjian, B. L., Lopes da Silva, F., et al. (2019). Epilepsy as a manifestation of a multistate network of oscillatory systems. *Neurobiol. Dis.* 130:104488. doi: 10.1016/j.nbd.2019.104488

Karanian, D. A., Karim, S. L., Wood, J. T., Williams, J. S., Lin, S., Makriyannis, A., et al. (2007). Endocannabinoid enhancement protects against kainic acid-induced seizures and associated brain damage. *J. Pharmacol. Exp. Ther.* 322, 1059–1066. doi: 10.1124/jpet.107.120147

Katona, I. (2015). Cannabis and endocannabinoid signaling in epilepsy. *Handb. Exp. Pharmacol.* 231, 285–316. doi: 10.1007/978-3-319-20825-1_10

Katona, I., and Freund, T. F. (2008). Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nat. Med.* 14, 923–930. doi: 10.1038/nm.f.1869

Katona, I., Sperlágh, B., Sík, A., Käfalvi, A., Vizi, E. S., Mackie, K., et al. (1999). Presynaptically Located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.* 19, 4544–4558. doi: 10.1523/jneurosci.19-11-04544.1999

Kawamura, Y., Fukaya, M., Maejima, T., Yoshida, T., Miura, E., Watanabe, M., et al. (2006). The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. *J. Neurosci.* 26, 2991–3001. doi: 10.1523/jneurosci.4872-05.2006

Klausberger, T., Marton, L. F., O'Neill, J., Huck, J. H. J., Dalezios, Y., Fuentealba, P., et al. (2005). Complementary roles of cholecystokinin- and parvalbumin-expressing GABAergic neurons in hippocampal network oscillations. *J. Neurosci.* 25, 9782–9793. doi: 10.1523/jneurosci.3269-05.2005

Klinzing, J. G., Niethard, N., and Born, J. (2019). Mechanisms of systems memory consolidation during sleep. *Nat. Neurosci.* 22, 1598–1610. doi: 10.1038/s41593-019-0467-3

Kreuz, T., Mormann, F., Andrzejak, R. G., Kraskov, A., Lehnertz, K., and Grassberger, P. (2007). Measuring synchronization in coupled model systems: a comparison of different approaches. *Physica D* 225, 29–42. doi: 10.1016/j.physd.2006.09.039

Kucewicz, M. T., Tricklebank, M. D., Bogacz, R., and Jones, M. W. (2011). Dysfunctional prefrontal cortical network activity and interactions following cannabinoid receptor activation. *J. Neurosci.* 31, 15560–15568. doi: 10.1523/jneurosci.2970-11.2011

Kudela, P., Franaszczuk, P. J., and Bergey, G. K. (2003). Changing excitation and inhibition in simulated neural networks: effects on induced bursting behavior. *Biol. Cybern.* 88, 276–285. doi: 10.1007/s00422-002-0381-7

Lima, I. V., de, A., Bellozi, P. M. Q., Batista, E. M., Vilela, L. R., Brandão, I. L., et al. (2020). Cannabidiol anticonvulsant effect is mediated by the PI3Kγ pathway. *Neuropharmacology* 176:108156. doi: 10.1016/j.neuropharm.2020.108156

Lisman, J. E., and Jensen, O. (2013). The θ-γ neural code. *Neuron* 77, 1002–1016.

Loscher, W. (2020). The holy grail of epilepsy prevention: preclinical approaches to antiepileptogenic treatments. *Neuropharmacology* 167:107605. doi: 10.1016/j.neuropharm.2019.04.011

Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A., and Kano, M. (2001). Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron* 31, 463–475. doi: 10.1016/s0896-6273(01)00375-0

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208

McNamara, J. O. (1994). Cellular and molecular basis of epilepsy. *J. Neurosci.* 14, 3413–3425. doi: 10.1523/jneurosci.14-06-03413.1994

Mechoulam, R. (1970). Marihuana chemistry. *Science* 168, 1159–1166. doi: 10.1126/science.168.3936.1159

Mechoulam, R., Hanuš, L. O., Pertwee, R., and Howlett, A. C. (2014). Early phytocannabinoid chemistry to endocannabinoids and beyond. *Nat. Rev. Neurosci.* 15, 757–764. doi: 10.1038/nrn3811

Medeiros, D. de C., and Moraes, M. F. D. (2014). Focus on desynchronization rather than excitability: a new strategy for intraencephalic electrical stimulation. *Epilepsy Behav.* 38, 32–36. doi: 10.1016/j.yebeh.2013.12.034

Medeiros, D. C., Oliveira, L. B., Mourão, F. A. G., Bastos, C. P., Cairasco, N. G., Pereira, G. S., et al. (2014). Temporal rearrangement of pre-ictal PTZ induced spike discharges by low frequency electrical stimulation to the amygdaloid complex. *Brain Stimul.* 7, 170–178. doi: 10.1016/j.brs.2013.11.005

Mizuseki, K., Sirota, A., Pastalkova, E., and Buzsáki, G. (2009). Theta oscillations provide temporal windows for local circuit computation in the entorhinal-hippocampal loop. *Neuron* 64, 267–280. doi: 10.1016/j.neuron.2009.08.037

Moraes, M. F. D., Chavali, M., Mishra, P. K., Jobe, P. C., and Garcia-Cairasco, N. (2005a). A comprehensive electrographic and behavioral analysis of generalized tonic-clonic seizures of GEPR-9s. *Brain Res.* 1033, 1–12. doi: 10.1016/j.brainres.2004.10.066

Moraes, M. F. D., Mishra, P. K., Jobe, P. C., and Garcia-Cairasco, N. (2005b). An electrographic analysis of the synchronous discharge patterns of GEPR-9s generalized seizures. *Brain Res.* 1046, 1–9. doi: 10.1016/j.brainres.2005.03.035

Morrison, P. D., Nottage, J., Stone, J. M., Bhattacharyya, S., Tunstall, N., Brenneisen, R., et al. (2011). Disruption of frontal theta coherence by Δ9-tetrahydrocannabinol is associated with positive psychotic symptoms. *Neuropsychopharmacology* 36, 827–836. doi: 10.1038/npp.2010.222

Nariai, H., Matsuzaki, N., Juhász, C., Nagasawa, T., Sood, S., Chugani, H. T., et al. (2011). Ictal high-frequency oscillations at 80-200 Hz coupled with delta phase in epileptic spasms. *Epilepsia* 52, e130–e134.

Naydenov, A. V., Horne, E. A., Cheah, C. S., Swinney, K., Hsu, K.-L., Cao, J. K., et al. (2014). ABHD6 blockade exerts antiepileptic activity in PTZ-induced seizures and in spontaneous seizures in R6/2 mice. *Neuron* 83, 361–371. doi: 10.1016/j.neuron.2014.06.030

Netoff, T. I., and Schiff, S. J. (2002). Decreased neuronal synchronization during experimental seizures. *J. Neurosci.* 22, 7297–7307. doi: 10.1523/jneurosci.22-16-07297.2002

O'Keefe, J., and Recce, M. L. (1993). Phase relationship between hippocampal place units and the EEG theta rhythm. *Hippocampus* 3, 317–330. doi: 10.1002/hipo.450030307

Patra, P. H., Barker-Haliski, M., White, H. S., Whalley, B. J., Glyn, S., Sandhu, H., et al. (2019). Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models. *Epilepsia* 60, 303–314. doi: 10.1111/epi.14629

Paz, J. T., and Huguenard, J. R. (2015). Microcircuits and their interactions in epilepsy: Is the focus out of focus? *Nat. Neurosci.* 18, 351–359. doi: 10.1038/nn.3950

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol. Rev.* 62, 588–631. doi: 10.1124/pr.110.003004

Perucca, P., and Gilliam, F. G. (2012). Adverse effects of antiepileptic drugs. *Lancet Neurol.* 11, 792–802.

Petersen, P. C., and Buzsáki, G. (2020). Cooling of medial septum reveals theta phase lag coordination of hippocampal cell assemblies. *Neuron* 107, 731–744.e3.

Quian Quiroga, R., Kraskov, A., Kreuz, T., and Grassberger, P. (2002). Performance of different synchronization measures in real data: a case study on electroencephalographic signals. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* 65:041903.

Quilichini, P., Sirota, A., and Buzsáki, G. (2010). Intrinsic circuit organization and theta-gamma oscillation dynamics in the entorhinal cortex of the rat. *J. Neurosci.* 30, 11128–11142. doi: 10.1523/jneurosci.1327-10.2010

Quinkert, A. W., Schiff, N. D., and Pfaff, D. W. (2010). Temporal patterning of pulses during deep brain stimulation affects central nervous system arousal. *Behav. Brain Res.* 214, 377–385. doi: 10.1016/j.bbr.2010.06.009

Robbe, D., and Buzsáki, G. (2009). Alteration of theta timescale dynamics of hippocampal place cells by a cannabinoid is associated with memory impairment. *J. Neurosci.* 29, 12597–12605. doi: 10.1523/jneurosci.2407-09.2009

Robbe, D., Montgomery, S. M., Thome, A., Rueda-Orozco, P. E., McNaughton, B. L., and Buzsaki, G. (2006). Cannabinoids reveal importance of spike timing coordination in hippocampal function. *Nat. Neurosci.* 9, 1526–1533. doi: 10.1038/nn1801

Santos-Valencia, F., Almazán-Alvarado, S., Rubio-Luviano, A., Valdés-Cruz, A., Magdaleno-Madrigal, V. M., and Martínez-Vargas, D. (2019). Temporally irregular electrical stimulation to the epileptogenic focus delays epileptogenesis in rats. *Brain Stimul.* 12, 1429–1438. doi: 10.1016/j.brs.2019.07.016

Schindler, K., Elger, C. E., and Lehnertz, K. (2007). Changes of EEG synchronization during low-frequency electric stimulation of the seizure onset zone. *Epilepsy Res.* 77, 108–119. doi: 10.1016/j.eplepsyres.2007.09.011

Schnitzler, A., and Gross, J. (2005). Normal and pathological oscillatory communication in the brain. *Nat. Rev. Neurosci.* 6, 285–296. doi: 10.1038/nrn1650

de Souza Silva, W., Maciel, R. M., and Cota, V. R. (2019). "Electrographic spectral signatures of animals submitted to pentylenetetrazole-induced seizures and treated with bilateral asynchronous non-periodic stimulation," in *Computational Neuroscience. LAWCN 2019. Communications in Computer and Information Science*, eds V. Cota, D. Barone, D. Dias, and L. Damázio (Cham: Springer), 258–266. doi: 10.1007/978-3-030-36636-0_19

Sohal, V. S., and Sun, F. T. (2011). Responsive neurostimulation suppresses synchronized cortical rhythms in patients with epilepsy. *Neurosurg. Clin. N. Am.* 22, 481–488. doi: 10.1016/j.nec.2011.07.007

Soltesz, I., Alger, B. E., Kano, M., Lee, S.-H., Lovinger, D. M., Ohno-Shosaku, T., et al. (2015). Weeding out bad waves: towards selective cannabinoid circuit control in epilepsy. *Nat. Rev. Neurosci.* 16, 264–277. doi: 10.1038/nrn3937

Sunderam, S., Gluckman, B., Reato, D., and Bikson, M. (2010). Toward rational design of electrical stimulation strategies for epilepsy control. *Epilepsy Behav.* 17, 6–22. doi: 10.1016/j.yebeh.2009.10.017

Udupa, K., and Chen, R. (2015). The mechanisms of action of deep brain stimulation and ideas for the future development. *Prog. Neurobiol.* 133, 27–49. doi: 10.1016/j.pneurobio.2015.08.001

Uhlhaas, P. J., Linden, D. E. J., Singer, W., Haenschel, C., Lindner, M., Maurer, K., et al. (2006). Dysfunctional long-range coordination of neural activity during Gestalt perception in schizophrenia. *J. Neurosci.* 26, 8168–8175. doi: 10.1523/jneurosci.2002-06.2006

Uhlhaas, P. J., and Singer, W. (2006). Neural synchrony in brain disorders: relevance for cognitive dysfunctions and pathophysiology. *Neuron* 52, 155–168. doi: 10.1016/j.neuron.2006.09.020

Uhlhaas, P. J., and Singer, W. (2012). Neuronal dynamics and neuropsychiatric disorders: toward a translational paradigm for dysfunctional large-scale networks. *Neuron* 75, 963–980. doi: 10.1016/j.neuron.2012.09.004

Unal, G., Joshi, A., Viney, T. J., Kis, V., and Somogyi, P. (2015). Synaptic targets of medial septal projections in the hippocampus and extrahippocampal cortices of the mouse. *J. Neurosci.* 35, 15812–15826. doi: 10.1523/jneurosci.2639-15.2015

Varela, F., Lachaux, J. P., Rodriguez, E., and Martinerie, J. (2001). The brainweb: phase synchronization and large-scale integration. *Nat. Rev. Neurosci.* 2, 229–239. doi: 10.1038/35067550

Vilela, L. R., Medeiros, D. C., Rezende, G. H., de Oliveira, A. C., Moraes, M. F., and Moreira, F. A. (2013). Effects of cannabinoids and endocannabinoid hydrolysis inhibition on pentylenetetrazole-induced seizure and electroencephalographic activity in rats. *Epilepsy Res.* 104, 195–202. doi: 10.1016/j.eplepsyres.2012.11.006

von Rüden, E. L., Bogdanovic, R. M., Wotjak, C. T., and Potschka, H. (2015). Inhibition of monoacylglycerol lipase mediates a cannabinoid 1-receptor dependent delay of kindling progression in mice. *Neurobiol. Dis.* 77, 238–245. doi: 10.1016/j.nbd.2015.03.016

von Stein, A., and Sarnthein, J. (2000). Different frequencies for different scales of cortical integration: from local gamma to long range alpha/theta synchronization. *Int. J. Psychophysiol.* 38, 301–313. doi: 10.1016/s0167-8760(00)00172-0

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and DeLorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920

Wang, X.-J. (2010). Neurophysiological and computational principles of cortical rhythms in cognition. *Physiol. Rev.* 90, 1195–1268. doi: 10.1152/physrev.00035.2008

Whittington, M. A., Traub, R. D., Kopell, N., Ermentrout, B., and Buhl, E. H. (2000). Inhibition-based rhythms: experimental and mathematical observations on network dynamics. *Int. J. Psychophysiol.* 38, 315–336. doi: 10.1016/s0167-8760(00)00173-2

Wilson, R. I., and Nicoll, R. A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 410, 588–592. doi: 10.1038/35069076

Womelsdorf, T., Schoffelen, J.-M., Oostenveld, R., Singer, W., Desimone, R., Engel, A. K., et al. (2007). Modulation of neuronal interactions through neuronal synchronization. *Science* 316, 1609–1612. doi: 10.1126/science.1139597

Wu, J., Yang, H., Peng, Y., Fang, L., Zheng, W., and Song, Z. (2013). The role of local field potential coupling in epileptic synchronization. *Neural Regeneration Res.* 8, 745–753.

Wyckhuys, T., Boon, P., Raedt, R., Van Nieuwenhuyse, B., Vonck, K., and Wadman, W. (2010). Suppression of hippocampal epileptic seizures in the kainate rat by Poisson distributed stimulation. *Epilepsia* 51, 2297–2304. doi: 10.1111/j.1528-1167.2010.02750.x

Wyeth, M. S., Zhang, N., Mody, I., and Houser, C. R. (2010). Selective reduction of cholecystokinin-positive basket cell innervation in a model of temporal lobe epilepsy. *J. Neurosci.* 30, 8993–9006. doi: 10.1523/jneurosci.1183-10.2010

Zhang, W., and Buckmaster, P. S. (2009). Dysfunction of the dentate basket cell circuit in a rat model of temporal lobe epilepsy. *J. Neurosci.* 29, 7846–7856. doi: 10.1523/jneurosci.6199-08.2009

Zheng, L., Feng, Z., Hu, H., Wang, Z., Yuan, Y., and Wei, X. (2020). The appearance order of varying intervals introduces extra modulation effects on neuronal firing through non-linear dynamics of sodium channels during high-frequency stimulations. *Front. Neurosci.* 14:397. doi: 10.3389/fnins.2020.00397

Zuardi, A. W. (2006). History of cannabis as a medicine: a review. *Rev. Bras. Psiquiatr.* 28, 153–157. doi: 10.1590/s1516-44462006000200015

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

frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
published: 17 November 2020
doi: 10.3389/fnbeh.2020.595315

Divergent Effects of Systemic and Intracollicular CB Receptor Activation Against Forebrain and Hindbrain-Evoked Seizures in Rats

Victor R. Santos[1,2†], *Robert Hammack*[1,3†], *Evan Wicker*[1], *Prosper N'Gouemo*[4,5,6] *and Patrick A. Forcelli*[1,5,7*]

[1]Department of Pharmacology and Physiology, Georgetown University School of Medicine, Washington, DC, United States, [2]Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Brazil, [3]Department of Cellular and Integrated Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States, [4]Department of Pediatrics, Georgetown University School of Medicine, Washington, DC, United States, [5]Interdisciplinary Program in Neuroscience, Georgetown University, Washington, DC, United States, [6]Department of Physiology and Biophysics, Howard University College of Medicine, Washington, DC, United States, [7]Department of Neuroscience, Georgetown University School of Medicine, Washington, DC, United States

OPEN ACCESS

Edited by:
Dolores E. López García,
University of Salamanca, Spain

Reviewed by:
Norberto Cysne Coimbra,
University of São Paulo, Brazil
Hiroki Toyoda,
Osaka University, Japan

***Correspondence:**
Patrick A. Forcelli
Paf22@georgetown.edu

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 16 August 2020
Accepted: 19 October 2020
Published: 17 November 2020

Citation:
Santos VR, Hammack R, Wicker E, N'Gouemo P and Forcelli PA (2020) Divergent Effects of Systemic and Intracollicular CB Receptor Activation Against Forebrain and Hindbrain-Evoked Seizures in Rats. Front. Behav. Neurosci. 14:595315. doi: 10.3389/fnbeh.2020.595315

Cannabinoid (CB) receptor agonists are of growing interest as targets for anti-seizure therapies. Here we examined the effect of systemic administration of the CB receptor agonist WIN 55,212-2 (WIN) against audiogenic seizures (AGSs) in the Genetically Epilepsy Prone Rat (GEPR)-3 strain, and against seizures evoked focally from the *Area Tempestas* (AT). We compared these results to the effect of focal administration of the CB1/2 receptor agonist CP 55940 into the deep layers of the superior colliculus (DLSC), a brain site expressing CB1 receptors. While systemic administration of WIN dose-dependently decreased AGS in GEPR-3s, it was without effect in the AT model. By contrast, intra-DLSC infusion of CP 55940 decreased seizures in both models. To determine if the effects of systemic WIN were dependent upon activation of CB1 receptors in the DSLC, we next microinjected the CB1 receptor antagonist SR141716, before WIN systemic treatment, and tested animals for AGS susceptibility. The pretreatment of the DLSC with SR141716 was without effect on its own and did not alter the anti-convulsant action of WIN systemic administration. Thus, while CB receptors in the DLSC are a potential site of anticonvulsant action, they are not necessary for the effects of systemically administered CB agonists.

Keywords: cannabinoid, superior colliculus (SC), area tempestas, piriform cortex, genetically epilepsy prone rat (GEPR)

INTRODUCTION

The epilepsies, as a group, are one of the most common neurological disorders and are associated with substantial morbidity, mortality, and economic burden. Identifying therapies to treat epilepsies can be a challenge due to the diversity of the condition. Epileptic seizures can arise in a variety of brain networks, resulting in differing semiology, pharmacosensitivity, and degree of

impairment—a drug that works for one type of seizure may be ineffective or exacerbate another. Studies suggested that the deep layers of the superior colliculus (DLSC) are part of the network that generates audiogenic seizures (AGSs), a form of reflex seizures triggered by intense sounds (Faingold and Randall, 1999b). While AGSs are uncommon in humans, they can serve as a model for brainstem seizure activity. In rats and mice, bilateral lesions of the superior colliculus, microinjection of GABA antagonist (Depaulis et al., 1990), *N*-methyl-D-aspartate (NMDA) antagonist (Faingold and Casebeer, 1999a; Raisinghani and Faingold, 2003) or alpha1-noradrenergic agonist (Faingold and Casebeer, 1999a) markedly attenuated AGS severity (Merrill et al., 2003). In a different approach, optogenetic activation of DLSC also attenuates seizures in Genetically Epilepsy-Prone Rat (GEPR) 3 substrain (Soper et al., 2016; Wicker et al., 2019). Activation of the DLSC also potently suppresses seizures in other models of epilepsy, including the maximal electroshock model (Redgrave et al., 1992; Gale et al., 1993), evoked and genetic absence seizure models (Nail-Boucherie et al., 2002, 2005; Soper et al., 2016); the *Area Tempestas* (AT) model of forebrain seizures (Soper et al., 2016), and the pentylenetetrazole model of generalized seizures (Weng and Rosenberg, 1992; Soper et al., 2016). Further elucidating the neurotransmitter systems in the networks for brainstem and forebrain seizures may offer novel therapeutic targets for the treatment of generalized seizures.

The endocannabinoid system has shown promise as a target for seizure control, but there are conflicting reports about the efficacy of cannabinoid (CB) receptor targeting compounds in suppressing seizures (Rosenberg et al., 2017). One of the most promising candidates are CB1 receptor agonists, which exert anticonvulsant effects against generalized tonic seizures in the maximal electroshock test (Luszczki et al., 2006; Florek-Luszczki et al., 2014b; Tutka et al., 2018), but produce mixed effects against generalized seizures evoked by pentylenetetrazole (Bahremand et al., 2008a; Andres-Mach et al., 2012; Vilela et al., 2013; Aghaei et al., 2015; Huizenga et al., 2017) and limbic seizures in the kainic acid model (Bojnik et al., 2012; Shubina et al., 2015). These models are characterized by different types of seizures: the former by tonic seizures that critically rely on brainstem networks (Merrill et al., 2003, 2005), and the later by limbic seizures that critically rely on forebrain networks (Browning et al., 1993).

The CB1 receptor is the major CB receptor found in the central nervous system, and is coupled to Gi signaling cascades; its activation is typically associated with decreased transmitter release. Endocannabinoid signaling mediated through presynaptic CB1 receptors reduces both glutamate and GABA release (Kathmann et al., 1999; Sullivan, 1999; Hájos et al., 2000), and therefore is a potent regulator of neuronal excitability. Somewhat paradoxically, in the context of seizure suppression, the CB1 receptor co-localizes with GABA neurons in many brain regions it has been suggested that the primary cell type that expresses CB1 receptor is inhibitory (Katona et al., 1999, 2001). This is of particular interest in the context of the DLSC, as *disinhibition* of the DLSC by silencing GABAergic inputs from the substantia nigra *pars reticulata* (SNpr) is also potently anticonvulsant (Wicker et al., 2019). Both the DLSC and the SNpr express CB1 receptors, with the SNpr displaying some of the highest receptor density in the brain (Herkenham et al., 1991). Thus, here, we sought to determine the effect of CB1 receptor agonists on brainstem seizures in GEPR-3 rats and forebrain seizures in the Area Tempestas model while comparing the effects of systemic and intra-DLSC delivery of CB1 agonist.

MATERIALS AND METHODS

Animals

Experiments were performed on male Sprague–Dawley (SD) purchased from Envigo (Frederick, MD, USA) rats and GEPR-3s obtained from a colony maintained at Georgetown University. Animals weighed 270–285 g at the time of surgery and were housed two per cage in the Georgetown University Division of Comparative Medicine under environmentally controlled conditions (12 h light/dark cycle, lights on between 6:00 AM and 6:00 PM; ambient temperature 23°C ± 1°C; controlled humidity) with food (Lab Diet, #5001) and water provided *ad libitum*. All the procedures and experiments were conducted following the Association for Assessment and Accreditation of Laboratory Animal Care standards, the Guide for the Care and Use of Laboratory Animals (Rowan, 1979), and were approved by the Georgetown University Care and Use Committee.

Surgery

Surgeries were performed as previously described (Santos et al., 2018). Briefly, rats were anesthetized with equithesin (2.8 mg/kg, i.p.) and placed into a stereotaxic frame (David Kopf, Tujunga, CA, USA). Surgery was conducted with the incisor bar at +5.0 mm above the interaural line according to the atlas of Pellegrino and Cushman (1967). For all microinjection experiments, a 22 g guide cannula (Plastics One, Roanoke, VA, USA) was paired with a 28 g internal cannula with the internal cannula extended 2 mm deeper than the guide. A screw cap was placed on the guide cannula to keep cannula tracks clean. Cannulae were implanted unilaterally into the AT (Piredda and Gale, 1985; 4.0 mm anterior to Bregma, 3.5 mm lateral to the midline, 5.5 mm ventral to the dura) and/or into the DLSC (5.0 mm posterior to Bregma, 2.5 mm lateral to the midline, and 4.5 mm ventral to the dura; Pellegrino and Cushman, 1967). Atlas panels shown in the Figures are reproduced in modified form from the Brain Maps 4.0 atlas (Swanson, 2018). Cannulae were fixed in place with jeweler's screws and dental acrylic. Following surgery, all animals were given at least 1 week to recover before behavioral testing.

Systemic Drug Administration

The CB1 receptor agonist, WIN [WIN 55212-2 mesylate (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl]pyrrolol[1,2, 3 de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone)], was obtained from Tocris Biosciences (Bristol, UK) and dissolved in 50% of DMSO and 0.9% saline. Solutions were prepared at a concentration of 2 mg/ml and drug or vehicle was administered by intraperitoneal (i.p.) injection at a volume of 1 ml/kg. The drug was administered 20 min before seizure testing.

CB Drug Microinfusion

The CB1/2 receptor agonist CP 55940 (CP) was dissolved in 50% DMSO-50% saline, and 26.5 nmol (in 0.5 μl) was microinfused bilaterally into the DLSC. We selected CP 55940 over WIN for microinjection, because it has a higher affinity for the CB1 receptor than does WIN. By contrast, we selected WIN for systemic administration because its anticonvulsant profile has been better characterized than that of systemic CP. The CB1 receptor antagonist SR 141716A (SR, rimonabant) was dissolved in 50% DMSO-50% saline, and 1 μg (4.3 nmol) in 0.5 μl was infused into the DLSC. These drug doses were chosen based on published reports (Sañudo-Peña et al., 2000; Citraro et al., 2013; Florek-Luszczki et al., 2014a). Drug microinfusions were performed essentially as we have previously described (Wicker and Forcelli, 2016; Wicker et al., 2019). In brief, rats were gently restrained and a 28-gauge internal cannula was inserted. Drug infusion was controlled by a syringe pump driving a Hamilton syringe programmed to deliver the drug at a rate of 0.2 μl/min. After the completion of the infusion, the internal cannula was left in place for at least 1 min to reduce drug reflux up the cannula track and then removed. Drug microinfusion was performed 5 min before seizure testing.

Piriform Cortex (Area Tempestas) Seizures

Twelve SD rats were used for these experiments. A stainless-steel guide cannula was stereotaxically implanted above the left AT as described above. Bicuculline methiodide (BMI; Sigma–Aldrich) was dissolved in 0.9% saline at a concentration of 1 mM, and a dose of 100–280 pmols (0.2 μl/min) was used to induce seizures. Bicuculline was injected immediately following intra-DLSC injections or 20 min following systemic WIN. Initially, a 100 pmol dose of bicuculine was microinjected into the AT; in the absence of seizure activity, the amount of bicuculline injected was increased (increments of 20 pmol) on a subsequent day until a seizure with the score of 3–5 on the modified Racine scale (see below) was reached. After AT injections were completed, SD rats were monitored for 60 min in a transparent plastic box, and behaviors were documented by an observer in real-time. In some SD rats, electroencephalogram (EEG) was used to monitor electrical activity correlated with behavioral seizures. During stereotaxic surgery, six holes were made in the skull so that six EEG screw wire electrodes could be screwed in for dura-mater contact. Placement of these screws was bilateral as follows: frontal lobe, parietal lobe, and cerebellum. These EEG wire electrodes were routed into an EEG pedestal (Plastics One, Roanoke, VA, USA) that was secured to the skull with dental acrylic. EEG recordings were performed in awake animals with the EEG pedestal coupled to an EEG preamplifier and amplifier (Pinnacle Technologies, Lawrence, KS, USA). The raw signal was routed to a PowerLab analog-to-digital converted and data were recorded with LabChart software (AD Instruments, Colorado Springs, CO, USA) with a 60 Hz low pass filter. After each experimental session, rats were returned to their home cage and given at least 48 h in between sessions. We used a modified Racine scale for seizure severity described as follows: 0.5 = jaw clonus, 1 = myoclonic jerks, 2 = single-arm forelimb clonus, 3 = bilateral forelimb clonus and facial clonus, 4 = bilateral forelimb and facial clonus with rearing, 5 = loss of balance after rearing with forelimb and facial clonus (Piredda and Gale, 1985; Cassidy and Gale, 1998).

Audiogenic Seizure Testing

GEPR-3 rats were first tested for response to AGSs by challenging them with 105–110 dB pure tones (Med Associates, St. Albans, VT, USA) for 60 s. In the cases of no seizure activity, the animals were given a 120 dB mixed sounds produced by an electrical bell. Following the establishment of baseline AGS score, the animals were given 60 min to recover and then tested again under a given experimental condition. The animals were then tested a final time for AGSs after an additional 20 min. Only GEPR-3s that exhibited AGSs with a score of 2 or greater were used in the pharmacological experiments. AGS seizure severity was classified into four stages as follows: 0 = no seizures, 1 = one episode of wild running seizures (WRS), 2 = two or more episodes of WRS, 3 = one episode of WRS followed by tonic-clonic seizures. Behavioral outcomes assessed included maximum seizure severity score reached, latency to seizure onset, and duration of the seizure. Seizure severity was scored by two observers in real-time and recorded with a video monitoring system (Med Associates, St. Albans, VT, USA).

Statistics and Data Analysis

Statistical analyses were performed in GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 25 (IBM). Non-parametric data and data that failed tests of normality (e.g., seizure severity, seizure frequency, and seizure count) were analyzed using the Wilcoxon Matched Pairs test for paired data or Friedman's test with Dunn's multiple comparison test.

RESULTS

We first evaluated the ability of systemic administration of WIN to attenuate AGSs in GEPR-3s; the testing strategy is shown in **Figure 1A**. GEPR-3s were first tested for a baseline AGS susceptibility. Thirty minutes later, GEPR-3s were injected with either vehicle or WIN (1, 1.5, or 2 mg/kg) and re-tested for AGS 30 min after injection. As shown in **Figure 1B**, the 1 mg/kg dose of WIN did not alter AGS responses (Friedman's test, $Q = 0.625$, $p = 0.94$). In all cases (Baseline 1, Vehicle, Baseline 2, and 1 mg/kg of WIN) the median seizure score was 3. We found a similar lack of effect following the 1.5 mg/kg dose of WIN (**Figure 1C**); the median seizure score for each group was 3, and thus did not differ as a function of treatment (Friedman's test, $Q = 5.0$, $p = 0.24$). Unlike the lower doses, 2 mg/kg of WIN produced a robust suppression of seizure activity (median seizure = 0), concurrent with mild sedation. This significant reduction in seizure severity ($Q = 24.97$, $p = 0.000016$, Friedman's test) was driven by the difference between the second baseline session and the 2 mg/kg treated post-test ($p = 0.0021$, Dunn's multiple comparison test). The 2 mg/kg dose also differed significantly from the vehicle ($p = 0.0073$). Importantly, the two baseline sessions did not differ

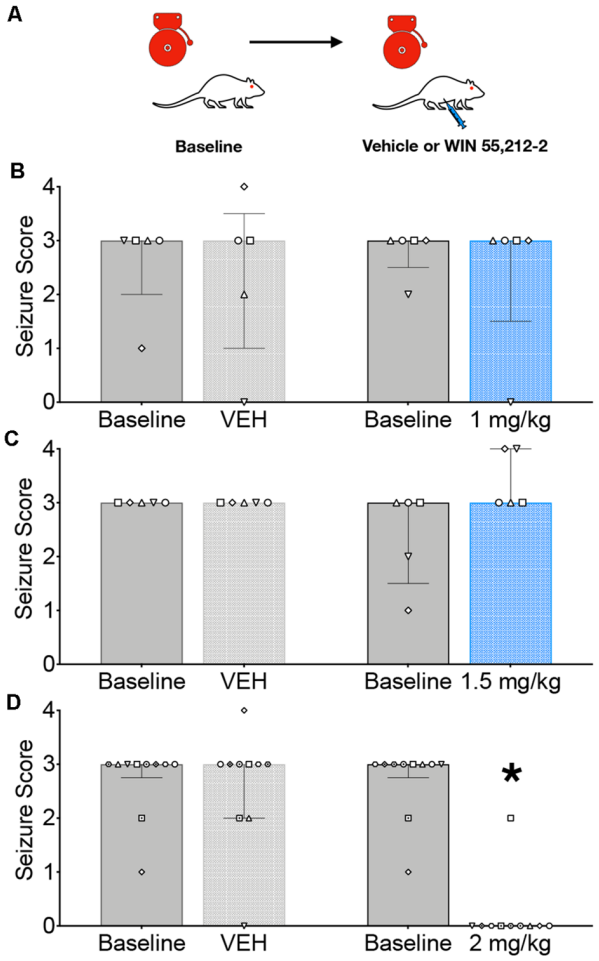


FIGURE 1 | Systemic administration of the CB1/2 receptor agonist, WIN 55,212-2 (WIN) attenuates audiogenic seizures in genetically epilepsy-prone rats (GEPRs). **(A)** Schematic of the testing scheme; animals were tested with a baseline test session before a vehicle-treated session. On a separate day, animals were tested on a baseline session and then with one of three doses of WIN. The testing order was counterbalanced across subjects. **(B)** Against audiogenic seizures (AGS) scores showing baseline sessions and comparing vehicle (left) and 1 mg/kg WIN (right). **(C)** AGS scores showing baseline sessions and comparing vehicle (left) and 1.5 mg/kg WIN (right). **(D)** AGS scores showing baseline sessions and comparing vehicle (left) and 2 mg/kg (right). Bars indicate median and interquartile range, symbols show individual subjects. *$p < 0.05$, according to Friedman test followed by Dunn's *post hoc* test.

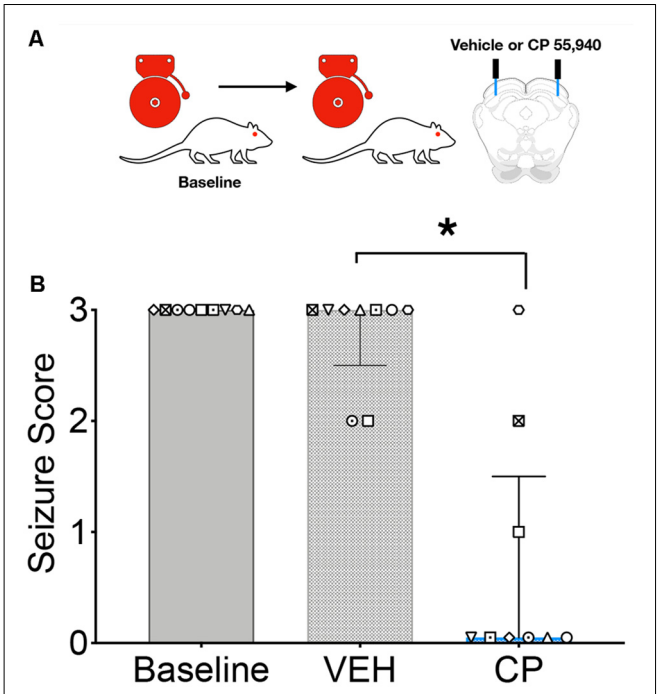


FIGURE 2 | Intracollicular injection of the CB1/2 receptor agonist, CP 55940 (CP) attenuates audiogenic seizures in GEPRs. **(A)** Schematic of the testing scheme; animals were tested with a baseline test session before a vehicle-treated session and a CP treated session. A baseline is shown only once, as in this experiment all animals displayed baseline scores of three across both tests. **(B)** Microinfusion of CP significantly decreased audiogenic seizure score as compared to vehicle (VEH) infusion into the deep layers of the superior colliculus (DLSC). Bars indicate median and interquartile range, symbols show individual subjects. *$p < 0.05$, according to Friedman test followed by Dunn's *post hoc* test.

from one another ($p > 0.999$), nor did the vehicle treatment differ from the baseline ($p > 0.999$).

We next sought to determine if focal activation of CB1 receptors in the DLSC would impair the effect of systemic drug administration; the testing scheme for these experiments is shown in **Figure 2A**. GEPR-3s were tested for baseline AGS susceptibility and then injected with vehicle or CP in a counterbalanced manner. During the un-injected (baseline) audiogenic susceptibility test and the vehicle injection test, the median seizure score was a 3. However, CP injection produced a significant suppression of seizure responses (median = 0; $Q = 15.08$, $p = 0.0002$; Friedman's test, **Figure 2B**). Baseline and vehicle-injected conditions did not differ from one another ($p > 0.999$, Dunn's multiple comparison test). CP injection, by contrast, significantly reduced AGS as compared both to the baseline session ($p = 0.0065$) and the vehicle-injected session ($p = 0.029$).

Given that focal delivery of CB1/2 agonist to the DLSC resulted in a suppression of seizures that was reminiscent of that seen after systemic administration of agonist, we next sought to determine if activation of CB1/2 receptors in the DLSC was necessary for the seizure-suppressive effects of systemic drug treatment. The experimental scheme is shown in **Figure 3A**. GEPR-3s were tested on a baseline AGS test and then retested following systemic administration of vehicle or WIN paired with an intra-DLSC injection of vehicle or the CB1 receptor antagonist SR141716. Due to premature implant loss, not all animals received all treatments; we, therefore, analyzed these sessions independently. When the systemic vehicle was paired with the intra-DLSC vehicle, seizure responses did not differ from the baseline session ($W = -11$, $p = 0.25$, **Figure 3B**). Similar to what we previously observed (**Figure 1**), systemic administration of WIN when paired with an intra-DLSC vehicle infusion produced a significant reduction

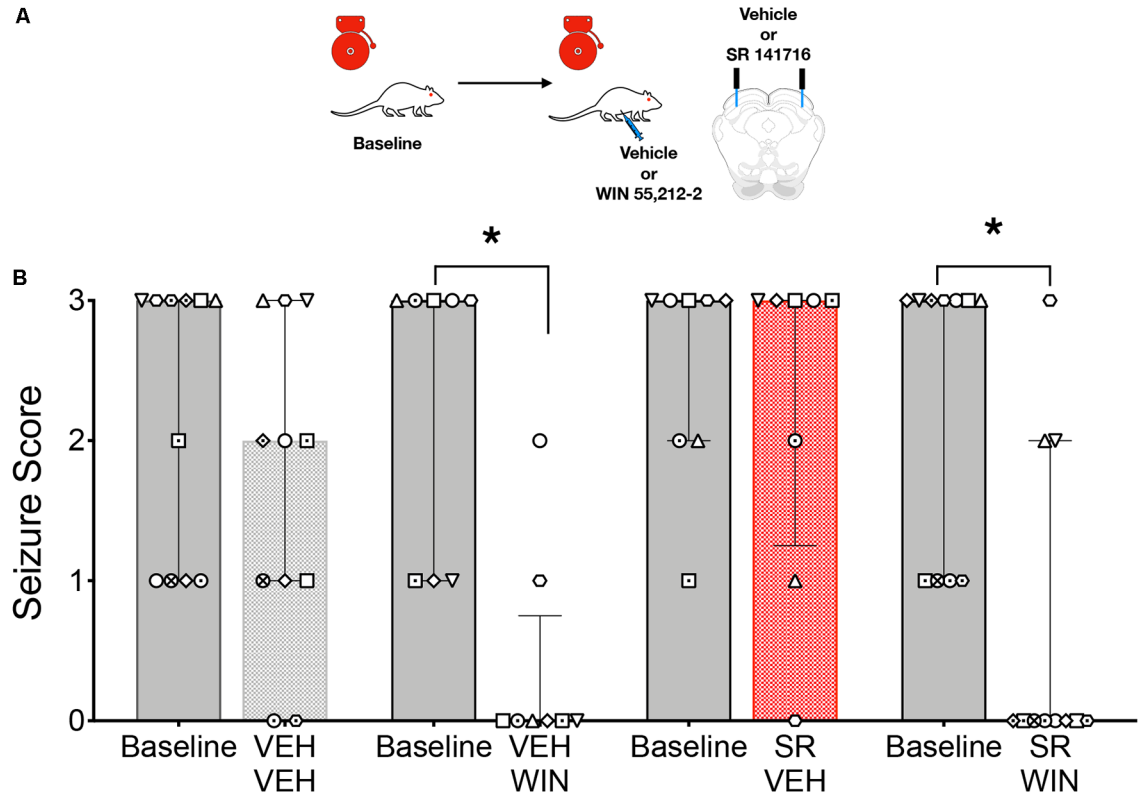


FIGURE 3 | Activation of collicular CB1/2 receptors is sufficient, but not necessary to account for the anticonvulsant effect of systemic WIN 55,212-2 (WIN) in GEPRs. **(A)** Schematic of the testing scheme; animals were tested with a baseline test session before each of the four test conditions. Animals received either vehicle (VEH) or SR 141716 (SR) infusion into the DLSC followed by VEH or WIN (2 mg/kg, i.p.). **(B)** AGS score as a function of drug treatments. WIN administration significantly reduced AGS severity as compared to the pre-administration baseline. SR administration was without effect on audiogenic seizures (AGSs) but failed to reverse the anticonvulsant action of WIN. Bars show median and interquartile range, symbols show individual subjects. *$p < 0.05$, according to Friedman test followed by Dunn's *post hoc* test.

in seizure severity when compared to the baseline session ($W = -36$, $p = 0.0078$). Intra-DLSC administration of the CB antagonist SR141716, when paired with systemic vehicle injection was without effect on seizure severity as compared to the baseline session ($W = -2$, $p = 0.75$). By contrast, we observed robust seizure suppression following systemic administration of WIN, paired with intra-DLSC administration of SR141716 ($W = -55$, $p = 0.002$). Thus, blockade of CB receptors in the DLSC did not alter the anticonvulsant action of WIN.

To determine if this anticonvulsant profile held for a seizure model reliant on forebrain networks, we examined the effect of systemic (**Figure 4A**) and intra-DLSC (**Figure 4B**) administration of CB1/2 agonist against seizures evoked focally from the AT. We used a high dose of WIN (2 mg/kg) for these experiments. We found that while this dose produced mild sedation, it did not suppress behavioral (**Figure 4B**) or electrographic (**Figure 4C**) seizures. Systemic injection of the vehicle was associated with a median seizure severity of 4.5, whereas WIN administration at the dose of 2 mg/kg was associated with a median seizure score of 5 ($W = 13$, $p = 0.41$). In contrast to the lack of effect following systemic administration of WIN, intra-DLSC injection of CP was associated with robust suppression of AT-evoked seizures. Following vehicle injection into the DLSC (**Figure 4D**), the median AT evoked seizure response was 4 (**Figure 4E**). By contrast, after the intra-DLSC injection of CP, the median response was 0.5, an effect that reached the level of statistical significance ($W = -28$, $p = 0.016$). Thus, unlike the case of AGSs, systemic CB agonists did not protect against AT-evoked seizures, but intra-DLSC activation of CB receptors potently suppressed behavioral (**Figure 4E**) and electrographic (**Figure 4F**) seizures.

DISCUSSION

Here we report a surprising dissociation between the effects of CB agonists when administered systemically and intra-DLSC across two models of experimental seizures. While systemic administration of the CB1/2 receptor agonist WIN exerted a dose-dependent suppression of AGSs in GEPR-3s, systemic administration was without effect against seizures evoked from AT. By contrast, intra-DLSC administration of the CB1 receptor agonist CP was effective in both models. Interestingly, while

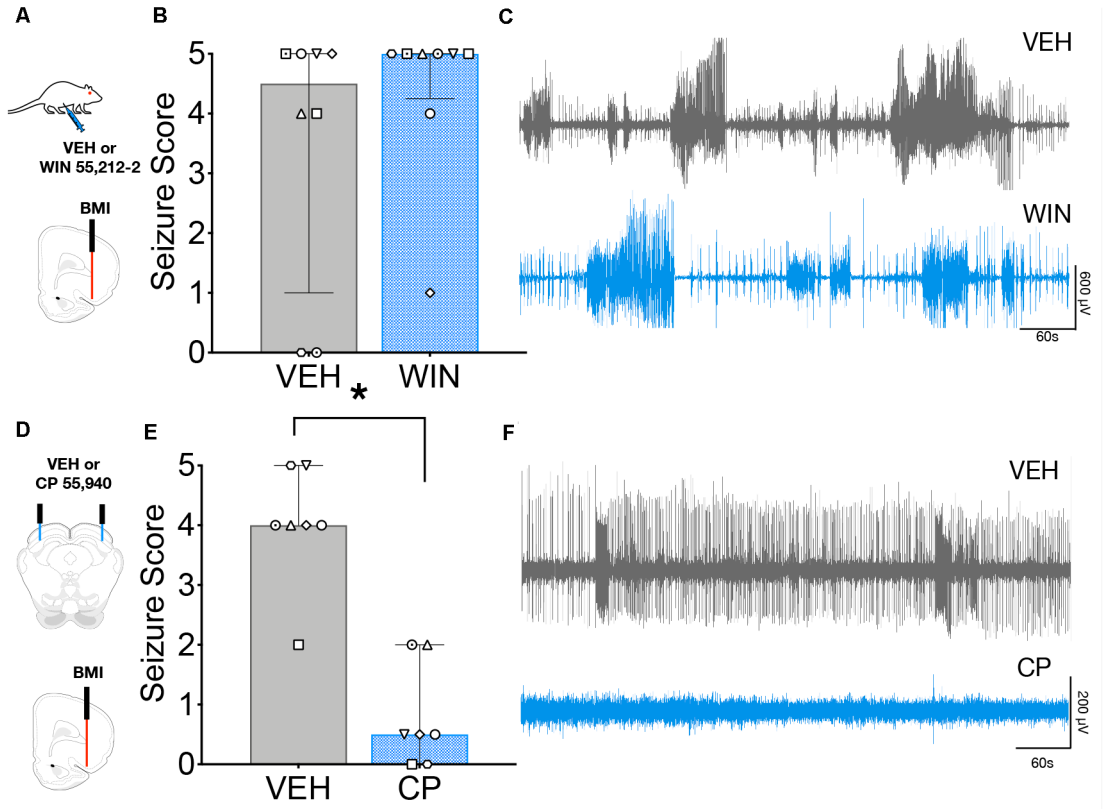


FIGURE 4 | Intracollicular, but not systemic cannabinoid (CB) receptor activation suppresses seizures evoked from Area Tempestas (AT). **(A)** Seizures were evoked by injection of the GABA-A receptor antagonist, bicuculline methiodide (BMI) into the AT after systemic administration of VEH or WIN 55,212-2. **(B)** Behavioral seizure severity under the vehicle (VEH) and WIN treated conditions did not differ. **(C)** Representative electrographic seizures from the same subject under VEH and WIN treated conditions, showing multiple high amplitude events and isolated interictal spiking. **(D)** Seizures were evoked by injection of BMI into the AT following either VEH or CP infusion into the DLSC. **(E)** Seizure severity was significantly reduced by CP infusion into the DLSC. **(F)** Representative electrographic activity following treatment of the DLSC with VEH (top) and CP (bottom) from the same subject, showing that behavioral seizure suppression co-occurred with suppression of electrographic seizure activity. Bars show median and interquartile range, symbols show individual subjects. *$p < 0.05$.

activation of the CB1 receptor in the DLSC was *sufficient* to suppress seizure activity, these CB1 receptors are *not necessary*—for the anticonvulsant action of WIN.

CB receptor agonists have been widely studied for anti-seizure effects across an array of models of seizures; this has been reviewed extensively elsewhere (Wallace et al., 2001; Skaper and Di Marzo, 2012; Cristino et al., 2020). Our results are generally consistent with previous reports examining the effect of WIN on seizure susceptibility in two other AGS models. For example, a single dose of WIN failed to suppress acute AGS in Krushinsky-Molodkina rats, it delays the development of audiogenic kindling suggestive of an antiepileptogenic effect (Vinogradova and van Rijn, 2015). Furthermore, chronic treatment with CB1 antagonist SR141716A worsens AGS (Vinogradova et al., 2011). While the GEPR-3 and the Krushinsky–Molodkina rat share phenotypic similarities, we cannot exclude a contribution of strain to the differences between published reports and our present studies. Our results are likewise consistent with the findings of Citraro et al. (2016) who reported that WIN reduced AGS severity in DBA/2 mice. Similarly, activation of CB1 receptors protected against acute clonic and generalized tonic-clonic seizures in pentylenetetrazole model (Bahremand et al., 2008b) and maximal electroshock test (Wallace et al., 2001; Luszczki et al., 2011; Florek-Luszczki et al., 2014b, 2015). Thus, activation of CB1 receptors is effective in suppressing seizures that require hindbrain circuitry.

In the present study, we found that systemic administration of WIN did not alter the occurrence of AT-evoked seizure. By contrast, systemic administration of WIN reduced the incidence of spontaneous seizures in the post-status epilepticus pilocarpine model (Wallace, 2003; Di Maio et al., 2015), and reduced the seizure severity of acute pilocarpine-induced status epilepticus (Colangeli et al., 2019; Di Maio et al., 2019). Moreover, WIN protects against acute temporal-lobe like seizures in the maximal dentate activation model (Carletti et al., 2016). The AT model differs from pilocarpine in both its focal nature (vs. systemic pilocarpine) and its pharmacology (GABA antagonist vs. muscarinic agonist). It also differs in the locus of seizure origination (piriform cortex) as compared to the maximal dentate activation model (dentate gyrus). Of particular note

is the dense expression of CB1 receptors on interneurons of the anterior piriform cortex (Terral et al., 2019), notably this area of dense receptor expression overlaps with the region functionally defined as AT. This raises the possibility that systemic administration of WIN may have further *disinhibited the* piriform cortex, negating anticonvulsant actions occurring at other sites in the brain.

We had hypothesized that the DLSC might be a critical site of action for CB-mediated anti-convulsant effects. This was based on: (1) the well-established role the DLSC plays in audiogenic seizures, and in particular, in the wild-running phase which is typical of the GEPR-3 strain (Merrill et al., 2005); (2) volumetric changes in the SC in GEPR-3 rats (Lee et al., 2018); (3) the interconnections between the substantia nigra pars reticulata and the DLSC and the density of CB1 receptors in the SNpr (Herkenham et al., 1991; Matsuda et al., 1992; Glass et al., 1997; Tsou et al., 1998); (4) the presence of CB1 immunopositive axons in the DLSC (Tsou et al., 1998; Sañudo-Peña et al., 2000); (5) the anticonvulsant effects of activation of the DLSC (Soper et al., 2016); and (6) the anticonvulsant effects of disinhibition of the SC through inhibition of nigrotectal projections (Wicker et al., 2019). Consistent with our hypothesis, we found that the focal application of CP to the DLSC suppressed both AGSs and AT-evoked seizures. These findings are, however, generally consistent with a *disinhibitory* action of CP in the DLSC. While we found that focal microinjection of CB1/2 agonist into the DLSC attenuated both AGSs and AT-evoked seizures, we found that the focal blockade of CB1 receptors in the DLSC was without effect on the anticonvulsant action of systemic agonist, suggesting an extra-DLSC site of the action of the agonist. A potential site of interest is the inferior colliculus that is critical in AGS initiation (see Faingold et al., 2014 for a review of audiogenic seizure networks), however, while there is an expression of CB1 receptors in the IC, the expression is less than that observed in the SC (Herkenham et al., 1991).

While the CB1 receptor is the predominant CB receptor in the brain, the CB2 receptor is also expressed in the brainstem (Van Sickle et al., 2005), cerebellum (Ashton et al., 2006; Suárez et al., 2008), and other brain regions (Gong et al., 2006). While selective CB1 receptor agonists and mixed CB1/CB2 receptor agonists have well-documented anticonvulsant effects, selective CB2 agonists are both less studied and less robust (for review see: Rosenberg et al., 2017). While WIN and CP are mixed CB1/2 agonists, the profile of anticonvulsant effects seen with WIN is similar to those of the highly selective CB1 agonist, ACEA (Rosenberg et al., 2017), suggesting that anticonvulsant effects are CB1, rather than CB2 mediated. Moreover, CB2 receptor expression in the SC (Gong et al., 2006; Duff et al., 2013) has been predominantly detected in the visual (superficial) as compared to the deep and intermediate layers, which are the sites associated with anticonvulsant action (Gale et al., 1993; Redgrave et al., 1993). Thus, it is unlikely that the anticonvulsant effects of intracollicular CP are mediated by CB2 receptors.

In sum, our data suggest that focal modulation of CB receptors within the DLSC controls both brainstems- and forebrain-evoked seizures, but also show that this site of action is not necessary for the anticonvulsant effect of systemic CB1/2 receptor agonists. While in the brainstem model, both systemic and intra-DLSC drug treatment reduced seizures, in the forebrain model, intra-DLSC drug infusion but not systemic drug treatment reduced seizures. These data add to our understanding of potential sites of action of CBs in the context of anti-seizure therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Georgetown University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

VS and RH contributed equally. VS, RH, PN'G, and PF designed research. VS, RH, and EW performed experiments. VS, RH, and PF analyzed data. VS, RH, and PF drafted the manuscript, which all authors edited. PN'G and PF obtained funding and supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by R01NS097762 from the National Institute of Neurological Disorders and Stroke to PF and R01 AA027660 from the National Institute on Alcohol Abuse and Alcoholism to PN'G. PF was supported in part by KL2TR001432 from the National Center for Advancing Translational Sciences.

REFERENCES

Aghaei, I., Rostampour, M., Shabani, M., Naderi, N., Motamedi, F., Babaei, P., et al. (2015). Palmitoylethanolamide attenuates PTZ-induced seizures through CB1 and CB2 receptors. *Epilepsy Res.* 117, 23–28. doi: 10.1016/j.eplepsyres.2015.08.010

Andres-Mach, M., Zolkowska, D., Barcicka-Klosowska, B., Haratym-Maj, A., Florek-Luszczki, M., and Luszczki, J. J. (2012). Effect of ACEA-a selective cannabinoid CB1 receptor agonist on the protective action of different antiepileptic drugs in the mouse pentylenetetrazole-induced seizure model. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 39, 301–309. doi: 10.1016/j.pnpbp.2012.07.001

Ashton, J. C., Friberg, D., Darlington, C. L., and Smith, P. F. (2006). Expression of the cannabinoid CB2 receptor in the rat cerebellum: an immunohistochemical study. *Neurosci. Lett.* 396, 113–116. doi: 10.1016/j.neulet.2005.11.038

Bahremand, A., Shafaroodi, H., Ghasemi, M., Nasrabady, S. E., Gholizadeh, S., and Dehpour, A. R. (2008a). The cannabinoid anticonvulsant effect on pentylenetetrazole-induced seizure is potentiated by ultra-low dose

naltrexone in mice. *Epilepsy Res.* 81, 44–51. doi: 10.1016/j.eplepsyres.2008.04.010

Bahremand, A., Shafaroodi, H., Ghasemi, M., Nasrabady, S. E., Gholizadeh, S., and Dehpour, A. R. (2008b). The cannabinoid anticonvulsant effect on pentylenetetrazole-induced seizure is potentiated by ultra-low dose naltrexone in mice. *Epilepsy Res.* 81, 44–51. doi: 10.1016/j.eplepsyres.2008.04.010

Bojnik, E., Turunç, E., Armağan, G., Kanıt, L., Benyhe, S., Yalçın, A., et al. (2012). Changes in the cannabinoid (CB1) receptor expression level and G-protein activation in kainic acid induced seizures. *Epilepsy Res.* 99, 64–68. doi: 10.1016/j.eplepsyres.2011.10.020

Browning, R., Maggio, R., Sahibzada, N., and Gale, K. (1993). Role of brainstem structures in seizures initiated from the deep prepiriform cortex of rats. *Epilepsia* 34, 393–407. doi: 10.1111/j.1528-1157.1993.tb02579.x

Carletti, F., Gambino, G., Rizzo, V., Ferraro, G., and Sardo, P. (2016). Involvement of TRPV1 channels in the activity of the cannabinoid WIN 55, 212-2 in an acute rat model of temporal lobe epilepsy. *Epilepsy Res.* 122, 56–65. doi: 10.1016/j.eplepsyres.2016.02.005

Cassidy, R. M., and Gale, K. (1998). Mediodorsal thalamus plays a critical role in the development of limbic motor seizures. *J. Neurosci.* 18, 9002–9009. doi: 10.1523/JNEUROSCI.18-21-09002.1998

Citraro, R., Russo, E., Ngomba, R. T., Nicoletti, F., Scicchitano, F., Whalley, B. J., et al. (2013). CB1 agonists, locally applied to the cortico-thalamic circuit of rats with genetic absence epilepsy, reduce epileptic manifestations. *Epilepsy Res.* 106, 74–82. doi: 10.1016/j.eplepsyres.2013.06.004

Citraro, R., Russo, E., Leo, A., Russo, R., Avagliano, C., Navarra, M., et al. (2016). Pharmacokinetic-pharmacodynamic influence of N-palmitoylethanolamine, arachidonyl-2′-chloroethylamide and WIN 55, 212-2 on the anticonvulsant activity of antiepileptic drugs against audiogenic seizures in DBA/2 mice. *Eur. J. Pharmacol.* 791, 523–534. doi: 10.1016/j.ejphar.2016.09.029

Colangeli, R., Di Maio, R., Pierucci, M., Deidda, G., Casarrubea, M., and Di Giovanni, G. (2019). Synergistic action of CB1 and 5-HT 2B receptors in preventing pilocarpine-induced status epilepticus in rats. *Neurobiol. Dis.* 125, 135–145. doi: 10.1016/j.nbd.2019.01.026

Cristino, L., Bisogno, T., and Di Marzo, V. (2020). Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nat. Rev. Neurol.* 16, 9–29. doi: 10.1038/s41582-019-0284-z

Depaulis, A., Liu, Z., Vergnes, M., Marescaux, C., Micheletti, G., and Warter, J. M. (1990). Suppression of spontaneous generalized non-convulsive seizures in the rat by microinjection of GABA antagonists into the superior colliculus. *Epilepsy Res.* 5, 192–198. doi: 10.1016/0920-1211(90)90038-w

Di Maio, R., Cannon, J. R., and Timothy Greenamyre, J. (2015). Post-status epilepticus treatment with the cannabinoid agonist WIN 55, 212-2 prevents chronic epileptic hippocampal damage in rats. *Neurobiol. Dis.* 73, 356–365. doi: 10.1016/j.nbd.2014.10.018

Di Maio, R., Colangeli, R., and Di Giovanni, G. (2019). WIN 55, 212-2 reverted pilocarpine-induced status epilepticus early changes of the interaction among 5-HT_{2C}/NMDA/CB_1 receptors in the rat hippocampus. *ACS Chem. Neurosci.* 10, 3296–3306. doi: 10.1021/acschemneuro.9b00080

Duff, G., Argaw, A., Cecyre, B., Cherif, H., Tea, N., and Zabouri, N. (2013). Cannabinoid receptor CB2 modulates axon guidance. *PLoS One* 8:e70849. doi: 10.1371/journal.pone.0070849

Faingold, C., and Casebeer, D. (1999a). Modulation of the audiogenic seizure network by noradrenergic and glutamatergic receptors of the deep layers of superior colliculus. *Brain Res.* 821, 392–399. doi: 10.1016/s0006-8993(99)01101-4

Faingold, C. L., and Randall, M. E. (1999b). Neurons in the deep layers of superior colliculus play a critical role in the neuronal network for audiogenic seizures: mechanisms for production of wild running behavior. *Brain Res.* 815, 250–258. doi: 10.1016/s0006-8993(98)01136-6

Faingold, C. L., Raisinghani, M., and N'Gouemo, P. (2014). "Neuronal networks in epilepsy: comparative audiogenic seizure networks," in *Neuronal Networks in Brain Function, CNS Disorders and Therapeutics*, eds C. L. Faingold and H. Blumenfeld (Sandiego, CA: Elsevier/Academic Press), 349–373.

Florek-Luszczki, M., Wlaz, A., Kondrat-Wrobel, M. W., Tutka, P., and Luszczki, J. J. (2014a). Effects of WIN 55, 212-2 (a non-selective cannabinoid CB1 and CB2 receptor agonist) on the protective action of various classical antiepileptic drugs in the mouse 6 Hz psychomotor seizure model. *J. Neural Transm.* 121, 707–715. doi: 10.1007/s00702-014-1173-7

Florek-Luszczki, M., Zagaja, M., and Luszczki, J. J. (2014b). Influence of WIN 55, 212-2 on the anticonvulsant and acute neurotoxic potential of clobazam and lacosamide in the maximal electroshock-induced seizure model and chimney test in mice. *Epilepsy Res.* 108, 1728–1733. doi: 10.1016/j.eplepsyres.2014.10.004

Florek-Luszczki, M., Zagaja, M., and Luszczki, J. J. (2015). Influence of arachidonyl-2′-chloroethylamide, a selective cannabinoid CB1 receptor agonist, on the anticonvulsant and acute side-effect potentials of clobazam, lacosamide and pregabalin in the maximal electroshock-induced seizure model and chimney test in mice. *Fundam. Clin. Pharmacol.* 29, 382–393. doi: 10.1111/fcp.12123

Gale, K., Pazos, A., Maggio, R., Japikse, K., and Pritchard, P. (1993). Blockade of GABA receptors in superior colliculus protects against focally evoked limbic motor seizures. *Brain Res.* 603, 279–283. doi: 10.1016/0006-8993(93)91248-q

Glass, M., Dragunow, M., and Faull, R. L. M. (1997). Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. *Neuroscience* 77, 299–318. doi: 10.1016/s0306-4522(96)00428-9

Gong, J.-P., Onaivi, E. S., Ishiguro, H., Liu, Q.-R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10–23. doi: 10.1016/j.brainres.2005.11.035

Hájos, N., Katona, I., Naiem, S. S., MacKie, K., Ledent, C., Mody, I., et al. (2000). Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur. J. Neurosci.* 12, 3239–3249. doi: 10.1046/j.1460-9568.2000.00217.x

Herkenham, M., Lynn, A. B., de Costa, B. R., and Richfield, E. K. (1991). Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. *Brain Res.* 547, 267–274. doi: 10.1016/0006-8993(91)90970-7

Huizenga, M. N., Wicker, E., Beck, V. C., and Forcelli, P. A. (2017). Anticonvulsant effect of cannabinoid receptor agonists in models of seizures in developing rats. *Epilepsia* 58, 1593–1602. doi: 10.1111/epi.13842

Kathmann, M., Bauer, U., Schlicker, E., and Göthert, M. (1999). Cannabinoid CB1 receptor-mediated inhibition of NMDA- and kainate-stimulated noradrenaline and dopamine release in the brain. *Naunyn Schmiedebergs Arch. Pharmacol.* 359, 466–470. doi: 10.1007/pl00005377

Katona, I., Sperlágh, B., Sík, A., Käfalvi, A., Vizi, E. S., Mackie, K., et al. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.* 19, 4544–4558. doi: 10.1523/JNEUROSCI.19-11-04544.1999

Katona, I., Rancz, E. A., Acsady, L., Ledent, C., Mackie, K., Hajos, N., et al. (2001). Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J. Neurosci.* 21, 9506–9518. doi: 10.1523/JNEUROSCI.21-23-09506.2001

Lee, Y., Rodriguez, O. C., Albanese, C., Santos, V. R., de Oliveira, J. A. C., Donatti, A. L. F., et al. (2018). Divergent brain changes in two audiogenic rat strains: a voxel-based morphometry and diffusion tensor imaging comparison of the genetically epilepsy prone rat (GEPR-3) and the wistar audiogenic rat (WAR). *Neurobiol. Dis.* 111, 80–90. doi: 10.1016/j.nbd.2017.12.014

Pellegrino, L. J., and Cushman, A. J. (1967). *A Stereotaxic Altasl of The Rat Brain.* New York, NY: Appleton-Century-Crofts.

Luszczki, J. J., Czuczwar, P., Cioczek-Czuczwar, A., and Czuczwar, S. J. (2006). Arachidonyl-2′-chloroethylamide, a highly selective cannabinoid CB1 receptor agonist, enhances the anticonvulsant action of valproate in the mouse maximal electroshock-induced seizure model. *Eur. J. Pharmacol.* 547, 65–74. doi: 10.1016/j.ejphar.2006.07.037

Luszczki, J. J., Andres-Mach, M., Barcicka-Klosowska, B., Florek-Luszczki, M., Haratym-Maj, A., and Czuczwar, S. J. (2011). Effects of WIN 55, 212-2 mesylate (a synthetic cannabinoid) on the protective action of clonazepam, ethosuximide, phenobarbital and valproate against pentylenetetrazole-induced clonic seizures in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1870–1876. doi: 10.1016/j.pnpbp.2011.07.001

Matsuda, L. A., Bonner, T. I., and Lolait, S. J. (1992). Cannabinoid receptors: which cells, where, how and why? *NIDA Res. Monogr.* 126, 48–56.

Merrill, M. A., Clough, R. W., Jobe, P. C., and Browning, R. A. (2003). Role of the superior colliculus and the intercollicular nucleus in the brainstem

seizure circuitry of the genetically epilepsy-prone rat. *Epilepsia* 44, 305–314. doi: 10.1046/j.1528-1157.2003.31802.x

Merrill, M. A., Clough, R. W., Jobe, P. C., and Browning, R. A. (2005). Brainstem seizure severity regulates forebrain seizure expression in the audiogenic kindling model. *Epilepsia* 46, 1380–1388. doi: 10.1111/j.1528-1167.2005.39404.x

Nail-Boucherie, K., Lê-Pham, B. T., Marescaux, C., and Depaulis, A. (2002). Suppression of absence seizures by electrical and pharmacological activation of the caudal superior colliculus in a genetic model of absence epilepsy in the rat. *Exp. Neurol.* 177, 503–514. doi: 10.1006/exnr.2002.7997

Nail-Boucherie, K., Lê-Pham, B. T., Marescaux, C., and Depaulis, A. (2005). Evidence for a role of the parafascicular nucleus of the thalamus in the control of epileptic seizures by the superior colliculus. *Epilepsia* 46, 141–145. doi: 10.1111/j.0013-9580.2005.30304.x

Piredda, S., and Gale, K. (1985). A crucial epileptogenic site in the deep prepiriform cortex. *Nature* 317, 623–625. doi: 10.1038/317623a0

Raisinghani, M., and Faingold, C. L. (2003). Identification of the requisite brain sites in the neuronal network subserving generalized clonic audiogenic seizures. *Brain Res.* 967, 113–122. doi: 10.1016/s0006-8993(02)04232-4

Redgrave, P., Simkins, M., Overton, P., and Dean, P. (1992). Anticonvulsant role of nigrotectal projection in the maximal electroshock model of epilepsy-I. Mapping of dorsal midbrain with bicuculline. *Neuroscience* 46, 379–390. doi: 10.1016/0306-4522(92)90059-b

Redgrave, P., Westby, G. W. M., and Dean, P. (1993). Functional architecture of rodent superior colliculus: relevance of multiple output channels. *Prog. Brain Res.* 95, 69–77. doi: 10.1016/s0079-6123(08)60358-1

Rosenberg, E. C., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Rowan, A. N. (1979). Guide for the care and use of laboratory animals. *J. Med. Primatol.* 8:128. doi: 10.1159/000460187

Santos, V. R., Kobayashi, I., Hammack, R., Danko, G., and Forcelli, P. A. (2018). Impact of strain, sex and estrous cycle on gamma butyrolactone-evoked absence seizures in rats. *Epilepsy Res.* 147, 62–70. doi: 10.1016/j.eplepsyres.2018.09.007

Sañudo-Peña, M. C., Tsou, K., Romero, J., Mackie, K., and Walker, J. M. (2000). Role of the superior colliculus in the motor effects of cannabinoids and dopamine. *Brain Res.* 853, 207–214. doi: 10.1016/s0006-8993(99)02291-x

Shubina, L., Aliev, R., and Kitchigina, V. (2015). Attenuation of kainic acid-induced status epilepticus by inhibition of endocannabinoid transport and degradation in guinea pigs. *Epilepsy Res.* 111, 33–44. doi: 10.1016/j.eplepsyres.2015.01.003

Skaper, S. D., and Di Marzo, V. (2012). Endocannabinoids in nervous system health and disease: the big picture in a nutshell. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 3193–3200. doi: 10.1098/rstb.2012.0313

Soper, C., Wicker, E., Kulick, C. V., N'Gouemo, P., and Forcelli, P. A. (2016). Optogenetic activation of superior colliculus neurons suppresses seizures originating in diverse brain networks. *Neurobiol. Dis.* 87, 102–115. doi: 10.1016/j.nbd.2015.12.012

Suárez, J., Bermúdez-Silva, F. J., Mackie, K., Ledent, C., Zimmer, A., and Cravatt, B. F. (2008). Immunohistochemical description of the endogenous cannabinoid system in the rat cerebellum and functionally related nuclei. *J. Comp. Neurol.* 509, 400–421. doi: 10.1002/cne.21774

Sullivan, J. M. (1999). Mechanisms of cannabinoid-receptor-mediated inhibition of synaptic transmission in cultured hippocampal pyramidal neurons. *J. Neurophysiol.* 82, 1286–1294. doi: 10.1152/jn.1999.82.3.1286

Swanson, L. W. (2018). Brain maps 4.0—Structure of the rat brain: an open access atlas with global nervous system nomenclature ontology and flatmaps. *J. Comp. Neurol.* 526, 935–943. doi: 10.1002/cne.24381

Terral, G., Busquets-Garcia, A., Varilh, M., Achicallende, S., Cannich, A., Bellocchio, L., et al. (2019). CB1 receptors in the anterior piriform cortex control odor preference memory. *Curr. Biol.* 29, 2455.e5–2464.e5. doi: 10.1016/j.cub.2019.06.041

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K., and Walker, J. M. (1998). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83, 393–411. doi: 10.1016/s0306-4522(97)00436-3

Tutka, P., Wlaź, A., Florek-Łuszczki, M., KoŁodziejczyk, P., Bartusik-Aebisher, D., and Łuszczki, J. J. (2018). Arvanil, olvanil, AM 1172 and LY 2183240 (various cannabinoid CB1 receptor agonists) increase the threshold for maximal electroshock-induced seizures in mice. *Pharmacol. Rep.* 70, 106–109. doi: 10.1016/j.pharep.2017.08.006

Van Sickle, M. D., Duncan, M., Kingsley, P. J., Mouihate, A., Urbani, P., Mackie, K., et al. (2005). Neuroscience: identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 310, 329–332. doi: 10.1126/science.1115740

Vilela, L. R., Medeiros, D. C., Rezende, G. H. S., de Oliveira, A. C. P., Moraes, M. F. D., and Moreira, F. A. (2013). Effects of cannabinoids and endocannabinoid hydrolysis inhibition on pentylenetetrazole-induced seizure and electroencephalographic activity in rats. *Epilepsy Res.* 104, 195–202. doi: 10.1016/j.eplepsyres.2012.11.006

Vinogradova, L. V., and van Rijn, C. M. (2015). Long-term disease-modifying effect of the endocannabinoid agonist WIN55, 212-2 in a rat model of audiogenic epilepsy. *Pharmacol. Rep.* 67, 501–503. doi: 10.1016/j.pharep.2014.12.002

Vinogradova, L. V., Shatskova, A. B., and van Rijn, C. M. (2011). Pro-epileptic effects of the cannabinoid receptor antagonist SR141716 in a model of audiogenic epilepsy. *Epilepsy Res.* 96, 250–256. doi: 10.1016/j.eplepsyres.2011.06.007

Wallace, M. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920

Wallace, M. J., Wiley, J. L., Martin, B. R., and DeLorenzo, R. J. (2001). Assessment of the role of CB1 receptors in cannabinoid anticonvulsant effects. *Eur. J. Pharmacol.* 428, 51–57. doi: 10.1016/s0014-2999(01)01243-2

Weng, X., and Rosenberg, H. C. (1992). Infusion of bicuculline methiodide into the tectum: model specificity of pro- and anticonvulsant actions. *Epilepsy Res.* 12, 1–8. doi: 10.1016/0920-1211(92)90085-8

Wicker, E., and Forcelli, P. A. (2016). Chemogenetic silencing of the midline and intralaminar thalamus blocks amygdala-kindled seizures. *Exp. Neurol.* 283, 404–412. doi: 10.1016/j.expneurol.2016.07.003

Wicker, E., Beck, V. C., Kulick-Soper, C., Kulick-Soper, C. V., Hyder, S. K., Campos-Rodriguez, C., et al. (2019). Descending projections from the substantia nigra pars reticulata differentially control seizures. *Proc. Natl. Acad. Sci. U S A* 116, 27084–27094. doi: 10.1073/pnas.1908176117

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 15 December 2020
doi: 10.3389/fnbeh.2020.611278

Cannabidiol Acts at 5-HT$_{1A}$ Receptors in the Human Brain: Relevance for Treating Temporal Lobe Epilepsy

Christopher Martínez-Aguirre[1], Francia Carmona-Cruz[1], Ana Luisa Velasco[2], Francisco Velasco[2], Gustavo Aguado-Carrillo[2], Manola Cuéllar-Herrera[2] and Luisa Rocha[1]**

[1]*Department of Pharmacobiology, Center for Research and Advanced Studies, Mexico City, Mexico,* [2]*Epilepsy Clinic, Hospital General de México Dr. Eduardo Liceaga, Mexico City, Mexico*

Experimental evidence indicates that cannabidiol (CBD) induces anxiolytic and antiepileptic effects through the activation of 5-HT$_{1A}$ receptors. These receptors are coupled to G$_{i/o}$ proteins and induce inhibitory effects. At present, the interaction of CBD with 5-HT$_{1A}$ receptors in the human brain is unknown. The aim of this study focused on evaluating the interaction between CBD and 5-HT$_{1A}$ receptors in cell membranes obtained from the hippocampus and temporal neocortex of autopsies and patients with drug-resistant mesial temporal lobe epilepsy (DR-MTLE). Cell membranes were isolated from the hippocampus and temporal neocortex of a group of patients with DR-MTLE who were submitted to epilepsy surgery (n = 11) and from a group of autopsies (n = 11). The [^{3}H]-8-OH-DPAT binding assay was used to determine the pharmacological interaction of CBD with 5-HT$_{1A}$ receptors. The [^{35}S]-GTPγS assay was used to investigate the CBD-induced activation of G$_{i/o}$ proteins through its action on 5-HT$_{1A}$ receptors. The CBD affinity (pK_i) for 5-HT$_{1A}$ receptors was similar for autopsies and patients with DR-MTLE (hippocampus: 4.29 and 4.47, respectively; temporal neocortex: 4.67 and 4.74, respectively). Concerning the [^{35}S]-GTPγS assay, no statistically significant changes were observed for both hippocampal and neocortical tissue ($p >$ 0.05) at low CBD concentrations (1 pM to 10 μM). In contrast, at high concentrations (100 μM), CBD reduced the constitutive activity of G$_{i/o}$ proteins of autopsies and DR-MTLE patients (hippocampus: 39.2% and 39.6%, respectively; temporal neocortex: 35.2% and 24.4%, respectively). These changes were partially reversed in the presence of WAY-100635, an antagonist of 5-HT$_{1A}$ receptors, in the autopsy group (hippocampus, 59.8%, $p < 0.0001$; temporal neocortex, 71.5%, $p < 0.0001$) and the group of patients with DR-MTLE (hippocampus, 53.7%, $p < 0.0001$; temporal neocortex, 68.5%, $p < 0.001$). Our results show that CBD interacts with human 5-HT$_{1A}$ receptors of the hippocampus and temporal neocortex. At low concentrations, the effect of CBD upon G$_{i/o}$ protein activation is limited. However, at high concentrations, CBD acts as an inverse agonist of 5-HT$_{1A}$ receptors. This effect could modify neuronal excitation and epileptic seizures in patients with DR-MTLE.

Keywords: serotonin, 5-HT$_{1A}$ receptor, hippocampus, mesial temporal lobe epilepsy, drug-resistant epilepsy, cannabidiol, temporal neocortex

OPEN ACCESS

Edited by:
Dolores E. López García, University of Salamanca, Spain

Reviewed by:
Inga Igorevna Poletaeva, Lomonosov Moscow State University, Russia
Rui Milton Patrício Da Silva Júnior, University of São Paulo, Brazil

****Correspondence:***
Luisa Rocha
lrocha@cinvestav.mx
Manola Cuéllar-Herrera
manolacuellar@yahoo.com.mx

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *28 September 2020*
Accepted: *23 November 2020*
Published: *15 December 2020*

Citation:
Martínez-Aguirre C, Carmona-Cruz F, Velasco AL, Velasco F, Aguado-Carrillo G, Cuéllar-Herrera M and Rocha L (2020) Cannabidiol Acts at 5-HT$_{1A}$ Receptors in the Human Brain: Relevance for Treating Temporal Lobe Epilepsy. Front. Behav. Neurosci. 14:611278. doi: 10.3389/fnbeh.2020.611278

INTRODUCTION

Cannabidiol (CBD), the main non-psychoactive component of *Cannabis* plants (Russo, 2017), has a terpenophenolic structure hydroxylated at carbons 1 and 3 (Jones et al., 1977). This structure gives CBD lipophilic properties, which allow its passage across the blood–brain barrier (Calapai et al., 2020).

CBD induces antiepileptic effects in humans and experimental models (Silvestro et al., 2019). In patients with Dravet- or Lennox-Gastaut syndromes, the administration of CBD reduces the frequency and severity of the seizures (Maa and Figi, 2014; Thiele et al., 2018; Lazaridis et al., 2019). In patients with drug-resistant temporal lobe epilepsy (DR-TLE), the coadministration of CBD with antiseizure drugs reduces the number and intensity of epileptic seizures (Cunha et al., 1980). CBD administration also decreases seizure activity and neuronal hyperexcitability in experimental models of temporal lobe epilepsy (Khan et al., 2018; Patra et al., 2019). Moreover, CBD produces anxiolytic effects in humans and experimental models (Shannon et al., 2019). CBD induces antidepressant effects that are more evident when tissue serotonin levels are high (Sales et al., 2018). These effects are partially explained because CBD interacts with 5-hydroxytryptamine1A (5-HT$_{1A}$) receptors (Magen et al., 2010). In cultured Chinese hamster ovary (CHO) cells expressing 5-HT$_{1A}$ receptors, CBD showed micromolar affinity in displacing [^{3}H]-8-OH-DPAT from 5-HT$_{1A}$ receptors, increased [^{35}S]-GTPγS binding in this $G_{i/o}$ protein-coupled receptor, and reduced cAMP concentration. According to these results, the authors concluded that CBD behaves as an agonist of 5-HT$_{1A}$ receptors (Russo et al., 2005). These effects were not reproduced when cell membranes obtained from rat brainstem were exposed to CBD. However, this phytocannabinoid enhanced the ability of 8-OH-DPAT, an agonist of 5-HT$_{1A}$ receptors, to stimulate [^{35}S]-GTPγS binding. These results suggest that CBD shows an allosteric interaction with 5-HT$_{1A}$ receptors (Rock et al., 2012).

At present, the interaction of CBD with 5-HT$_{1A}$ receptors in the human brain is not known. Indeed, changes of this interaction induced by drug-resistant epilepsy may represent a condition that augments or reduces the efficacy of CBD to control the seizure activity. The aim of this study focused on evaluating the interaction of CBD with 5-HT$_{1A}$ receptors in cell membranes obtained from the hippocampus and temporal neocortex of patients with drug-resistant mesial TLE (DR-MTLE). The results were compared with brain tissue with no neurological disorders obtained from autopsies.

MATERIALS AND METHODS

Patients with DR-MTLE

Patients with DR-MTLE underwent an extensive presurgical evaluation that included video electroencephalogram (EEG) and magnetic resonance imaging (MRI) in the Epilepsy Clinic of the General Hospital from Mexico Dr. Eduardo Liceaga. Four serial EEGs were conducted to determine the presence and location of epileptiform activity. T1–T2-weighted MRI served to identify mesial sclerosis. Patients with cortical dysplasia or neocortical TLE were excluded from the study. After the presurgical evaluation, 11 patients with DR-MTLE (five females and six males) were included in the present study. The scientific and ethics committees from all the institutions involved approved this experimental protocol (authorization number DI/15/403/03/32). Written informed consent was obtained from all participants.

A standard anterior temporal lobectomy ipsilateral to the epileptogenic zone was performed in every patient 48 h after the last seizure occurrence. During the surgical procedure, samples from the hippocampus and temporal neocortex were collected immediately after resection and stored at −70°C (**Table 1**).

Autopsies

Samples from the hippocampus and temporal neocortex were obtained from 11 autopsies (three females and eight males, 37.1 ± 17.8 years of age). Death was not associated with neurological disorders, for which brain tissues were analyzed and considered as controls. Autopsy samples were collected with a postmortem interval (PMI) of 15.91 ± 3.11 h. The samples were frozen immediately after resection and stored at −70°C. Autopsies were performed at the Institute of Forensic Sciences in Mexico City (**Table 2**).

Radioligand Displacement Assay

To assess the interaction of CBD with 5-HT$_{1A}$ receptors, we conducted radioligand displacement assays and evaluated the ability of CBD to displace a radioactively labeled ligand bound to these receptors. Cell membranes were obtained as previously described, with some modifications (Benyhe et al., 1997). Briefly, brain tissue (∼500 mg) was homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4). Subsequently, it was centrifuged at 15,000 rpm for 25 min at 4°C. The resulting pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) and incubated for 30 min at 35°C. At the end of the incubation, the preparation was centrifuged under the conditions previously indicated. The resultant pellet was resuspended in buffer (50 mM Tris–HCl, 1 mM EGTA, and 5 mM $MgCl_2 \bullet 6H_2O$, pH 7.4), and protein concentration was determined by the Bradford method (Bradford, 1976).

The radioligand displacement assay was performed in a final reaction volume of 500 μl that contained 250 μg of protein of the membrane suspension and increasing concentrations of CBD (10 nM to 10 mM). The assay was carried out in the presence of [^{3}H]-8-OH-DPAT at 0.7 nM. This ligand has a high affinity for 5-HT$_{1A}$ (K_i = 0.56) and low affinity for other receptors (K_i ranging from 41.9 to >10,000; Middlemiss and Fozard, 1983; Schlegel and Peroutka, 1986; Brown et al., 1990; Pauwels et al., 1996; Kleven et al., 1997). According to the K_i of [^{3}H]-8-OH-DPAT for the different receptors, we expected that the results obtained represent the interaction of this ligand on 5-HT$_{1A}$ receptors.

The mixture was incubated for 45 min at 35°C. Non-specific binding was determined in the presence of the 5-HT$_{1A}$ receptor antagonist WAY-100635 (10 μM). The reaction was terminated by rapid filtration on a Brandel M-48 multifilter through a Whatman GF/C glass fiber filter followed by three washes

TABLE 1 | Clinical data of patients with drug-resistant mesial temporal lobe epilepsy (DR-MTLE).

ID	Gender	Age (years)	Age of seizure onset (years)	Duration of epilepsy (years)	Frequency of seizures (per month)	Side of focus	Precipitating factors	ASD before surgery
HUM-138	F	37	4	33	75	Left	Febrile seizures in childhood	PHT/CBZ/VA/ OXC/CLN
HUM-154	F	9	3	6	7	Right	Febrile seizures in childhood	VA/TOP/CBZ/ PHB/LEV/LAM
HUM-155	M	31	12	19	20	Left	Febrile seizures in childhood	PHT/VA/CBZ/LAM
HUM- 161	M	19	0.16	19	2	Left	Febrile seizures in childhood	PHT/VA/CBZ/TOP
HUM-162	M	56	18	38	30	Right	Temporal-occipital AVM	CBZ/DZP/PHT/ PHB/LEV/VA
HUM-168	M	28	15	13	15	Bilateral	TBI and mother with epilepsy	CBZ/CLN/LEV
HUM-169	M	46	12	34	15	Right	TBI	PHT/CBZ
HUM-173	F	46	8	38	2	Right	Unknown	PHT/OXC
HUM-184	F	28	13	15	16	Bilateral	Unknown	LAM/VA/CBZ/LEV
HUM-192	M	41	32	9	10	Left	Father with epilepsy	LAM/VA/CBZ/LEV
HUM-193	F	22	11	11	4	Right	Febrile seizures in childhood	LAM/VA/LEV/TOP

ASD, antiseizure drugs; AVM, arteriovenous malformation; CBZ, carbamazepine; CLN, clonazepam; F, female; LAM, lamotrigine; LEV, levetiracetam; M, male; OXC, oxcarbazepine; PHB, phenobarbital; PHT, phenytoin; TBI, traumatic brain injury; TOP, topiramate; VA, valproic acid.

with ice-cold buffer (50 mM Tris–HCl, pH 7.4). Radioligand binding was determined as disintegrations per minute (DPM) values (Beckman LS6000SC scintillation counter), which were normalized with respect to the maximum binding. Data were fitted to a non-linear regression to determine the inhibitory concentration 50 (IC_{50}) with the model Y = Bottom + (Top − Bottom)/(1 + $10^{(\mathrm{LogIC50\text{-}X})*\mathrm{HillSlope}}$) using Prism software (GraphPad Software, Inc.). The same equation was used to determine the Hill coefficient, which gives information on the number of interacting sites and possible allosteric interactions (Prinz, 2010). The Cheng–Prusoff equation was used to determine the inhibition constant (K_i) considering the dissociation constant (K_d) of [^{3}H]-8-OH-DPAT equal to 0.46 nM (Cheng and Prusoff, 1973; Cusack et al., 1994). Data are expressed as the mean ± standard error of the mean (SEM).

[^{35}S]-GTPγS Binding Assay

5-HT$_{1A}$ receptors are highly expressed $G_{i/o}$ protein-coupled receptors that activate inhibitory pathways. Considering that CBD acts on several $G_{i/o}$ protein-coupled receptors, such as CB_1, CB_2, opioid, and 5-HT$_{1A}$, among others (Alves et al., 2020), we initially evaluated if CBD was able to activate the $G_{i/o}$ protein and if a specific antagonist to 5-HT$_{1A}$ receptors was able to prevent such effect. The [^{35}S]-GTPγS binding assay was used for this purpose.

TABLE 2 | Clinical data of autopsies.

ID	Gender	Age (years)	Cause of death	PMI (h)
A2	M	29	Polycontusion	18
A3	M	30	Ballistic trauma	14
A7	M	45	Suffocation	18
A8	M	73	Complications associated with diabetes	15
A10	M	36	Ballistic trauma	12
A11	F	12	Suffocation	14
A12	F	40	Ballistic trauma	20
A14	F	45	Unknown	10
A16	M	57	Myocardial infarction	18
A17	M	25	Penetrating wound in thorax	18
A21	M	16	Suffocation	18

F, female; h, hours; M, male; PMI, postmortem interval.

Brain tissue was homogenized in buffer (50 mM Tris–HCl, 1 mM EGTA, and 3 mM $MgCl_2 \bullet 6H_2O$, pH 7.4). The homogenate was centrifuged at 20,000 rpm for 45 min at 4°C. The resulting pellet was resuspended in buffer (50 mM Tris–HCl, 0.2 mM EGTA, 9 mM $MgCl_2 \bullet 6H_2O$, and 150 mM NaCl, pH 7.4) and centrifuged under the same conditions. The resulting pellet was resuspended in buffer once more, and protein concentration was determined as described above (see "Radioligand Displacement Assay" section).

The [^{35}S]-GTPγS binding assay was carried out as previously described (Spetea et al., 1998; Cuellar-Herrera et al., 2014), with minor variations. Briefly, cell membranes (10 μg of protein) were incubated at 30°C for 60 min in a reaction tube that contained Tris–EGTA buffer [Tris–50 mM HCl, 1 mM EGTA, 3 mM $MgCl_2 \bullet 6H_2O$, 100 mM NaCl, and 0.1% (w/v) albumin; pH 7.4], GDP (100 μM), [^{35}S]-GTPγS (100 μM), and increasing concentrations of CBD (1 pM to 100 μM). Total binding was measured in the absence of CBD. Non-specific binding was estimated in the presence of unlabeled GTPγS (100 μM). Data were analyzed as specific binding that resulted from subtracting non-specific binding from total binding. If any effect was obtained, WAY-100635 (100 μM) was included in the assay to determine the participation of 5-HT$_{1A}$ receptors. The reaction was terminated by rapid filtration on a Brandel M-48 multifilter through a Whatman GF/B glass fiber filters followed by three washes with ice-cold buffer (50 mM Tris–HCl and 5 mM $MgCl_2 \bullet 6H_2O$, pH 7.4). A concentration–effect curve was built

with the percentage of activation calculated considering the basal binding (in the absence of stimulation) as zero. Data were analyzed by a two-way ANOVA, taking group and CBD concentration as factors (concentration–effect curve), or a one-way ANOVA (effect of 100 μM in presence or absence of WAY-100635). p values < 0.05 were considered statistically significant.

RESULTS

CBD Acts on 5-HT$_{1A}$ Receptors in Human Brain Tissue

In the hippocampal tissue obtained from autopsies, CBD displaced [^{3}H]-8-OH-DPAT from its binding sites in a concentration-dependent manner (IC_{50} = 129.40 ± 9.40 μM; pK_i = 4.29 ± 0.03). In this tissue, the Hill coefficient was 3.37 ± 0.27. The radioligand displacement assay on the hippocampal tissue of patients with DR-MTLE revealed similar values (IC_{50} = 93.61 ± 18.25 μM, p = 0.1194; pK_i = 4.47 ± 0.09, p = 0.0748) as those observed in the autopsies. However, in the group of DR-MTLE patients, the Hill coefficient was higher (4.84 ± 0.35, $p < 0.05$) than in the group of autopsies.

In the temporal neocortex, we observed similar values in both DR-MTLE and autopsy groups (**Figure 1**). In the autopsy group, CBD displacement values (IC_{50} = 54.34 ± 3.66 μM; pK_i = 4.67 ± 0.03; Hill coefficient = 4.67 ± 0.93) were found within the same range of concentration as in the group of patients with DR-MTLE (IC_{50} = 46.84 ± 6.30 μM, p = 0.334; pK_i = 4.74 ± 0.05, p = 0.2643; Hill coefficient = 3.18 ± 0.57, p = 0.2091; **Table 3**).

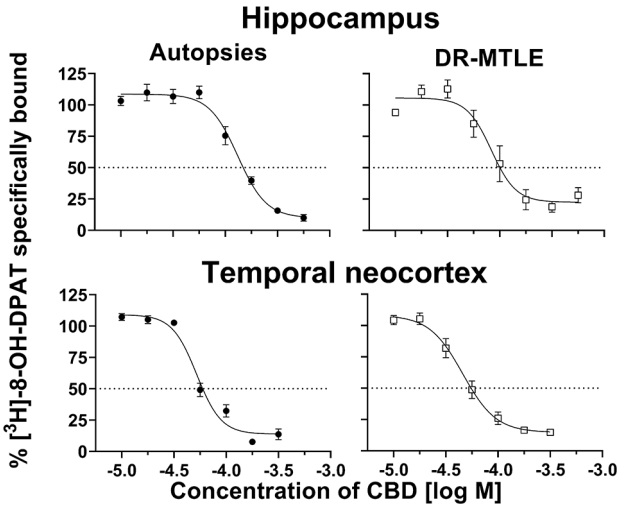


FIGURE 1 | Effects of cannabidiol (CBD) on specific binding of [^{3}H]-8-OH-DPAT to cell membranes obtained from the hippocampus (top panels) and temporal neocortex (bottom panels) of autopsies and patients with drug-resistant mesial temporal lobe epilepsy (MTLE; left and right panels, respectively). Symbols represent the mean ± Standard Error of the Mean (SEM) of five experiments. The dotted lines indicate 50% inhibition of specific binding. Curves were fitted to a model of four parameters (see "Materials and Methods" section).

CBD Modifies the Activity of $G_{i/o}$ Protein-Coupled Receptors

In the autopsies, CBD did not produce significant changes in the binding of [^{35}S]-GTPγS at low concentrations (1 pM to 10 μM, $p > 0.05$), neither in the hippocampus nor in the temporal neocortex. However, at high concentrations (100 μM), CBD reduced [^{35}S]-GTPγS binding, which was 39.2% and 35.2% lower in the hippocampus and temporal neocortex, respectively, than the basal levels ($p < 0.05$ and $p < 0.01$, respectively; **Figure 2**).

When evaluating the brain tissue of patients with DR-MTLE, the [^{35}S]-GTPγS assay revealed similar results as those observed in the group of autopsies. No changes were detected at low concentrations (1 pM to 10 μM, $p > 0.05$), whereas 100 μM CBD induced a significant reduction of [^{35}S]-GTPγS binding in the hippocampus and temporal neocortex (39.6% and 24.4% lower, respectively, when compared to basal binding levels, $p < 0.05$; **Figure 2**). In comparison with the autopsy group, a less evident [^{35}S]-GTPγS binding decrease induced by CBD was observed in the temporal neocortex of the DR-MTLE group ($p < 0.05$).

The decrease in the constitutive activity of $G_{i/o}$ proteins induced by a high concentration of CBD was partially reversed in the presence of WAY-100635 at 100 μM in both the autopsy group (hippocampus, 59.8%; $p < 0.0001$; temporal neocortex, 71.5%; $p < 0.0001$) and the DR-MTLE group (hippocampus, 53.7%; $p < 0.0001$; temporal neocortex, 68.5%; $p < 0.001$; **Figure 3**).

DISCUSSION

The radioligand displacement assay results support CBD affinity for 5-HT$_{1A}$ receptors in the brain tissue obtained from autopsies and patients with DR-MTLE. Furthermore, the experiments using [^{35}S]-GTPγS revealed that CBD decreased the constitutive activity of $G_{i/o}$ protein-coupled receptors at high concentrations and an antagonist of 5-HT$_{1A}$ receptors significantly reversed this effect.

Our experiments showed that CBD displaces [^{3}H]-8-OH-DPAT from its binding site on 5-HT$_{1A}$ receptors in a concentration-dependent manner in human hippocampal and neocortical samples. These findings support the affinity of CBD for 5-HT$_{1A}$ receptors in the human brain. Indeed, they are in agreement with previous studies indicating that the exposure to CBD at micromolar concentrations displaced [^{3}H]-8-OH-DPAT from its binding site in CHO cells expressing the human 5-HT$_{1A}$ receptor (Russo et al., 2005).

The present results indicate that CBD interacts with 5-HT$_{1A}$ receptors at high concentrations regardless of its lower affinity (pK_i ≈ 4.5) in comparison with other ligands such as serotonin (pK_i = 9.2), 8-OH-DPAT (pK_i = 8.0), or WAY-100635 (pK_i = 8.7; U.S. National Library of Medicine). However, the Hill coefficients obtained suggest that CBD is an allosteric modulator of 5-HT$_{1A}$ receptors (Hill, 1910; Goutelle et al., 2008). This condition may facilitate the binding of endogenous ligands and agonists to 5-HT$_{1A}$ receptors (May et al., 2007; Saleh et al., 2018). Indeed, the allosteric condition of CBD may represent a novel

TABLE 3 | Data obtained from the radioligand displacement assay in the hippocampus and temporal neocortex of autopsies and patients with drug resistance mesial lobe epilepsy.

Brain structure	Group	IC_{50} (μM)	K_i (μM)	pK_i	Hill coefficient
Hippocampus	Autopsies	129.40 ± 9.40	51.31 ± 3.73	4.29 ± 0.03	3.37 ± 0.27
	Patients	93.61 ± 18.25	37.12 ± 7.24	4.47 ± 0.09	4.84 ± 0.35*
Temporal neocortex	Autopsies	54.34 ± 3.66	21.55 ± 1.45	4.67 ± 0.03	4.67 ± 0.93
	Patients	46.84 ± 6.30	18.57 ± 2.50	4.74 ± 0.05	3.18 ± 0.57

*IC_{50}, inhibitory concentration 50; K_i, inhibition constant; pK_i, inverse logarithm of K_i. K_i was determined from IC_{50} according to the Cheng–Prusoff equation (1973): $K_i = IC_{50}/(1 + [radioligand]/K_D)$. Values are expressed as the mean ± SEM of five experiments. *$p < 0.05$ vs. autopsies.*

therapeutic strategy to influence the effects of 5-HT$_{1A}$ receptors (Azam et al., 2020).

Radioligand binding assay revealed differences in IC_{50} of CBD between hippocampus and temporal neocortex. It also showed the inversion of the Hill coefficient profile between autopsies and patients when temporal neocortex and hippocampus were compared. The results obtained suggest that activation of 5-HT$_{1A}$ receptors by CBD induces different functional consequences within these brain regions and depending on the pathological condition. It is known that variations of Hill coefficients are associated with conformational changes of the receptor and the number of binding sites (Colquhoun, 1998; Prinz, 2010). We found that the group of DR-MTLE patients showed a higher Hill coefficient in the hippocampus, the brain structure that mainly develops aberrant changes due to epilepsy (Bartolomei et al., 2008). This result indicates that epilepsy might be producing changes in the binding sites for CBD on the 5-HT$_{1A}$ receptors in the hippocampus (Lolkema and Slotboom, 2015). This finding is consistent with a previous study in which the Hill coefficient increase was associated with neuronal hyperexcitability and seizure activity due to a conformational change in the γ2 subunit of the GABA$_A$ receptor (Migita et al., 2013). Further binding kinetics studies are necessary to determine changes mediating the enhanced Hill coefficient in 5-HT$_{1A}$ receptors in the hippocampus of patients with DR-MTLE.

Regarding the [^{35}S]-GTPγS assay, low concentrations of CBD did not modify the activity of $G_{i/o}$ proteins in cell membranes obtained from the human brain, neither in autopsies nor in patients with DR-MTLE. These results

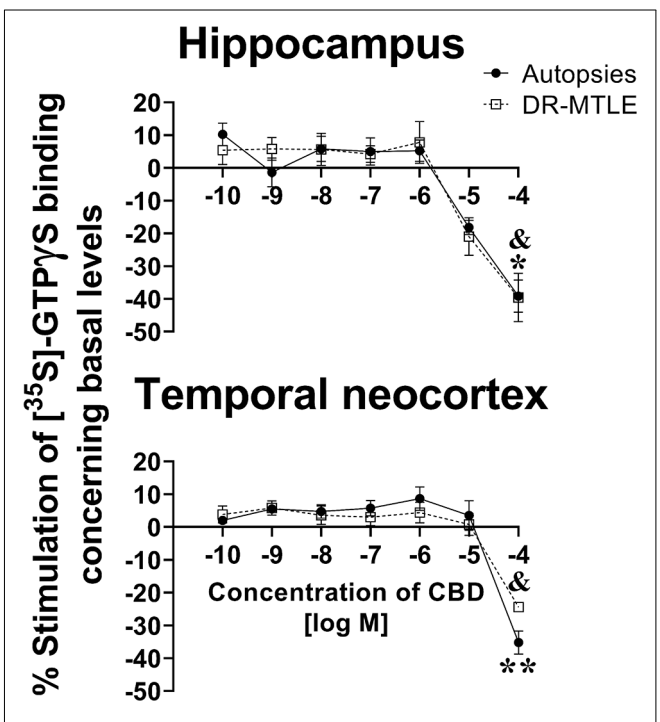


FIGURE 2 | Effect of increasing concentrations of CBD on [^{35}S]-GTPγS binding in cell membranes obtained from the hippocampus (top panels) and the temporal neocortex (bottom panels) of autopsies and patients with drug-resistant MTLE. Values are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ (autopsies vs. baseline values); $^{\&}p < 0.05$ MTLE vs. baseline values.

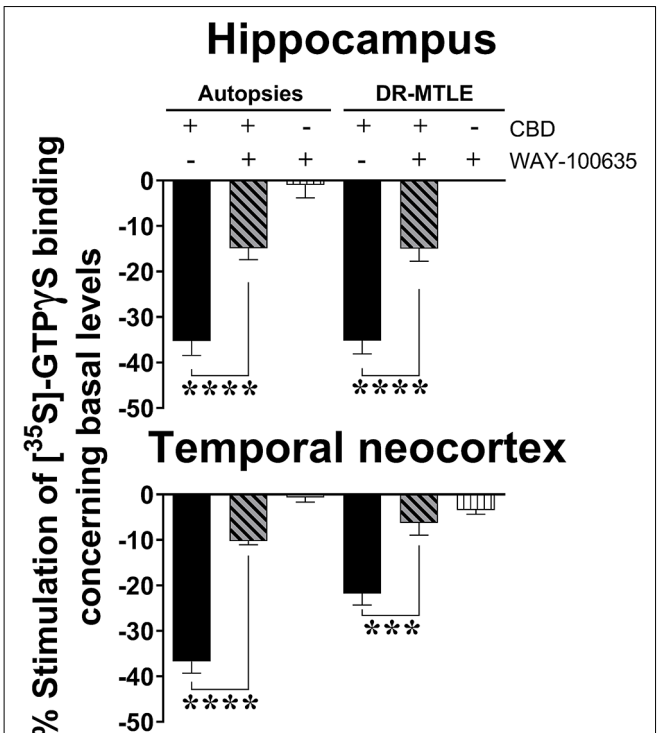


FIGURE 3 | Effect of CBD (100 μM) on the constitutive activity of $G_{i/o}$ proteins alone and combined with WAY100635 on [^{35}S]-GTPγS binding in cell membranes obtained from the hippocampus (top panel) and temporal neocortex (bottom panel) of autopsies and patients with drug-resistant MTLE. Values are expressed as the mean ± SEM. ***$p < 0.001$; ****$p < 0.0001$.

are similar to those previously reported by Rock et al. (2012), who described that $G_{i/o}$ protein-coupled receptors in rat brainstem membranes were not activated at low concentrations of CBD. This finding could be explained because the receptor–transducer selectivity was not evaluated. This is an important limitation of the experimental procedure used in the present study since the [^{35}S]-GTPγS assay evaluates all the interactions of $G_{i/o}$ protein-coupled receptors. Another possibility is that, at low concentrations of CBD, an equilibrium state between the activation and inhibition of $G_{i/o}$ proteins is achieved, which is a common condition for the $G_{i/o}$ protein-coupled receptors (Seyedabadi et al., 2019).

The constitutive activity of 5-HT_{1A} receptors is susceptible to the effect of inverse agonists (Newman-Tancredi et al., 1997; Milligan, 2003). We found that high concentrations of CBD (100 μM) decreased the binding of [^{35}S]-GTPγS to $G_{i/o}$ proteins below the baseline values. Therefore, CBD reduced the constitutive activity of receptors coupled to $G_{i/o}$ proteins. These changes were partially reversed when cell membranes were exposed to CBD in the presence of WAY100635, an antagonist of 5-HT_{1A} receptors. The results obtained indicated that at high concentrations, CBD modifies the constitutive activity of 5-HT_{1A} receptors acting as an inverse agonist.

Inverse agonists could indirectly increase the signalling of targeted receptors through the increase in the proportion of receptors on the cellular surface (Abbas et al., 2007; Kumar et al., 2019). Therefore, the continuous administration of inverse agonists of 5-HT_{1A} receptors induces antiallodynic effects due to inverse tolerance (Deseure et al., 2003). According to this information, the continuous administration of CBD as an inverse agonist of 5-HT_{1A} receptors may represent a therapeutic strategy to augment the signaling of these and other $G_{i/o}$ protein-coupled receptors involved in neuroprotection. Additional experiments are essential to support this notion.

It is known that CBD is an inverse agonist of other $G_{i/o}$ protein-coupled receptors, such as CB2 receptors (Thomas et al., 2007) and GPR3 and GPR6 orphan receptors (Laun and Song, 2017). In the present study, WAY-100635 partially blocked the CBD-induced decreased binding of [^{35}S]-GTPγS to $G_{i/o}$ proteins. WAY-100635 is considered the quintessential antagonist of the 5-HT_{1A} receptors (K_i = 2.2 nM). However, although with lower affinity values, it is also an antagonist of other receptors such as 5-HT_{2A} (K_i = 6260 nM), 5-HT_{2B} (K_i = 24 nM), and D2-like (K_i 16.4–940 nM; Chemel et al., 2006). Therefore, WAY-100635 could be blocking the constitutive activity of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, and D2-like receptors as well. On the other hand, [^{3}H]-8-OH-DPAT has low affinity for 5-HT_7 (Thomas et al., 1998) and α1 receptors (Yoshio et al., 2001). Then, some of the results obtained can involve the action of CBD on these receptors. Future experiments should be conducted to investigate the effects of CBD on binding and constitutive activity of different type of receptors in the brain of patients with epilepsy. These experiments should include kinetic binding studies and displacement experiments (pseudo-competition experiments) in the presence and absence of CBD.

5-HT_{1A} receptors play an important role in cerebral functions, and they are considered targets to develop novel therapeutic strategies. They show heterogeneous distribution, including pre- and postsynaptic localization, as well as cross-talk with different types of 5-HT and other neurotransmitter receptors. Dysfunction of 5-HT_{1A} receptors is associated with psychiatric disorders such as anxiety and depression (Popova and Naumenko, 2013). Experimental evidence indicates decline of 5-HT_{1A} receptor binding in the brain of patients with epilepsy (Toczek et al., 2003; Theodore et al., 2012). It is suggested that 5-HT_{1A} receptors on astrocytes represent a potential therapeutic target for the treatment of neurodegenerative disorders (Miyazaki and Asanuma, 2016). An important limitation of the present study is that the current methodology does not allow one to identify in which type of cells the 5-HT_{1A} receptors were evaluated. More experiments are needed to investigate the effects of CBD on specific brain cells and its therapeutic relevance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Hospital General de Mexico and Centro de Investigacion y de Estudios Avanzados. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LR conceived and designed the study and wrote the manuscript. CM-A carried out the experiments and analyzed the results. FC-C carried out the experiments. FV performed the neurosurgery of patients. AV and GA-C identified and evaluated the patients with epilepsy. MC-H collected samples, participated in the analysis of the results, and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was supported by the National Council of Science and Technology (Consejo Nacional de Ciencia y Tecnología, CONACyT; grant A3-S-26782 and scholarship 489763 to CM-A).

ACKNOWLEDGMENTS

We are thankful to HempMeds, LLC, USA, who donated the cannabidiol.

REFERENCES

Abbas, S. Y., Nogueira, M. I., and Azmitia, E. C. (2007). Antagonist-induced increase in 5-HT$_{1A}$-receptor expression in adult rat hippocampus and cortex. *Synapse* 61, 531–539. doi: 10.1002/syn.20399

Alves, P., Amaral, C., Teixeira, N., and Correia-da-Silva, G. (2020). Cannabis sativa: much more beyond Δ^9-tetrahydrocannabinol. *Pharmacol. Res.* 157:104822. doi: 10.1016/j.phrs.2020.104822

Azam, S., Haque, M., Jakaria, M., Jo, S., Kim, I., and Choi, D. (2020). G-Protein-coupled receptors in CNS: a potential therapeutic target for intervention in neurodegenerative disorders and associated cognitive deficits. *Cells* 9:506. doi: 10.3390/cells9020506

Bartolomei, F., Chauvel, P., and Wendling, F. (2008). Epileptogenicity of brain structures in human temporal lobe epilepsy: a quantified study from intracerebral EEG. *Brain* 131, 1818–1830. doi: 10.1093/brain/awn111

Benyhe, S., Farkas, J., Tó th, G., and Wollemann, M. (1997). Met5-enkephalin-Arg6-Phe7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in rat brain. *J. Neurosci. Res.* 48, 249–258. doi: 10.1002/(sici)1097-4547(19970501)48:3<249::aid-jnr7>3.0.co;2-f

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Brown, C., MacKinnon, A., McGrath, J., Spedding, M., and Kilpatrick, A. (1990). Heterogeneity of α2-adrenoceptors in rat cortex but not human platelets can be defined by 8-OH-DPAT, RU 24969 and methysergide. *Br. J. Pharmacol.* 99, 481–486. doi: 10.1111/j.1476-5381.1990.tb12954.x

Calapai, F., Cardia, L., Sorbara, E. E., Navarra, M., Gangemi, S., Calapai, G., et al. (2020). Cannabinoids, blood-brain barrier and brain disposition. *Pharmaceutics* 12:265. doi: 10.3390/pharmaceutics12030265

Chemel, B. R., Roth, B. L., Armbruster, B., Watts, V. J., and Nichols, D. E. (2006). WAY-100635 is a potent dopamine D4 receptor agonist. *Psychopharmacology* 188, 244–251. doi: 10.1007/s00213-006-0490-4

Cheng, Y., and Prusoff, W. H. (1973). Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108. doi: 10.1016/0006-2952(73)90196-2

Colquhoun, D. (1998). Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br. J. Pharmacol.* 125, 924–947. doi: 10.1038/sj.bjp.0702164

Cuellar-Herrera, M., Velasco, A. L., Velasco, F., Trejo, D., Alonso- Vanegas, M., Nuche- Bricaire, A., et al. (2014). Alterations of 5-HT$_{1A}$ receptor-induced G-protein functional activation and relationship to memory deficits in patients with pharmacoresistant temporal lobe epilepsy. *Epilepsy Res.* 108, 1853–1863. doi: 10.1016/j.eplepsyres.2014.09.013

Cunha, J. M., Carlini, E. A., Pereira, A. E., Ramos, O. L., Pimentel, C., Gagliardi, R., et al. (1980). Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology* 21, 175–185. doi: 10.1159/000137430

Cusack, B., Nelson, A., and Richelson, E. (1994). Binding of antidepressants to human brain receptors: focus on newer generation compounds. *Psychopharmacology* 114, 559–565. doi: 10.1007/BF02244985

Deseure, K., Koek, W., Adriaensen, H., and Colpaert, F. C. (2003). Continuous administration of the 5-hydroxytryptamine1A agonist (3-Chloro-4-fluoro-phenyl)-[4-fluoro-4-[[(5-methyl-pyridin-2-ylmethyl)-amino]-methyl]piperidin-1-yl]-methadone (F 13640) attenuates allodynia-like behavior in a rat model of trigeminal neuropathic pain. *J. Pharmacol. Exp. Ther.* 306, 505–514. doi: 10.1124/jpet.103.050286

Goutelle, S., Maurin, M., Rougier, F., Barbaut, X., Bourguignon, L., Ducher, M., et al. (2008). The Hill equation: a review of its capabilities in pharmacological modelling. *Fundam. Clin. Pharmacol.* 22, 633–648. doi: 10.1111/j.1472-8206.2008.00633.x

Hill, A. V. (1910). The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. *J. Physiol.* 40, 4–7. Available online at: http://jp.physoc.org/content/40/supplement/i.shortJ. Accessed November 17.

Jones, P. G., Falvello, L., Kennard, O., Sheldrick, G. M., and Mechoulam, R. (1977). Cannabidiol. *Acta Cryst.* B33, 3211–3214. doi: 10.1107/S0567740877010577

Khan, A. A., Shekh- Ahmad, T., Khalil, A., Walker, M. C., and Ali, A. B. (2018). Cannabidiol exerts antiepileptic effects by restoring hippocampal interneuron functions in a temporal lobe epilepsy model. *Br. J. Pharmacol.* 175, 2097–2115. doi: 10.1111/bph.14202

Kleven, M., Assié, M., and Koek, W. (1997). Pharmacological characterization of *in vivo* properties of putative mixed 5-HT$_{1A}$ agonist/5-HT(2A/2C) antagonist anxiolytics. II. Drug discrimination and behavioral observation studies in rats. *J. Pharmacol. Exp. Ther.* 282, 747–759.

Kumar, G. A., Sarkar, P., Jafurulla, M., Singh, S. P., Srinivas, G., Pande, G., et al. (2019). Exploring endocytosis and intracellular trafficking of the human serotonin$_{1A}$ receptor. *Biochemistry* 58, 2628–2641. doi: 10.1021/acs.biochem.9b00033

Laun, A. S., and Song, Z. H. (2017). GPR3 and GPR6, novel molecular targets for cannabidiol. *Biochem. Biophys. Res. Commun.* 490, 17–21. doi: 10.1016/j.bbrc.2017.05.165

Lazaridis, D., Eraikhuemen, N., Williams, K., and Lovince, J. (2019). Treatment of seizures associated with Lennox-Gastaut and Dravet syndromes: a focus on cannabidiol oral solution. *P T* 44, 255–266.

Lolkema, J. S., and Slotboom, D. J. (2015). The Hill analysis and co-ion-driven transporter kinetics. *J. Gen. Physiol.* 145, 565–574. doi: 10.1085/jgp.201411332

Maa, E., and Figi, P. (2014). The case for medical marijuana in epilepsy. *Epilepsia* 55, 783–786. doi: 10.1111/epi.12610

Magen, I., Avraham, Y., Ackerman, Z., Vorobiev, L., Mechoulam, R., and Berry, E. M. (2010). Cannabidiol ameliorates cognitive and motor impairments in bile-duct ligated mice *via* 5-HT$_{1A}$ receptor activation. *Br. J. Pharmacol.* 159, 950–957. doi: 10.1111/j.1476-5381.2009.00589.x

May, L. T., Leach, K., Sexton, P. M., and Christopoulos, A. (2007). Christopoulos, A. Allosteric modulation of G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* 47, 1–51. doi: 10.1146/annurev.pharmtox.47.120505.105159

Middlemiss, D., and Fozard, J. (1983). 8-Hydroxy-2-(di-n-propylamino)-tetralin discriminates between subtypes of the 5-HT$_{1A}$ recognition site. *Eur. J. Pharmacol.* 90, 151–153. doi: 10.1146/annurev.pharmtox.47.120505.105159

Migita, K., Yamada, J., Nikaido, Y., Shi, X., Kaneko, S., Hirose, S., et al. (2013). Properties of a novel GABAA receptor γ2 subunit mutation associated with seizures. *J. Pharmacol. Sci.* 121, 84–87. doi: 10.1254/jphs.12222sc

Milligan, G. (2003). Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol. Pharmacol.* 64, 1271–1276. doi: 10.1124/mol.64.6.1271

Miyazaki, I., and Asanuma, M. (2016). Serotonin 1A receptors on astrocytes as a potential target for the treatment of Parkinson's disease. *Curr. Med. Chem.* 23, 686–700. doi: 10.2174/0929867323666160122115057

Newman-Tancredi, A., Conte, C., Chaput, C., Spedding, M., and Millan, M. J. (1997). Inhibition of the constitutive activity of human 5-HT$_{1A}$ receptors by the inverse agonist, spiperone but not the neutral antagonist, WAY 100,635. *Br. J. Pharmacol.* 120, 737–739. doi: 10.1038/sj.bjp.0701025

Patra, P. H., Barker- Haliski, M., White, H. S., Whalley, B. J., Glyn, S., Sandhu, H., et al. (2019). Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models. *Epilepsia* 60, 303–314. doi: 10.1111/epi.14629

Pauwels, P., Palmier, C., Wurch, T., and Colpaert, F. (1996). Pharmacology of cloned human 5-HT$_{1D}$ receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence differentiating human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 353, 144–156. doi: 10.1007/BF00168751

Popova, N., and Naumenko, V. (2013). 5-HT$_{1A}$ receptor as a key player in the brain 5-HT system. *Rev. Neurosci.* 24, 1–14. doi: 10.1515/revneuro-2012-0082

Prinz, H. (2010). Hill coefficients, dose-response curves and allosteric mechanisms. *J. Chem. Biol.* 3, 37–44. doi: 10.1007/s12154-009-0029-3

Rock, E. M., Bolognini, D., Limebeer, C. L., Cascio, M. G., Anavi-Goffer, S., Fletcher, P. J., et al. (2012). Cannabidiol, a non-psychotropic component of cannabis, attenuates vomiting and nausea-like behaviour *via* indirect agonism of 5-HT$_{1A}$ somatodendritic autoreceptors in the dorsal raphe nucleus. *Br. J. Pharmacol.* 165, 2620–2634. doi: 10.1111/j.1476-5381.2011.01621.x

Russo, E. B. (2017). Cannabidiol claims and misconceptions. *Trends Pharmacol. Sci.* 38, 198–201. doi: 10.1016/j.tips.2016.12.004

Russo, E. B., Burnett, A., Hall, B., and Parker, K. K. (2005). Agonistic properties of cannabidiol at 5-HT$_{1A}$ receptors. *Neurochem. Res.* 30, 1037–1043. doi: 10.1007/s11064-005-6978-1

Saleh, N., Hucke, O., Kramer, G., Schmidt, E., Montel, F., Lipinski, R., et al. (2018). Multiple binding sites contribute to the mechanism of mixed agonistic and positive allosteric modulators of the cannabinoid CB1 receptor. *Angew. Chem. Int. Ed. Engl.* 57, 2580–2585. doi: 10.1002/anie.201708764

Sales, A. J., Crestani, C. C., Guimarã es, F. S., and Joca, S. R. L. (2018). Antidepressant-like effect induced by Cannabidiol is dependent on brain serotonin levels. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 86, 255–261. doi: 10.1016/j.pnpbp.2018.06.002

Seyedabadi, M., Ghahremani, M. H., and Albert, P. R. (2019). Biased signaling of G protein coupled receptors (GPCRs): molecular determinants of GPCR/transducer selectivity and therapeutic potential. *Pharmacol. Ther.* 200, 148–178. doi: 10.1016/j.pharmthera.2019.05.006

Shannon, S., Lewis, N., Lee, H., and Hughes, S. (2019). Cannabidiol in anxiety and sleep: a large case series. *Perm. J.* 23, 18–041. doi: 10.7812/TPP/18-041

Schlegel, J., and Peroutka, S. (1986). Nucleotide interactions with 5-HT$_{1A}$ binding sites directly labeled by [^{3}H]-8-hydroxy-2-(di)-*n*-propylamino) tetralin ([^{3}H]-8-OH-DPAT). *Biochem. Pharmacol.* 35, 1943–1949. doi: 10.1016/0006-2952(86)90725-2

Silvestro, S., Mammana, S., Cavalli, E., Bramanti, P., and Mazzon, E. (2019). Use of cannabidiol in the treatment of epilepsy: efficacy and security in clinical trials. *Molecules* 24:1459. doi: 10.3390/molecules24081459

Spetea, M., Monory, K., Tömbö ly, C., Tó th, G., Tzavara, E., Benyhe, S., et al. (1998). *In vitro* binding and signaling profile of the novel mu-opioid receptor agonist endomorphin 2 in rat brain membranes. *Biochem. Biophys. Res. Commun.* 250, 720–725. doi: 10.1006/bbrc.1998.9395

Theodore, W., Wiggs, E., Martinez, A., Dustin, I., Khan, O., Appel, S., et al. (2012). Serotonin 1A receptors, depression and memory in temporal lobe epilepsy. *Epilepsia* 53, 129–133. doi: 10.1111/j.1528-1167.2011.03309.x

Thiele, E. A., Marsh, E. D., French, J. A., Mazurkiewicz-Beldzinska, M., Benbadis, S. R., Joshi, C., et al. (2018). GWPCARE4 Study Group. Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 391, 1085–1096. doi: 10.1016/S0140-6736(18)30136-3

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., and Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists *in vitro*. *Br. J. Pharmacol.* 150, 613–623. doi: 10.1038/sj.bjp.0707133

Thomas, D. R., Gittins, S. A., Collin, L. L., Middlemiss, D. N., Riley, G., Hagan, J., et al. (1998). Functional characterisation of the human cloned 5-HT7 receptor (long form); antagonist profile of SB-258719. *Br. J. Pharmacol.* 124, 1300–1306. doi: 10.1038/sj.bjp.0701946

Toczek, M., Carson, R., Lang, L., Ma, Y., Spanaki, M., Der, M., et al. (2003). PET imaging of 5-HT$_{1A}$ receptor binding in patients with temporal lobe epilepsy. *Neurology* 60, 749–756. doi: 10.1212/01.wnl.0000049930.93113.20

Yoshio, R., Taniguchi, T., Itoh, H., and Muramatsu, I. (2001). Affinity of serotonin receptor antagonists and agonists to recombinant and native alpha1-adrenoceptor subtypes. *Jpn. J. Pharmacol.* 86, 189–195. doi: 10.1254/jjp.86.189

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 21 December 2020
doi: 10.3389/fnbeh.2020.602258

Cannabinoid Receptor Type 1 (CB1R) Expression in Limbic Brain Structures After Acute and Chronic Seizures in a Genetic Model of Epilepsy

Willian Lazarini-Lopes[1,2], *Rui M. P. da Silva-Júnior*[2,3], *Gabriel Servilha-Menezes*[2], *Raquel A. Do Val-da Silva*[1] and *Norberto Garcia-Cairasco*[1,2]*

[1] Neuroscience and Behavioral Sciences Department, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil, [2] Neurophysiology and Experimental Neuroethology Laboratory, Physiology Department, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil, [3] Department of Internal Medicine, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, Brazil

OPEN ACCESS

Edited by:
Patrizia Porcu,
Italian National Research Council, Italy

Reviewed by:
Arnau Busquets-Garcia,
Fundació Institut Mar d'Investigacions Mèdiques (IMIM), Spain
Zsofia Magloczky,
Institute of Experimental Medicine (MTA), Hungary

***Correspondence:**
Norberto Garcia-Cairasco
ngcairas@usp.br

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 02 September 2020
Accepted: 23 November 2020
Published: 21 December 2020

Citation:
Lazarini-Lopes W, da Silva Júnior RMP, Servilha-Menezes G, Do Val-da Silva RA and Garcia-Cairasco N (2020) Cannabinoid Receptor Type 1 (CB1R) Expression in Limbic Brain Structures After Acute and Chronic Seizures in a Genetic Model of Epilepsy. Front. Behav. Neurosci. 14:602258. doi: 10.3389/fnbeh.2020.602258

The endocannabinoid system (ECS) is related to several physiological processes, associated to the modulation of brain excitability, with impact in the expression of susceptibility and control of epileptic seizures. The cannabinoid receptor type 1 (CB1R) is widely expressed in the brain, especially in forebrain limbic structures. Changes in CB1R expression are associated with epileptic seizures in animal models and humans. The Wistar Audiogenic Rat (WAR) strain is a genetic model of epilepsy capable of mimicking tonic-clonic and limbic seizures in response to intense sound stimulation. The WAR strain presents several behavioral and physiological alterations associated with seizure susceptibility, but the ECS has never been explored in this strain. Therefore, the aim of the present study was to characterize CB1R expression in forebrain limbic structures important to limbic seizure expression in WARs. We used a detailed anatomical analysis to assess the effects of acute and chronic audiogenic seizures on CB1R expression in several layers and regions of hippocampus and amygdala. WARs showed increased CB1R immunostaining in the inner molecular layer of the hippocampus, when compared to control Wistar rats. Acute and chronic audiogenic seizures increased CB1R immunostaining in several regions of the dorsal hippocampus and amygdala of WARs. Also, changes in CB1R expression in the amygdala, but not in the hippocampus, were associated with limbic recruitment and limbic seizure severity in WARs. Our results suggest that endogenous alterations in CB1R immunostaining in WARs could be associated with genetic susceptibility to audiogenic seizures. We also demonstrated CB1R neuroplastic changes associated with acute and chronic seizures in the amygdala and hippocampus. Moreover, the present study brings important information regarding CB1R and seizure susceptibility in a genetic model of seizures and supports the relationship between ECS and epilepsy.

Keywords: CB1R expression, WAR strain, audiogenic seizures, amygdala, hippocampus

INTRODUCTION

The endocannabinoid system (ECS) is an important mechanism of biological signaling related to several physiological functions throughout the entire body (Ruiz de Azua and Lutz, 2019). In the epilepsy research scenario, the ECS has been associated with epileptic seizure susceptibility in both preclinical models of epilepsies and humans (Alger, 2004; Rosenberg et al., 2017; Lazarini-Lopes et al., 2020). Classically, the ECS comprises two G-protein-coupled receptors, the cannabinoid receptors type 1 (CB1R) and type 2 (CB2R), their endogenous ligands, called endocannabinoids (eCBs), anandamide and 2-arachidonoylglycerol (2-AG), besides the enzymes and proteins responsible for their synthesis, degradation, and transportation (Freund et al., 2003; Castillo et al., 2012). In the brain, the eCBs are synthetized "on-demand" at postsynaptic terminals and then activate CB1R located at presynaptic terminals, inhibiting neuronal firing (Lutz, 2004; Katona and Freund, 2008; Araque et al., 2017).

The CB1R is a Gi/o protein-coupled receptor widely expressed in the brain, especially in areas such as cerebellum, cortex, substantia nigra, and limbic structures (Herkenham et al., 1990; Tsou et al., 1998). Generally, CB1R regulate glutamatergic and GABAergic neurotransmission in presynaptic terminals (Katona et al., 2001; Hill et al., 2007; Turu and Hunyady, 2010), although postsynaptic CB1R modulation was also detected in the brain (Busquets-Garcia et al., 2018). CB1R are associated with epilepsies and several epilepsy-related comorbidities, such as anxiety, depression, and autism (Bhattacharyya et al., 2017; Hosie et al., 2018; Rocha et al., 2020). Additionally, changes in CB1R expression were observed in limbic brain sites after limbic seizures in animal models and humans with epilepsies (Wallace et al., 2003; Falenski et al., 2009; Maglóczky et al., 2010; Rocha et al., 2020). However, the effects of acute (brainstem) and chronic (limbic) audiogenic seizures on CB1R expression are unknown. Therefore, the role behind CB1R expression in preclinical models of epilepsies still needs to be explored.

The Wistar Audiogenic Rat (WAR) strain is a genetic model of epilepsy in which animals are capable of developing audiogenic seizures (AS) in response to intense sound stimulation (110–120 dB) (Garcia-Cairasco et al., 2017). The acute AS are modulated by brainstem sites and characterized by tonic-clonic behaviors (Garcia-Cairasco, 2002; Raisinghani and Faingold, 2003). However, during the chronic protocol of acoustic stimulation, the audiogenic kindling (AuK) (Marescaux et al., 1987), WARs, similar to Genetically epileptic-prone rats (GEPRs) (Naritoku et al., 1992) can develop limbic seizures dependent on forebrain limbic structures, such as the hippocampus and the amygdala, through an epileptogenic process called limbic recruitment (LR), with behavioral, EEG and histological correlates (Garcia-Cairasco et al., 1996; Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003; Galvis-Alonso et al., 2004). For these reasons, the AuK in WARs is considered a model of temporal lobe epilepsy (TLE) capable of mimicking limbic seizures (Moraes et al., 2000; Garcia-Cairasco et al., 2017), similar to those present in other models of TLE (Racine, 1972; Cavalheiro et al., 1991).

Although several physiological modifications have already been associated with seizure susceptibility in WARs (for a detailed review see Garcia-Cairasco et al., 2017) the ECS has never been characterized neither in WARs nor in any other audiogenic strain. Therefore, the present study was aimed to verify if the WAR strain, a genetic model of epilepsy, presents alterations in CB1R expression in the hippocampus and amygdala, some of the most important forebrain structures associated to limbic seizures expression. Additionally, we verified if acute and chronic audiogenic seizures could modulate CB1R expression in these limbic brain sites.

MATERIALS AND METHODS

Animals and Ethical Aspects

Male WARs ($n = 20$) and Wistar ($n = 5$) rats (4 months old) were provided by the Special Rat Strain Vivarium at the Ribeirão Preto School of Medicine and by the Central Vivarium of the University of São Paulo, Ribeirão Preto, respectively. During the entire experimental protocol, animals were maintained at the Animal Housing Facility located at the Physiology Department of the Ribeirão Preto School of Medicine. Animals were kept in acrylic cages (3-4 animals/cage), in a room with controlled temperature (23 ± 2°C) and light/dark cycle of 12/12 h (lights on at 6:00 a.m.), with food and water *ad libitum*.

The experimental protocol was approved by the Ethics Committee in Animal Research of the Ribeirão Preto School of Medicine at the University of São Paulo (Protocol number: 057/2017) and all efforts were made to minimize the animal's suffering.

Acute and Chronic Audiogenic Seizures

The protocol used to induce AS was similar to the one described in previous studies with WARs (Garcia-Cairasco et al., 1996; Moraes et al., 2000; Galvis-Alonso et al., 2004). Briefly, animals were placed into an acrylic cylindrical chamber (height: 32 cm, diameter: 30 cm) located at a soundproof chamber (45 × 45 × 40 cm). A small speaker was connected to a computer and placed on the top of the acrylic chamber. The sound (110–120 dB; 5–20 kHz) was manually triggered by the researcher and applied until the appearance of a tonic seizure, or for a maximum of 60 s.

To assess the effects of an acute brainstem (tonic-clonic) audiogenic seizure on CB1R expression, WARs were submitted to a single acoustic stimulation (WAR AS; $n = 5$). The AuK protocol was applied to investigate the effects of chronic seizures on CB1R expression in WARs (WAR AuK; $n = 10$). In the present study, 20 acoustic stimulations were applied during 10 days, every morning (8–9 a.m.) and afternoon (5–6 p.m.). Animal behavior was recorded in every acoustic stimulation for behavioral analysis. Control groups were composed by WARs submitted to the sham protocol of acoustic stimulation (with no sound) to assess the endogenous expression of CB1R in WARs ($n = 5$). Likewise, Wistar control rats ($n = 5$) were submitted to the sham protocol to access CB1R in a control non-epileptic strain.

Behavioral Analysis

The Categorized Severity Index (CSI) (Rossetti et al., 2006) and the Racine's scale (Racine, 1972) were used to measure brainstem and limbic seizure severity, respectively, in every acoustic stimulation (**Table 1**).

In the AuK protocol, behavioral criteria were applied to select animals that presented LR, the main feature of the AuK protocol (Naritoku et al., 1992; Garcia-Cairasco et al., 1996; Moraes et al., 2000). WARs that developed consistent (at least 3) and severe (Racine's scale 4–5) limbic seizures, were classified as WARs with LR. Otherwise, WARs that did not meet this criterion were classified as WAR with no limbic recruitment (NLR).

Tissue Processing and Immunohistochemistry

Twenty-four hours after the end of the protocol, animals were anesthetized with sodium thiopental (50 mg/kg; i.p.; Abbott, Brazil) and transcardially perfused with buffer (0.1 M phosphate buffered saline, pH 7.4, 350 ml), followed by 4% paraformaldehyde (pH 7.4, 350 ml) at room temperature. The brains were removed immediately after perfusion and post-fixed in 4% paraformaldehyde for 4 hours, then tissue was cryoprotected with sucrose solution (30%) at 4°C until sinking (48–72 h). Afterwards, brain tissue was frozen in isopentane and dry ice. Serial coronal sections (40 μm) of the dorsal hippocampus and the amygdala (−1.72 mm to −3.96) were cut according to rat brain atlas (Paxinos and Watson, 2005) on a cryostat (Microm HM-505-E, Microm International, Walldorf, Germany) at −20°C and were stored in cryoprotection solution (50% PBS, 30% ethylene glycol, 20% glycerol) at −20°C until immunohistochemical procedures.

Immunohistochemistry to CB1R was performed similar as previously described (Tsou et al., 1998; McDonald and Mascagni, 2001). Briefly, we used an antibody against the synthetic peptide MSVSTDTSAE AL, corresponding to the C terminal amino acids 461–472 of Human Cannabinoid Receptor I, Anti-Cannabinoid Receptor I Rabbit polyclonal antibody (1:1,000; Ab23703, lot. GR3239384-2, Abcam). For immunohistochemistry, brain sections were washed (five times) in PBS buffer (pH 7.4), permeabilized with Triton X-100 0.3% v/v (20 min) and placed into 0.1 M glycine (15 min). After washing in PBS, endogenous peroxidase activity was blocked in 2% H_2O_2 solution for 30 min and the sections were incubated in block buffer (BSA 2% w/v and 0.05% Triton X-100 v/v) for 2 h at room temperature. Primary antibody, diluted in block buffer, was applied overnight at 4°C. At the following day, tissue sections were washed in PBS and then incubated for 2 h, at room temperature, with biotinylated goat anti-rabbit IgG secondary antibody (1:1,000; BA-1000, lot. Zb0318, Vector) diluted in block buffer. Finally, tissues were washed in PBS and Tris-HCl (0.05 M; pH 7.6) and the immunoreactive antigenic sites were visualized using the 3,3′-diaminobenzidine (DAB) peroxidase (HRP) substrate kit with nickel (SK-4100, Vector). Nickel was used to intensifying the DAB reaction, avoiding possible misinterpretation in regions with scarce CB1R immunostaining. Specificity of each assay was tested by omitting the primary antibody. The slices were mounted on glass slides and coverslipped with Permount (Sigma, USA).

Image Processing and Analysis

CB1R immunostaining was assessed in Wistar and WAR rats (Wistar = 5; WAR = 5; WAR AS = 5; WAR AuK = 10). A mean of 7 sections were analyzed per animal. Images were acquired in a scanning microscope (Olympus BX61VS) with a 20x objective and all the parameters were kept the same in every image acquisition. The CB1R signal intensity was analyzed with the software imageJ (https://imagej.nih.gov/ij/) with Fiji (Schindelin et al., 2012; Schneider et al., 2012). For hippocampal layers and subregions, 3 rectangular standardized area (regions of interest, ROIs) with 2,500 μm^2 were randomly measured in each hippocampal analyzed area. In the amygdala subnuclei, the area of each ROI was 10,000 μm^2. The mean value of the Integrated Density (the product of area and mean gray value) was calculated using the mean value of 3 ROIs in each structure in every animal. For analysis of the total area, a manual ROI, covering the entire extension of the analyzed structure was used to assess the Integrated Density. Details of subgroup analysis are described in the **Supplementary Table 1**.

The heatmaps were generated using the ICY Bioimage software. The images were previously inverted and converted to gray scale, the colormap model was applied using the *Morgenstemning* colormap model as a template. The color scale bar was generated with the Color Bar 1.0.1.0 Local plugin developed by Stephane Dallongeville and available at http://icy.bioimageanalysis.org/plugin/color-bar/. The color scale bar represents the average intensity values with a variation from minimum 0 (black) to maximum 255 (white).

Statistical Analysis

One-way ANOVA followed by a *post-hoc* Tukey's test was used when multiples groups were compared. Behavioral and immunostaining results were expressed as mean ± standard error mean (SEM). Significant values were considered when $p < 0.05$. The software Graph Pad Prism 7.0 was used to develop the statistical analysis.

RESULTS

Behavioral Seizure Expression: Acute and Chronic Audiogenic Seizures

All WARs submitted to the AuK developed seizures during the protocol. Moreover, although the limbic seizures (as expected) were absent in the beginning of the AuK protocol, they became present in the chronic phase (**Figure 1A**). In the acute protocol of AS, 1 WAR expressed only wild running behaviors, and all the other WARs developed wild running followed by a tonic-clonic seizure (**Figure 1B**).

CB1R in the Dorsal Hippocampus

Firstly, we observed significant changes between experimental groups regarding CB1R immunostaining in the total area of the dorsal hippocampus ($F_{(3,21)} = 22.41$; $p < 0.0001$). *Post-hoc* analysis did not reveal endogenous differences of CB1R

TABLE 1 | Behavioral indexes used to analyze audiogenic seizures.

Categorized Severity Index (CSI)—Tonic-clonic seizures		Racine's scale—Limbic seizures	
0	No seizure	0	No seizure
1	One running	1	Facial and ears myoclonus
2	One wild running (running with jumping and atonic fall)	2	Head myoclonus
3	Two wild runnings	3	Forelimb myoclonus
4	Tonic convulsion	4	Forelimb myoclonus followed by elevation
5	Tonic seizures followed by generalized clonic convulsions	5	Forelimb myoclonus followed by elevation and fall
6	Head ventral flexion plus CSI 5		
7	Forelimb hyperextension plus CSI 6		
8	Hindlimb hyperextension plus CSI 7		

Categorized Severity Index used to analyze tonic-clonic seizures (Rossetti et al., 2006) and the Racine's scale used to analyze limbic seizure (Racine, 1972).

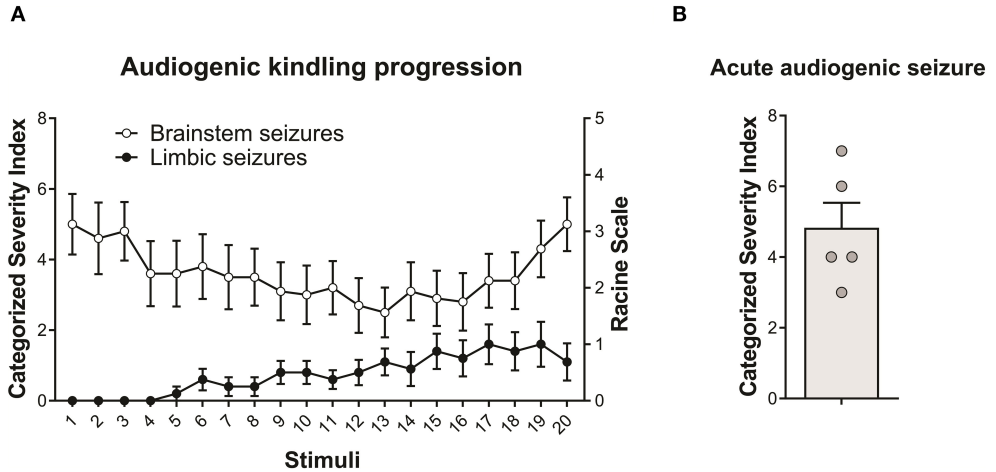


FIGURE 1 | Behavioral seizure expression in Wistar Audiogenic Rats (WAR). **(A)** Evolution of audiogenic seizures in animals submitted to chronic audiogenic seizures in the audiogenic kindling (AuK) protocol ($n = 10$). The Categorized Severity Index (Rossetti et al., 2006) was used to analyze brainstem seizures, while the Racine's scale (Racine, 1972) was used to analyze limbic seizures. It is possible to observe the presence of brainstem tonic-clonic seizures (white circles) in the beginning of the protocol and the development of limbic seizures (black circles) during the chronic phase of the protocol. **(B)** Mean of brainstem seizure severity in WARs submitted to a single acoustic stimulation ($n = 5$). Data are expressed as mean ± standard error mean (SEM).

immunostaining in WARs, when compared to control Wistar rats ($p > 0.05$). However, the acute AS and the AuK both increased CB1R immunostaining in the total hippocampal area of WARs ($p < 0.01$), when compared to Wistar and control WARs. Curiously, the increased CB1R immunostaining was higher in WARs submitted to an acute AS than in WARs submitted to chronic (kindled) seizures ($p < 0.01$). See **Figures 2A–C**.

In order to verify if changes in CB1R immunostaining observed in the total hippocampal area were associated with specific regions, we analyzed several hippocampal layers: stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum moleculare (SLM), in the dentate gyrus, superior molecular layer (sMO), inferior molecular layer (iMO), granular cell layer (GR), polymorph layer of the dentate gyrus (PoDG), stratum lucidum (SL), and fimbria (F). See **Figure 2A** for anatomical details. It is important to highlight that although the term hilus became the most used in this landscape, because the evaluation of our tissue at the dentate gyrus was based upon the optical density of CB1R in layers (molecular layer, granular cell layer, polymorph layer), not in regions, the term polymorph layer is the most appropriate (Scharfman and Myers, 2013).

WARs showed increased endogenous CB1R immunostaining when compared to Wistar control rats only in the iMO layer ($p < 0.05$). Acute AS increased CB1R immunostaining in all hippocampal layers of WARs, when compared to control WARs and Wistars ($p < 0.05$). The AuK increased CB1R in all layers, except in the F, of the WAR AuK group, when compared to Wistars ($p < 0.01$), but only in the SP, SR, sMO, GR, and iMO layers ($p < 0.05$), when compared to control WARs. Moreover, WAR AS showed increased CB1R immunostaining in all hippocampal layers, except the F, when compared to WAR AuK (**Figure 2D**).

CB1R in the Amygdala

Significant differences were observed between experimental groups regarding CB1R immunostaining in the total amygdala

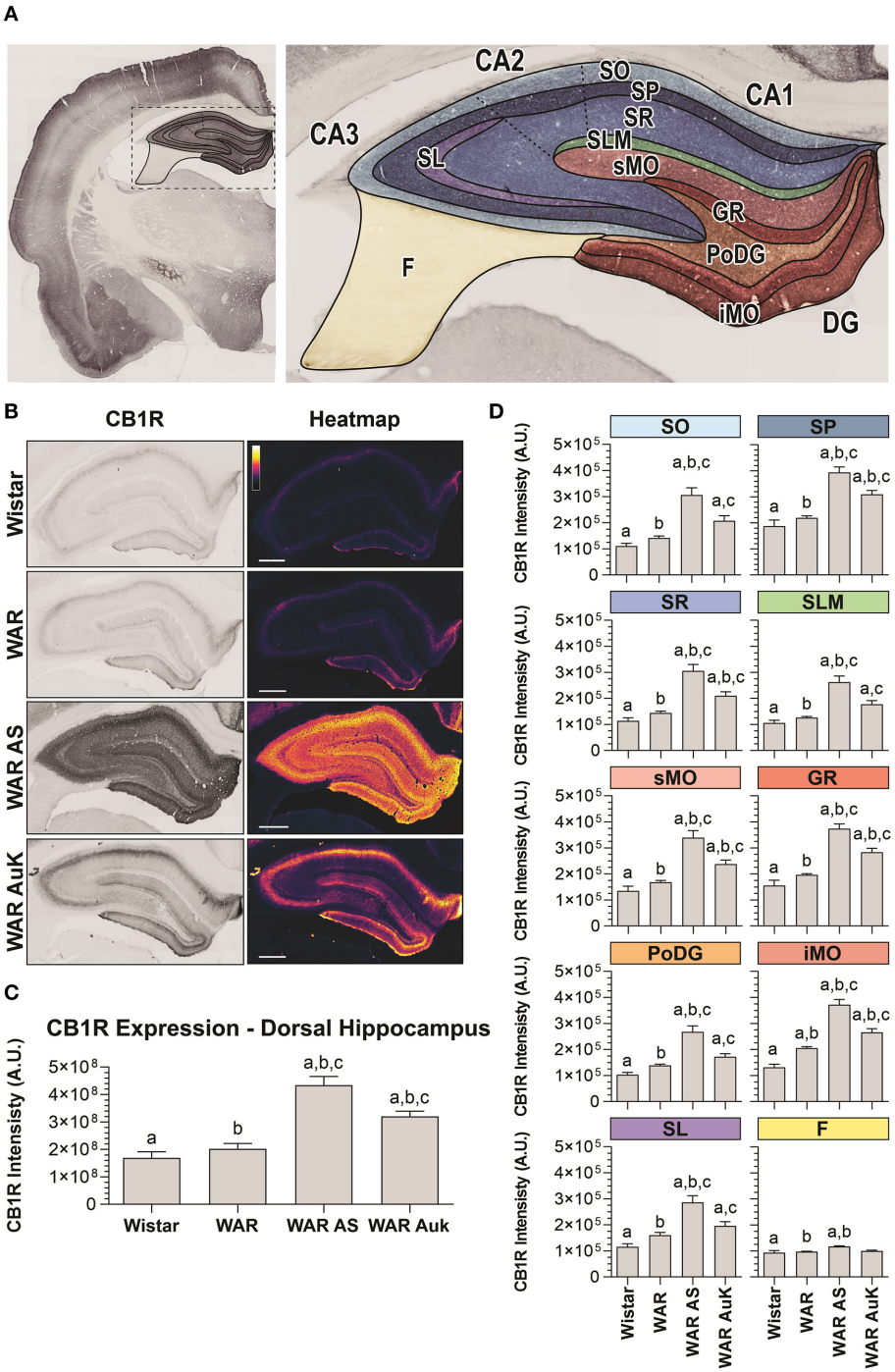


FIGURE 2 | CB1R immunostaining in the hippocampus. **(A)** Schematic representation of hippocampal regions and layers used to CB1R signal quantification: Stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum moleculare (SLM), superior molecular layer (sMO), inferior molecular layer (iMO), dentate gyrus granular cell layer (GR), polymorph layer of the dentate gyrus (PoDG), stratum lucidum (SL), fimbria (F). **(B)** Representative images of CB1R immunostaining in the dorsal hippocampus in different experimental groups (left column) and their corresponding heatmap (right column). **(C)** Quantification of CB1R intensity in the total hippocampal area of Wistar ($n = 5$), WAR ($n = 5$), WAR AS (after acute audiogenic seizure, $n = 5$), and WAR AuK (after audiogenic kindling, $n = 10$). **(D)** CB1R intensity in different hippocampal layers of Wistar ($n = 5$), WAR ($n = 5$), WAR AS ($n = 3$), and WAR AuK ($n = 10$). Data are expressed as mean ± standard error mean (SEM). Equal letters represent significant differences ($p < 0.05$) between groups: "a" in comparison to Wistar; "b" in comparison to WAR; "c" in comparison to WAR AS. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

area [$F_{(3,21)} = 7.93$; $p = 0.001$] (**Figures 3A–C**). Similarly, as occurred in the hippocampus, CB1R endogenous differences were not observed in the amygdala of WARs, when compared to control Wistars ($p > 0.05$). However, acute AS were capable of increasing CB1R immunostaining in the total area of the amygdala of WARs, when compared to Wistar and control WARs ($p < 0.01$). Additionally, it is worth to note that, although the mean of the total CB1R immunostaining in the amygdala of the WAR AuK group was higher than in control Wistar and WAR groups, these differences were not statistically significant ($0.05 < p < 0.1$). See **Figure 3C**.

We also analyzed CB1R immunostaining in specific amygdala nuclei: the lateral amygdala nucleus (LA), the basolateral amygdala nucleus (BLA), the basomedial amygdala nucleus (BMA), the central amygdala nucleus (CeA), and the medial amygdala nucleus (MeA). Here, it is worth to note that although the mean of endogenous CB1R signal intensity was slightly higher in all amygdaloid nuclei of control WARs in comparison to control Wistars, no statistical difference was observed ($p > 0.05$). Both acute AS and AuK were capable of increasing CB1R immunostaining in the LA, BLA, and BMA nuclei of WARs in comparison to control Wistar and WAR rats ($p < 0.05$). However, in the CeA and MeA, only the acute AS induced changes in CB1R expression, increasing CB1R immunostaining in comparison to control Wistars and WARs. No differences were observed between the WAR AS and WAR AuK groups. See **Figure 3D**.

Limbic Seizures Expression in WARs With and Without Limbic Recruitment

To verify if changes in CB1R immunostaining were associated to the LR with limbic seizures in WARs, we analyzed the limbic seizure expression during the AuK, and animals were classified as WARs with LR or WARs with NLR. Six WARs developed consistent limbic seizures (Racine's scale ≥ 4) during the AuK and were classified as recruited (LR) WARs. From the 4 remaining WARs, 3 of them never developed limbic seizures (NLR) and 1 WAR showed 2 limbic seizures (Racine's scale 2) during the AuK. For these reasons, these rats were classified as non-recruited WARs.

As expected, significant changes in the development of limbic seizures were observed in WARs LR compared to WARs NLR (**Figure 4A**). There is a statistical effect of the AuK progression (number of stimuli) on the limbic seizures expression [$F_{(19,152)} = 2{,}234$; $p = 0.0039$], with limbic seizures appearing after repeated stimulations. Similarly, significant differences in limbic seizures expression were associated with the presence of LR in WARs [$F_{(1,8)} = 24{,}96$; $p = 0.0011$], with recruited WARs showing increased limbic seizure frequency and severity during the AuK, when compared to non-recruited WARs ($p < 0.05$). Also, a statistical interaction between the AuK progression and limbic recruitment was observed [$F_{(19,152)} = 1{,}935$; $p = 0.0151$].

Hippocampus

Animals from the WAR AuK were divided into two different groups, WARs with LR and WARs with NLR, we observed that both groups of WARs chronically stimulated presented increased CB1R immunostaining in the total area of the dorsal hippocampus, when compared control WARs ($p < 0.05$), but no difference was observed between WAR LR and WAR NLR groups (**Figures 4B,C**). Several hippocampal layers and regions were analyzed to verify if changes in CB1R immunostaining in specific hippocampal sites could be associated with LR or not in WARs. WARs LR showed increased CB1 immunostaining in the SP, SR, sMO, GR, PoDG, and iMO ($p < 0.05$), while WARs NLR showed increased CB1 immunostaining in the SP, SR, SLM, sMO, GR, PoDG, iMO, and SL ($p < 0.05$). No difference was observed between WAR LR and WAR NLR in any hippocampal layer. See **Figure 4D**.

Hippocampal pyramidal neurons from the SP layer receive excitatory projections from several cortical areas. In their turn, hippocampal pyramidal neurons send information to dozens of other areas and subareas and they are closely associated with the epileptogenic process (Jay and Witter, 1991; Cenquizca and Swanson, 2007; Evans and Dougherty, 2018). For these reasons, we analyzed CB1R in SP throughout the Cornu Ammonis area (CA1, CA2, and CA3) of WARs submitted to the AuK (**Figures 5A,B**).

CA1 was divided in CA1a, CA1b, and CA1c and in all these subregions, CB1R immunostaining was increased in both, recruited and non-recruited WARs ($p < 0.05$) when compared control WARs. In the CA2, the same pattern of results was observed, with increased CB1R signal intensity in WARs NLR and WARs LR, when compared to control WARs ($p < 0.05$). Likewise, in all CA3 subregions CA3a, CA3b, and CA3c, CB1R immunostaining was increased in WARs NLR and WARs LR in comparison to control WARs ($p < 0.05$). However, no difference was observed in the fasciola cinereum (FC) of the hippocampus. Also, no difference was observed between WAR NLR and WAR LR (**Figure 5B**).

Amygdala

Similar as we did in the hippocampus, we verified if the AuK induced differences in CB1R expression in the amygdala of WAR LR and WAR NLR. After the AuK, CB1R immunostaining was increased in the total amygdala area of WARs NLR, when compared to control WARs and WARs LR ($p < 0.05$) (**Figures 6A,B**).

To verify if the changes observed in CB1R immunostaining between recruited and non-recruited WARs were associated with specific amygdala subregions, different amygdala nuclei were analyzed. We observed that WARs NLR showed increased CB1R immunostaining in all amygdaloid areas analyzed in comparison to control WARs ($p < 0.05$). Although in all amygdala structure analyzed the mean of CB1R intensity was higher in the WAR LR in comparison to control WARs, no significant difference was observed. Moreover, differences between recruited and non-recruited WARs were observed in specific regions. CB1R immunostaining was increased in the LA, BLA, BMA, and CeA ($p < 0.05$), but not in the MeA, of non-recruited WARs, when compared to recruited. See **Figure 6C**.

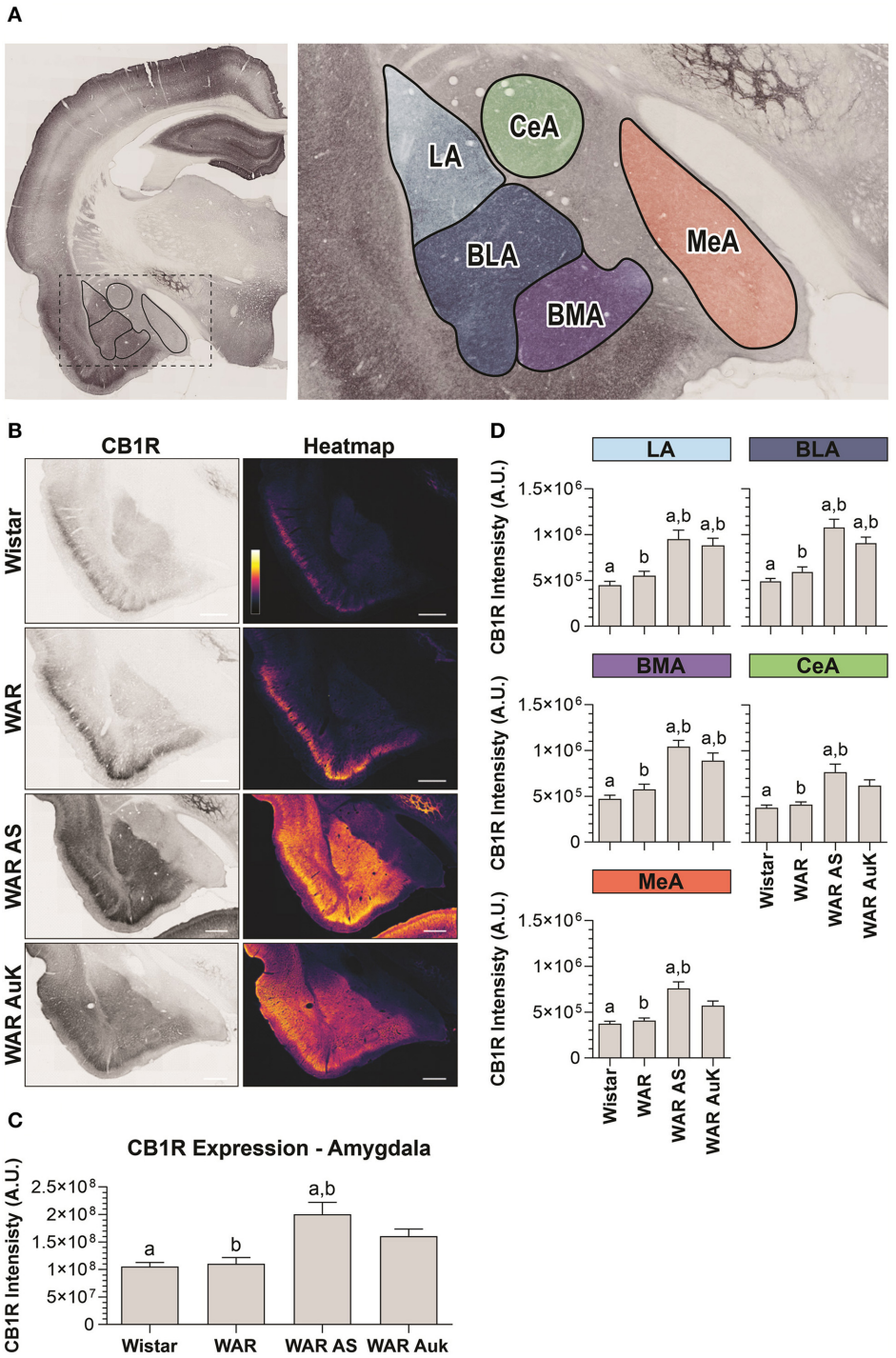


FIGURE 3 | CB1R immunostaining in the amygdala. **(A)** Schematic representation of the amygdala nuclei: lateral amygdala nucleus (LA), basolateral amygdala nucleus (BLA), basomedial amygdala nucleus (BMA), central amygdala nucleus (CeA), and medial amygdala nucleus (MeA). **(B)** Representative images of CB1R immunostaining in the amygdala in different experimental groups (left column) and their correspondent heatmap (right column). **(C)** CB1R immunostaining in the total area of the amygdala of Wistar ($n = 5$), WAR ($n = 5$), WAR AS (after acute audiogenic seizure, $n = 5$) and WAR AuK (after audiogenic kindling, $n = 10$). **(D)** CB1R intensity in different amygdala nuclei of Wistar ($n = 5$), WAR ($n = 5$), WAR AS ($n = 5$) and WAR AuK ($n = 10$). Data are expressed as mean ± standard error mean (SEM). Equal letters represent significant differences ($p < 0.05$) between groups: "a" in comparison to Wistar; "b" in comparison to WAR. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

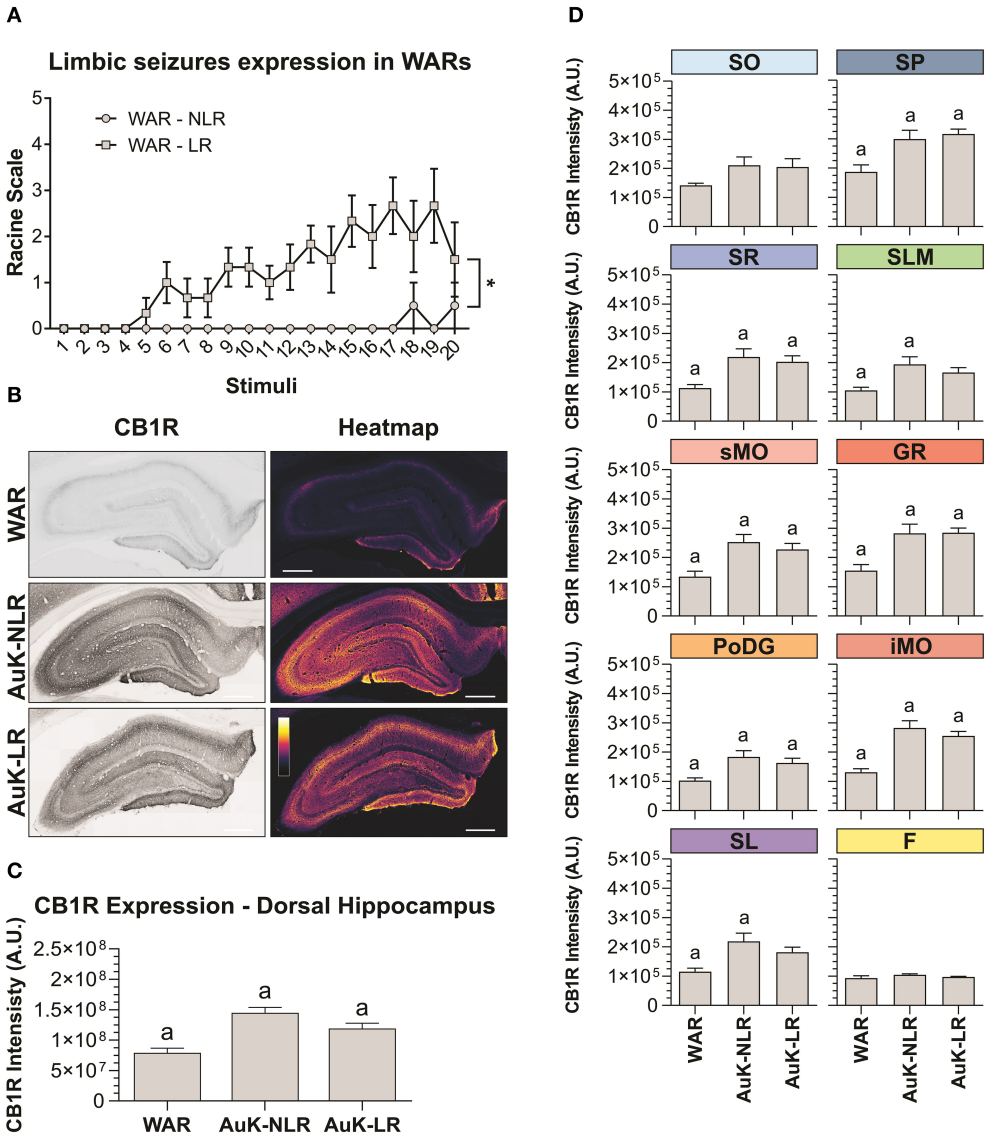


FIGURE 4 | CB1R immunostaining in the hippocampus after chronic seizures: WARs with limbic recruitment (LR) and WARs with no limbic recruitment (NLR). **(A)** Limbic seizure expression in WARs during the audiogenic kindling (AuK). Squares represent limbic seizure progression in WARs with limbic recruitment (LR). Circles represent limbic seizure progression in WARs with no limbic recruitment (NLR). Seizures were analyzed according to the Racine's scale (Racine, 1972). **(B)** Representative images of CB1R immunostaining in the dorsal hippocampus in different experimental groups (left column) and their correspondent heatmap (right column). **(C)** CB1 immunostaining in the total hippocampal area of WARs ($n = 5$) compared to WARs after the AuK with limbic recruitment (LR, $n = 6$) and with no limbic recruitment (NLR, $n = 4$). **(D)** CB1R signal intensity in different hippocampal layers. Stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum moleculare (SLM), superior molecular layer (sMO), inferior molecular layer (iMO), dentate gyrus granular cell layer (GR), polymorph layer of the dentate gyrus (PoDG), stratum lucidum (SL), fimbria (F). Data are expressed as mean ± standard error mean (SEM). Equal letters represent significant differences ($p < 0.05$) between groups: "a" in comparison to WAR. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

Finally, we observed a correlation between CB1R immunostaining and the maximum seizure severity. The correlation was observed only in the amygdala and was exclusively associated to limbic seizure severity. The correlation indicates that CB1R immunostaining decreases with the limbic seizure severity. These correlations were observed only in the LA, BLA, and BMA amygdala nuclei (**Figure 6D**).

DISCUSSION

In the present study, we observed increased CB1R immunostaining in specific limbic brain regions of WARs, a genetic model of epilepsy, when compared to control Wistar rats. Specifically, WARs showed increased endogenous CB1R immunostaining in the iMO layer of the dorsal hippocampus

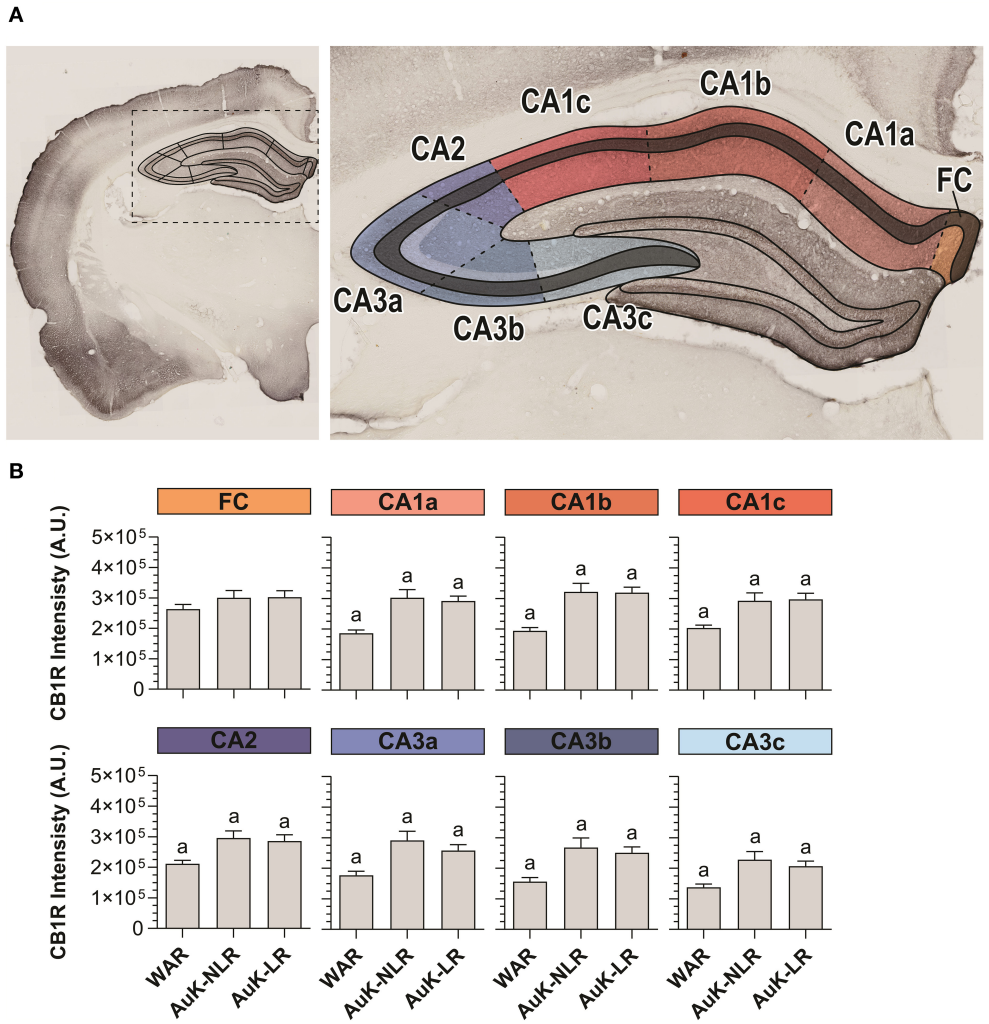


FIGURE 5 | CB1R immunostaining in stratum pyramidal (SP) layer throughout the Cornu Ammonis (CA) after chronic seizures. **(A)** Schematic representation of the Cornu Ammonis area (CA1, CA2, and CA3). **(B)** CB1R immunostaining in control WARs compared to WARs with limbic recruitment (LR) and WARs with no limbic recruitment (NLR) throughout the hippocampal CA area: fasciola cinereum (FC), CA1a, CA1b, CA1c, CA2, CA3a, CA3b, and CA3c. Data are expressed by mean ± standard error mean (SEM). Equal letters represent significant differences ($p < 0.05$) between groups: "a" in comparison to WAR. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

in comparison to Wistars. Additionally, we showed that both acute and chronic AS in WARs were capable of increasing CB1R immunostaining in several layers of the dorsal hippocampus, as well as in the amygdala. It is worth to mention that during the data analysis we observed intense CB1R immunostaining in many other brain sites such as hypothalamus, thalamus, and several cortical areas, like the piriform and motor cortices (**Supplementary Figure 1**). However, since our objective in the present study was to analyze CB1R immunostaining in limbic structures (hippocampus and amygdala) important to limbic seizure expression during the AuK, these data about CB1R expression in extra-limbic structures will not be discussed.

Previous studies have shown changes in CB1R expression in limbic brain structures in preclinical models of seizures and humans with TLE. Wallace et al. (2003) showed increased eCBs levels and CB1R expression in the hippocampus of rats submitted to the pilocarpine-induced *Status Epilepticus (SE)* model (Cavalheiro et al., 1991). Moreover, time-dependent effects on CB1R expression were also observed in the pilocarpine model of *SE*. Specifically, hippocampal CB1R expression was reduced in Sprague-Dawley rats during the acute phase, 4 days after pilocarpine injection, while CB1R expression increased in the chronic phase, 4 months after pilocarpine (Falenski et al., 2007, 2009). Similar results were observed in mice that developed severe seizures (Racine's scale 5) after pilocarpine injection. CB1R neuroplastic changes were observed throughout the hippocampus, although CB1R immunostaining was too intense to distinguish hippocampal layers (Karlócai et al., 2011).

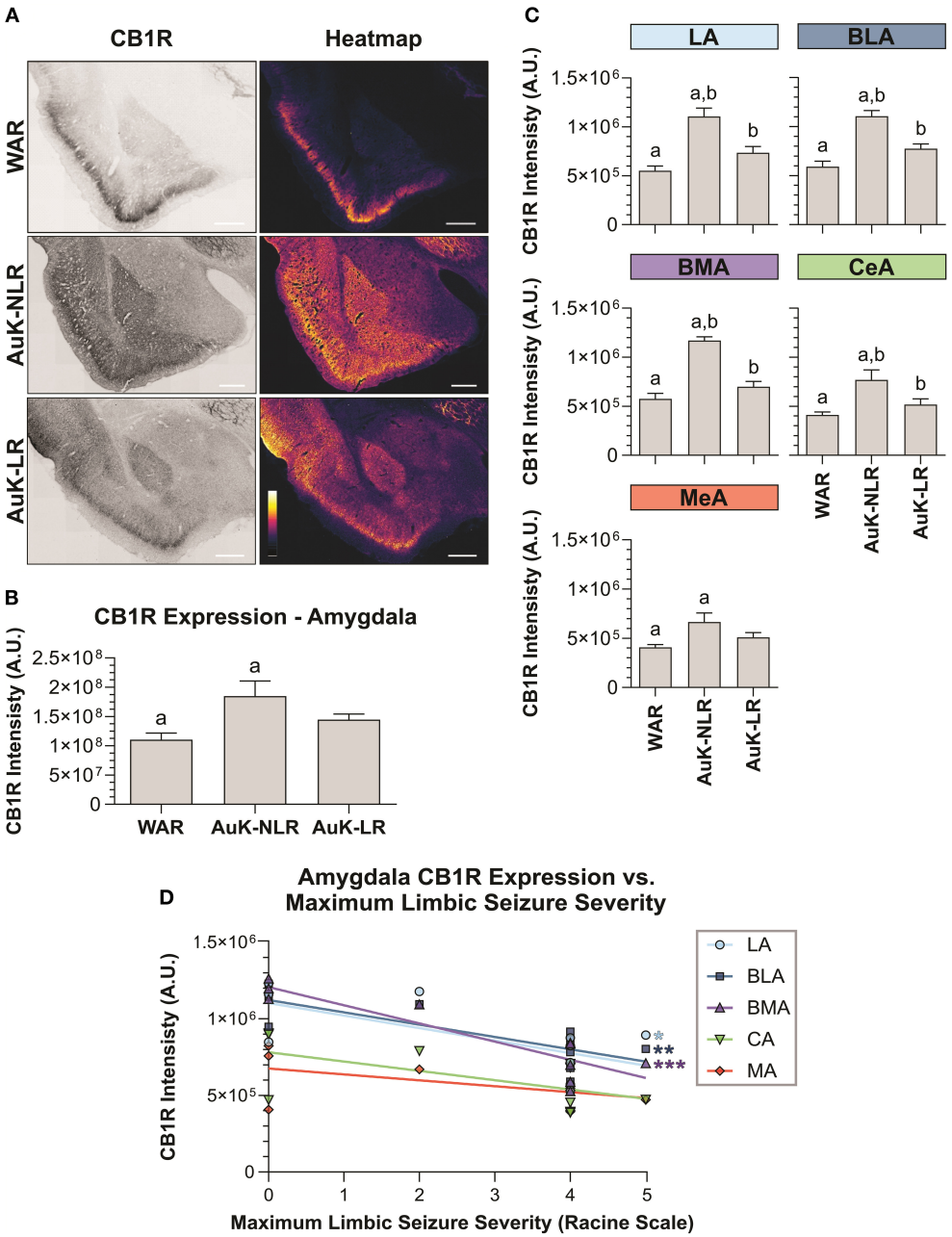


FIGURE 6 | CB1R immunostaining in the amygdala after chronic seizures. WARs with limbic recruitment (LR) and WARs with no limbic recruitment (NLR). **(A)** Representative images of CB1R immunostaining in the amygdala in different experimental groups (left column) and their correspondent heatmap (right column). **(B)** CB1R immunostaining in the total amygdala area of WARs ($n = 5$) compared with WARs after the AuK with limbic recruitment (LR, $n = 6$) and with no limbic recruitment (NLR, $n = 4$). **(C)** CB1R signal intensity in different amygdala nuclei: lateral amygdala nucleus (LA), basolateral amygdala nucleus (BLA), basomedial amygdala nucleus (BMA), central amygdala nucleus (CeA), and medial amygdala nucleus (MeA). **(D)** Correlation between CB1R immunostaining and maximum limbic seizure severity in different amygdala nuclei. Data are expressed by mean ± standard error mean (SEM). Equal letters represent significant differences ($p < 0.05$) between groups: "a" in comparison to WAR; "b" in comparison to WAR-NLR. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

Additionally, Karlócai et al. (2011) described a high increase of CB1R in the hippocampal SP layer, similarly as we showed in the present study after acute and chronic AS throughout the entire Cornu Ammonis area. Maglóczky et al. (2010) observed increased CB1R expression in hippocampal GABAergic terminals in tissues from sclerotic human brain and from mice submitted to the pilocarpine-induced *SE*. Considerable increase on CB1R expression was observed especially in the molecular

layer of the dentate gyrus (Maglóczky et al., 2010). The molecular layer of the dentate gyrus is an important region in the present study, not only because of the increased CB1R immunostaining in both sMO and iMO after acute and chronic AS, but also due to the endogenous increased expression observed in non-stimulated WARs in comparison to Wistars.

The main feature of the AuK is the forebrain and cortical recruitment associated with the development of behavioral clonic and limbic seizures in genetic susceptible animals (Marescaux et al., 1987; Naritoku et al., 1992; Romcy-Pereira and Garcia-Cairasco, 2003; Vinogradova, 2017). As we showed in the present study, during the AuK, the initially brainstem-dependent seizures, give rise to limbic seizures dependent on forebrain and limbic structures, like amygdala, cortex, and hippocampus (Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003; Galvis-Alonso et al., 2004; Poletaeva et al., 2017). Additionally, several behavioral and physiological changes are associated with seizure susceptibility and neuropsychiatric comorbidities in WARs and other audiogenic rodent strains (Castro et al., 2017; Garcia-Cairasco et al., 2017; Poletaeva et al., 2017; Aguilar et al., 2018). Furthermore, reduced GABAergic currents were observed in CA1 pyramidal neurons of WARs (Cunha et al., 2018) and volumetric increase was detected in limbic structures of WARs, like the dorsal hippocampus and amygdala, when compared to Wistars rats (Lee et al., 2018). These data indicate functional and anatomical alterations associated with epilepsies in WAR's limbic brain network.

Historically, before the genetic selection of the WARs, we had 10% susceptible Wistars, which became the parentals (founders) of the WARs strain (Garcia-Cairasco et al., 2017). Additionally, it is common to submit Wistar rats to chronic acoustic stimulation as a control for kindling in WARs and eliminate from the study those Wistars which eventually present audiogenic responses. Therefore, Wistar resistant rats submitted to the AuK are named as chronically stimulated (not kindled), because kindled animals mean those with behavioral and EEG seizures (Garcia-Cairasco et al., 2017). Studies with synchronized video-EEG, observed behavioral seizures concomitant to epileptic-like activity in the amygdala, hippocampus and cortex of WARs after the AuK protocol, but Wistars did not develop any EEG alteration in these forebrain structures after chronic acoustic stimulation (Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003). Moreover, it is worth noting that AuK induced a decrease in WAR's spatial memory retention in the Morris water maze test, but the chronic acoustic stimulation (no seizures) had no effect on Wistar's performance in the same memory test. Moreover, neurotransmission in the hippocampal Schaffer-collaterals fibers and their excitability were not affected by the chronic exposure to acoustic stimulation in Wistar rats (Cunha et al., 2015) and AuK-dependent limbic recruitment in WARs is not associated with any inflammatory process or oxidative stress, suggesting that this neuroplastic anatomical change is a "network expansion process," primarily linked to the genetic selection for the epilepsy phenotype in WARs (de Deus et al., 2020). Therefore, except for the small percentage of Wistar susceptible animals (usually discarded), acoustic stimulation does not induce audiogenic seizures in Wistar rats, as well as epileptic-like alterations or epilepsy-related comorbidities.

In the present study, we showed increased CB1R immunostaining in several hippocampal regions, including the SP layer throughout the entire Cornu Ammonis area (CA1–CA3), where hippocampal pyramidal neurons are located. Therefore, it is possible that changes in CB1R expression are related to deficits in GABA currents previously described in the hippocampus of WARs (Cunha et al., 2018). We know that CB1R agonist administration decreases GABAergic activity in hippocampal CA1 area and in the BLA (Hoffman and Lupica, 2000; Katona et al., 2001; Wilson and Nicoll, 2001). Additionally, cannabidiol (CBD) treatment for epilepsy protected animals from seizures and reduced hippocampal hyperexcitability increasing GABAergic currents (Kaplan et al., 2017). However, the direct relationship between CB1R and alterations in hippocampal GABA release observed in WARs still needs to be assessed.

Pharmacological manipulations of CB1R were associated with anticonvulsant effects in several models of epileptic seizures, but only a few studies assessed the role of CB1R in audiogenic models (Rosenberg et al., 2017; Lazarini-Lopes et al., 2020). Systemic activation of CB1R receptors presented protective effects against audiogenic seizures in the Krushinsky-Molodkina strain and in DBA/2 mice (Vinogradova and Van Rijn, 2015; Citraro et al., 2016). Conversely, antagonism of CB1R facilitated the appearance of clonic behavior during the AuK and induced the reappearance of seizures in animals that had previously developed resistance to audiogenic seizures (Vinogradova et al., 2011). Moreover, in WAG/Rij rats, a genetic model of absence seizures, with a subpopulation also susceptible to audiogenic seizures (Vinogradova, 2008), pharmacological activation of CB1R attenuated absence seizures, but the effects on AS were not assessed (Van Rijn et al., 2010; Citraro et al., 2013).

The ECS is a complex system capable of modulating many other mechanisms, for these reason we should not discard possible involvement of CB1R with glutamatergic neurons and/or astrocytes signaling (Monory et al., 2006; Maroso et al., 2016; Busquets-Garcia et al., 2018), especially because overexpression of glutamate receptors subunits have already been observed in the hippocampus of WARs after acute and chronic AS (Gitaí et al., 2010). Therefore, investigating not only the expression, but also the location of CB1R in specific cell types in genetic models of epilepsies can bring important information regarding the ECS and seizure control. Finally, other receptors related to endocannabinoid signaling, such as the transient receptor potential vanilloid 1 (TRPV1) and the GPR55, both activated by anandamide and associated with anticonvulsant effects (Van Der Stelt et al., 2005; Ryberg et al., 2007; Lazarini-Lopes et al., 2020), could be partially involved with CB1R modulation and should be further investigated in the genetic models of epilepsies.

Our results of increased CB1R expression in limbic brain regions are in agreement with other animal models and humans with TLE (Wallace et al., 2003; Falenski et al., 2007; Maglóczky et al., 2010; Karlócai et al., 2011; Rocha et al., 2020). We observed

increased endogenous CB1R expression in WARs in comparison to Wistar rats in the iMO of the hippocampus. Alterations in the iMO, such as increased granule cell dendrites, increased endings of apical dendrites, and increased neo-Timm staining, have already been described after pilocarpine-induced *SE* (Arisi and Garcia-Cairasco, 2007). Our results bring new insights about ECS alterations in the molecular layer of the hippocampus, reinforcing the role of this brain region in seizure susceptibility and control. Moreover, we suggest that endogenous increase of CB1R in the iMO of WARs could be associated with the genetic susceptibility to audiogenic seizures and the LR consequent to AuK.

Additionally, we bring important information regarding acute brainstem seizures and modulation of CB1R expression in limbic brain regions. Strikingly, CB1R was increased in hippocampus and amygdala after acute and chronic brainstem seizures. Our results suggest that in audiogenic models of seizures, brainstem seizures, either acute or chronic, are capable of modulating CB1R expression in a similar manner, increasing its expression in the amygdala and hippocampus. Additionally, differences in CB1R expression between WAR NLR and WAR LR were observed in the amygdala (LA, BLA, BMA, CeA), but not in the hippocampus. Also, a decrease of CB1R expression in the amygdala (LA, BLA, BMA) was correlated with increased limbic seizure severity. Curiously, these amygdala subnuclei are the main amygdaloid areas related to limbic recruitment in WARs and in other audiogenic strains, with histological and EEG alterations (Moraes et al., 2000; Galvis-Alonso et al., 2004; Tupal and Faingold, 2010). Therefore, these results suggest that the ECS could be associated with the epileptogenic process in WARs. Nonetheless, the functional consequences of these differences in CB1R expression still need to be investigated, especially because it is unclear if changes in CB1R expression were previously present in LR and NLR WARs before the beginning of the AuK. Additionally, the analysis of CB1R in different time-points during chronic seizures can bring important information about the progressive alterations in CB1R expression during chronic seizures.

The present study has some methodological limitations, such as the absence of EEG recordings in WARs, the lack of analysis of the CB1R functionality or the lack of evaluation of other ECS components. In addition to the assessment of CB1R in the current WAR brain sites (hippocampus and amygdala), directly related to limbic seizure generation, it would be interesting and necessary in the future, to evaluate the expression of CB1R at the sensory processing level, initially the auditory receptor and lower brainstem auditory structures, as well as nuclei of sensory-motor integration systems, the outcome for the manifestation of motor seizures (see details in Garcia-Cairasco et al., 2017). It is important to note that behavioral and immunohistochemical data were consistent in the present study and we showed the first evidence of endogenous changes in CB1R expression in WARs, a genetic model of epilepsy, which is able to mimick tonic-clonic seizures (acute protocol) and TLE (chronic protocol, kindling). It is important to note that we used a genetic model of epilepsy with no need of any chemical of electrical stimulation to induce seizures in WARs. Due to these features, genetic models of epilepsies with reflex seizures, like audiogenic strains, are important methodological approaches in the preclinical epilepsy research (Faingold et al., 2014; Garcia-Cairasco et al., 2017). Moreover, we applied a detailed anatomical analysis, which allowed the assessment of CB1R expression in specific brain regions and subregions. Only using this exhaustive and accurate methodology, it was possible to detect the changes in the iMO of the hippocampus of control WARs, as well as to identify specific regions of the amygdala where CB1R expression was correlated to limbic seizure severity.

Therefore, we conclude that WARs present increased endogenous CB1R expression in the iMO, when compared to control Wistar rats. Additionally, acute and chronic (kindled) audiogenic seizures increased CB1R expression in several hippocampal and amygdala regions. Furthermore, changes in CB1R expression in the amygdala, but not in the hippocampus, were correlated with limbic seizure severity in WARs. Our study is the first to characterize CB1R in the WAR strain, a genetic model of epilepsy with brainstem (acute protocol) and limbic (chronic protocol) seizures. Our results agree with previous studies, supporting that changes in the ECS may be related with the seizure susceptibility and its control. Further studies investigating ECS components in genetic models of epilepsies, such as WAR, GEPR, and WAG/Rij, may bring important information about seizure susceptibility and pharmacological control. Future mapping studies would also expand the search for brain sites of expression of CB1R, associated to epilepsies and epilepsy-related comorbidities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Animal Research of the Ribeirão Preto School of Medicine at the University of São Paulo (Protocol number: 057/2017).

AUTHOR CONTRIBUTIONS

WL-L, RV-S, RS-J, and NG-C conceived the original idea. WL-L conducted behavioral and immunohistochemical experiments, imaging protocols, and analysis. RV-S collected brain tissue. RS-J and GS-M performed immunohistochemical analysis, image processing, and prepared figures. WL-L prepared figures and wrote the manuscript. NG-C made important intellectual contributions and obtained funding. All authors reviewed, discussed, and approved the manuscript.

FUNDING

This work was supported by São Paulo Research Foundation (FAPESP) grants: 14/50891-1, 19/05957-8, 19/00849-2, and 2019/02787-4. National Council for Scientific and Technological Development (CNPq) grants: 465458/2014-9 and 305883/2014-3 (NG-C). WL-L holds Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Finance code 001) and CAPES-Print (Process no. 88887.370299/2019-00) grants.

ACKNOWLEDGMENTS

The authors thank Dr. Norberto Cysne Coimbra for the assistance in the neuroanatomical studies. We also thank José Antônio Cortes de Oliveira for the assistance in the genetic selection and maintenance of the Wistar Audiogenic Rat (WAR) colony for more than 30 years. The authors also thank the Multiuser Laboratories on Confocal at the Cell and Molecular Biology Department, Ribeirão Preto School of Medicine, University of São Paulo.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.602258/full#supplementary-material

Supplementary Figure 1 | Representative images of CB1R immunostaining in extra-limbic brain structures. Different experimental groups (left column) and their correspondent heatmap (right column) of CB1R immunostaining. It is possible to observe intense CB1R immunostaining in several brain areas, besides the hippocampus and amygdala, such as the hypothalamus, the thalamus, and several cortical areas. Scale bar: 500 μm. Color code scale (8 bits image): 0–255 (min–max).

Supplementary Table 1 | Number of animals and sections used to analyze CB1R immunostaining in each experimental group.

REFERENCES

Aguilar, B. L., Malkova, L., N'Gouemo, P., and Forcelli, P. A. (2018). Genetically epilepsy-prone rats display anxiety-like behaviors and neuropsychiatric comorbidities of epilepsy. *Front. Neurol.* 9:476. doi: 10.3389/fneur.2018.00476

Alger, B. E. (2004). Endocannabinoids and their implications for epilepsy. *Epilepsy Curr.* 4, 169–173. doi: 10.1111/j.1535-7597.2004.04501.x

Araque, A., Castillo, P. E., Manzoni, O. J., and Tonini, R. (2017). Synaptic functions of endocannabinoid signaling in health and disease. *Neuropharmacology* 124, 13–24. doi: 10.1016/j.neuropharm.2017.06.017

Arisi, G. M., and Garcia-Cairasco, N. (2007). Doublecortin-positive newly born granule cells of hippocampus have abnormal apical dendritic morphology in the pilocarpine model of temporal lobe epilepsy. *Brain Res.* 1165, 126–134. doi: 10.1016/j.brainres.2007.06.037

Bhattacharyya, S., Egerton, A., Kim, E., Rosso, L., Riano Barros, D., Hammers, A., et al. (2017). Acute induction of anxiety in humans by delta-9-tetrahydrocannabinol related to amygdalar cannabinoid-1 (CB1) receptors. *Sci. Rep.* 7:15025. doi: 10.1038/s41598-017-14203-4

Busquets-Garcia, A., Bains, J., and Marsicano, G. (2018). CB 1 receptor signaling in the brain: extracting specificity from ubiquity. *Neuropsychopharmacology* 43, 4–20. doi: 10.1038/npp.2017.206

Castillo, P. E., Younts, T. J., Chávez, A. E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron* 76, 70–81. doi: 10.1016/j.neuron.2012.09.020

Castro, G. P., Medeiros, D. de C., Guarnieri, L. de O., Mourão, F. A. G., Pinto, H. P. P., Pereira, G. S., et al. (2017). Wistar audiogenic rats display abnormal behavioral traits associated with artificial selection for seizure susceptibility. *Epilepsy Behav.* 71, 243–249. doi: 10.1016/j.yebeh.2015.08.039

Cavalheiro, E. A., Leite, J. P., Bortolotto, Z. A., Turski, W. A., Ikonomidou, C., and Turski, L. (1991). Long-term effects of pilocarpine in rats: structural damage of the brain triggers kindling and spontaneous i recurrent seizures. *Epilepsia* 32, 778–782. doi: 10.1111/j.1528-1157.1991.tb05533.x

Cenquizca, L. A., and Swanson, L. W. (2007). Spatial organization of direct hippocampal field CA1 axonal projections to the rest of the cerebral cortex. *Brain Res. Rev.* 56, 1–26. doi: 10.1016/j.brainresrev.2007.05.002

Citraro, R., Russo, E., Leo, A., Russo, R., Avagliano, C., Navarra, M., et al. (2016). Pharmacokinetic-pharmacodynamic influence of N-palmitoylethanolamine, arachidonyl-2′-chloroethylamide and WIN 55,212-2 on the anticonvulsant activity of antiepileptic drugs against audiogenic seizures in DBA/2 mice. *Eur. J. Pharmacol.* 791, 523–534. doi: 10.1016/j.ejphar.2016.09.029

Citraro, R., Russo, E., Scicchitano, F., Van Rijn, C. M., Cosco, D., Avagliano, C., et al. (2013). Antiepileptic action of N-palmitoylethanolamine through CB1 and PPAR-α receptor activation in a genetic model of absence epilepsy. *Neuropharmacology* 69, 115–126. doi: 10.1016/j.neuropharm.2012.11.017

Cunha, A. O. S., Ceballos, C. C., De Deus, J. L., Pena, R. F. D. O., De Oliveira, J. A. C., Roque, A. C., et al. (2018). Intrinsic and synaptic properties of hippocampal CA1 pyramidal neurons of the Wistar Audiogenic Rat (WAR) strain, a genetic model of epilepsy. *Sci. Rep.* 8:10412. doi: 10.1038/s41598-018-28725-y

Cunha, A. O. S., de Oliveira, J. A. C., Almeida, S. S., Garcia-Cairasco, N., and Leão, R. M. (2015). Inhibition of long-term potentiation in the schaffer-CA1 pathway by repetitive high-intensity sound stimulation. *Neuroscience* 310, 114–127. doi: 10.1016/j.neuroscience.2015.09.040

de Deus, J. L., Amorim, M. R., de Barcellos Filho, P. C. G., de Oliveira, J. A. C., Batalhão, M. E., Garcia-Cairasco, N., et al. (2020). Inflammatory markers in the hippocampus after audiogenic kindling. *Neuroscience Letters* 721, 134830. doi: 10.1016/j.neulet.2020.134830

Evans, M. C., and Dougherty, K. A. (2018). Carbamazepine-induced suppression of repetitive firing in CA1 pyramidal neurons is greater in the dorsal hippocampus than the ventral hippocampus. *Epilepsy Res.* 145, 63–72. doi: 10.1016/j.eplepsyres.2018.05.014

Faingold, C. L., Raisinghani, M., and N'Gouemo, P. (2014). Neuronal networks in epilepsy: comparative audiogenic seizure networks. *Neuronal Netw. Brain Funct. CNS Disord. Ther.* 2014, 349–373. doi: 10.1016/B978-0-12-415804-7.00026-5

Falenski, K. W., Blair, R. E., Sim-Selley, L. J., Martin, B. R., and DeLorenzo, R. J. (2007). Status epilepticus causes a long-lasting redistribution of hippocampal cannabinoid type 1 receptor expression and function in the rat pilocarpine model of acquired epilepsy. *Neuroscience* 146, 1232–1244. doi: 10.1016/j.neuroscience.2007.01.065

Falenski, K. W., Carter, D. S., Harrison, A. J., Martin, B. R., Blair, R. E., and DeLorenzo, R. J. (2009). Temporal characterization of changes in hippocampal cannabinoid CB1 receptor expression following pilocarpine-induced status epilepticus. *Brain Res.* 1262, 64–72. doi: 10.1016/j.brainres.2009.01.036

Freund, T. F., Katona, I., and Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev.* 83, 1017–1066. doi: 10.1152/physrev.00004.2003

Galvis-Alonso, O. Y., Cortes De Oliveira, J. A., and Garcia-Cairasco, N. (2004). Limbic epileptogenicity, cell loss and axonal reorganization induced by audiogenic and amygdala kindling in wistar audiogenic rats (WAR strain). *Neuroscience* 125, 787–802. doi: 10.1016/j.neuroscience.2004.01.042

Garcia-Cairasco, N. (2002). A critical review on the participation of inferior colliculus in acoustic-motor and acoustic-limbic networks involved in the expression of acute and kindled audiogenic seizures. *Hear. Res.* 168, 208–222. doi: 10.1016/S0378-5955(02)00371-4

Garcia-Cairasco, N., Umeoka, E. H. L., and Cortes de Oliveira, J. A. (2017). The Wistar Audiogenic Rat (WAR) strain and its contributions to epileptology and

related comorbidities : History and perspectives. *Epilepsy Behav.* 71, 250–273. doi: 10.1016/j.yebeh.2017.04.001

Garcia-Cairasco, N., Wakamatsu, H., Oliveira, J. A. C., Gomes, E. L. T., Del Bel, E. A., and Mello, L. E. A. M. (1996). Neuroethological and morphological (Neo-Timm staining) correlates of limbic recruitment during the development of audiogenic kindling in seizure susceptible Wistar rats. *Epilepsy Res.* 26, 177–192. doi: 10.1016/S0920-1211(96)00050-2

Gitaí, D. L. G., Martinelli, H. N., Valente, V., Pereira, M. G. A. G., Oliveira, J. A. C., Elias, C. F., et al. (2010). Increased expression of GluR2-flip in the hippocampus of the wistar audiogenic rat strain after acute and kindled seizures. *Hippocampus* 20, 125–133. doi: 10.1002/hipo.20590

Herkenham, M., Lynn, A. B., Litrle, M. D., Johnsont, M. R., Melvin, L. S., De Costa, B. R., et al. (1990). Cannabinoid receptor localization in brain. *Neurobiology* 87, 1932–1936. doi: 10.1073/pnas.87.5.1932

Hill, E. L., Gallopin, T., Férézou, I., Cauli, B., Rossier, J., Schweitzer, P., et al. (2007). Functional CB1 receptors are broadly expressed in neocortical GABAergic and glutamatergic neurons. *J. Neurophysiol.* 97, 2580–2589. doi: 10.1152/jn.00603.2006

Hoffman, A. F., and Lupica, C. R. (2000). Mechanisms of cannabinoid inhibition of GABA(A) synaptic transmission in the hippocampus. *J. Neurosci.* 20, 2470–2479. doi: 10.1523/JNEUROSCI.20-07-02470.2000

Hosie, S., Malone, D. T., Liu, S., Glass, M., Adlard, P. A., Hannan, A. J., et al. (2018). Altered amygdala excitation and CB1 receptor modulation of aggressive behavior in the neuroligin-3R451C mouse model of autism. *Front. Cell. Neurosci.* 12:234. doi: 10.3389/fncel.2018.00234

Jay, T. M., and Witter, M. P. (1991). Distribution of hippocampal CA1 and subicular efferents in the prefrontal cortex of the rat studied by means of anterograde transport of Phaseolus vulgaris-leucoagglutinin. *J. Comp. Neurol.* 313, 574–586. doi: 10.1002/cne.903130404

Kaplan, J. S., Stella, N., Catterall, W. A., and Westenbroek, R. E. (2017). Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11229–11234. doi: 10.1073/pnas.1711351114

Karlócai, M. R., Tóth, K., Watanabe, M., Ledent, C., Juhász, G., Freund, T. F., et al. (2011). Redistribution of CB1 cannabinoid receptors in the acute and chronic phases of pilocarpine-induced epilepsy. *PLoS ONE* 6:e27196. doi: 10.1371/journal.pone.0027196

Katona, I., and Freund, T. F. (2008). Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nat. Med.* 14, 923–930. doi: 10.1038/nm.f.1869

Katona, I., Rancz, E. A., Acsády, L., Ledent, C., Mackie, K., Hájos, N., et al. (2001). Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J. Neurosci.* 21, 9506–9518. doi: 10.1523/JNEUROSCI.21-23-09506.2001

Lazarini-Lopes, W., Do Val-da Silva, R. A., da Silva-Júnior, R. M. P., Leite, J. P., and Garcia-Cairasco, N. (2020). The anticonvulsant effects of cannabidiol in experimental models of epileptic seizures: from behavior and mechanisms to clinical insights. *Neurosci. Biobehav. Rev.* 111, 166–182. doi: 10.1016/j.neubiorev.2020.01.014

Lee, Y., Rodriguez, O. C., Albanese, C., Santos, V. R., Cortes de Oliveira, J. A., Donatti, A. L. F., et al. (2018). Divergent brain changes in two audiogenic rat strains: a voxel-based morphometry and diffusion tensor imaging comparison of the genetically epilepsy prone rat (GEPR-3) and the Wistar Audiogenic Rat (WAR). *Neurobiol. Dis.* 111, 80–90. doi: 10.1016/j.nbd.2017.12.014

Lutz, B. (2004). On-demand activation of the endocannabinoid system in the control of neuronal excitability and epileptiform seizures. *Biochem. Pharmacol.* 68, 1691–1698. doi: 10.1016/j.bcp.2004.07.007

Maglóczky, Z., Tóth, K., Karlócai, R., Nagy, S., Eross, L., Czirják, S., et al. (2010). Dynamic changes of CB1-receptor expression in hippocampi of epileptic mice and humans. *Epilepsia* 51, 115–120. doi: 10.1111/j.1528-1167.2010.02624.x

Marescaux, C., Vergnes, M., Kiesmann, M., Depaulis, A., Micheletti, G., and Warter, J. M. (1987). Kindling of audiogenic seizures in wistar rats : an EEG study. *Exp. Neurol.* 168, 160–168. doi: 10.1016/0014-4886(87)90290-1

Maroso, M., Szabo, G. G., Kim, H. K., Alexander, A., Bui, A. D., Lee, S. H., et al. (2016). Cannabinoid control of learning and memory through HCN channels. *Neuron* 89, 1059–1073. doi: 10.1016/j.neuron.2016.01.023

McDonald, A. J., and Mascagni, F. (2001). Localization of the CB1 type cannabinoid receptor in the rat basolateral amygdala: High concentrations in a subpopulation of cholecystokinin-containing interneurons. *Neuroscience* 107, 641–652. doi: 10.1016/S0306-4522(01)00380-3

Monory, K., Massa, F., Egertová, M., Eder, M., Blaudzun, H., Westenbroek, R., et al. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51, 455–466. doi: 10.1016/j.neuron.2006.07.006

Moraes, M. F. D., Galvis-Alonso, O. Y., and Garcia-Cairasco, N. (2000). Audiogenic kindling in the Wistar rat: a potential model for recruitment of limbic structures. *Epilepsy Res.* 39, 251–259. doi: 10.1016/S0920-1211(00)00107-8

Naritoku, D. K., Mecozzi, L. B., Aiello, M. T., and Faingold, C. L. (1992). Repetition of audiogenic seizures in genetically epilepsy-prone rats induces cortical epileptiform activity and additional seizure behaviors. *Exp. Neurol.* 115, 317–324. doi: 10.1016/0014-4886(92)90197-X

Paxinos, G., and Watson, C. (2005). *The Rat Brain in Stereotaxic Coordinates*, 5th edn. San Diego, CA: Academic Press.

Poletaeva, I. I., Surina, N. M., Kostina, Z. A., Perepelkina, O. V., and Fedotova, I. B. (2017). The krushinsky-molodkina rat strain: the study of audiogenic epilepsy for 65 years. *Epilepsy Behav.* 71, 130–141. doi: 10.1016/j.yebeh.2015.04.072

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalogr. *Clin. Neurophysiol.* 32, 281–294. doi: 10.1016/0013-4694(72)90177-0

Raisinghani, M., and Faingold, C. L. (2003). Identification of the requisite brain sites in the neuronal network subserving generalized clonic audiogenic seizures. *Brain Res.* 967, 113–122. doi: 10.1016/S0006-8993(02)04232-4

Rocha, L. L., Cinar, R., Guevara-Guzmán, R., Alonso Vanegas, M. A., San Juan Orta, D., Martínez-Juárez, I. E., et al. (2020). Endocannabinoid system and cannabinoid 1 receptors in patients with pharmacoresistant temporal lobe epilepsy and comorbid mood disorders. *Front. Behav. Neurosci.* 14:52. doi: 10.3389/fnbeh.2020.00052

Romcy-Pereira, R. N., and Garcia-Cairasco, N. (2003). Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or fluoro-jade staining. *Neuroscience* 119, 533–546. doi: 10.1016/S0306-4522(03)00191-X

Rosenberg, E. C., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Rossetti, F., Rodrigues, M. C. A., de Oliveira, J. A. C., and Garcia-Cairasco, N. (2006). EEG wavelet analyses of the striatum-substantia nigra pars reticulata-superior colliculus circuitry: audiogenic seizures and anticonvulsant drug administration in wistar audiogenic rats (War strain). *Epilepsy Res.* 72, 192–208. doi: 10.1016/j.eplepsyres.2006.08.001

Ruiz de Azua, I., and Lutz, B. (2019). Multiple endocannabinoid-mediated mechanisms in the regulation of energy homeostasis in brain and peripheral tissues. *Cell. Mol. Life Sci.* 76, 1341–1363. doi: 10.1007/s00018-018-2994-6

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N. O., Leonova, J., et al. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br. J. Pharmacol.* 152, 1092–1101. doi: 10.1038/sj.bjp.0707460

Scharfman, H. E., and Myers, C. E. (2013). Hilar mossy cells of the dentate gyrus: a historical perspective. *Front. Neural Circ.* 6:106. doi: 10.3389/fncir.2012.00106

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K., and Walker, J. M. (1998). Immunohistochemical distribuition of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83, 393–411. doi: 10.1016/S0306-4522(97)00436-3

Tupal, S., and Faingold, C. (2010). Inhibition of adenylyl cyclase in amygdala blocks the effect of audiogenic seizure kindling in genetically epilepsy-prone rats. *Neuropharmacology* 59, 107–111. doi: 10.1016/j.neuropharm.2010.04.011

Turu, G., and Hunyady, L. (2010). Signal transduction of the CB1 cannabinoid receptor. *J. Mol. Endocrinol.* 44, 75–85. doi: 10.1677/JME-08-0190

Van Der Stelt, M., Trevisani, M., Vellani, V., De Petrocellis, L., Moriello, A. S., Campi, B., et al. (2005). Anandamide acts as an intracellular messenger amplifying Ca2+ influx via TRPV1 channels. *EMBO J.* 24, 3026–3037. doi: 10.1038/sj.emboj.7600784

Van Rijn, C. M., Gaetani, S., Santolini, I., Badura, A., Gabova, A., Fu, J., et al. (2010). WAG/Rij rats show a reduced expression of CB1 receptors in thalamic nuclei and respond to the CB1 receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. *Epilepsia* 51, 1511–1521. doi: 10.1111/j.1528-1167.2009.02510.x

Vinogradova, L. V. (2008). Audiogenic kindling in wistar and WAG/Rij rats: kindling-prone and kindling-resistant subpopulations. *Epilepsia* 49, 1665–1674. doi: 10.1111/j.1528-1167.2008.01617.x

Vinogradova, L. V. (2017). Audiogenic kindling and secondary subcortico-cortical epileptogenesis: behavioral correlates and electrographic features. *Epilepsy Behav.* 71, 142–153. doi: 10.1016/j.yebeh.2015.06.014

Vinogradova, L. V., Shatskova, A. B., and Van Rijn, C. M. (2011). Pro-epileptic effects of the cannabinoid receptor antagonist SR141716 in a model of audiogenic epilepsy. *Epilepsy Res.* 96, 250–256. doi: 10.1016/j.eplepsyres.2011.06.007

Vinogradova, L. V., and Van Rijn, C. M. (2015). Long-term disease-modifying effect of the endocannabinoid agonist WIN55,212-2 in a rat model of audiogenic epilepsy. *Pharmacol. Rep.* 67, 501–503. doi: 10.1016/j.pharep.2014.12.002

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and Delorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920

Wilson, R. I., and Nicoll, R. A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 410, 588–592. doi: 10.1038/35069076

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

frontiers in Behavioral Neuroscience

REVIEW
published: 12 January 2021
doi: 10.3389/fnbeh.2020.610484

Cannabinoids: A New Perspective on Epileptogenesis and Seizure Treatment in Early Life in Basic and Clinical Studies

*Angélica Vega-García[1], Iris Feria-Romero[2], Anais García-Juárez[3], Ana Ch. Munguia-Madera[2], Alexia V. Montes-Aparicio[2], Esli Zequeida-Muñoz[4], Estefany Garcia-Albavera[5] and Sandra Orozco-Suárez[2]**

[1]Departamento de Fisiología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, [2]Unidad de Investigación Médica en Enfermedades Neurológicas, Hospital de Especialidades, "Dr. Bernardo Sepúlveda", Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, IMSS, Ciudad de México, Mexico, [3]División de Ciencias Biológicas y Ambientales, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Guadalajara, Mexico, [4]Facultad de Medicina, Universidad Autónoma de Guerrero, Acapulco, Mexico, [5]Facultad de Biología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico

OPEN ACCESS

Edited by:
Dolores E. López García,
University of Salamanca, Spain

Reviewed by:
Orlando Jorge Castellano,
University of Salamanca, Spain
Alan Talevi,
National University of La Plata, Argentina

***Correspondence:**
Sandra Orozco-Suárez
sorozco5@hotmail.com

Specialty section:
This article was submitted to journal Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 26 September 2020
Accepted: 26 November 2020
Published: 12 January 2021

Citation:
Vega-García A, Feria-Romero I, García-Juárez A, Munguia-Madera ACh, Montes-Aparicio AV, Zequeida-Muñoz E, Garcia-Albavera E and Orozco-Suárez S (2021) Cannabinoids: A New Perspective on Epileptogenesis and Seizure Treatment in Early Life in Basic and Clinical Studies. Front. Behav. Neurosci. 14:610484. doi: 10.3389/fnbeh.2020.610484

Neural hyperexcitability in the event of damage during early life, such as hyperthermia, hypoxia, traumatic brain injury, status epilepticus, or a pre-existing neuroinflammatory condition, can promote the process of epileptogenesis, which is defined as the sequence of events that converts a normal circuit into a hyperexcitable circuit and represents the time that occurs between the damaging event and the development of spontaneous seizure activity or the establishment of epilepsy. Epilepsy is the most common neurological disease in the world, characterized by the presence of seizures recurring without apparent provocation. Cannabidiol (CBD), a phytocannabinoid derived from the subspecies *Cannabis sativa* (*CS*), is the most studied active ingredient and is currently studied as a therapeutic strategy: it is an anticonvulsant mainly used in children with catastrophic epileptic syndromes and has also been reported to have anti-inflammatory and antioxidant effects, supporting it as a therapeutic strategy with neuroprotective potential. However, the mechanisms by which CBD exerts these effects are not entirely known, and the few studies on acute and chronic models in immature animals have provided contradictory results. Thus, it is difficult to evaluate the therapeutic profile of CBD, as well as the involvement of the endocannabinoid system in epileptogenesis in the immature brain.

Abbreviations: CNS, central nervous system; ABHD6, α-β-hydrolase domain 6; ACEA, arachidonyl-2′-chloroethylamide; AEA, anandamide; eCBS, endocannabinoid system; eCB-endocannabinoide CBD, cannabidiol; CBDV, cannabidivarin; CB1R, cannabinoid type 1 receptor; CB2R, cannabinoid type 2; GluR, glutamate receptor; DAGL, diacylglycerol lipase; Δ^9-THC, Δ^9-tetrahydrocannabinol; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; FAAH, fatty acid amide hydrolase; GABA γ, aminobutyric acid; GAD, glutamate decarboxylase; GPR, G- protein-coupled receptor; KA, kainic acid; MAGL, monoacylglycerol lipase; MDA, maximal dentate activation; MES, maximal electroshock; NAPE-PLD, N-acylphosphatidylethanolamine-hydrolyzing phospholipase D; PMSF, phenylmethane sulfonyl fluoride; PTZ, pentylenetetrazole; TRPV1, transient receptor potential vanilloid receptor (type 1); NMDArs, N-methyl-d-aspartate receptor; AMPArs AMPA-type glutamate receptors; FS, febrile seizures; SE, *Status epilepticus*; TBI, traumatic brain injury; PN, post natal; eCB, endocannabinoid system; CCK, colecistoquina; Ca^{2+}, calcium; K^+, potassium; Cl^-, chlorine; Cox-2, cyclooxigenase 2; 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, N-arachidonoyl-ethanolamine or anandamide; CNS, central nervous system; Cox, cyclooxygenase; IL-1β, interleukin-1β; LOX, lipoxygenase.

Therefore, this review focuses on the collection of scientific data in animal models, as well as information from clinical studies on the effects of cannabinoids on epileptogenesis and their anticonvulsant and adverse effects in early life.

Keywords: cannabinoids, epileptogenesis, neurodevelopment, neuroprotection, anti-inflammatory, pharmacokinetics

INTRODUCTION

Seizure disorders are common during childhood; they are causes of morbidity (Glass et al., 2018), and a large percentage of them have a poor response to current first-line anticonvulsant drugs (ADs; Glass et al., 2012). In the postnatal neurodevelopmental period, neuronal excitability is predominantly mediated by the glutamatergic and GABAergic activity system, and it promotes the processes of growth, plasticity, synaptogenesis, and organization of neural networks essential for the adult stage (Ben-Ari, 2002; Ben-Ari and Holmes, 2006; Rakhade and Jensen, 2009). Thus, the immature brain is highly susceptible to developing neuronal hyperexcitability under pathological conditions such as hyperthermia, hypoxia-ischemia, traumatic brain injury (TBI), or a pre-existing neuroinflammatory condition that in turn facilitates the development of seizure activity and the establishment of *status epilepticus* (*SE*; Pitkänen et al., 2015; Suchomelova et al., 2015).

Additionally, the early exposure to many ADs, is a significant risk for brain development such as PB, DFH, and valproate, and has been related to developmental disorders (Bittigau et al., 2002; Forcelli et al., 2011; Kaushal et al., 2016; Al-Muhtasib et al., 2018). Furthermore, clinical evidence indicates that gestational exposure to ADs can also lead to deficits in cognitive function (Meador et al., 2012). For this reason, the need to identify new drugs, Cannabidiol (CBD), and its propyl cannabidivarin (CBDV) analog, has aroused interest in the treatment of epilepsy in early life. While CBD was recently approved for the treatment of refractory childhood epilepsies (Abu-Sawwa et al., 2020), little is known about the efficacy and safety of compounds derived from *Cannabis sativa* (*CS*) in the early stage of development (Rosenberg et al., 2017). Here we address this issue through a systematic evaluation of the cannabinoid literature investigating multiple therapeutic targets, some of which were tested in early developmental seizure models, including data from our laboratory as well as clinical evidence.

Cannabinoids

CS is an herbaceous plant native to central Asia that is widely distributed in a variety of habitats and altitudes and is a unique species of its kind. Some authors recognize *C. ruderalis* and *C. indica* as separate species or subspecies of *CS*; however, a monospecific criterion has been adopted in many of the nontaxonomic publications, since all the groups of plants that have been included within the genus are interfertile and their morphological diversity shows a diffuse and continuous pattern (Etienne, 2014). This plant has been known for its medicinal and textile uses that date back to more than 5,000 years ago. Currently, a total of 545 constituents of cannabis have been identified, of which 104 are phytocannabinoids classified into 11 types: (−)-Δ^9-trans-tetrahydrocannabinol (Δ^9 THC) type, (−)-Δ^8-trans-tetrahydrocannabinol (Δ^8-THC) type, cannabigerol (CBG) type, cannabichrome (CBC) type, cannabidiol (CBD) type, cannabidiol (CBND) type, cannabielsoin (CBE) type, cannabicyclol (CBL) type, cannabinol (CBN) type, cannabitriol (CBT) type, and miscellaneous cannabinoid type. Additional compounds include flavonoids, steroids, phenanthrenes, xanthones, and sugars, among others (Pertwee, 2008; El Sohly et al., 2016). Phytocannabinoids are terpenophenolic products that exhibit a 21- to a 22-carbon skeleton, some of which are breakdown products of other cannabinoids. The predominant compounds in the plant are tetrahydrocannabinol acid (THCA), cannabidiolic acid (CBDA), and cannabinolic acid (CBNA), followed by cannabigerolic acid (CBGA), cannabichromenic acid (CBCA), and cannabinodiolic acid (CBNDA). Although THCA is the most important compound in drug-type cannabis and CBDA in fiber-type cannabis, it is also worth noting that CBCA predominates in young plants and decreases with maturation (Andre et al., 2016). Nevertheless, *CS* is not the only natural source of cannabinoids, as, in the Radula and Helichrysum gender, the presence of cannabinoid-type terpenophenolics has also been reported; however, little is known about these compounds (Mahmoud and Waseem, 2014; Andre et al., 2016). Currently, the pharmacological actions of the psychotropic cannabinoids Δ^9-THC, Δ^8-THC, CBN, and Δ^9-THCV and the non-psychotropic phytocannabinoids CBC, CBD, CBDA, CBG, THCA, THCVA, CBGA, CBDV and CBGV have been well documented. Also, pharmacological actions of the nonphytocannabinoid component (E)-β-caryophyllene, which is a sesquiterpene, have also been described (Mechoulam and Gaoni, 1965; Pertwee, 2008; Mechoulam and Parker, 2013; Pertwee and Cascio, 2014).

Endocannabinoid System

The endocannabinoid system (eCBs) was discovered by Mechoulam and Gaoni (1965), who isolated the active compound (Δ^9-THC); in 1990, they discovered the binding site of this cannabinoid, oriented their search for additional receptors, and described the abundant CB1 receptor (CB1R) in the central nervous system (CNS) with a similar density to the GABA and glutamate receptors. Years later, the CB2 receptor (CB2R), abundant in tissues of the immune system, was described, followed by the functioning of its endogenous ligands, the endocannabinoids 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl ethanolamide or anandamide (AEA; Andre et al., 2016). Similarly, how the endocannabinoids 2-AG and anandamide or AEA are synthesized from fatty acids from the remodeling of the cell lipid membrane and are produced

according to the individual's bodily demands was described shortly thereafter (Di Marzo and Piscitelli, 2015). Anandamide is produced by the action of the enzyme *N*-acyltransferase (NAT), which produces *N*-arachidonoyl phosphatidylethanolamine (NArPE). Phospholipase D generates a family of compounds, the arachidonoyl glycerol ethanolamine (FAE) family, which includes 2-AG and anandamide. When these compounds are released, their degradation occurs through metabolism by enzymatic hydrolysis, where fatty acid amide hydrolase (FAAH) intervenes in the degradation of anandamide and monoacylglycerol lipase (MAGL). Finally, 2-AG is metabolized into glycerol and arachidonic acid during postsynaptic cell recapture. Anandamide and 2-AG can also bind to plasma-circulating albumin and have distant effects (Patel et al., 2017). NAT is regulated by calcium and cAMP cyclic adenosine monophosphate and is selectively stimulated by cellular depolarization or by the action of the metabotropic receptors for glutamic acid, dopamine, or acetylcholine (Lu and Mackie, 2016; Patel et al., 2017).

The abovementioned endocannabinoids act as agonists of these receptors and are G protein-coupled to the endocannabinoid CB1R and CB2R. G proteins inhibit adenylate cyclase activation by reducing cAMP concentrations and interfering with the activity of cAMP-dependent protein kinase (PKA). The same G protein activates mitogen activation-dependent protein kinase (MAPK); both PKA and MAPK are involved in the selective expression of genes. The activation of the CB1R and CB2R inhibits L-, N-, P-, and Q-type voltage-dependent Ca^{2+} channels, which reduces the entry of Ca^{2+} and stimulates the endogenous K+ rectifier channels at the neuronal level by allowing the release of ions, resulting in hyperpolarization. Interactions with other receptors, such as GPR55, GPCR, and TRPV1 (in this case acting as an antagonist), have also been described, in which the endocannabinoids act as neuromodulators (Castillo et al., 2012; Alexandre et al., 2020; **Figure 1**). The CB1R is expressed throughout the CNS, particularly in the hippocampus on mossy fibers of the granule cells of the dentate gyrus (DG), wherein the mature stage, eCBs regulate the efficiency of inhibitory synapses during periods of sustained depolarization at the postsynaptic level through increased intracellular Ca^{2+}, which activates the synthesis of endocannabinoids, mainly in pyramidal neurons of the hippocampus (Herkenham et al., 1990, 1991; Katona et al., 1999, 2006). These eCB are fat-soluble, which allows their diffusion through the plasma membrane as well as the synaptic cleft in a retrograde manner towards the neighboring presynaptic terminals, favoring the inhibitory activity mediated by the GABAergic system (Katona et al., 1999, 2006; Kawamura et al., 2006; Monory et al., 2006).

Endocannabinoid System in Postnatal Neurodevelopment

It has been shown that during critical periods of neurodevelopment, eCBs participate in the maturation of corticolimbic circuits, where excitatory neuronal activity is essential for the processes of synaptogenesis and neuronal plasticity (Harkany et al., 2008), directed by the glutaminergic system, which constitutes most synapses; likewise, ion channels and glutamate transporters and receptors are expressed at levels that promote the activity of excitatory networks (Sanchez et al., 2001; Jensen, 2009). In rodents, during the first week, from 0 to 7 post natal (PN) days, a period analogous to a 36-week, premature human, the GABAergic system participates in excitatory neurotransmission, mediated by an increase in the K^+/Cl cotransporter NKCC1 and downregulation of KCC2. The neuronal depolarization is generated by intracellular Cl^- increases and activates L-type voltage-sensitive Ca^{2+} channels (L-VSCC) and NMDARs, which increases the flux of intracellular Ca^{2+} and activates signaling cascades directed at intracellular trophic activity, neuritic growth, neural network formation, and synaptogenesis (Ben-Ari, 2002, 2014; Galanopoulou, 2008). During this period of neurodevelopment, CB1R is located in the terminals of mossy fibers in the CA3 subfield of the hippocampus, mainly in GABAergic and cholecystokinin (CCK) circuits, reaching a maximum concentration at the day of 4 PN, where CB1Rs are coupled to G proteins. In these terminals, CB1Rs suppresses the release of GABA, reducing the excitatory activity of GABA and exerting a role as a regulator of excitatory neuronal activity important for the development of neuronal circuits (Bernard et al., 2005; Fernández-Ruiz et al., 2005).

The eCBs modulate neuronal activity by regulating retrograde signaling, which participates in short-term synaptic plasticity, also known as suppression of depolarization-induced inhibition (DSI) and excitation (DSE). It is estimated that eCB levels, specifically those of AEA during development, determine the direction of synaptic plasticity (Kreitzer and Regehr, 2001; Castillo et al., 2012). From PN day 8, GABA begins to exert its inhibitory activity due to the increase in expression of the KCC2 cotransporter, which decreases intracellular Cl^- levels, favoring neuronal hyperpolarization and the inhibitory activity of eCBs (Ben-Ari, 2002, 2014; Galanopoulou, 2008). Then, from PN days 10–14 in the rat, which corresponds to the first 36 months of human life, GluRs are regulated by glia, and ionotropic glutamate receptors (iGluRs) are linked to ion channels that allow the flow of sodium (Na^+), potassium (K^+) and Ca^{2+} ions to different degrees depending on the receptor subunit. The main GluRs are NMDARs, AMPARs, and KARs (Benítez-Diaz et al., 2003; Herlenius and Lagercrantz, 2004; Rakhade and Jensen, 2009). During this period, NMDARs are expressed differently in the brain, with an increase in the expression of NR2B and NR3A subunits, which are not very sensitive to Mg^{+2} ions, which increases the activation of the receptor and increases intracellular Ca^{2+} flux, thereby promoting neuronal hyperexcitability and long-term potentiation (LTP; Ben-Ari, 2002, 2014; Qu et al., 2003; Coulter, 2006).

The NMDARs distributed in the immature brain are mainly found in the hippocampus at the postsynaptic level, but some are also present at the presynaptic level and in astrocytes (Lee et al., 2010; Szczurowska and Mareš, 2013; Skowroń ska et al., 2019). The glutamate receptors AMPARs and KARs are permeable to Ca^{2+} ions when GluR2 expression is relatively low or absent (Romjin et al., 1991; Tyzio et al., 1999; Jensen, 2009); KARs are also permeable to Ca^{2+} when the GluR5 and GluR6 subunits are absent. Additionally, metabotropic glutamate receptors

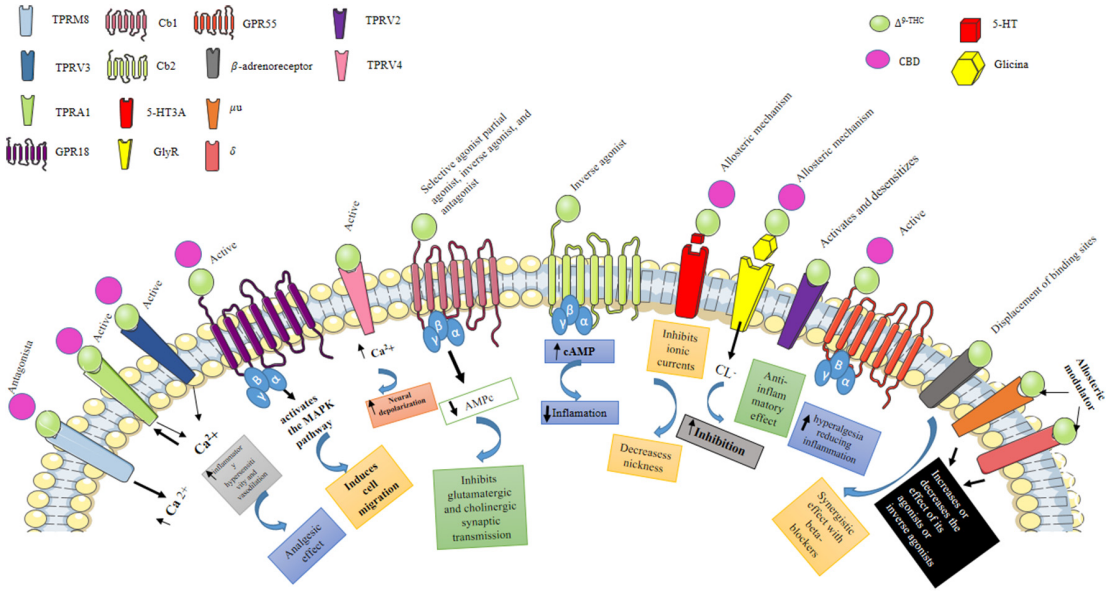


FIGURE 1 | Pharmacological effects of Δ^9-THC and CBD. Δ^9-THC is the main psychoactive component of *C. sativa*, which can behave as a selective agonist, partial agonist, inverse agonist, and antagonist of the Cb1 receptor, while when activating the Cb2 receptor it behaves as an inverse agonist. Activation of the Cb1 and Cb2 receptors stimulates GTPγS binding to cell membranes and inhibits cyclic AMP production. Also, Δ^9-THC can inhibit $5HT^{3A}$ receptor-mediated currents induced by 5-hydroxytryptamine (5HT); antagonizing receptor activation, possibly through an allosteric mechanism, by this same mechanism, Δ^9-THC and CBD can enhance the activation of GlyR expressed in central tegmental area (ATV) neurons. Additionally, Δ^9-THC activates the TRPV3 and TRPV4 receptors, which are nonselective calcium-permeable cation channels that, when activated, raise intracellular Ca^{2+} and consequently cause neuronal depolarization. Transient receptor potential (TRP) channels are a group of membrane proteins involved in the transduction of a large number of stimuli. Unlike Δ^9-THC, CBD does not activate CB1 and CB2 receptors, which likely accounts for its lack of psychotropic activity. However, CBD interacts with many other, nonendocannabinoid signaling systems. It is a "multi-target" drug. At low micromolar to submicromolar concentrations, CBD is a blocker of the equilibrative nucleoside transporter (ENT), the orphan G-protein-coupled receptor GPR55, and the TRP of melastatin type 8 (TRPM8) channel. At higher micromolar concentrations, CBD activates the TRP of vanilloid type 1 (TRPV1) and 2 (TRPV2) channels while also inhibiting cellular uptake and fatty acid amide hydrolase—catalyzed degradation of anandamide.

(mGluRs) are coupled to a G protein (GTP) and mediate slow synaptic responses (Jensen, 2009; Ben-Ari, 2014), which favor activation of CB1R expressed in glutamatergic neurons to modulate excitatory and inhibitory neurotransmission. This process is essential in the remodeling of inhibitory circuits during adolescence, a period from PN day 30 in rodents, where the regulation of glutamatergic and GABAergic activity are of vital importance for the formation, maturity, and elimination of synapses, mainly at the level of the prefrontal cortex (PFC), which has greater activity compared with the adult stage, a period from PN day 60 in rodents (Clancy et al., 2001; Auvin and Dupuis, 2014; Dow-Edwards and Silva, 2017). During adolescence, eCBs participate in the formation of neural networks in the prefrontal cortex and maintain the interaction between the amygdala, hippocampus, and hypothalamus, which is responsible for cognitive and emotional development since endocannabinoids are responsible for the normal response to stress and the regulation of neuronal excitation and inhibition (Schonhofen et al., 2018; Cheung et al., 2019).

The expression of CB2R in cells of the immune system at the peripheral level has been reported in $CD4^+$, $CD8^+$ T and B lymphocytes, natural killer cells, monocytes, and polymorphonuclear neutrophils (Atwood and Mackie, 2010). CB2R is also expressed in the microglia, which during postnatal neurodevelopment maintain amoeboid forms that allow them to actively participate in the phagocytosis of apoptotic cell debris, in the induction of apoptosis in other cells during the formation of functional neural circuits, and the removal of residual myelin, as well as in the formation and expansion of neural networks that imply the pruning of synapses at the level of the cerebral cortex, hippocampus, cerebellum, and amygdala (Berdyshev, 2000; Bessis et al., 2007; Atwood and Mackie, 2010; Onaivi et al., 2012). In adult rats, however, the expression of CB2R decreases as the microglia become inactive (Stella, 2004; Fernández-Ruiz et al., 2005; Caiati et al., 2012). Additionally, the expression of CB2R has been reported in glutamatergic and GABAergic neurons in the DG and CA1 subfield of the hippocampus at the level of the pyramidal stratum and stratum radiatum to a lesser degree than CB1R, which is observed mainly in the cerebral cortex, hippocampus, amygdala, and cerebellum (Brusco et al., 2008; Onaivi et al., 2012).

Astrocytes and microglia are responsible for the surveillance and modulation of the immune response and are the main cytokine producers in the CNS (Baud and Saint-Faust, 2019). Here, CB2R has been reported to participate as a neuromodulator of the neuroactive molecules ON, glutamate, PGs, and neurotrophins in the glia and vascular endothelium, which participate in neurodevelopmental processes, homeostatic mechanisms such as sleep, and activation of signaling pathways involved in neuronal plasticity and synaptogenesis; however,

the mechanisms responsible for its participation in eCBs during postnatal neurodevelopmental processes are not yet entirely clear (Devinsky et al., 2013; Schonhofen et al., 2018; Cheung et al., 2019).

Endocannabinoid System in Epileptogenesis in the Immature Brain

The term epileptogenesis is defined as a sequence of events that convert a normal circuit into a hyperexcitable circuit (Pitkänen et al., 2015). Epileptogenesis refers to the development of tissue capable of generating spontaneous seizures, which result in an epileptic condition or in the progression of established epilepsy (Pitkänen and Engel, 2014), whereby it is a continuous and multifactorial process in which the eCBs participate in the modulation of neuronal activity, neuronal migration, axonal growth and guidance, synaptic plasticity, and the neuroinflammatory response (Schonhofen et al., 2018; Cheung et al., 2019). It has been established that damage to eCB signaling in the early stages of neurodevelopment can favor the process of epileptogenesis and the establishment of epilepsy in later stages (**Figure 2**).

During the latent period, the participation of eCBs has been described in different cascades of events defined as acute, subacute, and chronic (Kadam et al., 2010). The induction of early genes (IEGs), including Fos, Jun, Egr4, Egr1, Homer 1, Nurr77, and Arc, occurs during acute changes as a result of intense and repeated synaptic activity during seizure activity, which promotes increased intracellular Ca^{2+} flux by NMDARs (Herdegen and Leah, 1998). Upregulation of IEGs can pathologically modulate synaptic function, lowering the threshold to neuronal hyperexcitability (Rakhade and Jensen, 2009). The levels of intracellular Ca^{2+} immediately alters synaptic efficiency mechanisms, such as increasing the postsynaptic density and dendritic spines, grouping NMDARs and AMPARs, and increasing glutamate synthesis (Haglid et al., 1994; Sanchez et al., 2001), where the participation of eCBs plays an important pathophysiological role in modifying excitatory and inhibitory synaptic neurotransmission in the brain (Rosenberg et al., 2017). During postnatal neurodevelopment, CB1R located in presynaptic cells, mainly in the granule cells of the hippocampus, regulates excitatory activity; additionally, endocannabinoids can also act through interactions with other types of receptors, mainly on G protein-coupled receptors, such as GPRSS and the Transient receptor potential (TRP) cation channel subfamily V member 1" or "vanilloid receptor 1" (TRPV1), which participate in neuronal hyperexcitability, favoring intracellular Ca^{2+} flux (Bhaskaran and Smith, 2010; Schonhofen et al., 2018; Cheung et al., 2019).

As we have indicated, the synthesis of endocannabinoids is mediated by an increase in intracellular Ca^{2+} generated by neuronal hyperexcitability and the demand for membrane phospholipid diacylglycerol lipase (DAGL) and for glutamate and acetylcholine (Wallace et al., 2003; Rosenberg et al., 2017). The eCBs passively diffuse through presynaptic cells in a retrograde manner to bind orthosterically to and activate CBR1 acylphosphatidyl ethanolamine-hydrolyzing phospholipase D (NAPE-PLD) to form 2-AG and AEA, in the same way, endocannabinoids are also synthesized from the activation of metabotropic receptors and thus inhibit the release of glutamate or GABA, a process defined as depolarization-induced suppression of inhibition (DSI) or excitation (DSE), respectively (Armstrong et al., 2009). In models of febrile seizures at PN day 10, alterations to these DSI and DSE mechanisms have been reported, which are estimated to interfere with the maturation of the GABAergic system and persist in later stages through the generation of hyperexcitable circuits (Bernard et al., 2005).

Similarly, it has been reported that eCB signaling has an important role in epileptogenesis, because, after the dissociation from CB1R, eCB 2-AG and AEA are catabolized by the enzymes monoacylglycerol lipase (MAGL) and alpha-beta hydrolase domain containing 6 (CABHD6) or through fatty acid amide hydrolase (FAAH), respectively, and activation of TRPV1 by AEA can trigger increased glutamate release after intracellular Ca^{2+} concentrations are increased (Wallace et al., 2003; Rosenberg et al., 2017). This process activates calcineurin (PPP3C or serine-threonine protein phosphatase 2B), which, during intense seizure activity, activates calcium-calmodulin-phosphatase to promote the dephosphorylation and subsequent endocytosis of $GABA_A$ (Blair et al., 2004). These phenomena reduce the inhibitory potential and lead to the gradual loss of GABAergic inhibitory networks (Kurz et al., 2001; Blair et al., 2004; Rakhade and Jensen, 2009; Semple et al., 2020). In the immature brain, calcineurin activation increases the phosphorylation of Kv2.1 ion channels (also known as KCNB1) and promotes their expression in the postsynaptic membrane, allowing prolonged neuronal depolarization (Kurz et al., 2001; Rakhade and Jensen, 2009).

Similarly, protein kinase C, which is dependent on protein kinase type II and calcium-calmodulin-phosphatase, increases within minutes after the induction of *SE* in immature rats, which allows an increase in the phosphorylation of serine 831 of GluR1 and serine 880 of GluR2. This process promotes the endocytosis of GluR2 and increases the permeability to Ca^{2+} in AMPARs, which generates an increase in seizure susceptibility in later or adult stages (Rice and De Lorenzo, 1998; Sanchez et al., 2001; Rakhade and Jensen, 2009). Additionally, it has been reported that astrocyte activation, immediate to neuronal damage, increases extracellular K^+ concentrations, which in turn facilitates continuous neuronal hyperexcitability, a triggering factor for epileptogenesis (Jabs et al., 1997; Jensen, 2009).

Subacute changes in the latent period are established from hours to days after neuronal damage (**Figure 2**), in which a rapid increase occurs in NGF and BDNF, which together with Trk alter the modulation of the maturation of the KCC2 cotransporter, promoting GABA-mediated inhibitory activity mainly in DG granular cells, which in turn alters the modulating function of eCBs on the GABAergic system to favor long-term neuronal hyperexcitability (Chen et al., 2003, 2007). However, the cellular mechanisms by which CB1R and CB2R act on short- and long-term changes in the seizure threshold are still unknown, particularly in mesiotemporal structures consisting of mainly the hippocampus and the cerebral cortex in the immature brain (Chen et al., 2007).

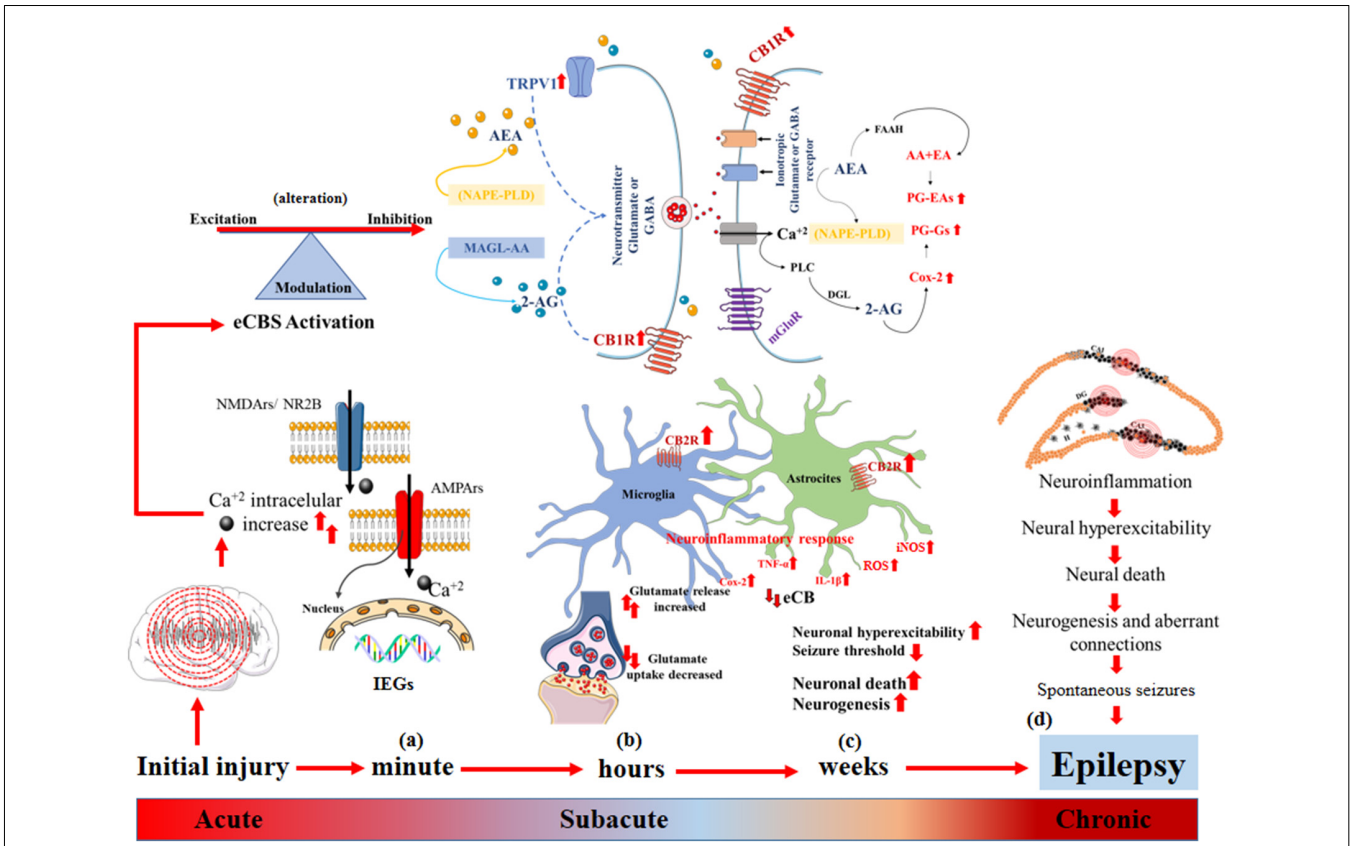


FIGURE 2 | Epileptogenesis and endocannabinoid system (eCBs). **(A)** Acute changes: increased intracellular Ca^{2+} flux, induction of early genes (IEGs) that alter synaptic function, decreased threshold to neuronal hyperexcitability, alteration of eCBs that modulates the balance between excitatory and inhibitory neurotransmission. **(B)** Sub-acute changes: synthesis of endocannabinoids (eCB) mediated by the increase in intracellular Ca^{2+}, neuronal hyperexcitability and the demand for membrane phospholipids diacylglycerol lipase (DAGL) and *N*-acyl phosphatidyl ethanolamine-hydrolyzing phospholipase D (NAPE-PLD) to form 2-AG and AEA, respectively, and their degradation after the dissociation of CB1R and the activation of TRPTV1 by AEA, which triggers greater glutamate release and increases in intracellular Ca^{2+}. The eCBs degrade rapidly, and 2-AG and AEA are catabolized by the enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), respectively, which generate AA, PGs-Eas, PG-Gs, and Cox-2 to increase convulsive susceptibility, activation of astrocytes and microglia and increases in CB2R that promote the release of pro-inflammatory proteins IL1-β, Cox-2, TNF-α and iNOS, generating neuroinflammation, neuronal hyperexcitability, and neuronal death. **(C,D)** Chronic changes: neuronal death, the perpetuation of the neuroinflammatory response and dysregulation of eCBs that promote neurogenesis, formation of aberrant connections, expression of spontaneous seizures, and epilepsy.

The neuroinflammatory response activated by microglia and astrocytes, the main response responsible for the synthesis and release of pro-inflammatory cytokines such as IL1-β, activates the IL-1/TLR signaling pathway, where an increase in pro-inflammatory proteins occur. These proteins include Cox-2, an enzyme that catalyzes the production of PGs from AA and increases seizure susceptibility (Chen et al., 2001, 2007; Jiang et al., 2004; Feng et al., 2016), and TNF-α, which generates an increase in the expression of AMPAR and a decrease in GluR2 through the expression of TNF-R1 on the neuronal membrane surface, which in turn causes increased permeability of immature neurons to Ca^{2+} (Beattie et al., 2002; Stellwagen et al., 2005). The activity of TNF-α has been shown to result in the endocytosis of $GABA_A$ receptors, mainly their β 2/3 subunits, in the pyramidal neurons of CA1 and interneurons of the DG, promoting neuronal excitation (Wang et al., 2000; Balosso et al., 2005; Stellwagen et al., 2005; Shimada et al., 2014) and leading to neurodegeneration and neuronal death in the CA1 and CA3 regions of the immature hippocampus (Rizzi et al., 2003; Kawaguchi et al., 2005).

In the same way, the rapid increase in Cox-2 mRNA expression after *SE* induced by KA itself induces neurodegeneration and neuronal death in the immature hippocampus, mainly the CA1 and CA3 subfields, at 12 and 24 h post *SE* (De Simoni et al., 2000; Rizzi et al., 2003; Kawaguchi et al., 2005). This finding supports that the neuroinflammatory response precedes neuronal damage (Ravizza et al., 2005; Joseph and Levine, 2006), and this neuronal damage is associated with an increase in Cox-2 and the subsequent production of PGs and ROS, activation of the oxidative stress mechanism, and mitochondrial dysfunction (Gobbo and O'Mara, 2004); thus, it has been reported that the eCBs participate as a substrate of the Cox-2 enzyme for the synthesis of PGs (Nomura et al., 2011; Ruhaak et al., 2011). Similarly, experimental models of *SE* induced by KA and Li-Pilo in PN 12 day rats have reported a rapid increase in ROS and mitochondrial markers of oxidative

damage, including NT-3, 4-HNE and carbonylated proteins, as well as the formation of free radicals 24 h post-*SE*, which are responsible for neurodegeneration in the subfields of CA1, CA3 and DG of the hippocampus, cerebral cortex and thalamus (Patel and Li, 2003; Folbergrová et al., 2010).

The contribution of mitochondrial dysfunction on complex I of the respiratory chain NADH (Cock, 2002; Kudin et al., 2002) favor the development of neurodegenerative disorders, such as epilepsy, due to the excessive release of glutamate, which causes an alteration in the reuptake mechanisms of this neurotransmitter and an increase in intracellular Ca^{2+} due to the saturation of the regulatory mechanisms mediated by the Na^{+}/Ca^{2+} exchanger and the Ca^{2+} buffer proteins (Tretter et al., 2004).This causes the mitochondria to capture the excess Ca^{2+} in the mitochondrial matrix, which induces the depolarization of the membrane by the partial inhibition of the chemiosmotic potential and by the accumulation of positive charges in the mitochondrial matrix (Tretter et al., 2004). This sustained overload produces an irreversible depolarization through the activation of the mitochondrial transition pore, a pathway through which Ca^{2+} returns to the cytosol, causing the collapse of the mitochondrial chemiosmotic potential and a reduction in the synthesis of ATP (Murchison and Griffith, 2000). The decrease in ATP generates metabolic dysfunction, ROS production, activation of proteases, phospholipases, iNOS, and endonucleases, and inhibition of protein synthesis. Furthermore, NO can act as a retrograde messenger, enhancing the excitotoxic effect of glutamate and increasing its release from presynaptic terminals (Lorigados et al., 2013). Likewise, it has been reported that complex I dysfunction may also be the result of an increase in carbonylated proteins during the subacute phase post-*SE* induced by Li-Pilo and that this alteration persists up to 5 weeks, a period in which spontaneous convulsive activity is observed (Folbergrová et al., 2010).

Likewise, Cox-2 and TNF-α act directly on receptors and ion channels and indirectly by modulating extracellular glutamate reuptake systems *via* the reduction of selective transporters to glutamate GLT-1, mediated by astrocytes, mainly in CA1 and DG of the hippocampus. Additionally, the activation of iNOS, which promotes the synthesis of NO, increases the release of glutamate and substance P, which preserves the activation of cytokines together with the increase in the affinity of NMDARs and AMPARs, which supports the finding that the activation of the neuroinflammatory response by microglia and astrocytes increases the vulnerability of the immature hippocampus to neuronal hyperexcitability and that these changes depend on the age of development (De Simoni et al., 2000; Rizzi et al., 2003; Viviani et al., 2003; Ravizza et al., 2005; Stellwagen et al., 2005; Bessis et al., 2007; Chen et al., 2013). The participation of CB2R, which is overexpressed by microglia in the neuroinflammatory response in response to neuronal damage and the infiltration of cells of the immune system into the brain parenchyma (Sagredo et al., 2009; Bouchard et al., 2012), induces chronic CB2R activation, which increases excitatory neurotransmission (Li and Kim, 2015) and decreases inhibitory neurotransmission (Morgan et al., 2009). The CB2R, which are mostly expressed in microglia, have been reported to participate in the neuroinflammatory response in experimental models in rats, where they increase 2-AG and CB1R in the hippocampus after *SE* (Wallace et al., 2003); however, the increase in 2-AG in the seizure model in mice shows a decrease in seizure activity (Sugaya et al., 2016).

Chronic changes (**Figure 2**) can be observed over 2–12 weeks in rodents and from months to years in humans. It has been reported that during the first 2 weeks of life, the resistance to excitotoxic damage in the immature brain is relative since the amount of Ca^{2+} that enters a pyramidal neuron is directly related to the age of PN development, where in the first 3 days of life, glutamate minimally increases intracellular Ca^{2+}. Conversely, between PN days 10 and 25, intracellular Ca^{2+} increases markedly due to recurrent seizure activity, leading to neuronal death in the CA1 subfield of the hippocampus, the area most vulnerable to excitotoxic damage, due to an increase in NMDARs and AMPARs (Kubová et al., 2001, 2012; Kubová and Mareš, 2013). Additionally, an alteration in the immature glutamatergic system that results in the overexpression of GluR2 and its activity on the intracellular Ca^{2+} flux through AMPARs and KARs, a condition that pathologically persists during the adolescent stage mainly in the prefrontal cortex, favors the formation of hyperexcitable circuits. Similarly, prolonged positive regulation has been reported in the expression of the CB1R and in the suppression of the inhibition induced by depolarization, which can cause alterations in neuronal hyperexcitability throughout life (Chen et al., 2003; Bernard et al., 2005).

Anticonvulsant and Neuroprotective Effect of CBD on Experimental Models of *Status Epilepticus* and Epilepsy

It has been reported that CBD has a low affinity for CB1R and CB2, and at high levels, it can act as an indirect CB1R antagonist as evaluated by a wide range of experimental models of seizures and epilepsy in adult rats. The evaluation of high doses of CBD, up to 300 mg/kg i.p., in the hippocampal kindling model reveals a reduction in amplitude of the discharge and an increase in the after-discharge threshold (ADT; Turkanis et al., 1979; Ghovanloo et al., 2018). However, in the lamotrigine drug-resistant tonsillar kindling model, CBD does not show anticonvulsant or neuroprotective effects (Klein et al., 2017). Though, in both models, potential neuroprotective effects were identified at low vs. high doses of CBD, which increased the neurotoxic effects of this active ingredient (Patra et al., 2019). Conversely, no anticonvulsant effects of repeated CBD administration have been reported (Rosenberg et al., 2017). The neuroprotective effects of CBD have been identified when administered after *SE* induced by intrahippocampal pilocarpine microinjection and in *in vitro* models in rat hippocampal slices, in which CBD decreases the amplitude and duration of the epileptiform activity induced by 4-aminopyridine (Jones et al., 2010; Franco and Perucca, 2019). However, despite the experimental existence of the anticonvulsant effects of CBD, the responsible mechanisms remain unclear, and few studies have examined its anticonvulsant effect in the immature stage (**Table 1**).

TABLE 1 | Effect of cannabinoids in experimental models of epileptic seizures.

Compound	Type of study	Effect	Mechanism	Reference
CBD (HU-320)	*In vivo* Mouse CIA model. *In vitro* Mouse macrophages and RAW 267.7 cells.	Antiinflammatory	Inhibition of IL-1β, pro-inflammatory cytokines, and TNF-α.	Burstein (2015)
CBD	*In vivo* Hypoxia-ischemia model in newborn pigs.	Neuroprotective	Increasing eCB levels through CB2 receptors and as a $5HT_{1A}$ agonist.	Leo et al. (2016)
CBD	*In vivo* Electrophysiology model in female and male Wistar rats, free of Mg^2 and 4-AP.	Anticonvulsant	Multi-electrodes in hippocampus.	Leo et al. (2016)
	In vitro Male Wistar Rat PTZ Seizure Model.	Anticonvulsant	Displacing the selective SR141716A receptor antagonist.	Jones et al. (2010)
CBD	*In vitro* Mouse fibroblasts and human B lymphoblastoid serum.	Antioxidant	Pathway not mediated by receptors. It antagonizes oxidative stress, and the consequent cell death induced by the retinoid anhydroretinol.	Booz (2011)
CBD	*In vivo* BCCAO model in male mice.	Neuroprotective	Decreased expression of GFAP 7.	Mori et al. (2017)
CBD	*In vivo* In RVM of rats treated with CFA.	Antiinflammatory	Regulation of the release of pro-inflammatory and anti-inflammatory cytokines.	Bouchet and Ingram (2020)
CB_2 synthetic	*In vivo* EM Viral Model (Theiler).	Antiinflammatory	Modulation in cytokines of the IL-12 family.	Correa et al. (2007)
THC THC-A CBD CBDA	*In vitro* Human colon adenocarcinoma. HT 29 cell culture.	Antiinflammatory	Cox inhibition	Ruhaak et al. (2011)
CBD	*In vitro/in vivo* AD model.	Neuroprotective	Nitrite levels reduction.	Mecha et al. (2016)
CBD	*In vivo* HI model in newborn mice.	Neuroprotective	Decreased glutamate levels, IL-6, TNF-α, and Cox-2.	Castillo et al. (2012)
CBD	*In vivo* Ischemic damage model.	Antiinflammatory	HMGB1 decrease in microglia.	Hayakawa et al. (2009)
CBD	*In vivo* HI model in newborn Wistar rats.	Neuroprotective	Modulation of the expression of oxidative stress and inflammation.	Pazos et al. (2012)
CBD	*In vivo* Hippocampal kindling in adult rats.	Anticonvulsant	CB1R antagonism	Turkanis et al. (1979)
CBD	*In vitro* Model of SE by intrahippocampal pilocarpine.	Neuroprotective	Decreased amplitude and duration of epileptiform activity.	Jones et al. (2010)

(Continued)

TABLE 1 | Continued

Compound	Type of study	Effect	Mechanism	Reference
CBD	*In vitro* Cultivo de células HEK 293A y células ST Hdh.	Anticonvulsant	Modulatory effect on neuronal hyperexcitability through GPR 55 antagonism.	Laprairie et al. (2015)
CBD	*In vivo* Human coronary artery atherosclerosis.	Antiinflammatory	Inhibition in the pro-inflammatory pathway of NF-kB, decreased nitrotyrosine, iNOS, ICAM1, and VECAM1.	Rajesh et al. (2007)
CBD	*In vivo* Rat uveitis model.	Antiinflammatory	Modulation of macrophage and microglial function. Nitrogen activating protein kinase activation.	El-Remessy et al. (2008)
CBD	Lymph node cells from mice.	Antiinflammatory	Decreased expression of IL-1β, TNFα, and MAPKs.	Malfait et al. (2000)

CBD cannabidiol; HU-320, modified CBD structure; CIA, collagen-induced arthritis; $5HT_{1a}$, 5-hydroxytryptamine; 4-AP, 4-aminopiridina; PTZ, pentilenetetrazol; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1hpyrazole-3-carboxamide; BCCAO, bilateral common carotid artery, occlusion; GFAP, glial fibrillary acidic protein; RVM, rostral ventromedial medulla; CFA, complete Freund's adjuvant; EM, esclerosis multiple; THC, tetrahydrocannabinol; THC-A, tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; CBG, cannabigerol; AD, Alzheimer's disease; HI, hypoxic-ischemic; HMGB1, high mobility group box 1; CB1R, cannabinoid type 1 receptor; SE, status epilepticus; KA, kainic acid; PN, post natal; GPR 55, G protein-coupled receptor 55; NF-KB, nuclear transcription factor kappa B; iNOS, inducible nitric oxide synthase; ICAM1, intercellular adhesion molecules; VECAM1, vascular adhesion molecules; IL-1β, interleukin-1β; TNFα, tumor necrosis factor y; MAPKs, mitogen-activated protein kinase.

Therefore, we decided to carry out a pilot study to evaluate the anticonvulsant effect of CBD for 12 days PN on the model of *SE* induced by KA 3 mg/kg delivered *via* an intraperitoneal route, which showed that the oral route of low doses (20 and 25 mg/kg) of CBD 30 min before KA caused behavioral arrest; however, administration of higher doses (30 and 35 mg/kg) of CBD resulted in changes in motor behavior such as startles, wet dog shakes and lower limb myoclonus. During the evaluation of the anticonvulsant effect, compared with the KA group, the 20 and 25 mg/kg CBD groups showed a significant increase in *SE* initiation latency ($p < 0.0001$), while the 30 and 35 mg/kg CBD groups showed significant reductions in *SE* initiation latency ($p < 0.01$ and $p < 0.001$, respectively). Conversely, in the high-dose groups of 40, 60, 80, and 90 mg/kg CBD administered 30 min before KA, chewing, wet dog shaking, and unilateral myoclonus as well as scratching movements with the lower extremities were observed. Although CBD induced these behaviors, the anticonvulsant effect in the 40 and 60 mg/kg groups was efficient in significantly reducing the *SE* initiation latency ($p < 0.01$) relative to the KA group; however, in the 80 and 100 mg/kg of CBD groups, an increase in the severity of *SE* was observed (**Figure 3**). Motor behavior has been previously reported in experimental models of seizures and KA-induced SE in 10–14-day-old immature PN rats (Vega-García et al., 2020), which indicates that in SE models, an increase in the dose has opposite effects to those in adults (Jones et al., 2010; Hill et al., 2014; Klein et al., 2017). Agreeing with this, Anderson et al. (2020) in the $Scn1a^{+/-}$ mouse model of Dravet syndrome, detected that subchronic oral administration of Δ^9-THC or CBD alone did not affect spontaneous seizure frequency or mortality while, surprisingly, their co-administration (70 mg Δ^9-THC + 3,500 mg CBD/kg chow) increased the severity of spontaneous seizures and overall mortality. The authors point out the detrimental outcome might simply be explained by a significant pharmacokinetic interaction between CBD and Δ^9-THC, as plasma and brain concentrations of both Δ^9-THC and CBD were dramatically increased, which has been demonstrated in mice and rats in other epilepsy models (Sofia et al., 1976; Chan et al., 1996).

Additionally, the anticonvulsant effect was examined in a Dravet syndrome model in voltage-gated sodium channel Nav1-knockout mice. Treatment with CBD from PN days 21–27 decreased the severity and number of seizures induced by hyperthermia as well as the number of spontaneous seizures (Kaplan et al., 2017). However, the mechanisms responsible for the anticonvulsant effect of CBD remain unclear. Previous experimental studies have reported that the anticonvulsant effect of CBD is linked to the modulating effects on neuronal hyperexcitability through the antagonism of G protein-coupled receptor 55 (GPR55), of which CBD is a negative allosteric modulator (Laprairie et al., 2015), desensitization of TRPV1 (Chen and Hackos, 2015) and inhibition of adenosine reuptake by blocking equilibrative nucleotide transporter 1 (ENT1), which increases the concentration of extracellular adenosine (Pandolfo et al., 2011). These mechanisms decrease intracellular Ca^{2+} flux and regulate neuronal hyperexcitability. These effects have also been tested in GPR55- and TRPV1-knockout mice (Klein et al., 2017; Stott et al., 2018; Franco and Perucca, 2019).

Moreover, the activity of CBD has been identified in blocking voltage-gated sodium channels and T-type Ca^{2+} channels and in the modulation of VDAC1 (Ghovanloo et al., 2018). Similarly, the interaction of CBD with voltage-gated potassium channels has been shown to have an effect on glycine α1 and α3 receptors and on the modulation of TNF-α (Gaston and Friedman, 2017; Gaston and Szafarski, 2018). It has been reported that CBD modulates the activity of the immune system in a

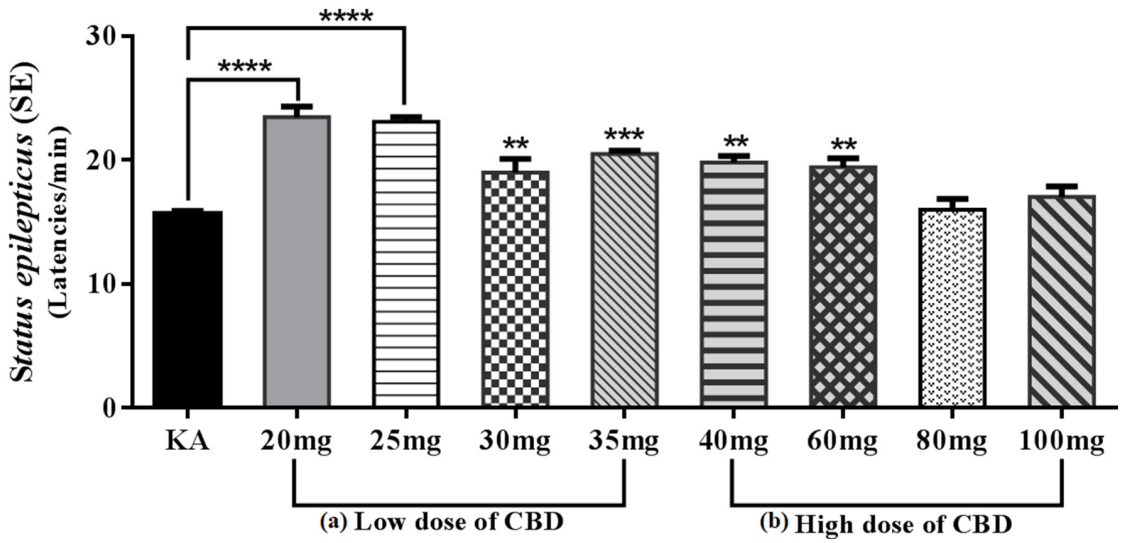


FIGURE 3 | Graph showing the mean ± SE of the latencies of *Status epilepticus* (SE) induced by KA. **(A)** Low doses: the 20 and 25 mg/kg CBD groups showed an increase of SE latencies with a significant difference ****$p < 0.0001$ compared with the KA group. However, the 30 and 35 mg/kg groups of CBD showed a reduction in SE latencies though with a significant difference, **$p < 0.01$ and ***$p < 0.001$, respectively, compared with the KA group. **(B)** The high-dose 40 and 60 mg/kg groups of CBD showed an increase in the latencies of SE with a significant difference, **$p < 0.01$ compared with the KA group. The 80 and 100 mg/kg CBD groups did not show significant differences compared with the KA group. One-way ANOVA followed by Bonferroni's *post hoc* test, $p < 0.05$ (unpublished data).

concentration-dependent manner (Pertwee, 2008), showing that the anti-inflammatory, antioxidant and neuroprotective effects of CBD are attributed to its agonist activity on CB1R and CB2R (Ibeas et al., 2015). Also, CBD has been shown to act as an inhibitor of fatty acid amide hydrolase (FAAH), an enzyme involved in the degradation of eCB (Capasso et al., 2008). Likewise, CBD acts as a competitive inhibitor of adenosine uptake *via* ENT1 in microglia and increases exogenous adenosine, which activates the A2A receptor (Liou et al., 2008; Haskó et al., 2009); nevertheless, the mechanism of action of the A2A receptor on the modulation of the inflammatory response activated by microglia is unclear (Haskó et al., 2009; Rosenberg et al., 2017).

Pretreatment with CBD attenuates the formation of ROS and the mitochondrial damage generated by the increase in glutamate. Similarly, in models of artery sclerosis, treatment with CBD inhibits the pro-inflammatory pathway of NF-kB and decreases nitrotyrosine and iNOS, while in the endothelial cells of human coronary arteries, it decreases adhesion molecules ICAM-1 and VECAM-1. In the same way, CBD attenuates the transendothelial migration of monocytes (Rajesh et al., 2007). The antioxidant effect of CBD has been reported in a model of nephropathy induced by cisplatin, reducing ROS and generating NAPDH oxidases, iNOS and NT-3, which reduce cell death and improve kidney function (Pan et al., 2009). Therefore, it is suggested that the antioxidant effect of CBD acts at the mitochondrial level (Booz, 2011). Additionally, CBD has been reported to act as an antagonist of the GPR55 receptor, which decreases intracellular Ca^{2+} flux and modulates neuronal hyperexcitability (Rosenberg et al., 2017).

There are few reports on the neuroprotective effects of CBD in developing epilepsy models. In newborn Wistar rats that underwent IH injury (10% oxygen for 120 min after carotid artery electrocoagulation), CBD (1 mg/kg single dose, 1 mg/kg every 24 h for 72 h, or 1 mg/kg every 8 h for 72 h) modulated the expression of oxidative stress and inflammation, which reduced neuronal death and produced greater functional recovery (Pazos et al., 2012). Similarly, in a sciatic nerve section model of 2 PN day rats that received treatment with a single dose of 15 or 30 mg/kg CBD, 30% synaptic preservation was observed by immunohistochemical analysis (obtained by synaptophysin staining). The administration of CBD decreased astroglial and microglial reactions by 30 and 27%, respectively, and reduced the number of apoptotic cells mainly in the intermediate zone of the spinal cord (Pérez et al., 2013).

CLINICAL STUDIES

Cannabis-Derived Approved Drugs

While there are many descriptive and anecdotal reports on the benefits of cannabinoids, few cannabinoid drugs are regulated for clinical use. The most important licensed cannabis-based drugs are described herein: (a) dronabinol (Marinol®) was approved by the FDA on May 31, 1985, for nausea and vomiting control, associated with cancer chemotherapy that had not responded adequately to conventional antiemetic treatments. First marketed in 1986, on December 22, 1992, Marinol® was approved for the relief of anorexia associated with weight loss in patients with HIV-AIDS (human immunodeficiency virus/acquired immunodeficiency syndrome). At the time of its approval, the FDA recognized that dronabinol Δ^9-THC was considered to be the psychoactive component of marijuana. Marinol® is also licensed for use in other countries, such

as Canada and Germany. Generic forms of dronabinol (first approved in 2011) have now been approved in the US (Wright and Guy, 2014); (b) nabilone (Cesamet® dl-3-(1,1-dimethyl heptyl)-6,6ab 7, 8, 10, 10a alpha-hexahydro-1-hydroxy-6,6-dimethyl-9H-dibenzo pyran-9-(one) is a synthetic analog of Δ^9 THC that was developed in the 1970s long before the target receptor was identified. It has a low nanomolar affinity for the CB1 receptor and a somewhat reduced affinity for the CB2 receptor. It was initially approved by the FDA in 1985 for chemotherapy-induced nausea and vomiting that do not respond to conventional antiemetics. The drug was marketed in the United States until 2006 (Wright and Guy, 2014); (c) nabiximols (Sativex®) differs from the other authorized cannabinoids insofar as it comprises an extract of the cannabis plant. It is formulated as a sublingual/oromucosal spray, every 100 μl of which provides 2.7 mg of Δ^9 THC and 2.5 mg of CBD. It was approved for the first time in Canada in 2005 under legislation that allows the conditional approval of a new drug in areas of high unmet medical need and in the presence of very promising clinical data. Its approval in the USA was delayed until the end of 2010 and early 2011 when Savitex was approved by the UK and Spain following a decentralized procedure. It is licensed as second-line therapy for the relief of spasticity in multiple sclerosis (MS); in some other countries, it may also be prescribed for the treatment of neuropathic pain in people with MS and for the treatment of cancer-related pain, where it is used as an opioid supplement (Wright and Guy, 2014); and (d) epidiolex, is the first plant-derived, purified pharmaceutical-grade CBD medication. It was approved in the USA by the FDA on June 25, 2018 (Sekar and Pack, 2019). Its approval for patients ≥2 years of age with DS or LGS markedly altered the treatment of medically refractory seizures in these disorders. Epidiolex is a CBD only component with no Δ^9THC, and the psychoactive component of cannabis responsible for appetite stimulation and euphoria sensation (Abu-Sawwa et al., 2020; Specchio et al., 2020).

Pharmacokinetics of Cannabinoids

To date, few clinical studies have been conducted in pediatric patients with difficult-to-control epilepsy and oral administration with cannabidiol solutions (CBD) to determine the pharmacokinetic parameters. In this review, data were obtained from a population with an age range between 0–20 years (Devinsky et al., 2018a; Wheless et al., 2019). The pharmacokinetics of cannabinoids is divided into four phases: absorption, distribution, metabolism, and excretion, but they are modified by interactions with other drugs. The pharmacokinetics of these compounds are divided into four phases: absorption, distribution, metabolism, and excretion, but they are modified by interactions with other drugs.

Absorption

The absorption rate of cannabinoids is determined by the route of administration of the drug and its formulation. Administration through oral formulations of any cannabinoid or food presents variable absorption and extensive first-pass hepatic metabolism. The administration of oral formulations presents a variable absorption depending on whether it is a single dose or repeated doses, as well as an extensive first-pass hepatic metabolism. The geometric mean of the time to maximum plasma concentration (T_{max}) of cannabidiol ranged from 2 to 4 h after the administration of a single dose in a concentration range from 5–20 mg/kg without previous consumption of food. Thus, the route of administration of this pharmaceutical form has a slow absorption with a wide absorption phase. Concerning the administration of repeated doses (7 days), the T_{max} of cannabidiol was detected between 2–3 h in a concentration range between 10–40 mg/kg/day (Wheless et al., 2019). Sublingual administration is very similar to oral administration. Oro-mucosal or spray preparations of Δ^9THC—CBD (1:1) allow rapid absorption, whereas higher plasma concentrations are reached compared with oral administrations, but inhaled Δ^9 THC leads to lower concentrations in the plasma (Karschner et al., 2011; Pertwee and Cascio, 2014).

Distribution

Once absorbed, CBD can be detected in plasma. The geometric means of the maximum plasma concentration (C_{max}) were 29.12, 47.19, and 103.7 ng/ml with a single dose administration of 5, 10, and 20 mg/kg, respectively. Also, an increase in the geometric means of C_{max} of 3.12, 2.67, and 3.03 times was observed in repeated doses of 10, 20, and 40 mg/kg for 7 days compared with the single dose. The geometric averages of the area under the plasma concentration curve at time 0–12 h [AUC (0–12 h)] were 122, 243.6, and 473.5 ng·h/ml with the administration of a single dose of 5, 10, and 20 mg/kg, respectively. Additionally, an increase of 4.15, 3.43, and 4.45 times in AUC(0-τ) was observed with the administration of repeated doses of 10, 20, and 40 mg/kg for 7 days compared with the single-dose (Wheless et al., 2019).

Metabolism

Both CBD and its metabolites 7-OH CBD and 7-COOH-CBD are metabolized in the liver and can induce the expression of CYP1A1, CYP1A2, CYP2C9, and CYP2D6 during prolonged periods of administration (Greene and Saunders, 1974; Bornheim et al., 1992; Watanabe et al., 2007). Pharmacokinetic parameters of 7-OH CBD and the geometric averages of T_{max} 7-OH CBD were similar to the T_{max} of CBD after the administration of a single dose of CBD in a concentration range from 5–20 mg/kg, with repeated dosing for 7 days. Nevertheless, the T_{max} of 7-OH CBD was detected at 2 h. The geometric means of C_{max} of 7-OH CBD were 22.03, 34.56, and 71.7 ng/ml with the administration of a single dose of 5, 10, and 20 mg/kg of CBD, respectively. However, the C_{max} of 7-OH CBD increased to 2.97, 2.81, and 3.04 with repeated doses of 10, 20, and 40 mg/kg for 7 days compared with the single dose. Additionally, a decrease in the percentage of the coefficient of variation (CV%) was observed compared with a single dose. The geometric averages of AUC (0–12 h) of 7-OH CBD were 104, 202.4, and 381.9 ng h/ml with the administration of a single dose of 5, 10, and 20 mg/kg of CBD, respectively. The AUC(0-τ) of 7-OH CBD increased 4.12, 3.25, and 4.42 times with the administration of repeated doses of 10, 20, and 40 mg/kg CDB for 7 days compared with the single dose. Finally, the geometric averages of the metabolite to parent

(7-OH cannabidiol/cannabidiol) ratio of AUC(0-τ) was 0.8, 0.75, and 0.76 with the administration of repeated doses of 10, 20, and 40 mg/kg for 7 days, respectively (Wheless et al., 2019).

In another study, the mean plasma concentrations of CBD and its metabolites 6-OH-CBD and 7-COOH-CBD were detected after the administration of a single dose (1.25 mg/kg) or multiple doses (5, 10, or 20 mg/kg/day) of CBD. The most abundant circulating metabolite was 7-COOH-CBD. At the end of the treatment, the AUC(0-τ) of 7-COOH-CBD was 13–17 times higher than the AUC(0-τ) of CBD. The CV% was considered to be high to moderate (20–121%; Devinsky et al., 2018b).

Elimination

The geometric averages of the apparent terminal half-life ($t_{1/2}$) of CBD were 26.4, 29.6, and 19.5 h with the administration of a single dose of 5, 10, and 20 mg/kg, respectively. The geometric averages of the apparent total body clearance after oral administration at steady state (CLss/F) were 9.9, 12.3, and 9.5 L/h/kg, with the administration of repeated doses of 10, 20, and 40 mg/kg for 7 days, respectively (Wheless et al., 2019). Regarding the pharmacokinetic parameters of its metabolite 7-OH CBD, the geometric means of $t_{1/2}$ were 18.4, 25.6, and 14.2 h with the administration of a single dose of 5, 10, and 20 mg/kg, respectively (Wheless et al., 2019). The metabolites of 7-OH-CBD are excreted in the feces and, to a lesser extent, the urine (Ohlsson et al., 1986).

During interactions with other drugs, the pharmacokinetic parameters of CBD change significantly with the administration of clobazam (CBZ). Therefore, it is often necessary to reduce clobazam due to excessive sedation. Pediatric patients who received repeated doses of 40 mg/kg for 7 days of CBD and CBZ had a 2.36-fold increase in CBD AUC(0-τ) and a 2.5-fold increase in CL/F compared with those who did not receive CBZ (Wheless et al., 2019). Conversely, CBD inhibits CYP2C19, and CYP3A4, which catalyze the metabolism of norclobazam (nCBZ). nCBZ is detected at high plasma concentrations (500% 300% mean increase) compared with the increase detected in CBZ (60% 80%) in a concentration range from 20–25 mg/kg/day of CBD and 0.18–2.24 mg/kg/day of CBZ (Geffrey et al., 2015).

Clinical Evidence of Cannabinoids in the Treatment of Pediatric Epilepsy

Clinical studies reported below assess the safety and/or efficacy of CBD in addition to common AEDs. Most of these studies enrolled pediatric patients (0.5–17 years) with diagnoses of genetically based epilepsy, Dravet syndrome, Lennox Gastaut, febrile seizures, and seizures associated with tuberous sclerosis are the most difficult epilepsies to treat, and the latest generation of antiepileptic drugs offers limited control for these diseases (Devinsky et al., 2017; Lattanzi et al., 2018, 2019). The use of *CS* in an empirical way has been documented for a long time for the treatment of epilepsy, and in most cases, reports provide a subjective perception of benefit. Despite the positive effects of the use of medicinal cannabis CBD for the control of epileptic seizures, there is controversy in the use of CBD at the pediatric level. The first trials examining purified CBD (Epidiolex) were launched as an expanded access program (EAP) in 2014 for patients with refractory epilepsy. Szaflarski et al. (2018) published provisional data for 600 patients who used CBD during 96 weeks, revealing a reduction of seizure events by 51% mainly in cases related to Dravet syndrome, Lennox Gastaut, and tuberous sclerosis complex (reviewed by Silvestro et al., 2019).

Given the increase in the use of handcrafted cannabinoids in pediatric epilepsy and the lack of studies providing data on their safety and usefulness, a prospective investigation was carried out in 32 children with refractory epilepsy. CBD was administered at a dose of 10 mg/kg/day for 12 weeks, and the frequency of seizures and serum CBD levels were evaluated. There was a 50% reduction in the frequency of seizures during the final weeks compared with the initial ones (Knupp et al., 2019). In another open comparative study on the use of CBD to treat epilepsy, 55 patients aged 1 and 30 years with CDKL5 deficiency disorder and Aicardi, Doose, and Dup15q syndromes were included. The patients were treated in 11 institutions from January 2014 to December 2016, and a decrease in the frequency of seizures was reported from the start of CBD treatment. The dose used ranged between 10 and 20 mg/kg/day orally, and this open-label trial provided class III evidence for the long-term safety and efficacy of CBD administration in patients with refractory epilepsy (Devinsky et al., 2014, 2016, 2018a,b; Elliott et al., 2018).

In a clinical trial of children aged 1–17 years with refractory epilepsy (60), an oral solution of CBD at doses between 5, 10, and 20 mg/kg/day was administered for 12 weeks as a complement to their regimen of antiepileptic drugs. A 50% decrease in seizure frequency was observed, no serious adverse effects were found, and CBD was well tolerated (Wheless et al., 2019). In most trials, CBD is administered orally as an oil solution. In open trials, maximum doses of 25 mg/kg/day have been used in controlled studies, and higher doses of up to 50 mg/kg/day were also used; however, studies on Lennox Gastaut syndrome have shown that a significant proportion of children respond to doses of 10 mg/kg/day. Therefore, a "slow start" and "scale as appropriate" strategy is recommended, beginning with 5 mg/kg/day, increasing to 10 mg/kg/day after 2 weeks, reviewing the clinical response and adverse effects, remaining with the dose if it is effective, and otherwise increasing the dose of 5 mg/kg/day according to tolerance up to a maximum of 20–25 mg/kg/day (Treat et al., 2017; Devinsky et al., 2018b; Arzimanoglou et al., 2020).

In the previous systematic review, published in August 2017, examined 22 clinical trials, only five of which were controlled clinical trials that included a total of 795 children. The greatest evidence was for its use as an antiemetic, analgesic, and antiepileptic agent (Wong and Wilens, 2017). Currently, only two synthetic products derived from cannabis are approved by the FDA of the United States: dronabinol and nabilone. Both contain Δ^9–THC as the main cannabinoid and are specified for the treatment of cancer comorbidities and anorexia associated with patients with HIV/AIDS (Pertwee and Cascio, 2014; Wong and Wilens, 2017).

To date, in Uruguay, the only pharmaceutical specialty registered by the MS is epifatan as 2% or 5% oral solution (drops), which contains 2 g or 5 g of CBD, respectively, and

less than 0.1% THC and tetrahydrocannabinolic acid (THCA) every 100 ml. Most reports concerning the therapeutic effect of CBD in drug-resistant epilepsy have been conducted in children and adolescents (Wong and Wilens, 2017), thus providing the greatest amount of available scientific evidence. In recent decades, various investigations have been carried out on the use of cannabis in the treatment of refractory epilepsy in children, especially in epileptic syndromes such as DS, Doose, and LGS (Wong and Wilens, 2017).

In the same way, Tzadok et al. (2016) in a retrospective review of a cohort of 74 children and adolescents with drug-resistant epilepsy reported that CBD reduced the frequency of seizures in 89% of patients. In a survey of 19 parents of children with treatment-resistant epilepsy, Porter and Jacobson (2013) found that CBD treatment of 117 children with drug-resistant epilepsy reduced the seizure frequency in 84% of the patients. Conversely, in a case series of six children with drug-resistant epilepsy, dronabinol has also been reported to reduce seizures in two patients. However, most of the studies have shown inconsistency in terms of the control of the variables and absence of a placebo group, making it difficult to generate an accurate conclusion, in addition to secondary effects such as drowsiness, diarrhea, and decreased appetite (Tzadok et al., 2016). Additionally, in controlled trials, Δ^9THC most commonly led to side effects of drowsiness and dizziness, with greater severity associated with higher doses (Wang et al., 2008). Likewise, cannabis overdose has been reported with multiple adverse effects, among which are reports of seizures among young children, which may be due to the toxicity of high-dose Δ^9 THC (Wang et al., 2008; Mechoulam and Parker, 2013).

However, clinical investigations have methodological limitations, such as the small population of children included, the short follow-up period, and the methodological design. Nevertheless, they have reported a reduction between 57% and 84% in the frequency and intensity of epileptic seizures, with a greater impact on DS and LGS (Wong and Wilens, 2017). Few studies have examined medical cannabinoids for the treatment of seizures in children and adolescents, and they have reported that CBD reduces the frequency of seizures in the pediatric population with resistance to initial treatment in epilepsy of different etiologies (Devinsky et al., 2017; Huntsman et al., 2020). Additionally, since 2014, CBD has been administered in a continuously EAP. In a review of 119 pediatric epilepsy patients, Treat et al. (2017) reported that oral CS extracts improved seizures in 49% of the cohort. In a second study, the oral CS extracts reduced the frequency and intensity of seizures in 57% of 75 patients with drug-resistant epilepsy (Treat et al., 2017).

In addition to the lack of studies on CS, the long-term risks associated with medical CBD in pediatric patients limits our understanding of the mechanism of action and its secondary effects on neurodevelopment and epileptogenesis (Mechoulam and Parker, 2013). However, evidence has shown that onset of CS use before the age of 16 years has a relationship with cognitive and verbal learning deficits and poor psychomotor performance and attention, similar to later onset of CS, which have been associated with poorer attention, executive functioning, memory and verbal performance (Solowij et al., 2011). Similarly, frequent and recreational CS use before the age of 15 years is associated with an increased risk of depression, confusion, and subsequent suicidal tendencies. In contrast, cannabis use in early adolescence shows a greater relationship with the early onset of psychotic disorders (Hayatbakhsh et al., 2007; Claudet et al., 2017). When using CBD as an adjuvant with other antiepileptic drugs, there was an increase in liver enzymes, jaundice, and thrombocytopenia, but these alterations were resolved when the antiepileptic was discontinued. An interaction is observed between the use of CBD and clobazam, and the main adverse reactions to the use of CBD are fatigue and diarrhea; however, an increase in appetite has been documented in some patients (Geffrey et al., 2015). There is evidence of the efficacy of a large percentage of CBD in epilepsy of different etiologies despite the few studies conducted to date. However, some of them have a risk of bias or poor follow-up by caregivers of the patients. Therefore, more protocols or experimental studies are suggested with internationally approved administration doses according to age, weight, and specific indication. Such studies will provide a broader picture to not only test the efficacy of drugs in epilepsy but also study the adverse effects of drugs derived from cannabis.

CONCLUSIONS

Evidence describing the effects of the major cannabinoids that do not act as CBR ligands, in particular cannabidiol and cannabidivarin, reveals consistently beneficial therapeutic effects in preclinical models of seizures, epilepsy, epileptogenesis, and neuroprotection. The emerging results of clinical trials in humans have methodological limitations, such as the small population of children included, the short follow-up period, and the methodological design, although, in most of them, they have reported a reduction in the frequency and intensity of seizures, in the drug-resistant pediatric population of different etiologies. What is clear is that the beneficial effects in epilepsy for the immature brain vary compared to the adult brain, due to neurodevelopmental processes. Therefore, more protocols or experimental studies should be performed with a stricter, broader, internationally approved administration dose according to age, weight, and specific indication.

ETHICS STATEMENT

All procedures, including the use, management and care of animals, adhered to the guidelines of and were approved by the Ethics and Research Committee of the Hospital of Specialties in the National Medical Center Century XXI, Mexican Social Security Institute (IMSS) and Mexican Official Standard [1999]; NOM-062-ZOO, 1999.

AUTHOR CONTRIBUTIONS

AV-G developed the topic of epileptogenesis, performed the experiments with kainic acid and evaluated the latencies with the support of AM-M. IF-R, AM-A, and EZ-M developed the clinical studies. AG-J reviewed the derivatives of Cannabis

and participated in the images. EG-A developed with AV-G the effects of CD on neurodevelopment. SO-S developed the idea, the topics to be considered, organized and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the IMSS health research fund, FIS/IMSS/PROT/PRIO/19/107 number grant for the included experimental part and the correction of the English style.

REFERENCES

Abu-Sawwa, R., Scutt, B., and Park, Y. (2020). Emerging use of epidiolex (Cannabidiol) in epilepsy. *J. Pediatr. Pharmacol. Ther.* 25, 485–499. doi: 10.5863/1551-6776-25.6.485

Alexandre, J., Carmo, H., Carvalho, F., and Pedro Silva, J. (2020). Synthetic cannabinoids and their impact on neurodevelopmental processes. *Addict. Biol.* 25:e12824. doi: 10.1111/adb.12824

Al-Muhtasib, N., Sepulveda-Rodriguez, A., Vicini, S., and Forcelli, P. A. (2018). Neonatal phenobarbital exposure disrupts GABAergic synaptic maturation in rat CA1 neurons. *Epilepsia* 59, 333–344. doi: 10.1111/epi.13990

Anderson, L. L., Low, I. K., McGregor, I. S., and Arnold, J. C. (2020). Interactions between cannabidiol and Δ^9-tetrahydrocannabinol in modulating seizure susceptibility and survival in a mousae model of Dravet syndrome. *Br. J. Pharmacol.* 177, 4261–4274. doi: 10.1111/bph.15181

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). Cannabis sativa: the plant of the thousand one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Armstrong, C., Morgan, R. J., and Soltesz, I. (2009). Pursuing paradoxical proconvulsant prophylaxis for epileptogenesis. *Epilepsia* 50, 1657–1669. doi: 10.1111/j.1528-1167.2009.02173.x

Arzimanoglou, A., Bland, U. B., Cross, J. H., Gil-Nagel, A., Lagae, L., Landmark, C. J., et al. (2020). Epilepsy and cannabidiol: a guide to treatment. *Epileptic Disord.* 22, 1–14. doi: 10.1684/epd.2020.1141

Atwood, B. K., and Mackie, K. (2010). CB2: a cannabinoid receptor with an identity crisis. *Br. J. Pharmacol.* 160, 467–479. doi: 10.1111/j.1476-5381.2010.00729.x

Auvin, S., and Dupuis, N. (2014). Outcome of status epilepticus. What do we learn from animal data? Prolonged epileptic seizures: identification and treatment. *Epileptic Disord.* 16, S37–S43. doi: 10.1684/epd.2014.0670

Balosso, S., Ravizza, T., Perego, C., Peschon, J., Campbell, I. L., De Simoni, M. G., et al. (2005). Tumor necrosis factor-alpha inhibits seizures in mice *via* p75 receptors. *Ann. Neurol.* 57, 804–812. doi: 10.1002/ana.20480

Baud, O., and Saint-Faust, M. (2019). Neuroinflammation in the developing brain: risk factors, involvement of microglial cells, and implication for early anesthesia. *Anesth. Analg.* 128, 718–725. doi: 10.1213/ANE.0000000000004032

Beattie, E. C., Stellwagen, D., Morishita, W., Bresnahan, J. C., Ha, B. K., Von Zastrow, M., et al. (2002). Control of synaptic strength by glial TNF α. *Science* 295, 2282–2285. doi: 10.1126/science.1067859

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat. Rev. Neurosci.* 3, 728–739. doi: 10.1038/nrn920

Ben-Ari, Y. (2014). The GABA excitatory/inhibitory developmental sequence: a personal journey. *Neuroscience* 279, 187–219. doi: 10.1016/j.neuroscience.2014.08.001

Ben-Ari, Y., and Holmes, G. L. (2006). Effects of seizures on developmental processes in the immature brain. *Lancet Neurol.* 5, 1055–1063. doi: 10.1016/S1474-4422(06)70626-3

Benítez-Diaz, P., Miranda-Contreras, L., Mendoza-Briceño, R. V., Peña-Contreras, Z., and Palacios-Prü, E. (2003). Prenatal and postnatal contents of amino acid neurotransmitters in mouse parietal cortex. *Dev. Neurosci.* 25, 366–374. doi: 10.1016/j.ridd.2020.103793

Berdyshev, E. V. (2000). Cannabinoid receptors and the regulation of immune response. *Chem. Phys. Lipids.* 108, 169–190. doi: 10.1016/s0009-3084(00)00195-x

Bernard, C., Milh, M., Morozov, Y. M., Ben-Ari, Y., Freund, T. F., and Gozlan, H. (2005). Altering cannabinoid signaling during development disrupts neuronal activity. *Proc. Natl. Acad. Sci. U S A* 102, 9388–9393. doi: 10.1073/pnas.0409641102

Bessis, A., Béchade, C., Bérnard, D., and Roumier, A. (2007). Microglial control of neuronal death and synaptic properties. *Glia* 55, 233–238. doi: 10.1002/glia.20459

Bhaskaran, M. D., and Smith, B. N. (2010). Effects of TRPV1 activation on synaptic excitation in the dentate gyrus of a mouse model of temporal lobe epilepsy. *Exp. Neurol.* 223, 529–536. doi: 10.1016/j.expneurol.2010.01.021

Bittigau, P., Sifringer, M., Genz, K., Reith, E., Pospischil, D., Govindarajalu, S., et al. (2002). Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc. Natl. Acad. Sci. U S A* 99, 15089–15094. doi: 10.1073/pnas.222550499

Blair, R. E., Sombati, S., Lawrence, D. C., McCay, B. D., and De Lorenzo, R. J. (2004). Epileptogenesis causes acute and chronic increases in $GABA_A$ receptor endocytosis that contributes to the induction and maintenance of seizures in the hippocampal culture model of acquired epilepsy. *J. Pharmacol. Exp. Ther.* 310, 871–880. doi: 10.1124/jpet.104.068478

Booz, G. W. (2011). Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. *Free Radic. Biol. Med.* 51, 1054–1061. doi: 10.1016/j.freeradbiomed.2011.01.007

Bornheim, L. M., Lasker, J. M., and Raucy, J. L. (1992). Human hepatic microsomal metabolism of δ1-tetrahydrocannabinol. *Drug Metab. Dispos.* 20, 241–246.

Bouchard, J., Truong, J., Bouchard, K., Dunkelberger, D., Desrayaud, S., Moussaoui, S., et al. (2012). Cannabinoid receptor 2 signaling in peripheral immune cells modulates disease onset and severity in mouse models of Huntington's disease. *J. Neurosci.* 32, 18259–18268. doi: 10.1523/JNEUROSCI.4008-12.2012

Bouchet, C. A., and Ingram, S. L. (2020). Cannabinoids in the descending pain modulatory circuit: role in inflammation. *Pharmacol. Ther.* 209:107495. doi: 10.1016/j.pharmthera.2020.107495

Brusco, A., Tagliaferro, P., Saez, T., and Onaivi, E. S. (2008). Postsynaptic localization of CB2 cannabinoid receptors in the rat hippocampus. *Synapse* 62, 944–949. doi: 10.1002/syn.20569

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg. Med. Chem.* 23, 1377–1385. doi: 10.1016/j.bmc.2015.01.059

Caiati, M. D., Sivakumaran, S., Lanore, F., Mulle, C., Richard, E., Verrier, D., et al. (2012). Developmental regulation of CB1-mediated spike-time dependent depression at immature mossy fiber-CA3 synapses. *Sci. Rep.* 2:285. doi: 10.1038/srep00285

Capasso, R., Borrelli, F., Aviello, G., Romano, B., Scalisi, C., Capasso, F., et al. (2008). Cannabidiol, extracted from *Cannabis sativa*, selectively inhibits inflammatory hypermotility in mice. *Br. J. Pharmacol.* 154, 1001–1008. doi: 10.1038/bjp.2008.177

Castillo, P. E., Younts, T. J., Chávez, A. E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron* 76, 70–81. doi: 10.1016/j.neuron.2012.09.020

Chan, P. C., Sills, R. C., Braun, A. G., Haseman, J. K., and Bucher, J. R. (1996). Toxicity and carcinogenicity of δ^9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. *Fundam. Appl. Toxicol.* 30, 109–117. doi: 10.1006/faat.1996.0048

Chen, J., and Hackos, D. H. (2015). TRPA1 as a drug target—promise and challenges. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 388, 451–463. doi: 10.1007/s00210-015-1088-3

Chen, K., Aradi, I., Thon, N., Eghbal-Ahmadi, M., Baram, T. Z., and Soltesz, I. (2001). Persistently modified h-channels after complex febrile seizures convert the seizure-induced enhancement of inhibition to hyperexcitability. *Nat. Med.* 7, 331–337. doi: 10.1038/85480

Chen, K., Neu, A., Howard, A. L., Foldy, C., Echegoyen, J., Hilgenberg, L., et al. (2007). Prevention of plasticity of endocannabinoid signaling inhibits persistent limbic hyperexcitability caused by developmental seizures. *J. Neurosci.* 27, 46–58. doi: 10.1523/JNEUROSCI.3966-06.2007

Chen, K., Ratzliff, A., Hilgenberg, L., Gulyas, A., Freund, T. F., Smith, M., et al. (2003). Long-term plasticity of endocannabinoid signaling induced by developmental febrile seizures. *Neuron* 39, 599–611.doi: 10.1016/s0896-6273(03)00499-9

Chen, Y.-H., Kuo, T.-T., Chu, M.-T., Ma, H.-I., Chiang, Y.-H., and Huang, E. Y.-K. (2013). Postnatal systemic inflammation exacerbates impairment of hippocampal synaptic plasticity in an animal seizure model. *Neuroimmunomodulation* 20, 223–232. doi: 10.1159/000348440

Cheung, K. A., Peiris, H., Wallace, G., Holland, O. J., and Mitchell, M. D. (2019). The interplay between the endocannabinoid system, epilepsy and cannabinoids. *Int. J. Mol. Sci.* 20:6079. doi: 10.3390/ijms20236079

Clancy, B., Darlington, R. B., and Finlay, B. L. (2001). Translating developmental time across mammalian species. *Neuroscience* 105, 7–17. doi: 10.1016/s0306-4522(01)00171-3

Claudet, I., Le Breton, M., Bréhin, C., and Franchitto, N. (2017). A 10-year review of cannabis exposure in children under 3-years of age: do we need a more global approach? *Eur. J. Pediatr.* 176, 553–556. doi: 10.1007/s00431-017-2872-5

Cock, H. R. (2002). The role of mitochondria and oxidative stress in neuronal damage after brief and prolonged seizures. *Prog. Brain Res.* 135, 187–196. doi: 10.1016/S0079-6123(02)35018-0

Correa, F., Docagne, F., Mestre, L., Loría, F., Hernangómez, M., Borrell, J., et al. (2007). Cannabinoid system and neuroinflammation: implications for multiple sclerosis. *Neuroimmunomodulation* 14, 182–187. doi: 10.1159/000110644

Coulter, D. A. (2006). Endocannabinoids: a critical regulator of activity in the developing brain. *Epilepsy Curr.* 6, 18–19. doi: 10.1111/j.1535-7511.2005.00082.x

De Simoni, M. G., Perego, C., Ravizza, T., Moneta, D., Conti, M., Marchesi, F., et al. (2000). Inflammatory cytokines and related genes are induced in the rat hippocampus by limbic status epilepticus. *Eur. J. Neurosci.* 12, 2623–2633. doi: 10.1046/j.1460-9568.2000.00140.x

Devinsky, O., Cilio, M. R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* 55, 791–802. doi: 10.1111/epi.12631

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al. (2017). Trial of cannabidiol for drug-resistant seizures in the Dravet syndrome. *N. Engl. J. Med.* 376, 2011–2020. doi: 10.1056/NEJMoa1611618

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurol.* 15, 270–278. doi: 10.1016/S1474-4422(15)00379-8

Devinsky, O., Patel, A. D., Thiele, E. A., Wong, M. H., Appleton, R., Harden, C. L., et al. (2018a). Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome. *Neurology* 90, e1204–e1211. doi: 10.1212/WNL.0000000000005254

Devinsky, O., Verducci, C., Tiele, E., Laux, L., Patel, A. D., Filloux, F., et al. (2018b). Open-label use of highly purified CBD (Epidiolex®) in patients with CDKL5 deficiency disorder and Aicardi, Dup15q, and Doose syndromes. *Epilepsy Behav.* 86, 131–137. doi: 10.1016/j.yebeh.2018.05.013

Devinsky, O., Vezzani, A., Najjar, S., De Lanerolle, N. C., and Rogawski, M. A. (2013). Glia and epilepsy: excitability and inflammation. *Trends Neurosci.* 36, 174–184. doi: 10.1016/j.tins.2012.11.008

Di Marzo, V., and Piscitelli, F. (2015). The endocannabinoid system and its modulation by phytocannabinoids. *Neurotherapeutics* 12, 692–698. doi: 10.1007/s13311-015-0374-6

Dow-Edwards, D., and Silva, L. (2017). Endocannabinoids in brain plasticity: cortical maturation, HPA axis function and behavior. *Brain Res.* 1654, 157–164. doi: 10.1016/j.brainres.2016.08.037

Elliott, J., DeJean, D., Clifford, T., Coyle, D., Potter, B., Skidmore, B., et al. (2018). Cannabis for pediatric epilepsy: protocol for a living systematic review. *Syst. Rev.* 7:95. doi: 10.1186/s13643-018-0761-2

El-Remessy, A. B., Tang, Y., Zhu, G., Matragoon, S., Khalifa, Y., Liu, E. K., et al. (2008). Neuroprotective effects of cannabidiol in endotoxin-induced uveitis: critical role of p38 MAPK activation. *Mol. Vis.* 14, 2190–2203.

El Sohly, E. A., Mehmedic, Z., Foster, S., Gon, C., Chandra, S., and Church, J. C. (2016). Changes in cannabis potency over the last 2 decades (1995–2014): analysis of current data in the united states. *Biol. Psychiatry* 79, 613–619. doi: 10.1016/j.biopsych.2016.01.004

Etienne, M. (2014). "The chemical phenotypes (chemotypes) of cannabis," in *Hand Book of Cannabis*, ed R. Pertwee (University Press), 144–165.

Feng, B., Tang, Y., Chen, B., Xu, C., Wang, Y., Dai, Y., et al. (2016). Transient increase of interleukin-1β after prolonged febrile seizures promotes adult epileptogenesis through long-lasting upregulating endocannabinoid signaling. *Sci. Rep.* 6:21931. doi: 10.1038/srep21931

Fernández-Ruiz, J., Berrendero, F., Hernández, M. L., and Ramos, J. A. (2005). The endogenous cannabinoid system and brain development. *Trends Neurosci.* 23, 14–20. doi: 10.1016/s0166-2236(99)01491-5

Folbergrová, J., Jesina, P., Haugvicová, R., Lisý, V., and Houstek, J. (2010). Sustained deficiency of mitochondrial complex I activity during long periods of survival after seizures induced in immature rats by homocysteic acid. *Neurochem. Int.* 56, 394–403. doi: 10.1016/j.neuint.2009.11.011

Forcelli, P. A., Kim, J., Kondratyev, A., and Gale, K. (2011). Pattern of antiepileptic drug-induced cell death in limbic regions of the neonatal rat brain. *Epilepsia* 52, e207–e211. doi: 10.1111/j.1528-1167.2011.03297.x

Franco, V., and Perucca, E. (2019). Pharmacological and therapeutic properties of cannabidiol for epilepsy. *Drugs* 79, 1435–1454. doi: 10.1007/s40265-019-01171-4

Galanopoulou, A. S. (2008). $GABA_A$ receptors in normal development and seizures: friends or foes? *Curr. Neuropharmacol.* 6, 1–20. doi: 10.2174/157015908783769653

Gaston, T. E., and Friedman, D. (2017). Pharmacology of cannabinoids in the treatment of epilepsy. *Epilepsy Behav.* 70, 313–318. doi: 10.1016/j.yebeh.2016.11.016

Gaston, T. E., and Szafarski, J. P. (2018). Cannabis for the treatment of epilepsy: an update. *Curr. Neurol. Neurosci. Rep.* 8:73. doi: 10.1007/s11910-018-0882-y

Geffrey, A. L., Pollack, S. F., Bruno, P. L., and Thiele, E. A. (2015). Drug-drug interaction between clobazam and cannabidiol in children with refractory epilepsy. *Epilepsia* 56, 1246–1251. doi: 10.1111/epi.13060

Ghovanloo, M. R., Shuart, N. G., Mezeyova, J., Dean, R. D., Ruben, P. C., and Goodchild, S. J. (2018). Inhibitory effects of cannabidiol on voltage-dependent sodium currents. *J. Biol. Chem.* 293, 16546–16558. doi: 10.1074/jbc.RA118.004929

Glass, H. C., Grinspan, Z. M., and Shellhaas, R. A. (2018). Outcomes after acute symptomatic seizures in neonates. *Semin. Fetal Neonatal Med.* 23, 218–222. doi: 10.1016/j.siny.2018.02.001

Glass, H. C., Kan, J., Bonifacio, S. L., and Ferriero, D. (2012). Neonatal seizures: treatment practices among term and preterm infants. *Pediatr. Neurol.* 46, 111–115. doi: 10.1016/j.pediatrneurol.2011.11.006

Gobbo, O. L., and O'Mara, S. M. (2004). Post-treatment, but not pre-treatment, with the selective cyclooxygenase-2 inhibitor celecoxib markedly enhances functional recovery from kainic acid-induced neurodegeneration. *Neuroscience* 125, 317–327. doi: 10.1016/j.neuroscience.2004.01.045

Greene, M. L., and Saunders, D. R. (1974). Metabolism of tetrahydrocannabinol by the small intestine. *Gastroenterology* 66, 365–372. doi: 10.1016/S0016-5085(74)80135-6

Haglid, K. G., Wang, S., Qiner, Y., and Hamberger, A. (1994). Excitotoxicity. Experimental correlates to human epilepsy. *Mol Neurobiol.* 9, 259–263. doi: 10.1007/BF02816125

Harkany, T., Keimpema, E., Barabás, K., and Mulder, J. (2008). Endocannabinoid functions controlling neuronal specification during brain development. *Mol. Cell. Endocrinol.* 286, S84–S90. doi: 10.1016/j.mce.2008.02.011

Haskó, G., Csóka, B., Németh, Z. H., Vizi, E. S., and Pacher, P. (2009). A2B adenosine receptors in immunity and inflammation. *Trends Immunol.* 30, 263–270. doi: 10.1016/j.it.2009.04.001

Hayakawa, K., Irie, K., Sano, K., Watanabe, T., Higuchi, S., Enoki, M., et al. (2009). Therapeutic time window of cannabidiol treatment on delayed ischemic

damage *via* high-mobility group box1-inhibiting mechanism. *Biol. Pharm. Bull.* 32, 1538–1544. doi: 10.1248/bpb.32.1538

Hayatbakhsh, M. R., Najman, J. M., Jamrozik, K., Mamun, A. A., Alati, R., and Bor, W. (2007). Cannabis and anxiety and depression in young adults: a large prospective study. *J. Am. Acad. Child Adolesc. Psychiatry* 46, 408–417. doi: 10.1097/chi.0b013e31802dc54d

Herdegen, T., and Leah, J. D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox and CreB/ATF proteins. *Brain Res. Rev.* 28, 370–490. doi: 10.1016/s0165-0173(98)00018-6

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative *in vitro* autoradiographic study. *J Neurosci.* 11, 563–583. doi: 10.1523/JNEUROSCI.11-02-00563.1991

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R., et al. (1990). Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. U S A* 87, 1932–1936. doi: 10.1073/pnas.87.5.1932

Herlenius, E., and Lagercrantz, H. (2004). Development of neurotransmitter systems during critical periods. *Exp. Neurol.* 190, S8–S21. doi: 10.1016/j.expneurol.2004.03.027

Hill, A. J., Jones, N. A., Smith, I., Hill, C. L., Williams, C. M., Stephens, G. J., et al. (2014). Voltage-gated sodium (NaV) channel blockade by plant cannabinoids does not confer anticonvulsant effects *per se*. *Neurosci. Lett.* 566, 269–274. doi: 10.1016/j.neulet.2014.03.013

Huntsman, R. J., Wai, R., and Shackelford, A. E. (2020). Cannabis for Pediatric Epilepsy. *J. Clin. Neurophysiol.* 37, 2–8. doi: 10.1097/WNP.0000000000000641

Ibeas, B. C., Chen, T., Nunn, A. V., Bazelot, M., Dallas, M., and Whalley, B. J. (2015). Molecular targets of cannabidiol in neurological disorders. *Neurotherapeutics* 12, 699–730. doi: 10.1007/s13311-015-0377-3

Jabs, R., Paterson, I. A., and Walz, W. (1997). Qualitative analysis of membrane currents in glial cells from normal and gliotic tissue *in situ*: down-regulation of Na^+ current and lack of P2 purinergic responses. *Neuroscience* 81, 847–860. doi: 10.1016/s0306-4522(97)00207-8

Jensen, E. F. (2009). Neonatal seizures: an update on mechanisms and management. *Clin. Perinatol.* 36:881. doi: 10.1016/j.clp.2009.08.001

Jiang, J., Borisenko, G. G., Osipov, A., Martin, I., Chen, R., Shvedova, A. A., et al. (2004). Arachidonic acid-induced carbon-centered radicals and phospholipid peroxidation in cyclo-oxygenase-2-transfected PC12 cells. *J. Neurochem.* 90, 1036–1049. doi: 10.1111/j.1471-4159.2004.02577.x

Jones, N. A., Hill, A. J., Smith, I., Bevan, S. A., Williams, C. M., Whalley, B. J., et al. (2010). Cannabidiol displays antiepileptiform and antiseizure properties *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.* 332, 569–577. doi: 10.1124/jpet.109.159145

Joseph, E. K., and Levine, J. D. (2006). Mitochondrial electron transport in models of neuropathic and inflammatory pain. *Pain* 121, 105–114. doi: 10.1016/j.pain.2005.12.010

Kadam, S. D., White, A. M., Staley, K. J., and Dudek, F. E. (2010). Continuous electro encephalographic monitoring with radio-telemetry in a rat model of perinatal hypoxia-ischemia reveals progressive post-stroke epilepsy. *J. Neurosci.* 30, 404–415. doi: 10.1523/JNEUROSCI.4093-09.2010

Kaplan, J. S., Stella, N., Catterall, W. A., and Westenbroek, R. E. (2017). Cannabidiol attenuates seizures and social defcits in a mouse model of Dravet syndrome. *Proc. Natl. Acad. Sci. U S A* 114, 11229–11234. doi: 10.1073/pnas.1711351114

Karschner, E. L., Darwin, W. D., Goodwin, R. S., Wright, S., and Huestis, M. A. (2011). Plasma cannabinoid pharmacokinetics following controlled oral δ^9-tetrahydrocannabinol and oromucosal cannabis extract administration. *Clin. Chem.* 57, 66–75. doi: 10.1373/clinchem.2010.152439

Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E. S., Mackie, K., et al. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.* 19, 4544–4558. doi: 10.1523/JNEUROSCI.19-11-04544.1999

Katona, I., Urban, G. M., Wallace, M., Ledent, C., Jung, K. M., Piomelli, D., et al. (2006). Molecular composition of the endocannabinoid system at glutamatergic synapses. *J. Neurosci.* 26, 5628–5637. doi: 10.1523/JNEUROSCI.0309-06.2006

Kaushal, S., Tamer, Z., Opoku, F., and Forcelli, P. A. (2016). Anticonvulsant drug-induced cell death in the developing white matter of the rodent brain. *Epilepsia* 57, 727–734. doi: 10.1111/epi.13365

Kawaguchi, K., Hickey, R. W., Rose, M. E., Zhu, L., Chen, J., and Graham, S. H. (2005). Cyclooxygenase-2 expression is induced in rat brain after kainate-induced seizures and promotes neuronal death in CA3 hippocampus. *Brain Res.* 1050, 130–137. doi: 10.1016/j.brainres.2005.05.038

Kawamura, Y., Fukaya, M., Maejima, T., Yoshida, T., Miura, E., Watanabe, M., et al. (2006). The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. *J. Neurosci.* 26, 2991–3001. doi: 10.1523/JNEUROSCI.4872-05.2006

Klein, B. D., Jacobson, C. A., Metcalf, C. S., Smith, M. D., Wilcox, K. S., Hampson, A. J., et al. (2017). Evaluation of cannabidiol in animal seizure models by the Epilepsy Therapy Screening Program (ETSP). *Neurochem. Res.* 42, 1939–1948. doi: 10.1007/s11064-017-2287-8

Knupp, K. G., Rice, J. D., Helmkamp, L. J., Galinkin, J., Sempio, C., Jost, K., et al. (2019). Prospective evaluation of oral cannabis extracts in children with epilepsy. *Seizure* 72, 23–27. doi: 10.1016/j.seizure.2019.09.007

Kreitzer, A. C., and Regehr, W. G. (2001). Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron.* 29, 717–727. doi: 10.1016/s0896-6273(01)00246-x

Kubová, H., Druga, R., Lukasiuk, K., Suchomelová, L., Haugvicová, R., Jirmanová, I., et al. (2001). Status epilepticus causes necrotic damage in the mediodorsal nucleus of the thalamus in immature rats. *J. Neurosci.* 21, 3593–3599. doi: 10.1523/JNEUROSCI.21-10-03593.2001

Kubová, H., Lukasiuk, K., and Pitkänen, A. (2012). New insight on the mechanisms of epileptogenesis in the developing brain. *Adv. Tech. Stand. Neurosurg.* 39, 3–44. doi: 10.1007/978-3-7091-1360-8_1

Kubová, H., and Mareš, P. (2013). Are morphologic and functional consequences of status epilepticus in infant rats progressive? *Neuroscience* 235, 232–249. doi: 10.1016/j.neuroscience.2012.12.055

Kudin, A., Tatiana, A., Jan, K., Seyfried, S. V., Beck, H., Elger, C. E., et al. (2002). Seizure-dependent modulation of mitochondrial oxidative phosphorylation in rat hippocampus. *Eur. J. Neurosci.* 15, 1105–1114. doi: 10.1046/j.1460-9568.2002.01947.x

Kurz, J. E., Sheets, D., Parsons, J. T., Rana, A., Delorenzo, R. J., and Churn, S. B. (2001). A significant increase in both basal and maximal calcineurin activity in the rat pilocarpine model of status epilepticus. *J. Neurochem.* 78, 304–315. doi: 10.1046/j.1471-4159.2001.00426.x

Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., and Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi: 10.1111/bph.13250

Lattanzi, S., Brigo, F., Trinka, E., Zaccara, G., Cagnetti, C., Del Giovane, C., et al. (2018). Efficacy and safety of cannabidiol in epilepsy: a systematic review and meta-analysis. *Drugs* 78, 1791–1804. doi: 10.1007/s40265-018-0992-5

Lattanzi, S., Trinka, E., Russo, E., Striano, P., Citraro, R., Silvestrini, M., et al. (2019). Cannabidiol as adjunctive treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome. *Drugs Today* 55, 177–196. doi: 10.1358/dot.2019.55.3.2909248

Lee, M. C., Ting, K. K., Adams, S., Brew, B. J., Chung, R., and Guillemin, G. J. (2010). Characterization of the expression of NMDA receptors in human astrocytes. *PLoS One* 5:e14123. doi: 10.1371/journal.pone.0014123

Leo, A., Russo, E., and Elia, M. (2016). Cannabidiol and epilepsy: rationale and therapeutic potential. *Pharmacol. Res.* 107, 85–92. doi: 10.1016/j.phrs.2016.03.005

Li, Y., and Kim, J. (2015). Neuronal expression of CB2 cannabinoid receptor mRNAs in the mouse hippocampus. *Neuroscience* 311, 253–267. doi: 10.1016/j.neuroscience.2015.10.041

Liou, G. I., Auchampach, J. A., Hillard, C. J., Zhu, G., Yousufzai, B., Mian, S., et al. (2008). Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A2A adenosine receptor. *Invest. Ophthalmol. Vis. Sci.* 49, 5526–5531. doi: 10.1167/iovs.08-2196

Lorigados, L., Orozco, S., Morales, L., Estupiñán, B., García, I., and Rocha, L. (2013). Excitotoxicidad y muerte neuronal en la epilepsia. *Biotecnología Aplicad.* 30, 1–8. doi: 10.2174/1381612811319380009

Lu, H. C., and Mackie, K. (2016). An introduction to the endogenous cannabinoid system. *Biol. Psychiatry.* 79, 516–525. doi: 10.1016/j.biopsych.2015.07.028

Mahmoud, E., and Waseem, G. (2014). "Constituents of cannabis sativa," in *Hand Book of Cannabis*, ed R. Pertwee (University Press Oxford Scholarship), 46–66.

Malfait, A. M., Gallily, R., Sumariwalla, P. F., Malik, A. S., Andreakos, E., Mechoulam, R., et al. (2000). The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. U S A.* 97, 9561–9566. doi: 10.1073/pnas.160105897

Meador, K. J., Baker, G. A., Browning, N., Cohen, M. J., Bromley, R. L., Clayton-Smith, J., et al. (2012). Effects of fetal antiepileptic drug exposure: outcomes at age 4.5 years. *Neurology* 78, 1207–1214. doi: 10.1212/WNL.0b013e318250d824

Mecha, M., Carrillo-Salinas, F. J., Feliú, A., Mestre, L., and Guaza, C. (2016). Microglia activation states and cannabinoid system: therapeutic implications. *Pharmacol. Ther.* 166, 40–55. doi: 10.1016/j.pharmthera.2016.06.011

Mechoulam, R., and Gaoni, Y. (1965). The isolation and structure of cannabinolic cannabidiolic and cannabigerolic acids. *Tetrahedron* 21, 1223–1229. doi: 10.1016/0040-4020(65)80064-3

Mechoulam, R., and Parker, L. A. (2013). The endocannabinoid system and the brain. *Annu. Rev. Psychol.* 64, 21–47. doi: 10.1146/annurev-psych-113011-143739

Monory, K., Massa, F., Egertová, M., Eder, M., Blaudzun, H., Westenbroek, R., et al. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51, 455–466. doi: 10.1016/j.neuron.2006.07.006

Morgan, N. H., Stanford, I. M., and Woodhall, G. L. (2009). Functional CB2 type cannabinoid receptors at CNS synapses. *Neuropharmacology* 57, 356–368. doi: 10.1016/j.neuropharm.2009.07.017

Mori, M. A., Meyer, E., Soares, L. M., Milani, H., Guimarães, F. S., and de Oliveira, R. M. W. (2017). Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 75, 94–105. doi: 10.1016/j.pnpbp.2016.11.005

Murchison, D., and Griffith, W. H. (2000). Mitochondria buffer non-toxic calcium loads and release calcium through the mitochondrial permeability transition pore and sodium/calcium exchanger in rat basal forebrain neurons. *Brain Res.* 854, 139–151. doi: 10.1016/s0006-8993(99)02297-0

Nomura, D. K., Morrison, B. E., Blankman, J. L., Long, J. Z., Kinsey, S. G., Marcondes, M. C. G., et al. (2011). Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. *Science* 334, 809–813. doi: 10.1126/science.1209200

Ohlsson, A., Lindgren, J. E., Andersson, S., Agurell, S., Gillespie, H., Hollister, L. E., et al. (1986). Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration. *Biomed. Environ. Mass Spectrom.* 13, 77–83. doi: 10.1002/bms.1200130206

Onaivi, E. S., Ishiguro, H., Gu, S., and Qing-Rong, L. (2012). CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. *J. Psychopharmacol.* 26, 92–103. doi: 10.1177/0269881111400652

Pan, H., Mukhopadhyay, P., Rajesh, M., Patel, V., Mukhopadhyay, B., Gao, B., et al. (2009). Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation and cell death. *J. Pharmacol. Exp. Ther.* 328, 708–714. doi: 10.1124/jpet.108.147181

Pandolfo, P., Silveirinha, V., dos Santos-Rodrigues, A., Venance, L., Ledent, C., Takahashi, R. N., et al. (2011). Cannabinoids inhibit the synaptic uptake of adenosine and dopamine in the rat and mouse striatum. *Eur. J. Pharmacol.* 655, 38–45. doi: 10.1016/j.ejphar.2011.01.013

Patel, S., Hill, M. N., Cheer, J. F., Wotjak, C. T., and Holmes, A. (2017). The endocannabinoid system as a target for novel anxiolytic drugs. *Neurosci. Biobehav. Rev.* 76, 56–66. doi: 10.1016/j.neubiorev.2016.12.033

Patel, M., and Li, Q. Y. (2003). Age dependence of seizure-induced oxidative stress. *Neuroscience* 118, 431–437. doi: 10.1016/s0306-4522(02)00979-x

Patra, P. H., Barker-Haliski, M., White, H. S., Whalley, B. J., Glyn, S., Sandhu, H., et al. (2019). Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models. *Epilepsia* 60, 303–314. doi: 10.1111/epi.14629

Pazos, M. R., Cinquina, V., Gómez, A., Layunta, R., Santos, M., Fernández-Ruiz, J., et al. (2012). Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. *Neuropharmacology* 63, 776–783. doi: 10.1016/j.neuropharm.2012.05.034

Pérez, M., Benitez, S. U., Cartarozzi, L. P., Del Bel, E., Guimarães, F. S., and Oliveira, A. L. R. (2013). Neuroprotection and reduction of glial reaction by cannabidiol treatment after sciatic nerve transection in neonatal rats. *Eur. J. Neurosci.* 38, 3424–3434. doi: 10.1111/ejn.12341

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. *Br. J. Pharmacol.* 153, 199–215. doi: 10.1038/sj.bjp.0707442

Pertwee, R. G., and Cascio, M. G. (2014). "Known pharmacological actions of delta-9-tetrahydrocannabinol and of four other chemical constituents of cannabis that activate cannabinoid receptors," in *Hand Book of Cannabis*, ed R. Pertwee (UK: University of Aberdeen) 167–185.

Pitkänen, A., and Engel, J., Jr. (2014). Review Past and present definitions of epileptogenesis and its biomarkers. *Neurotherapeutics* 11, 231–241. doi: 10.1007/s13311-014-0257-2

Pitkänen, A., Lukasiuk, K., Dudek, F. E., and Staley, K. J. (2015). Epileptogenesis. *Cold Spring Harb. Perspect. Med.* 5:a022822. doi: 10.1101/cshperspect.a022822

Porter, B. E., and Jacobson, C. (2013). Report of a parent survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. *Epilepsy Behav.* 29, 574–577. doi: 10.1016/j.yebeh.2013.08.037

Qu, Y., Vadivelu, S., Choi, L., Liu, S., Lu, A., Lewis, B., et al. (2003). Neurons derived from embryonic stem (ES) cells resemble normal neurons in their vulnerability to excitotoxic death. *Exp. Neurol.* 184, 326–336. doi: 10.1016/j.expneurol.2003.07.001

Rajesh, M., Mukhopadhyay, P., Bátkai, S., Haskó, G., Liaudet, L., Drel, V. R., et al. (2007). Cannabidiol attenuates high glucose-induced endothelial cell inflammatory response and barrier disruption. *Am. J. Physiol. Heart Circ. Physiol.* 293, H610–H619. doi: 10.1152/ajpheart.00236.2007

Rakhade, S. N., and Jensen, F. E. (2009). Epileptogenesis in the immature brain: emerging mechanisms. *Nat. Rev. Neurol.* 5:380. doi: 10.1038/nrneurol.2009.80

Ravizza, T., Rizzi, M., Perego, C., Richichi, C., Veliskova, J., Moshe, S. L., et al. (2005). Inflammatory response and glia activation in developing rat hippocampus after status epilepticus. *Epilepsia* 5, 113–117. doi: 10.1111/j.1528-1167.2005.01006.x

Rice, A. C., and De Lorenzo, R. J. (1998). NMDA receptor activation during status epilepticus is required for the development of epilepsy. *Brain Res.* 782, 240–247. doi: 10.1016/s0006-8993(97)01285-7

Rizzi, M., Perego, C., Aliprandi, M., Richichi, C., Ravizza, T., Colella, D., et al. (2003). Glia activation and cytokine increase in rat hippocampus by kainic acidinduced status epilepticus during postnatal development. *Neurobiol. Dis.* 14, 494–503. doi: 10.1016/j.nbd.2003.08.001

Romjin, H., Hofman, M., and Gramsberg, A. (1991). At what age is the developing cerebral cortex of the rat comparable to that the full-term newborn baby? *Early Human dev.* 26, 61–67. doi: 10.1016/0378-3782(91)90044-4

Rosenberg, E., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Ruhaak, L. R., Felth, J., Karlsson, P. C., Rafter, J. J., Verpoorte, R., and Bohlin, L. (2011). Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from Cannabis sativa. *Biol. Pharm. Bull.* 34, 774–778. doi: 10.1248/bpb.34.774

Sagredo, O. S., González, I., Aroyo, M. R., Pazos, C., Benito, I., Lastres-Becker, J. P., et al. (2009). Cannabinoid CB2 receptor agonists protect the striatum against malonate toxicity: relevance for Huntington's disease. *Glia* 57, 1154–1167. doi: 10.1002/glia.20838

Sanchez, R. M., Koh, S., Rio, C., Wang, C., Lamperti, E. D., Sharma, D., et al. (2001). Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced

seizures. *J. Neurosci.* 21, 8154–8163. doi: 10.1523/JNEUROSCI.21-20-08154.2001

Schonhofen, P., Bristot, I. J., Crippa, J. A., Hallak, J. E. C., Zuardi, W. Z., Parsons, R. B., et al. (2018). Cannabinoid–based therapies and brain development: potential harmful efect of early modulation of the endocannabinoid system. *CNS Drugs* 32, 697–712. doi: 10.1007/s40263-018-0550-4

Sekar, K., and Pack, A. (2019). Epidiolex as adjunct therapy for treatment of refractory epilepsy: a comprehensive review with a focus on adverse effects. *F1000Res.* 8:234.doi: 10.12688/f1000research.16515.1

Semple, B. D., Dill, L. K., and O'Brien, T. J. (2020). Immune challenges and seizures: how do early life insults influence epileptogenesis? *Front. Pharmacol.* 11:2.doi: 10.3389/fphar.2020.00002

Shimada, T., Takemiya, T., Sugiura, H., and Yamagata, K. (2014). Role of inflammatory mediators in the pathogenesis of epilepsy. *Mediators Inflamm.* 2014:901902. doi: 10.1155/2014/901902

Silvestro, S., Mammana, S., Cavalli, E., Bramanti, P., and Mazzon, E. (2019). Use of cannabidiol in the treatment of epilepsy: efficacy and security in clinical trials. *Molecules* 24:1459.doi: 10.3390/molecules24081459

Skowrońska, K., Obara-Michlewska, M., Zielińska, M., and Albrecht, J. (2019). NMDA receptors in astrocytes: in search for roles in neurotransmission and astrocytic homeostasis. *Int. J. Mol. Sci.* 20:309. doi: 10.3390/ijms20020309

Sofia, R. D., Solomon, T. A., and Barry, H., 3rd (1976). Anticonvulsant activity of δ^9-tetrahydrocannabinol compared with three other drugs. *Eur. J. Pharmacol.* 35, 7–16. doi: 10.1016/0014-2999(76)90295-8

Solowij, N., Jones, K. A., Rozman, M. E., Davis, S. M., Ciarrochi, J., Heaven, C. L., et al. (2011). Verbal learning and memory in adolescent cannabis users, alcohol users and non-users. *Psychopharmacology* 216, 131–144. doi: 10.1007/s00213-011-2203-x

Specchio, N., Pietrafusa, N., and Cross, H. (2020). Source of cannabinoids: what is available, what is used, and where does it come from? *Epileptic Disord.* 22, 1–9. doi: 10.1684/epd.2019.1121

Stella, N. (2004). Cannabinoid signaling in glial cells. *Glia* 48, 267–277. doi: 10.1002/glia.20084

Stellwagen, D., Beattie, E. C., Seo, J. Y., and Malenka, R. C. (2005). Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. *J. Neurosci.* 25, 3219–3228. doi: 10.1523/JNEUROSCI.4486-04.2005

Stott, N. K., Jones, C., Bazelot, N., and Whalley, B. J. (2018). "The proposed multimodal mechanism of action of cannabidiol in epilepsy: modulation of intracellular calcium and adenosine-mediated signaling", *American Epilepsy Society Annual Meeting*, New Orleans, LA. Available online at: https://www.aesnet.org/meetings_events/annual_meeting_abstracts/view/554059.

Suchomelova, L., Lopez-Meraz, M. L., Niquet, J., Kubova, H., and Wasterlain, C. G. (2015). Hyperthermia aggravates status epilepticus-induced epileptogenesis and neuronal loss in immature rats. *Neuroscience* 305, 209–224. doi: 10.1016/j.neuroscience.2015.08.006

Sugaya, Y., Yamazaki, M., Uchigashima, M., Kobayashi, K., Watanabe, M., Sakimura, K., et al. (2016). Crucial roles of the endocannabinoid 2-arachidonoylglycerol in the suppression of epileptic seizures. *Cell Rep.* 16, 1405–1415. doi: 10.1016/j.celrep.2016.06.083

Szaflarski, J. P., Bebin, E. M., Comi, A. M., Patel, A. D., Joshi, C., Checketts, D., et al. (2018). Long-term safety and treatment effects of cannabidiol in children and adults with treatment-resistant epilepsies: expanded access program results. *Epilepsia* 59, 1540–1548. doi: 10.1111/epi.14477

Szczurowska, E., and Mareš, P. (2013). NMDA and AMPA receptors: development and status epilepticus. *Physiol. Res.* 1, 21–38. doi: 10.33549/physiolres.932662

Treat, L., Chapman, K. E., Colborn, K. L., and Knupp, K. G. (2017). Duration of use of oral cannabis extract in a cohort of pediatric epilepsy patients. *Epilepsia* 58, 123–127. doi: 10.1111/epi.13617

Tretter, L., Sipos, I., and Vizi, A. V. (2004). Initiation of neuronal damage by complex I deficiency and oxidative stress in Parkinson's disease. *Neurochem. Res.* 29, 569–577. doi: 10.1023/b:nere.0000014827.94562.4b

Turkanis, S. A., Smiley, K. A., Borys, H. K., Olsen, D. M., and Karler, R. (1979). An electrophysiological analysis of the anticonvulsant action of cannabidiol on limbic seizures in conscious rats. *Epilepsia* 20, 351–363. doi: 10.1111/j.1528-1157.1979.tb04815.x

Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H., and Anikszejn, L. (1999). The establishment of GABAergic and Glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates whit the development of the apical dendrite. *J. Neurosci.* 19, 10372–10382. doi: 10.1523/JNEUROSCI.19-23-10372.1999

Tzadok, M., Uliel-Siboni, S., Linder, I., Kramer, U., Epstein, O., Menascu, S., et al. (2016). CBD-enriched medical cannabis for intractable pediatric epilepsy: the current Israeli experience. *Seizure* 35, 41–44. doi: 10.1016/j.seizure.2016.01.004

Vega-García, A., Rocha, L., Guevara-Guzmán, R., Guerra-Araiza, C., Feria-Romero, I., Gallardo, J. M., et al. (2020). Magnolia officinalis reduces inflammation and damage induced by recurrent status epilepticus in immature rats. *Curr. Pharm. Des.* 26, 1388–1401. doi: 10.2174/1381612826666200320121813

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., et al. (2003). Interleukin-1β enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J. Neurosci.* 23, 8692–8700. doi: 10.1523/JNEUROSCI.23-25-08692.2003

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and DeLorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizures frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920

Wang, S., Cheng, Q., Malik, S., and Yang, J. (2000). Interleukin-1beta inhibits gamma-aminobutyric acid type A (GABAA) receptor current in cultured hippocampal neurons. *J. Pharmacol. Exp Ther.* 292, 497–504.

Wang, T., Collet, J. P., Shapiro, S., and Ware, M. A. (2008). Adverse effects of medical cannabinoids: a systematic review. *CMAJ* 178, 1669–1678. doi: 10.1503/cmaj.071178

Watanabe, K., Yamaori, S., Funahashi, T., Kimura, T., and Yamamoto, I. (2007). Cytochrome P450 enzymes involved in the metabolism of tetrahydrocannabinols and cannabinol by human hepatic microsomes. *Life Sci.* 80, 1415–1419. doi: 10.1016/j.lfs.2006.12.032

Wheless, J. W., Dlugos, D., Miller, I., Oh, D. A., Parikh, N., Phillips, S., et al. (2019). Pharmacokinetics and tolerability of multiple doses of pharmaceutical-grade synthetic cannabidiol in pediatric patients with treatment-resistant epilepsy. *CNS Drugs* 33, 593–604. doi: 10.1007/s40263-019-00624-4

Wong, S., and Wilens, T. (2017). Medical cannabinoids in children and adolecents: a systematic review. *Pediatrics* 140, 1–16. doi: 10.1542/peds.2017-1818

Wright, S., and Guy, G. (2014). "Licensed cannabis-based medicines: benefits and risks," in *Handbook of Cannabis*, ed R. Pertwee (New York, NY: Oxford University), 483–498.

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 20 January 2021
doi: 10.3389/fnbeh.2020.611780

Drug-Resistant Temporal Lobe Epilepsy Alters the Expression and Functional Coupling to Gαi/o Proteins of CB1 and CB2 Receptors in the Microvasculature of the Human Brain

*María de los Ángeles Nuñez-Lumbreras[1], José Luis Castañeda-Cabral[1], María Guadalupe Valle-Dorado[1], Vicente Sánchez-Valle[2], Sandra Orozco-Suárez[3], Rosalinda Guevara-Guzmán[4], Iris Martínez-Juárez[5], Mario Alonso-Vanegas[5,6], Fruzsina Walter[7], Maria A. Deli[7], Francia Carmona-Cruz[1] and Luisa Rocha[1]**

[1] Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados, Mexico City, Mexico, [2] Departamento de Farmacología, Centro de Investigación y de Estudios Avanzados, Mexico City, Mexico, [3] Unidad de Investigación Médica en Enfermedades Neurológicas, Hospital de Especialidades, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Mexico City, Mexico, [4] Departamento de Fisiología, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico, [5] Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez (INNNMVS), Mexico City, Mexico, [6] Centro Internacional de Cirugía de Epilepsia, Hospital HMG-Coyoacán, Mexico City, Mexico, [7] Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

OPEN ACCESS

Edited by:
Dolores E. López García,
University of Salamanca, Spain

Reviewed by:
Ricardo Gómez-Nieto,
University of Salamanca, Spain
Luiz E. Mello,
Federal University of São Paulo, Brazil

***Correspondence:**
Luisa Rocha
lrocha@cinvestav.mx

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 29 September 2020
Accepted: 10 December 2020
Published: 20 January 2021

Citation:
Nuñez-Lumbreras MÁ, Castañeda-Cabral JL, Valle-Dorado MG, Sánchez-Valle V, Orozco-Suárez S, Guevara-Guzmán R, Martínez-Juárez I, Alonso-Vanegas M, Walter F, Deli MA, Carmona-Cruz F and Rocha L (2021) Drug-Resistant Temporal Lobe Epilepsy Alters the Expression and Functional Coupling to Gαi/o Proteins of CB1 and CB2 Receptors in the Microvasculature of the Human Brain. Front. Behav. Neurosci. 14:611780. doi: 10.3389/fnbeh.2020.611780

Cannabinoid receptors 1 and 2 (CB1 and CB2, respectively) play an important role in maintaining the integrity of the blood–brain barrier (BBB). On the other hand, BBB dysfunction is a common feature in drug-resistant epilepsy. The focus of the present study was to characterize protein expression levels and Gαi/o protein-induced activation by CB1 and CB2 receptors in the microvascular endothelial cells (MECs) isolated from the brain of patients with drug-resistant mesial temporal lobe epilepsy (DR-MTLE). MECs were isolated from the hippocampus and temporal neocortex of 12 patients with DR-MTLE and 12 non-epileptic autopsies. Immunofluorescence experiments were carried out to determine the localization of CB1 and CB2 receptors in the different cell elements of MECs. Protein expression levels of CB1 and CB2 receptors were determined by Western blot experiments. [^{35}S]-GTPγS binding assay was used to evaluate the Gαi/o protein activation induced by specific agonists. Immunofluorescent double-labeling showed that CB1 and CB2 receptors colocalize with tight junction proteins (claudin-5, occludin, and zonula occludens-1), glial fibrillary acidic protein and platelet-derived growth factor receptor-β. These results support that CB1 and CB2 receptors are expressed in the human isolated microvessels fragments consisting of MECs, astrocyte end feet, and pericytes. The hippocampal microvasculature of patients with DR-MTLE presented lower protein expression of CB1 and CB2 receptors (66 and 43%, respectively; $p < 0.001$). However, its Gαi/o protein activation was with high efficiency (CB1, 251%, $p < 0.0008$; CB2, 255%, $p < 0.0001$). Microvasculature of temporal neocortex presented protein overexpression of CB1 and CB2 receptors (35 and 41%, respectively; $p < 0.01$). Their coupled Gαi/o protein activation was with higher efficiency for CB1 receptors (103%, $p < 0.006$), but lower potency ($p < 0.004$) for CB2 receptors.

The present study revealed opposite changes in the protein expression of CB1 and CB2 receptors when hippocampus (diminished expression of CB1 and CB2) and temporal neocortex (increased expression of CB1 and CB2) were compared. However, the exposure to specific CB1 and CB2 agonists results in high efficiency for activation of coupled Gαi/o proteins in the brain microvasculature of patients with DR-MTLE. CB1 and CB2 receptors with high efficiency could represent a therapeutic target to maintain the integrity of the BBB in patients with DR-MTLE.

Keywords: mesial temporal lobe epilepsy, drug-resistance, blood-brain barrier, endocannabinoid system, CB1 receptors, CB2 receptors

INTRODUCTION

The blood–brain barrier (BBB) regulates the access of molecules, drugs, neurotoxins, and pathogens from the bloodstream into the cerebral parenchyma. The close interaction between microvascular endothelial cells (MECs), the main components of the BBB, and other elements of the neurovascular unit (astrocytes, pericytes, neurons, and basement membrane) plays a significant role in the homeostasis of the central nervous system (Stanimirovic and Friedman, 2012). MECs present tight junctions, which participate in the control of transcytosis. Tight junctions involve the interaction of transmembrane (occludin, claudins, and junctional adhesion molecules), as well as cytoplasmic [zonula occludens (ZO)] proteins (Hawkins and Davis, 2005). Changes in the expression and interaction of these proteins facilitate BBB disruption (Kook et al., 2012).

Studies indicate that cannabinoid receptors 1 and 2 (CB1 and CB2, respectively) are expressed in different components of the BBB (Molina-Holgado et al., 2002; Golech et al., 2004; Zong et al., 2017). The activation of CB1 receptors prevents downregulation of ZO-1, claudin-5, and junctional adhesion molecules-1 proteins and the consequent BBB breakdown in cocultures of human brain MECs and astrocytes (Lu et al., 2008). In turn, the activation of CB2 receptors increases the transendothelial electrical resistance and the expression of tight junction proteins in MECs and reduces neuroinflammation effects (Ramirez et al., 2012). In autopsy brain samples from patients with multiple sclerosis, the BBB overexpressed CB2 receptors in chronic inactive plaques (Zhang et al., 2011), a finding that supports their protective role.

Epilepsy, a neurological disorder characterized by a permanent predisposition to spontaneous and recurrent seizures (Fisher, 2015), is associated with BBB dysfunction (van Vliet et al., 2014; Broekaart et al., 2018). Recent studies from our research group have shown that the neocortical microvasculature from patients with drug-resistant mesial temporal lobe epilepsy (DR-MTLE) exhibited lower protein expression levels of occludin and ZO-1 as compared to control tissues obtained from autopsies, whereas the protein expression levels of the vascular endothelial growth factors (VEGF-A, VEGFR-2) and claudin-5 were increased (Castañeda-Cabral et al., 2020a). Furthermore, the protein expression levels of the proinflammatory cytokines interleukin 1β and tumor necrosis factor α (TNF-α) and its receptor TNF-R1, as well as the nitric oxide synthase, a marker of oxidative stress, were increased in the microvasculature of DR-MTLE patients (Castañeda-Cabral et al., 2020b). These changes suggest important alterations in the integrity of the BBB that may contribute to the pathogenesis of epilepsy.

Considering that CB1 and CB2 receptors play an important role in the integrity of BBB, it is proposed that epilepsy modifies protein expression levels and/or signal transduction pathways mediated by these receptors in the microvasculature of the human brain. Experiments were designed to evaluate protein expression levels and signaling of CB1 and CB2 receptors in the microvasculature of the hippocampus (epileptogenic area) and temporal neocortex (the brain area involved in the spread of seizure activity) of patients with DR-MTLE. The influence of clinical conditions was investigated through correlation analysis.

MATERIALS AND METHODS

Subjects

Brain tissue was obtained from adult patients with DR-MTLE ($n = 12$) who underwent surgery through the Epilepsy Surgery Program of the National Institute of Neurology and Neurosurgery "Manuel Velasco Suárez" (INNNMVS) in Mexico City. All the neurosurgeries were carried out by the same neurosurgeon (MAV). Patients with DR-MTLE showed the following clinical data: age of subjects, 30.2 ± 2.5 years; age at seizure onset, 10.0 ± 2.3 years; epilepsy duration, 20.8 ± 3.3 years; and frequency of seizures, 7.9 ± 1.6 per month (**Table 1**). Preoperative evaluation for each patient included video electroencephalogram, magnetic resonance imaging, and single-photon emission computed tomography. Subsequently, patients underwent an anterior lobectomy ipsilateral to the epileptic focus. During the surgical procedure, hippocampal and temporal neocortex biopsies were collected immediately after resection, frozen on dry ice, and stored at −70°C until processed. The scientific and ethics committees from INNNMVS and Cinvestav approved the protocol for this experimental study (reference numbers of approval: 101/18 and 055/2018, respectively). Written informed consent was obtained from all participants.

Samples from the hippocampus and temporal neocortex obtained from 12 autopsies, died of causes not associated with neurological disorders, were evaluated as controls. The mean age of the autopsy subjects (41.0 ± 4.8 years) was not significantly different from that of the patients with DR-MTLE ($p > 0.05$).

TABLE 1 | Clinical data of patients with drug-resistance mesial temporal lobe epilepsy.

Patient	Age (years)	Gender	Age at seizure onset (years)	Epilepsy duration (years)	Frequency of seizures (per month)	ASD treatment before surgery
416	18	F	9	9	4	CBZ, TPM, CNZ, LVT
428	45	M	8	37	3	PHE, VPA, CBZ, OXC, LMG, TPM, CNZ
429	34	M	1	33	10	PHE, CBZ, TPM, CNZ, CLB, LCS
434	32	F	3	29	4	PHE, OXC, LMG, TPM, LVT
454	26	F	2	26	5	CBZ, OXC
457	35	F	20	15	4	PHE, VPA, OXC
470	30	F	6	24	4	CBZ, TPM, CNZ, LVT
474	44	F	20	24	12	CBZ, CZP
516	17	F	16	1	16	PHE, LVT
517	25	M	24	1	20	VPA, LVT
534	29	F	6	23	8	CBZ, LVT
545	27	M	5	22	5	VPA, CBZ, OXC, TPM

ASD, antiseizure drug; CBZ, carbamazepine; CLB, clobazam; CNZ, clonazepam; CZP, clorazepate; F, female; LCS, lacosamide; LMG, lamotrigine; LVT, levetiracetam; M, male; OXC, oxcarbazepine; PHE, phenytoin; TPM, topiramate; VPA, valproic acid.

TABLE 2 | Clinical data of non-epileptic autopsies.

Autopsy	Age (years)	Gender	Cause of death	PMI (hours)
A1	29	M	Abdominal trauma	13
A2	29	M	Polytrauma	18
A6	49	F	Asphyxia	18
A7	45	M	Asphyxia	18
A8	73	M	Diabetes complications	15
A9	33	F	Thoracic trauma	12
A11	12	F	Asphyxia	14
A12	40	F	Ballistic trauma	20
A14	45	F	Unknown	10
A16	57	M	Heart attack	18
A17	25	F	Thoracic trauma	18
A18	55	M	Thoracolumbar trauma	15

F, female; M, male; PMI, postmortem interval.

Autopsy samples were collected with a postmortem interval of 14.8 ± 3.7 h (**Table 2**). The samples were frozen immediately after resection and stored at −70°C. Autopsies were performed at the Institute of Forensic Sciences in Mexico City. The quality of autopsies was verified by evaluating the integrity of mRNA of each sample.

Isolation of Human Brain Microvessels

Brain microvessels were obtained from frozen human brain tissue according to a modified version of the protocol described previously by Veszelka et al. (2007) (for further details, see Castañeda-Cabral et al., 2020a). Using this procedure, we previously have found that the isolated microvessels from frozen brain tissue contain MECs, pericytes, and astrocytes end feet (Castañeda-Cabral et al., 2020a). All these cells are also a source of CB1 and CB2 receptors (Benyó et al., 2016).

Briefly, brain tissue was homogenized in Ringer HEPES (RH) buffer solution (150 mM NaCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5.2 mM KCl, 2.8 mM glucose, 5 mM HEPES, 6 mM $NaHCO_3$, pH 7.4) in a proportion of 4 mL per gram of tissue (4 mL/g of tissue). The homogenates were centrifuged at 1,000 × *g* for 15 min, at 4°C. The obtained pellet was resuspended in 17.5% RH-dextran and then centrifuged at 1,500 × *g* for 15 min, at 4°C. The resulting pellet was resuspended in 2 mL of 1% RH–bovine serum albumin (BSA), whereas the supernatant was centrifuged twice under the same conditions until obtaining three pellets. All the pellets were filtered with a 100-μm mesh. The obtained supernatant was filtered again with a 40-μm mesh. The filtrate was centrifuged at 1,000 × *g*, for 10 min, at 4°C. Subsequently, the pellet was collected in an Eppendorf tube. A sample of each pellet was analyzed using an inverted microscope (Leica DMi1) to evaluate the characteristics of the isolated microvessels. Subsequently, the expression of CD34, a valuable marker for the identification and characterization of capillary endothelial cells, was analyzed in MECs by immunofluorescence experiments. For this purpose, a fraction of the pellet was mounted on slides previously coated with poly-L-lysine and allowed to dry at room temperature for 24 h. The tissue was fixed with cold acetone for 90 min at 4°C and subsequently permeabilized with phosphate-buffered saline (PBS)—0.25% Triton X-100 for 15 min at 4°C. Blocking was performed using 3% BSA-PBS for 50 min at 4°C. Slides were incubated overnight (4°C) with an anti-CD34 antibody followed by incubation with fluorescence-labeled secondary antibody (Alexa Fluor 488, goat anti–rabbit immunoglobulin G, 1:200) for 2 h at 4°C. Subsequently, slides were washed with PBS and incubated with Hoechst 33342 (1:5,000, cat. H3570, lot 1724829, Thermo Fisher Scientific, USA) for 5 min at room temperature for nuclear staining. Finally,

slides were mounted with Vectashield mounting medium for fluorescence (cat. CB-1000; Vector Laboratories Inc., USA) and analyzed by confocal microscopy (Eclipse TE2000 microscope, Nikon, Japan). Images were obtained with a Spot RT digital camera (Diagnostic Instruments, USA).

Once the isolation of microvessels was confirmed, each pellet was divided into three fractions for the following experiments: (a) immunofluorescence analysis, (b) Western blot analysis, and (c) [^{35}S]-GTPγS binding assay. The pellets were stored at −70°C until further use.

Immunofluorescence Staining of CB1 and CB2 Receptors in the Human Brain Microvessels

Immunofluorescence experiments were carried out to localize CB1 and CB2 receptors in the different cell elements of human brain microvessels. Their expression was analyzed alone, as well as their colocalization with glial fibrillary acidic protein (GFAP), platelet-derived growth factor receptor-β (PDGFR-β), and tight junction proteins (claudin-5, occludin, and ZO-1). For this purpose, a fraction of the pellet previously obtained was mounted on slides coated with poly-L-lysine and allowed to dry at room temperature for 24 h. The tissue was fixed with cold acetone for 90 min at 4°C and subsequently permeabilized with PBS-0.25% Triton X-100 for 15 min at 4°C. Blocking was performed using 3% BSA-PBS for 50 min at 4°C. Slides containing microvessels followed an overnight primary antibody incubation at 4°C and were exposed to secondary antibody for 2 h at 4°C. Particulars for each antibody, such as primary and secondary antibody information as well as dilutions, are listed in **Supplementary Table 1**. Subsequently, slides were washed with PBS and incubated with Hoechst 33342 (1:5,000, cat. H3570, lot 1724829, Thermo Fisher Scientific, USA) for 5 min at room temperature for nuclear staining. Finally, slides were mounted with Vectashield mounting medium for fluorescence (cat. CB-1000, Vector Laboratories Inc., USA) and analyzed by confocal microscopy (Eclipse TE2000 microscope, Nikon, Japan). Images were obtained with a Spot RT digital camera (Diagnostic Instruments, USA). As negative control for specificity of staining, the immunohistochemical assay was carried out with the primary antibody omitted that resulted in the absence of immunolabeling. Also, images of Z-stack (17 planes each μm) were used for protein localization. NIS-Elements v.4.50 and ImageJ v.1.50i software were used for protein colocalization.

Evaluation of CB1 and CB2 Protein Expression by Western Blot

The pellet was homogenized in RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100, pH 7.5) and a mixture of protease inhibitors (Complete Roche Diagnostics GmbH, Germany). Protein quantification was performed by the Bradford method (Protein Assay Dye, cat. 5000006, Bio-Rad Laboratories, USA). MEC samples (15 μg/lane) were loaded in Laemmli sample buffer (cat. 1610737, Bio-Rad Laboratories, USA) and separated in 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis gel (85 V for 30 min and 100 V for 2 h) using running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3; cat. 1610723, Bio-Rad Laboratories, USA). Subsequently, the proteins were transferred onto polyvinylidene difluoride membranes (Immun-blot, cat. 1620264, Bio-Rad Laboratories, USA). After electroblotting, membranes were incubated for 1 h at room temperature with 5% blocking solution (Blot-QuickBlocker, cat. WB57, EMD Millipore, USA) dissolved in TBS-T buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5). Subsequently, membranes were incubated overnight at 4°C with the corresponding primary antibody. Membranes were washed with TBS-T and incubated for 1 h with appropriate horseradish peroxidase–coupled secondary antibody (**Supplementary Table 1**). Blots were developed using Clarity Western ECL Blotting Substrates (Bio-Rad Laboratories, USA) according to the manufacturer's indications. Scanning of the immunoblots was performed, and the density of bands was estimated using ImageJ v.1.50i software. The chemiluminescent data were normalized using β-actin as a constitutive protein, resulting in a relative ratio expression. Each sample was evaluated by duplicate.

Evaluation of the Gαi/o Protein Activation Induced by CB1 and CB2 Receptors in Human Microvessels

CB1 and CB2 receptors are composed of seven transmembrane α-helix domains and a C-terminal domain (73 and 59 amino acids, respectively) (Matsuda et al., 1990; Munro et al., 1993), which couple to Gαi/o proteins to induce their cell effects (Abood and Martin, 1996). The interaction of specific agonists with these receptors facilitates the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) in the Gαi/o subunit. For the present study, a binding assay was used to evaluate the efficacy and potency of specific CB1 and CB2 agonists to increase the binding of [^{35}S]-GTPγS, a radiolabeled non-hydrolyzable GTP analog, to Gαi/o proteins.

Cell membranes of microvessels were obtained according to the following procedure. The pellet was resuspended, homogenized in a buffer solution (2 mM Tris–EDTA, 320 mM sucrose, 5 mg/mL BSA, 5 mM $MgCl_2$; pH 7.4), and centrifuged three times at 1,000 × *g* for 10 min, at 4°C. The first and second supernatants were collected in a tube and centrifuged at 39,000 × *g* for 30 min at 4°C, whereas the third supernatant was discarded. The resulting pellet was resuspended in a buffer solution (50 mM Tris–HCl, 2 mM Tris–EDTA, 3 mM $MgCl_2$, pH 7.4). A fraction of the pellet was used for protein quantification according to the Bradford method (Protein Assay Dye, cat. 5000006, Bio-Rad Laboratories, USA).

Cell membranes were used for binding assays as previously described (Catani and Gasperi, 2016), with some modifications. A total of 8 μg of membrane protein per sample was incubated (60 min, 37°C) in 0.8 mL of buffer solution (50 mM Tris–HCl, 2 mM Tris–EDTA, 3 mM $MgCl_2$, 5 mg/mL BSA, 50 μM phenylmethylsulfonyl fluoride; pH 7.4), [^{35}S]-GTPγS (1,500–2,000 counts per minute), GDP (100 μM), and increasing concentrations (10^{-9}-10^{-4}) of a selective agonist for CB1

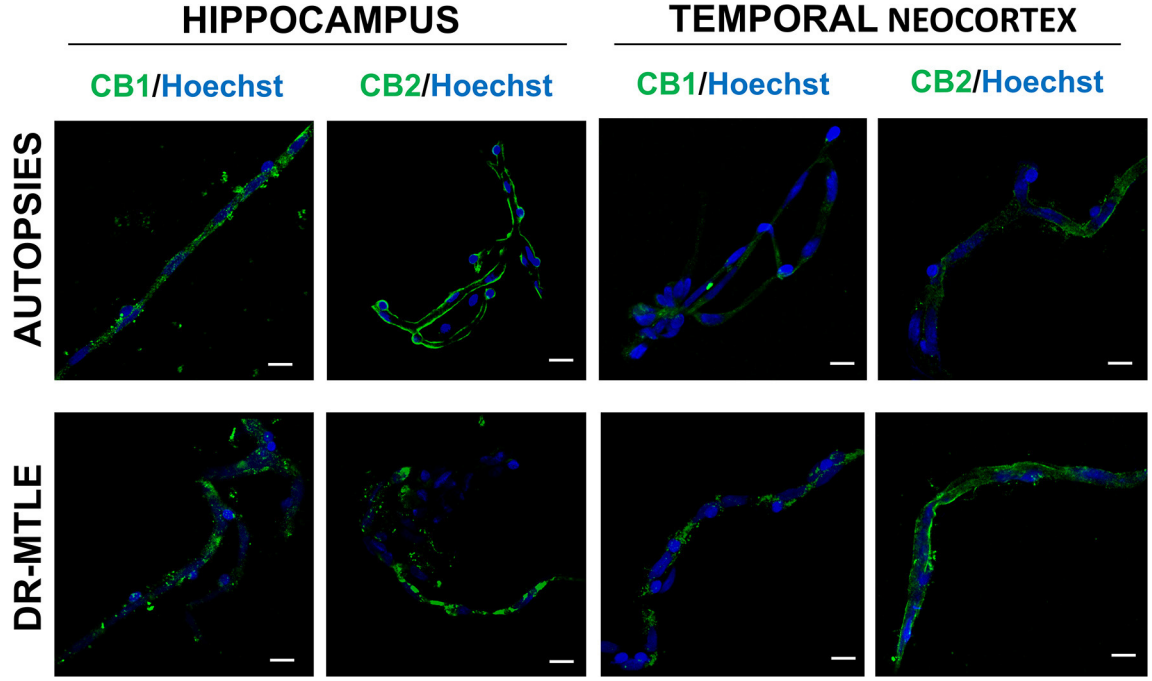


FIGURE 1 | Immunofluorescence staining of CB1 and CB2 receptor proteins in microvessels isolated from the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE. Cell nuclei were stained with Hoechst (in blue). In the group of patients with DR-MTLE, a low staining of CB1 and CB2 receptors can be observed in the hippocampus, whereas both receptors are highly expressed in the temporal neocortex. All scale bars are 20 μm.

(methanandamide cat. M186 Sigma–Aldrich) or CB2 receptors (CB65 cat. 2663 Tocris). The total binding was estimated in the absence of the specific agonist, whereas non-specific binding was obtained in the presence of unlabeled GTPγS (100 μM). The specific binding was calculated by subtracting the non-specific binding from the total binding. The reaction was initiated by adding [^{35}S]-GTPγS and was terminated by filtration of the samples through Whatman GF/B glass fiber filters and using a Brandel M-48 multifilter. Filters were washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4) and 1 mg/mL BSA (pH 7.4) and dried. The filters were immersed in the Sigma Fluor™ scintillation liquid and 500 μL of 0.1% Triton X-100 (cat. 9002-93-1, Sigma–Aldrich). The radioactivity in the filters was estimated in counts per minute using a Beckman detector LS6000SC. The final values expressed in fmol/mg of protein activation were analyzed by non-linear regression to determine the receptor coupling efficiency or maximum stimulation (Emax) induced by the different agonists and its potency (pEC50). Each sample was evaluated by triplicate.

Statistical Analysis

Student *t* test was used to evaluate the results obtained from the different experiments. Pearson correlation coefficients were calculated to determine the possible influence of clinical conditions on the experimental results. Values were expressed as the mean ± SEM. GraphPad Software Inc. (v.6.01 USA) was used for the statistical analysis.

RESULTS

Identification of CB1 and CB2 Receptors in Human Microvessels by Immunofluorescence Staining

The immunofluorescence images and their Z-stack analysis confirmed that receptors CB1 and CB2 proteins were expressed in the brain microvessels obtained from patients with DR-MTLE and non-epileptic autopsies (**Figure 1**; **Supplementary Videos 1–4**). Immunofluorescent double-labeling showed that CB1 and CB2 receptors colocalize with claudin-5, occludin, and ZO-1 proteins (**Figures 2–4**). Immunofluorescent experiments also displayed that CB1 and CB2 receptors colocalize with GFAP and PDGFR-β (**Figure 5**). These results support that both CB receptors are expressed in the human isolated microvessels fragments consisting of MECs with tight junction proteins, astrocyte end feet, and pericytes. Quantification of the immunofluorescence staining images was not carried out as the procedure used was qualitative and based on observations by a histologist.

Protein Expression Levels of CB1 and CB2 Receptors and Functional Coupling to Gαi/o Proteins in Brain Microvessels of Autopsies

Western blot experiments showed that CB1 and CB2 receptors were expressed in brain microvessels of non-epileptic autopsies.

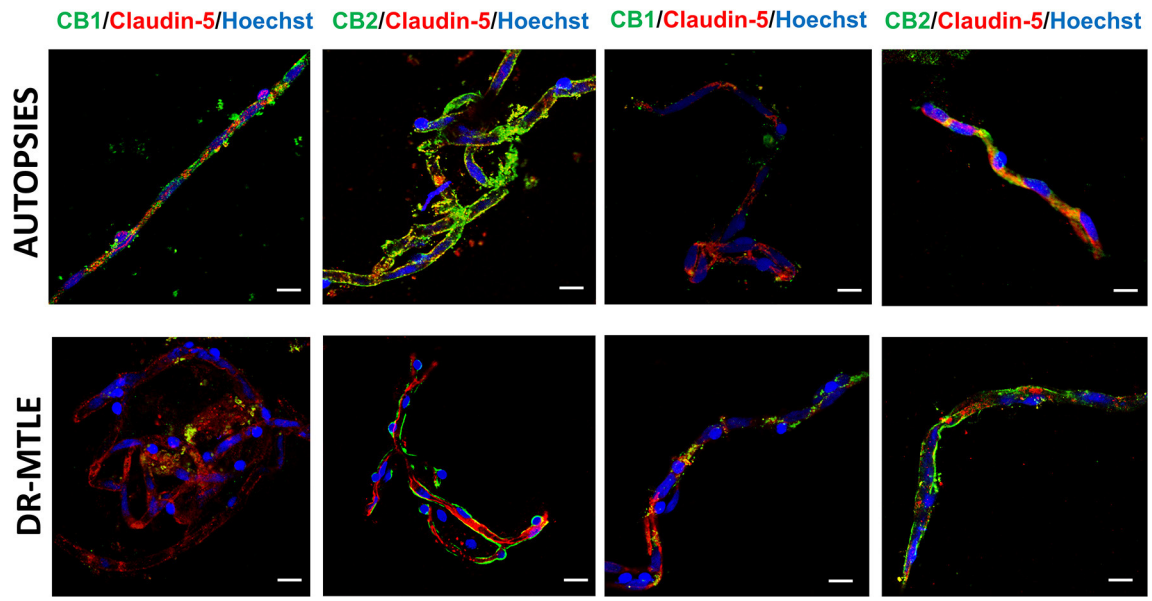


FIGURE 2 | Double-labeling immunofluorescence staining of claudin-5 and CB1 or CB2 in MECs isolated from the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE. Cell nuclei were stained with Hoechst (in blue). Notice the high claudin-5 staining colocalization with low (hippocampus) and high (temporal neocortex) CB1 and CB2 staining in MECs of patients with DR-MTLE. All scale bars are 20 μm.

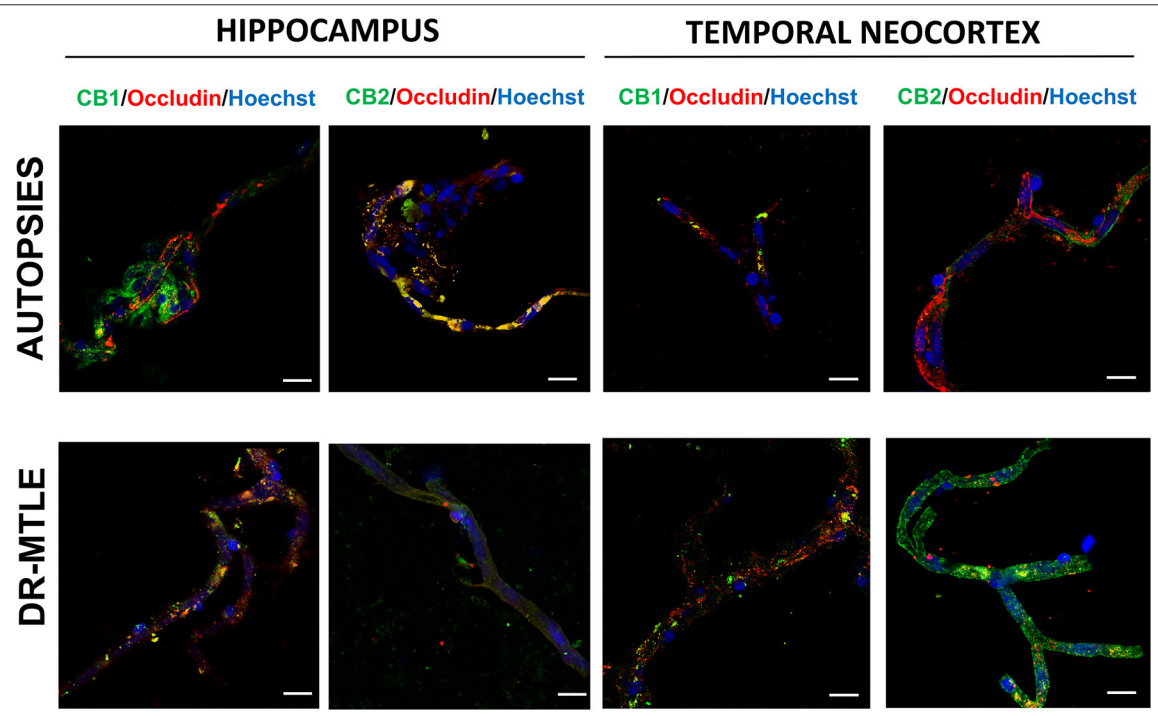


FIGURE 3 | Double-labeling immunofluorescence staining of occludin and CB1 or CB2 in MECs isolated from the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE. Cell nuclei were stained with Hoechst (in blue). Notice the low occludin staining colocalization with low (hippocampus) and high (temporal neocortex) CB1 and CB2 staining in MECs of patients with DR-MTLE. All scale bars are 20 μm.

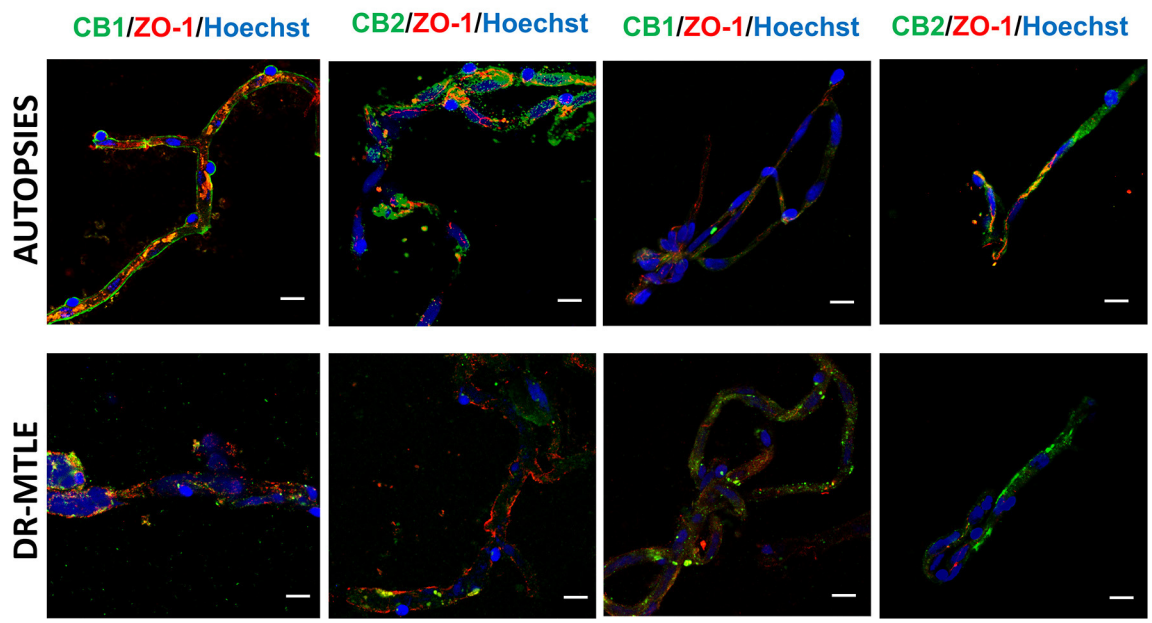


FIGURE 4 | Double-labeling immunofluorescence staining of zonula occludens-1 (ZO-1) and CB1 or CB2 in MECs isolated from the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE. Cell nuclei were stained with Hoechst (in blue). Notice the low ZO-1 staining colocalization with low (hippocampus) and high (temporal neocortex) CB1 and CB2 staining in MECs of patients with DR-MTLE. All scale bars are 20 μm.

Protein expression of CB1 and CB2 receptors was as follows: hippocampus, 0.62 ± 0.09 and 1.25 ± 0.08, respectively; temporal neocortex, 0.93 ± 0.07 and 0.84 ± 0.04, respectively (**Figure 6**). The binding of [^{35}S]-GTPγS induced by the exposure of specific agonists to CB1 and CB2 receptors in microvessels of the hippocampus and temporal neocortex of autopsies was concentration-dependent (**Figure 7**). Emax and pEC_{50} values attained are indicated in **Table 3**. No significant correlations were detected between the results obtained and the age of subjects or the postmortem interval (**Table 4**). According to these results, data obtained from the autopsy group were considered as the control condition.

DR-MTLE Modifies the CB1 and CB2 Protein Expression and Functional Coupling to Gαi/o Proteins in Human Microvessels

Concerning the hippocampal microvessels of patients with DR-MTLE, Western blot experiments showed lower protein expression of CB1 and CB2 receptors (66 and 43%, respectively; $p < 0.001$) (**Figure 6**). This effect was associated with lower potency (pEC_{50}) for CB2 receptors ($p < 0.035$) revealed by the binding assay. Despite this condition, the activation of CB1 and CB2 receptors resulted in a higher Gαi/o protein activation efficiency (Emax) (CB1, 251%, $p < 0.0008$ vs. autopsies; CB2, 255%, $p < 0.0001$ vs. autopsies) (**Figure 7**; **Table 3**).

The correlation analysis showed that CB1 receptors with the highest potency were found in the hippocampal microvessels of the oldest patients ($r = 0.887$, $p < 0.001$ vs. autopsies) and patients with the longest duration of the illness ($r = 0.6509$, $p < 0.02$ vs. autopsies) (**Table 4**).

Concerning the microvessels obtained from the temporal neocortex of patients with DR-MTLE, the Western blot evaluation showed high expression of CB1 receptors (35%, $p < 0.01$ vs. autopsies) (**Figure 6**). This effect was associated with higher efficiency for CB1-induced Gαi/o protein activation (103%, $p < 0.006$ vs. autopsies) revealed by the binding assay (**Figure 7**). Concerning CB2 receptors, protein overexpression was found with Western blot experiments (41%, $p < 0.01$ vs. autopsies). Despite this effect, the efficiency of CB2-induced Gαi/o protein activation was similar to autopsies ($p < 0.2639$ vs. autopsies), but with lower potency ($p < 0.0004$ vs. autopsies) (**Figure 7**; **Table 3**).

Opposite changes were found when hippocampus (diminished expression of CB1 and CB2) and temporal neocortex (increased expression of CB1 and CB2) of patients with DR-MTLE were compared. However, the comparison of the Gαi/o protein activation efficiency induced by the activation of CB1 and CB2 receptors in the epileptic hippocampus (513 ± 93 and 384 ± 49 fmol/mg protein, respectively) was similar when compared with the propagation area (temporal neocortex, 676 ± 105 and 339.7 ± 30 fmol/mg protein, respectively) (**Table 3**). According to this information, the Gαi/o protein activation efficiency in the

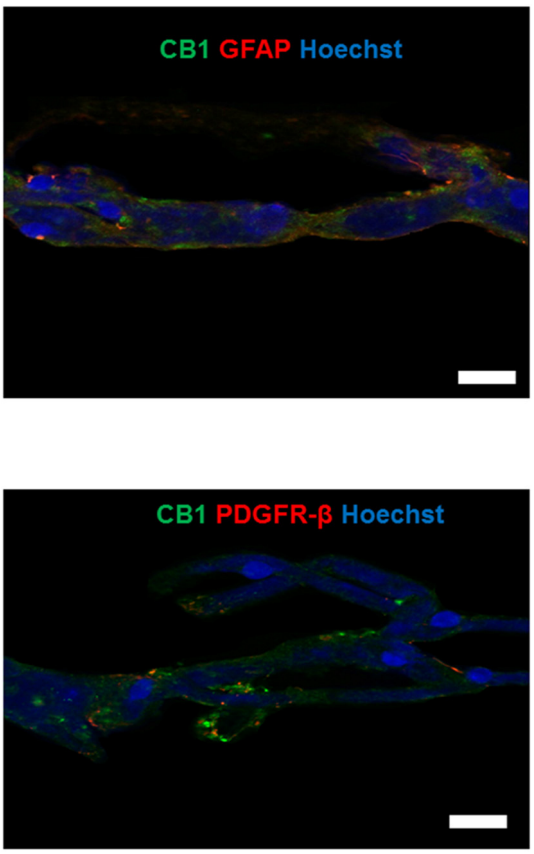


FIGURE 5 | Upper panel: double-labeling immunofluorescence staining of CB1 receptors and GFAP in microvessels isolated from the hippocampus of a non-epileptic autopsy. Lower panel: double-labeling immunofluorescence staining of CB1 receptors and PDGFR-β in microvessels isolated from the temporal neocortex of a patient with DR-MTLE. Cell nuclei were stained with Hoechst (in blue). All scale bars are 20 μm.

hippocampus was similar to the temporal neocortex of patients with DR-MTLE.

Correlation analysis indicated that cortical microvessels of patients with DR-MTLE with the highest seizure frequency showed CB2 receptors with the lowest efficiency ($r = -0.7807$, $p < 0.0077$) and the highest potency ($r = 0.6327$, $p < 0.0496$) to induce Gαi/o protein activation. Correlation analysis also revealed that a highest number of antiseizure drugs received before the epilepsy surgery correlated with the highest protein expression of CB1 in the temporal neocortex and the lowest protein expression of CB2 receptors in the hippocampus (**Table 4**).

DISCUSSION

The results indicated that cerebral microvessels of patients with DR-MTLE and non-epileptic autopsies expressed CB1 and CB2 receptors, and their occupation by specific agonists induced the activation of Gαi/o proteins.

In the present study, we used an optimized protocol that allowed isolating microvessels from frozen brain tissue. The isolated microvessels contained MECs, pericytes (PDGFR-β), and astrocytes end feet (GFAP) (Castañeda-Cabral et al., 2020a; present study). Pericytes and astrocytes are also a source of CB1 and CB2 receptors (Benyó et al., 2016). Then, we cannot discard the influence of these cell elements in the results obtained.

The criteria we used to include autopsy tissue as control were according to the quality of mRNA of each sample. In addition, β-actin was used as a loading control for the Western blot experiments. The expression of mRNA and β-actin was always within the range of detection. We also investigated the correlation of the values obtained from the different experiments with the postmortem interval and the age of the subjects to determine their possible influence on the experimental results. According to our analysis, the postmortem interval or age of autopsies did not influence the results obtained from autopsies. However, we cannot completely confirm that the controls did not have neurodegenerative disorders. This situation represents a limitation of the present study.

Microvessels from the hippocampus and temporal neocortex of patients with DR-MTLE showed a higher efficiency to induce Gαi/o protein activation as a consequence of CB1 agonist exposure when compared to non-epileptic autopsies. In addition, CB1 receptors presented higher potency in the hippocampus of the patients with a longer duration of epilepsy. These findings suggest that the neurotransmission mediated by CB1 receptors is higher in the BBB of patients with DR-MTLE. This effect was detected in the hippocampus despite lower protein expression, suggesting an adaptive change in the proximal downstream signaling mediated by these receptors.

Binding assays revealed a higher efficiency of CB2 receptor-induced Gαi/o protein activation in microvessels of epileptic hippocampus regardless of low protein expression. These results indicated that CB2 receptors are more efficiently coupled to signal transduction mechanisms in the hippocampal BBB of patients with DR-MTLE. The functional coupling of CB2 receptors to Gαi/o proteins in the hippocampal and cortical BBB of patients with DR-MTLE was found to be lower than the potency parameter in the control autopsies. However, correlation analysis indicated that the cortical microvessels of patients with the highest seizure frequency showed the lowest efficiency but the highest potency for CB2 receptor-induced Gαi/o protein activation. These results indicated that the potency of the functional coupling of CB2 receptors might augment as a response to high seizure frequency.

Overall, these results suggest a differential role for CB1 and CB2 receptors in the hippocampal and cortical BBB of patients with DR-MTLE. Indeed, opposite changes were detected when hippocampus (diminished expression of CB1 and CB2) and temporal neocortex (increased expression of CB1 and CB2) were compared. This situation can be associated to clinical conditions. Concerning this issue, we found that the highest number of antiseizure drugs received before the surgery correlated with the lowest protein expression of CB2 receptors in the hippocampus and the highest protein expression of CB1 receptors in the temporal neocortex. However, the Gαi/o protein activation

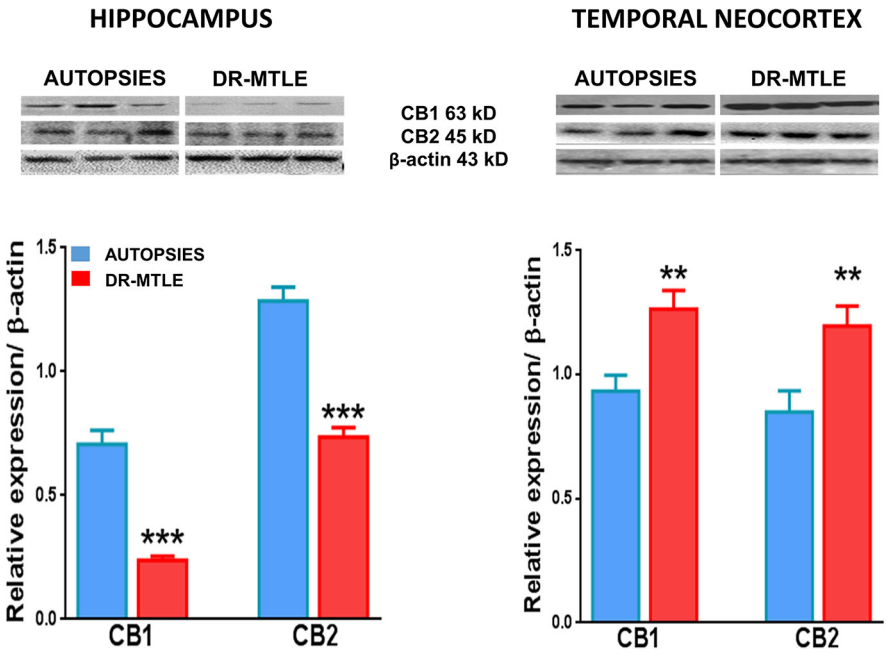


FIGURE 6 | CB1 and CB2 protein expression in human microvessels obtained from non-epileptic autopsies and patients with DR-MTLE. Upper panels: Representative Western blots of CB1 and CB2 receptor proteins in microvessels isolated from the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE. Lower panels: Representation of the densitometric analysis. The results are expressed as a percentage of the optical density (O.D.) ratio of CB1 and CB2-immunostained bands to those of β-actin. Data are presented as mean ± standard error. $^{**}p < 0.01$, $^{***}p < 0.001$.

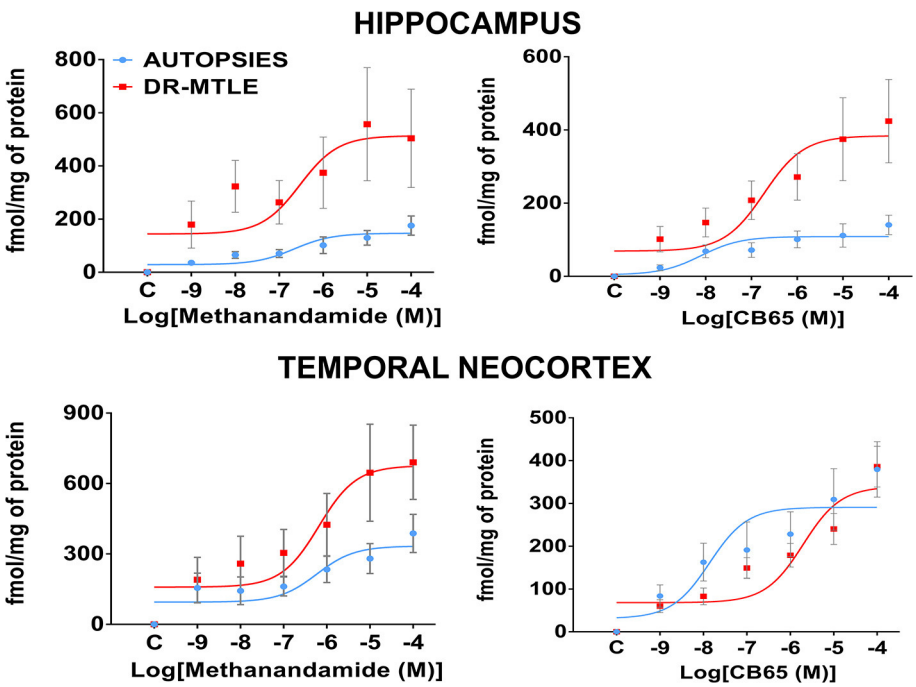


FIGURE 7 | Gαi/o protein activation induced by agonists of CB1 and CB2 receptors in the human microvessels. Specific [^{35}S]-GTPγS binding to cell membranes obtained from microvessels of the hippocampus and the temporal neocortex of non-epileptic autopsies and patients with DR-MTLE as a function of increasing concentrations of methanandamide (CB1 agonist) and CB65 (CB2 agonist). Each point represents the mean ± SE of the individual percentage stimulation over the basal values. Values are expressed as fmol/mg of protein.

efficiency induced by the activation of CB1 and CB2 receptors in the hippocampus was similar when compared with temporal neocortex. Then, we can conclude that the Gαi/o protein activation efficiency in the epileptogenic area (hippocampus) was similar to the propagation zone (temporal neocortex) despite opposite changes in protein expression. Further studies are essential to determine the influence of clinical factors in the expression and function of CB1 and CB2 receptors in patients with epilepsy.

Some studies support the protecting role of CB1 and CB2 receptors in the integrity of BBB (Lu et al., 2008; Zhang et al., 2011; Ramirez et al., 2012). Indeed, the activation of CB1 and CB2 receptors by 2-arachidonoylglycerol, an endocannabinoid, contributes to maintaining the integrity of the BBB following a brain insult (Piro et al., 2018). On the other hand, microvessels of patients with DR-MTLE overexpress VEGF-A and its receptor VEGFR-2 (Castañeda-Cabral et al., 2020a). These changes may facilitate angiogenesis (Marchi and Lerner-Natoli, 2013), neuroinflammation, and increased BBB permeability (Gorter et al., 2015; Baruah et al., 2020). Activation of cannabinoid receptors is considered a strategy to attenuate VEGF signaling and chronic inflammation and thereby diminishing neoangiogenesis (Schley et al., 2009; Staiano et al., 2016; Sathyapalan et al., 2017). However, the high signal transduction efficiency mediated by the activation of cannabinoid receptors (present study) does not avoid BBB impairment associated with epilepsy (van Vliet et al., 2014; Broekaart et al., 2018). The lack of protective effects mediated by CB1 and CB2 receptors in the microvasculature of patients with DR-MTLE can be related to low tissue levels of endocannabinoids such as 2-arachidonoylglycerol (Rocha et al., 2020).

Nanocarriers have been designed to overcome the BBB and deliver drugs to the brain parenchyma (Naz and Siddique, 2020). However, BBB represents a valuable target in the treatment of neurodegenerative disorders. The present study indicates that brain microvessels with high signal transmission mediated by

TABLE 3 | Maximum stimulation (Emax) and potency (pEC_{50}) values for methanandamide (CB1 agonist) and CB65 (CB2 agonist) inducing [^{35}S]GTPγS binding in microvessels of the hippocampus and temporal neocortex of non-epileptic autopsies and patients with DR-MTLE.

Receptor, brain area, and group	Emax fmol/mg prot	*p* <	pEC_{50}	*p* <
CB1 hippocampus autopsies	146 ± 11	0.0008*	−6.65 ± 0.34	0.8687
CB1 hippocampus DR-MTLE	513 ± 93		−6.52 ± 0.7	
CB2 hippocampus autopsies	108 ± 10	0.0001*	−8.1 ± 0.37	0.035*
CB2 hippocampus DR-MTLE	384 ± 49		−6.7 ± 0.49	
CB1 neocortex autopsies	333 ± 45	0.006*	−6.23 ± 0.46	0.9459
CB1 neocortex DR-MTLE	676 ± 105		−6.18 ± 0.5	
CB2 neocortex autopsies	290.9 ± 29	0.2639	−7.86 ± 0.39	0.0002*
CB2 neocortex DR-MTLE	339.7 ± 30		−5.7 ± 0.26	

*Emax, maximum stimulation; pEC50, −log of half-effective concentration (potency); DR-MTLE, drug-resistant mesial temporal lobe epilepsy. *p < 0.05.*

TABLE 4 | Correlations between clinical data and results obtained from the evaluation of CB1 and CB2 receptors.

Clinical data	CB1 receptor						CB2 receptor					
	Protein expression		Emax		pEC_{50}		Protein expression		Emax		pEC_{50}	
	Hipp	Cx	Hipp	Cx	Hipp	Cx	Hipp	Cx	Hipp	Cx	Hipp	Cx
Patients												
Age (years)	−0.145	0.280	−0.026	−0.355	0.887***	−0.200	−0.033	−0.451	0.136	0.083	−0.210	−0.615
Age of seizure onset (years)	0.0452	−0.309	−0.406	−0.277	−0.070	0.204	0.275	0.127	−0.499	−0.377	0.040	0.037
Duration of epilepsy (years)	−0.123	0.224	0.191	−0.136	0.651*	−0.399	−0.273	−0.422	0.379	0.330	−0.075	−0.473
Frequency of Seizures (per month)	0.287	−0.051	−0.079	0.166	−0.445	0.115	−0.106	0.608	−0.130	−0.781**	0.278	0.633*
No. of ASD before surgery	0.103	0.644*	0.531	−0.188	−0.349	0.167	−0.643*	−0.265	0.318	0.064	0.092	0.231
Autopsies												
Age (years)	0.351	0.542	−0.178	−0.325	0.357	−0.297	−0.329	−0.428	−0.085	0.134	0.235	−0.447
PMI (h)	0.101	0.120	−0.274	0.409	0.472	0.370	−0.144	−0.129	0.426	−0.488	0.066	0.598

ASD, antiseizure drugs; Emax, maximum stimulation; Hipp, hippocampus; Cx, cortex; pEC50, −log of half-effective concentration (potency); PMI, postmortem interval. Values represent Pearson correlation coefficient value (r).
**p < 0.05, **p < 0.01, ***p < 0.001.*

CB1 and CB2 receptors may represent a novel therapeutic target to preserve the integrity of BBB of patients with DR-MTLE.

On the other hand, experimental evidence suggests that the activation of CB1 and CB2 receptors could induce neurotoxicity, a condition that may depend on the chronicity of the disorder (Di Marzo, 2008; Fowler et al., 2010; Vendel and de Lange, 2014). Neurodegenerative disorders studies also indicate that the activation of CB1 and CB2 receptors may augment the migration of immune cells (Miller and Stella, 2008) and thus facilitate oxidative stress (Mukhopadhyay et al., 2010), inflammation, and the disruption of BBB (Persidsky et al., 2006). Studies indicate that activation of CB1 and CB2 receptors augments leukocyte accumulation, inflammation, and neovascularization (Guabiraba et al., 2013). Our results revealed that patients with DR-MTLE showed a high signal transduction efficiency mediated by the activation of CB1 and CB2 receptors, a condition that may facilitate inflammation and neovascularization in the hippocampus and temporal neocortex. Further studies are essential to determine if the high-efficiency coupling to the signal transduction mechanisms mediated by CB1 and CB2 receptors facilitates the impairment of the BBB in patients with DR-MTLE.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Instituto Nacional de Neurologia and Neurocirugia and Centro de Investigación y de Estudios Avanzados. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LR designed the study and organized the manuscript. MN-L carried out the Western blot and binding [^{35}S]-GTPγS assay experiments. JC-C carried out the immunofluorescence experiments. MV-D, FW, and MD designed the procedure for isolating microvessels from the frozen human brain. VS-V participated in the Western blot experiments. SO-S participated in the analysis of immunofluorescence experiments. RG-G obtained and evaluated the autopsy samples. IM-J identified and assessed the patients with epilepsy. MA-V did the neurosurgery of patients. FC-C participated in the [^{35}S]-GTPγS assay experiments. All authors contributed to the article and approved the submitted version.

FUNDING

MN-L received support from Consejo Nacional de Ciencia y Tecnología (CONACyT) (scholarship 347414). LR received support from CONACyT (grant A3-S-26782).

ACKNOWLEDGMENTS

We thank the technical assistance of Dr. Vadim Pérez Koldenkova from the National Laboratory for Advanced Microscopy-IMSS, Centro Médico Nacional Siglo XXI.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.611780/full#supplementary-material

REFERENCES

Abood, M. E., and Martin, B. R. (1996). Molecular neurobiology of the cannabinoid receptor. *Int. Rev. Neurobiol.* 39, 197–221. doi: 10.1016/S0074-7742(08)60667-4

Baruah, J., Vasudevan, A., and Köhling, R. (2020). Vascular integrity and signaling determining brain development, network excitability, and epileptogenesis. *Front. Physiol.* 10:1583. doi: 10.3389/fphys.2019.01583

Benyó, Z., Ruisanchez, É., Leszl-Ishiguro, M., Sándor, P., and Pacher, P. (2016). Endocannabinoids in cerebrovascular regulation. *Am. J. Physiol. Heart Circ. Physiol.* 310, H785–H801. doi: 10.1152/ajpheart.00571.2015

Broekaart, D. W. M., Anink, J. J., Baayen, J. C., Idema, S., de Vries, H. E., Aronica, E., et al. (2018). Activation of the innate immune system is evident throughout epileptogenesis and is associated with blood-brain barrier dysfunction and seizure progression. *Epilepsia* 59, 1931–1944. doi: 10.1111/epi.14550

Castañeda-Cabral, J. L., Colunga-Durán, A., Ureña-Guerrero, M. E., Beas-Zárate, C., Nuñez-Lumbreras, M. L. A., Orozco-Suárez, S., et al. (2020a). Expression of VEGF- and tight junction-related proteins in the neocortical microvasculature of patients with drug-resistant temporal lobe epilepsy. *Microvasc. Res.* 132:104059. doi: 10.1016/j.mvr.2020.104059

Castañeda-Cabral, J. L., Ureña-Guerrero, M. E., Beas-Zárate, C., et al. (2020b). Increased expression of proinflammatory cytokines and iNOS in the neocortical microvasculature of patients with temporal lobe epilepsy. *Immunol. Res.* 68, 169–176. doi: 10.1007/s12026-020-09139-3

Catani, V. M., and Gasperi, V. (2016). Assay of CB1 receptor binding. *Methods Mol. Biol.* 1412, 41–55. doi: 10.1007/978-1-4939-3539-0_5

Di Marzo, V. (2008). Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discov.* 7, 438–455. doi: 10.1038/nrd2553

Fisher, R. (2015). Redefining epilepsy. *Curr. Opin. Neurol.* 28, 130–135. doi: 10.1097/WCO.0000000000000174

Fowler, C. J., Rojo, M. L., and Rodriguez-Gaztelumendi, A. (2010). Modulation of the endocannabinoid system: neuroprotection or neurotoxicity? *Exp. Neurol.* 224, 37–47. doi: 10.1016/j.expneurol.2010.03.021

Golech, S. A., McCarron, R. M., Chen, Y., Bembry, J., Lenz, F., Mechoulam, R., et al. (2004). Human brain endothelium: coexpression and function of vanilloid and endocannabinoid receptors. *Brain Res. Mol. Brain Res.* 132, 87–92. doi: 10.1016/j.molbrainres.2004.08.025

Gorter, J. A., van Vliet, E. A., and Aronica, E. (2015). Status epilepticus, blood-brain barrier disruption, inflammation, and epileptogenesis. *Epilepsy Behav.* 49, 13–16. doi: 10.1016/j.yebeh.2015.04.047

Guabiraba, R., Russo, R. C., Coelho, A. M., Ferreira, M. A., Lopes, G. A., Gomes, A. K., et al. (2013). Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation, and neovascularization in a model of sponge-induced inflammatory angiogenesis. *Inflamm. Res.* 62, 811–821. doi: 10.1007/s00011-013-0638-8

Hawkins, B. T., and Davis, T. P. (2005). The blood-brain barrier neurovascular unit in health and disease. *Pharmacol. Rev.* 57, 173–185. doi: 10.1124/pr.57.2.4

Kook, S. Y., Hong, H. S., Moon, M., Ha, C. M., Chang, S., and Mook-Jung, I. (2012). Aβ1–4_2-RAGE interaction disrupts tight junctions of the blood-brain barrier via Ca^{2+}-calcineurin signaling. *J. Neurosci.* 32, 8845–8854. doi: 10.1523/JNEUROSCI.6102-11.2012

Lu, T. S., Avraham, H. K., Seng, S., Tachado, S. D., Koziel, H., Makriyannis, A., et al. (2008). Cannabinoids inhibit HIV-1 Gp120-mediated insults in brain microvascular endothelial cells. *J. Immunol.* 181, 6406–6416. doi: 10.4049/jimmunol.181.9.6406

Marchi, N., and Lerner-Natoli, M. (2013). Cerebrovascular remodeling and epilepsy. *Neuroscientist* 19, 304–312. doi: 10.1177/1073858412462747

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 346, 561–564. doi: 10.1038/346561a0

Miller, A. M., and Stella, N. (2008). CB2 receptor-mediated migration of immune cells: it can go either way. *Br. J. Pharmacol.* 153, 299–308. doi: 10.1038/sj.bjp.0707523

Molina-Holgado, F., Molina-Holgado, E., Guaza, C., and Rothwell, N. J. (2002). Role of CB_1 and CB_2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. *J. Neurosci. Res.* 67, 829–836. doi: 10.1002/jnr.10165

Mukhopadhyay, P., Rajesh, M., Bátkai, S., Patel, V., Harvey-White, J., Mukhopadhyay, B., et al. (2010). CB1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes. *Cardiovasc. Res.* 85, 773–784. doi: 10.1093/cvr/cvp369

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 365, 61–65. doi: 10.1038/365061a0

Naz, F., and Siddique, Y. H. (2020). Nanotechnology: its application in treating neurodegenerative diseases. *CNS Neurol. Disord. Drug Targets*. 19:1. doi: 10.2174/1871527319666200916121515

Persidsky, Y., Ramirez, S. H., Haorah, J., and Kanmogne, G. D. (2006). Blood-brain barrier: structural components and function under physiologic and pathologic conditions. *J. Neuroimmune Pharmacol.* 1, 223–236. doi: 10.1007/s11481-006-9025-3

Piro, J. R., Suidan, G. L., Quan, J., Pi, Y., O'Neill, S. M., Ilardi, M., et al. (2018). Inhibition of 2-AG hydrolysis differentially regulates blood brain barrier permeability after injury. *J. Neuroinflammation* 15, 1–142. doi: 10.1186/s12974-018-1166-9

Ramirez, S. H., Haskó, J., Skuba, A., Fan, S., Dykstra, H., McCormick, R., et al. (2012). Activation of cannabinoid receptor 2 attenuates leukocyte-endothelial cell interactions and blood-brain barrier dysfunction under inflammatory conditions. *J. Neurosci.* 32, 4004–4016. doi: 10.1523/JNEUROSCI.4628-11.2012

Rocha, L., Cinar, R., Guevara-Guzmán, R., Alonso-Vanegas, M., San-Juan, D., Martínez-Juárez, I., et al. (2020). Endocannabinoid system and cannabinoid 1 receptors in patients with pharmacoresistant temporal lobe epilepsy and comorbid mood disorders. *Front. Behav. Neurosci.* 14, 1–10. doi: 10.3389/fnbeh.2020.00052

Sathyapalan, T., Javed, Z., Kilpatrick, E. S., Coady, A. M., and Atkin, S. L. (2017). Endocannabinoid receptor blockade increases vascular endothelial growth factor and inflammatory markers in obese women with polycystic ovary syndrome. *Clin. Endocrinol. (Oxf.)* 86, 384–387. doi: 10.1111/cen.13239

Schley, M., Ständer, S., Kerner, J., Vajkoczy, P., Schüpfer, G., Dusch, M., et al. (2009). Predominant CB2 receptor expression in endothelial cells of glioblastoma in humans. *Brain Res. Bull.* 79, 333–337. doi: 10.1016/j.brainresbull.2009.01.011

Staiano, R. I., Loffredo, S., Borriello, F., Iannotti, F. A., Piscitelli, F., Orlando, P., et al. (2016). Human lung-resident macrophages express CB1 and CB2 receptors whose activation inhibits the release of angiogenic and lymphangiogenic factors. *J. Leukoc. Biol.* 99, 531–540. doi: 10.1189/jlb.3HI1214-584R

Stanimirovic, D., and Friedman, A. (2012). Pathophysiology of the neurovascular unit: disease cause or consequence? *J. Cereb. Blood Flow Metab.* 32, 1207–1221. doi: 10.1038/jcbfm.2012.25

van Vliet, E. A., Aronica, E., and Gorter, J. A. (2014). Role of blood-brain barrier in temporal lobe epilepsy and pharmacoresistance. *Neuroscience* 277, 455–473. doi: 10.1016/j.neuroscience.2014.07.030

Vendel, E., and de Lange, E. C. (2014). Functions of the CB1 and CB 2 receptors in neuroprotection at the level of the blood-brain barrier. *Neuromolecular Med.* 16, 620–642. doi: 10.1007/s12017-014-8314-x

Veszelka, S., Pásztói, M., Farkas, A. E., Krizbai, I., Ngo, T. K., Niwa, M., et al. (2007). Pentosan polysulfate protects brain endothelial cells against bacterial lipopolysaccharide-induced damages. *Neurochem. Int.* 50, 219–228. doi: 10.1016/j.neuint.2006.08.006

Zhang, H., Hilton, D. A., Hanemann, C. O., and Zajicek, J. (2011). Cannabinoid receptor and N-acyl phosphatidylethanolamine phospholipase D–evidence for altered expression in multiple sclerosis. *Brain Pathol.* 21, 544–557. doi: 10.1111/j.1750-3639.2011.00477.x

Zong, Y., Zhou, X., Cheng, J., Yu, J., Wu, J., and Jiang, C. (2017). Cannabinoids regulate the diameter of pericyte-containing retinal capillaries in rats. *Cell. Physiol. Biochem.* 43, 2088–2101. doi: 10.1159/000484193

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 22 January 2021
doi: 10.3389/fnbeh.2020.612624

Behavioral and Molecular Effects Induced by Cannabidiol and Valproate Administration in the GASH/Sal Model of Acute Audiogenic Seizures

Giselda Cabral-Pereira[1,2], *David Sánchez-Benito*[1,2], *Sandra M. Díaz-Rodríguez*[1,2,3], *Jaime Gonçalves*[1,2,3], *Consuelo Sancho*[1,2,4], *Orlando Castellano*[1,2,3], *Luis J. Muñoz*[5], *Dolores E. López*[1,2,3] *and Ricardo Gómez-Nieto*[1,2,3]*

[1] *Institute of Neuroscience of Castilla y León (INCYL), University of Salamanca, Salamanca, Spain,* [2] *Institute for Biomedical Research of Salamanca (IBSAL), University of Salamanca, Salamanca, Spain,* [3] *Department of Cell Biology and Pathology, Faculty of Medicine, University of Salamanca, Salamanca, Spain,* [4] *Department of Physiology and Pharmacology, Faculty of Medicine, University of Salamanca, Salamanca, Spain,* [5] *Animal Research and Service Center, University of Salamanca, Salamanca, Spain*

OPEN ACCESS

Edited by:
Simona Cabib,
Sapienza University of Rome, Italy

Reviewed by:
Carlos De Cabo,
Complejo Hospitalario Universitario de Albacete, Spain
Hideo Hagihara,
Fujita Health University, Japan

***Correspondence:**
Ricardo Gómez-Nieto
richard@usal.es

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *30 September 2020*
Accepted: *09 December 2020*
Published: *22 January 2021*

Citation:
Cabral-Pereira G, Sánchez-Benito D, Díaz-Rodríguez SM, Gonçalves J, Sancho C, Castellano O, Muñoz LJ, López DE and Gómez-Nieto R (2021) Behavioral and Molecular Effects Induced by Cannabidiol and Valproate Administration in the GASH/Sal Model of Acute Audiogenic Seizures. Front. Behav. Neurosci. 14:612624. doi: 10.3389/fnbeh.2020.612624

Despite evidence that supports cannabidiol (CBD) as an anticonvulsant agent, there remains controversy over the antiseizure efficacy, possible adverse effects, and synergistic interactions with classic antiepileptics such as valproate (VPA). The genetic audiogenic seizure hamster from the University of Salamanca (GASH/Sal) is a reliable experimental model of generalized tonic–clonic seizures in response to intense sound stimulation. The present study examines the behavioral and molecular effects of acute and chronic intraperitoneal administrations of VPA (300 mg/kg) and CBD (100 mg/kg) on the GASH/Sal audiogenic seizures, as well as the coadministration of both drugs. The GASH/Sal animals were examined prior to and after the corresponding treatment at 45 min, 7 days, and 14 days for seizure severity and neuroethology, open-field behaviors, body weight variations, and various hematological and biochemical parameters. Furthermore, the brain tissue containing the inferior colliculus (so-called epileptogenic nucleus) was processed for reverse transcription–quantitative polymerase chain reaction analysis to determine the treatment effects on the gene expression of neuronal receptors associated with drug actions and ictogenesis. Our results indicated that single dose of VPA helps prevent the animals from getting convulsions, showing complete elimination of seizures, whereas 7 days of chronic VPA treatment had few effects in seizure behaviors. Acute CBD administration showed subtle attenuation of seizure behaviors, increasing seizure latency and decreasing the duration of the convulsion phase, but without entirely seizure abolition. Chronic CBD treatments had no significant effects on sound-induced seizures, although some animals slightly improved seizure severity. Acute and chronic CBD treatments have no significant adverse effects on body weight, hematological parameters, and liver function, although locomotor activity was reduced. The combination of VPA and CBD did not alter the therapeutic outcome of the VPA monotherapy, showing no apparent synergistic effects. As compared

to sham animals, chronic treatments with CBD caused abnormal mRNA expression levels for *Trpv1*, *Adora1*, *Slc29a1*, and *Cnr1* genes, whereas no differences in gene expression were found for *Htr1a* and *Sigmar1*. Our study shed light on the behavioral and molecular effects of CBD and VPA on the GASH/Sal model and constituted the basis to develop further studies on the pharmacological effects of CBD and its interactions with other anticonvulsants.

Keywords: animal models, antiepileptic drugs, cannabis, epilepsy, gene expression, inferior colliculus (IC), valproic acid, drug interactions

INTRODUCTION

Epilepsy syndromes are considered neurological disorders characterized by an enduring predisposition to generate epileptic seizures that can be accompanied by a wide spectrum of behavioral and neuropsychiatric conditions (Kanner et al., 2012; Fisher et al., 2014). Among the myriad of epilepsy symptoms, seizures are one of the most important and notable signs that are associated with the abnormal excessive or synchronous neuronal activity in the brain (Fisher et al., 2005). Despite the numerous antiseizure drugs with different modes of action, a large number of patients confirm pharmacoresistance, that is, the failure to achieve seizure control with a trial of an anticonvulsant medication given at the appropriate dosage (Kwan et al., 2009; Sharma et al., 2015). Uncontrolled seizure is closely linked to an increased risk of pharmacoresistance that is the major responsible for boosting mortality, and no promising therapeutic treatments are currently available to prevent the emergence of pharmacoresistance (reviewed in Sharma et al., 2015). Thus, the high incidence of epilepsy (more than 60 million people worldwide) and the increasing number of antiseizure drug–resistant patients (∼40% of the cases) call for the encouragement of evaluating new anticonvulsant compounds. Toward this end, experimental animal models of epilepsies and seizures have proven to be one of the biggest assets in the research programs that focused on the discovery and development of new antiseizure drugs (Nef, 2001; Löscher, 2011). In this context, cannabidiol (CBD), the major non-psychoactive phytocannabinoid present in the *Cannabis sativa* plant, has been increasingly attracting therapeutic interest, particularly because of its anticonvulsive actions and favorable side effect profile in preclinical models of seizures and epilepsy (Devinsky et al., 2014; Ibeas Bih et al., 2015). Among all of the animal models used to study the anticonvulsant properties of CBD, there is less evidence for CBD's effects on audiogenic seizure (AGS) models of genetic origin (reviewed in Lazarini-Lopes et al., 2020). AGSs are frequent in rodents that exhibited generalized clonic or tonic–clonic convulsive muscle contractions caused by excessive or abnormal neuronal firing in response to intense sound stimulation. It is well-established that acutely induced AGSs depend on brainstem substrates (Faingold, 1999; Garcia-Cairasco, 2002), whereas repetitions of AGS (after an audiogenic kindling protocol) further recruit limbic brain regions (Romcy-Pereira and Garcia-Cairasco, 2003; Dutra et al., 2009). In contrast to traditional models of epilepsy induced by chemical or electrical means, the genetically AGS rodents offer several advantages: (1) the AGS susceptibility is inherited and does not require any experimental procedure to become susceptible, thus avoiding incompatibilities with antiseizure drugs administration; (2) the innate occurring seizures can be elicited at will by an investigator, as the specific trigger is a sound; and (3) the substantial characterization of behavioral, cellular, and molecular alterations available that potentiates their usefulness in antiepileptic drug screening and elucidating mechanisms underlying ictogenesis (reviewed in Kandratavicius et al., 2014; Bosque et al., 2019). The genetic AGS hamster from Salamanca (GASH/Sal) exhibits susceptibility to sound-induced seizures and is the only susceptible hamster strain that is scientific research available (Muñoz et al., 2017). The GASH/Sal has recently gathered attention for the amount of interesting studies related to the neuroethological (Barrera-Bailón et al., 2013, 2017), electrophysiological (Carballosa-Gonzalez et al., 2013), neurochemical (Prieto-Martín et al., 2017), molecular (López-López et al., 2017; Díaz-Casado et al., 2020; Díaz-Rodríguez et al., 2020), and morphological (Sánchez-Benito et al., 2017, 2020) substrates underlying AGS. GASH/Sal animals, from 2 to 4 months of age at which susceptibility reached its maximum, undergoes generalized tonic–clonic seizures that are characterized by a short latency period after loud acoustic stimulation, followed by phases of wild running, convulsions, and stupor (Muñoz et al., 2017). Anticonvulsant drugs such as valproic acid (VPA), phenobarbital, and lamotrigine are highly effective in suppressing sound-induced seizures in the GASH/Sal (Barrera-Bailón et al., 2013, 2017), and hence GASH/Sal animals are suitable to examine the efficacy of antiepileptic agents (Werner and Coveñas, 2017). The overall goal of the present study was to make a preliminary determination of the potential anticonvulsant effects of CBD treatment in the GASH/Sal model following acute and chronic intraperitoneal administration, as well as the possible adverse effects on the hematologic profile, liver function, body weight, general locomotor activity levels, and emotionality. As no data exist regarding synergistic combinations of anticonvulsant agents with CBD in genetically AGS models, we also study the effects of concomitant treatment with VPA, an effective antiepileptic drug in the GASH/Sal model (Barrera-Bailón et al., 2013). Thus, by using a neuroethological approach (Garcia-Cairasco et al., 1996, 2004), we carried out a quantitative behavioral analysis of the AGS phases after the treatments with VPA, CBD, or the combination of both drugs. As occurred in other AGS rodent models, activation of the auditory pathway

is required for seizure development in the GASH/Sal model (Garcia-Cairasco, 2002; Muñoz et al., 2017). In fact, the innate AGS susceptibility of the GASH/Sal model lies in an upward spread of abnormal glutamatergic transmission throughout the primary acoustic pathway to the inferior colliculus, a critical integration center in the auditory midbrain that is considered the epileptogenic region (Sánchez-Benito et al., 2020). Furthermore, recent studies have found disrupted gene expression profiles in the inferior colliculus of the GASH/Sal under free-seizure conditions and after sound-induced seizures that are associated with seizure susceptibility and altered regulation of neuronal excitability (López-López et al., 2017; Damasceno et al., 2020; Díaz-Casado et al., 2020; Díaz-Rodríguez et al., 2020). In view of this, it becomes of interest to compare patterns of gene expression after each of the treatments in the inferior colliculus of the GASH/Sal, particularly when CBD is coadministered with VPA. Among the large variety of protein channel and receptors targeted by the CBD (reviewed in Franco and Perucca, 2019), we selected those involved in the regulation of neuronal excitability and mechanism of CBD action. At the completion of each chronic drug treatment, we therefore determined the gene expression levels of the following neuronal receptors: the transient receptor potential of vanilloid type 1 (*Trpv1*), the 5-hydroxytryptamine (serotonin) receptor 1A (*5-Htr1a*), the sigma non-opioid intracellular receptor 1 (*Sigmar1*), the adenosine A1 receptor (*Adora1*), the equilibrative nucleoside transporter 1 (*Slc29a1*), and the cannabinoid receptor 1 (*Cnr1*). Overall, our results provide valuable information on the behavioral and molecular effects of VPA, CBD, and the combination of both drugs on the acute AGS of the GASH/Sal model. From a technical point of view, the present study is highly valuable as it combines a multitechnical approach to generate correlated results at behavioral and molecular levels that constituted the basis for further analysis of the CBD effects and its interactions with other anticonvulsant agents.

MATERIALS AND METHODS

Experimental Animals and Ethical Statement

A total of 38 GASH/Sal animals from the inbred strain maintained at the vivarium of the University of Salamanca (USAL, Spain) were used in this study. All the GASH/Sal animals were males, 4 months of age, as it is stated that the GASH/Sal strain exhibits the maximum susceptibility to seizures from 2 to 4 months of age (Muñoz et al., 2017). All procedures and experimental protocols were performed in accordance with the guidelines of the European Communities Council Directive (2010/63/UE) for the care and use of laboratory animals and approved by the Bioethics Committee of the University of Salamanca (application no. 380). All efforts were made to minimize the number of animals and their suffering. The animals were maintained in Eurostandard Type III cages (Tecniplast, Italy), with Lignocel bedding (Rettenmaier Iberica), 14/10 light–dark cycle, and 22–24°C room temperature with *ad libitum* access to food (Tecklad Global 2918 irradiated diet) and water. Communities of three to four animals were housed in groups until the beginning of the study, when they were individually caged 24 h prior to drug administration. To avoid the influence of the circadian rhythm, each of the different tests was performed at the same time of day.

Experimental Design and Drug Administration

The series of experiments were performed at the same time and by the same experimenters. The experimental design is shown in **Figure 1**. The GASH/Sal animals were divided into four groups (six animals per group): (1) GASH/Sal animals that received the vehicle (Cremophor-based) treatment (the so-called sham group); (2) GASH/Sal animals that received VPA treatment at 300 mg/kg; (3) GASH/Sal animals that received CBD treatment at 100 mg/kg; and (4) GASH/Sal animals with concomitant treatment of VPA (300 mg/kg) and CBD (100 mg/kg). Prior to the initiation of corresponding vehicle or drug treatments, all animals were subjected to the different tests (open-field and blood biochemical analyses) to measure the baseline pre-treatment parameters, and they further underwent the seizure stimulation protocol to confirm that all of them developed a complete AGS (**Figure 1**). The drugs used in the experiments were stored in a freezer (at ∼-20°C), protected from light and freshly prepared immediately prior to injection. The VPA (Depakine®, Sanofi Aventis) has been successfully used in a previous study showing anticonvulsant effects in GASH/Sal animals at its peak plasma concentrations, which was 30 to 45 min after the intraperitoneal injections (Barrera-Bailón et al., 2013). According to this study, VPA had 75% protective effect against seizures at doses of 300 mg/kg; therefore, we precisely selected this dosage to determine possible synergistic (additive or antagonistic) effects when administered in combination with CBD. CBD, extracted from cannabis plants, was subsequently purified and provided by RiverForce Partners Inc. (Boston, MA, USA). Purity for the phytocannabinoid was >99% (according to the supplier's information certificate). The CBD powder was suspended in Cremophor® RH 40/ethanol/saline in a ratio of 1:2:17 and mixed by Vortex shaker followed by sonication. The CBD dose of 100 mg/kg was selected based on the results of a separate experiment performed in 14 GASH/Sal animals, in which we determined the timing of the maximal levels of CBD by measuring the serum concentration of the drug after a single intraperitoneal administration. The results of this pharmacokinetic evaluation are shown in **Figure 3**. All vehicle and drug treatments were injected intraperitoneally. To determine the effects of the corresponding acute drug administrations, all groups of animals were examined 45 min after the first intraperitoneal injection for AGS severity (**Figure 1**). To determine the effects of chronic drug administrations, the animals with VPA treatment received repeated intraperitoneal injections of VPA at a dosage of 300 mg/kg every 24 h for 2 weeks, the animals with CBD treatment received repeated intraperitoneal injections of CBD at a dosage of 100 mg/kg every 12 h for 2 weeks, and the animals with the combined CBD and VPA treatment received repeated intraperitoneal injections of VPA and CBD at the corresponding

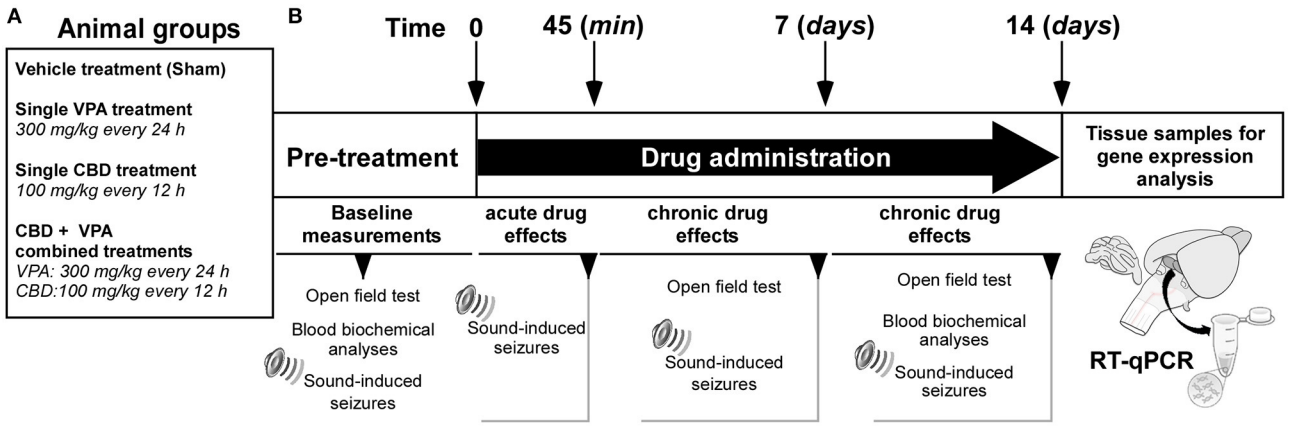


FIGURE 1 | Experimental design and timeline. **(A)** Animal groups and drug treatments used in the study. **(B)** Experimental time schedules. Basal measurements were conducted prior to the initiation of the treatments. Acute effects of each drug treatment on sound-induced seizures were evaluated 45 min after the first intraperitoneal administration. Chronic effects of each drug treatment on sound-induced seizures were evaluated after 7 and 14 days of drug administration. Tissue samples for gene expression analyses in the inferior colliculus were obtained at the final time point.

dosage used in the single drug treatments (**Figure 1**). In all animal groups at 7 days, effects of each drug treatment were evaluated for open-field test and seizure severity. Furthermore, the effects of chronic drug administrations were also evaluated in all animal groups at 14 days (the final time point) for open-field test, blood biochemical analyses, and AGS severity. All these tests for evaluating the effects of chronic drug administration were carried out 8 h after the first intraperitoneal administration of the day (at 7:30 a.m.) and in an orderly manner leaving 24 h in between. Tissue samples for gene expression analyses in the inferior colliculus were obtained at the final time point (**Figure 1**).

Open-Field Test

The open-field test was used for assessment of exploratory behavior and general locomotor activity in the GASH/Sal before and after the treatments with vehicle, VPA, CBD, or both CBD and VPA (CBD+VPA) at the protocol-defined time points, detailed in **Figure 1**. The protocols of the open-field test were applied the same way for all treatment groups, and the experiments were conducted between 3:30 and 5:30 p.m. at the same light intensity and temperature conditions, in which the animals were housed. The handling of the animals was carried out by the same investigator, and the field was carefully cleaned with a disinfectant alcohol-based solution and thoroughly dried after each animal. The test was performed prior to the loud sound stimulation, and every GASH/Sal animal underwent video recording during every open-field assessment. The open-field box was made of white acrylic plastic with a circular arena of 80 cm in diameter and rounded by a 30 cm-high wall. The arena was subdivided into three zones: central, intermediate, and external, each occupying one third of the total surface area. At the time of testing, individual animals were removed from its home cage and immediately placed into the center of the field. The performance and paths of the animals were recorded for 10 min using a digital video camera set at 1.20 m above the ground. The ANY-maze software (Stoelting Co., v. 6.16) was used to automatically analyze the animal's locomotor and exploratory activity (time spent in activity, distance traveled, and number of rearings) and emotional performance (time spent in central zone and number of groomings).

Blood Extraction and Sample Preparation

Sample collection and preparation were carried out following a similar procedure previously described by Barrera-Bailón et al. (2013) at protocol-defined time points, detailed in **Figure 1**. The blood was extracted from the cranial vena cava under inhalation anesthesia (induction: 4% isoflurane and 1 L/min O_2; maintenance: 3% isoflurane and 0.4 L/min O_2), immediately stored in plastic tubes, and right after obtaining the sample, equal volume of blood was replaced by receiving subcutaneous hydration of 0.9% saline. At each time point, a specific amount of blood was prepared according to the type and nature of the analysis to be carried out. The 200 μL samples of blood were drawn for measuring the CBD serum levels and kept at room temperature for 1 h and subsequently on ice during 30 min until a clot was formed. Then, these blood samples were centrifuged for 20 min at 4,000 rpm at 4°C. The supernatant (serum) was collected in aliquots of 100 μL and frozen until the pharmacokinetic analysis of CBD concentrations. For the hematological assessment, 500 μL of blood was collected in K3-EDTA–coated sample bottles and immediately analyzed after drawing each sample to prevent errors in cell counting. For the analysis of blood biochemistry, the collected volume was 400 μL that was kept in the plastic tube, without anticoagulant substances, at room temperature for 30 min until a clot was formed. Subsequently, these blood samples were centrifuged at 4°C for 10 min at 10,000 rpm, and then the serum was stored at −20°C until the moment of the biochemical analysis.

Serum Concentrations of CBD

A separate set of experiments was initially carried out to determine the serum concentrations levels of CBD after

intraperitoneal administration of 100 mg/kg. For this purpose, 14 GASH/Sal animals followed the blood extraction and preparation protocol described above at various times after administration of the CBD: 15, 30, 45, 60, 120, 240, 480, 720, and 1,440 min. The blood samples were analyzed with the high-performance liquid chromatography (HPLC)/mass spectrometry method at the Mass Spectrometry Service of the USAL. The equipment used consisted of a Waters Acquity UPLC HT® system (Waters Corporation, Milford, MA, USA) equipped with a binary pump, an online degasser, and a thermostatic column compartment for the HPLC assay and a tandem quadrupole Waters Xevo TQS micro system (Waters Corporation) for the mass spectrophotometry assay. The mobile phase consisted of A (water containing 0.1% formic acid) and B (acetonitrile) and was delivered at a flow rate of 0.7 mL/min to an Acquity UPLC BEH C-18 column (particle size: 1.7 μm, diameter: 2.1 mm, length: 50 mm) (Waters Corporation, Milford, MA, USA). The method of elution for the CBD was carried out using the following gradient program: 95% A and 60% B at 0.00 min, 90% A and 10% B at 0.35 min, 75% A and 25% B at 0.99 min, 0% A and 100% B at 1.00–1.90 min, and 95% A and 5% B at 1.91 min. The pharmacokinetic profile after CBD intraperitoneal administration is shown in **Figure 3**.

Body Weight and Hematological and Biochemical Analysis

The body weight of the animals belonging to each experimental group was measured three times a week to determine the possible side effects of each treatment on weight gain, as well as on food and water intake. Hematological analysis of the blood samples was performed at the end of the chronic treatments by using an automated hematology analyzer (ADVIA® 120 Hematology System, Bayer, Germany). The hematological parameters that were evaluated included hemoglobin concentration, hematocrit, and the number of red blood cells, white blood cells, and platelets per microliter. To assess the biochemical markers of liver function, the following biochemical assays were performed in the serum samples: hepatic enzymes such as aspartate aminotransferase and alanine aminotransferase, as well as bilirubin, serum albumin, and total protein. The concentrations of those biochemical parameters were estimated using standard laboratory kits (Spotchem II Liver-1 kit, #33925, Menarini Diagnostic PAIS), as per manufacturer's instructions, and the spectrophotometric reading was made in a continuous flow system by using the automated Spotchem EZ analyzer (SP-4430). All these procedures were performed in the Experimental Animal Facility of the University of Salamanca.

Stimulation for Sound-Induced Seizures

The AGSs were induced by intense acoustic stimulation 45 min after the acute drug administration and 8 h after the last drug administration for analyzing the chronic drug effects (**Figure 1**). The sound stimulation protocol for triggering AGS in the GASH/Sal animals followed the same procedure previously used by Barrera-Bailón et al. (2013, 2017). The animals were placed in a cylindrical acrylic arena (height: 50 cm, diameter: 37 cm) and allowed to acclimate for 1 min. The animals were then exposed to a continuous white noise of 0 to 18 kHz and an intensity of 115 to 120 dB, to induce AGSs. Exposure continued until the initiation of wild-running phase or until 20 s had elapsed, whichever came first. The recorded sound was created using a high-pass filter (>500 Hz, Bruel & Kjaer #4134 microphone and preamplifier #2619), digitized at 44.1 kHz, and played by a computer-coupled amplifier (Fonestar MA-25T, Revilla de Camargo, Spain) and speaker (Beyma T2010, Valencia, Spain) located above the arena.

Video Recordings, Seizure Severity Index, and Neuroethological Analysis

Video recordings of seizure activity in the GASH/Sal animals began 1 min prior to the sound exposure and continued until the animal recovered from the stupor. To assess the intensity and severity of the AGS in the GASH/Sal animals, we followed the same tools and behavioral scoring previously described by Barrera-Bailón et al. (2013). Briefly, the analysis of each recording session was based on the behavioral repertoire of the animals during the seizure at three time windows: 1 min before the starting of the acoustic stimulation (presound), the first 30 s during the acoustic stimulation (sound), and 1 min after the acoustic stimulation (postsound). The severity index used to assess the overall intensity of the seizures in the GASH/Sal has been used previously (Garcia-Cairasco et al., 2004; Rossetti et al., 2006; Barrera-Bailón et al., 2013) and is presented in **Figure 2A**. After each animal was assessed for seizure severity, we divided the animals into two groups for further analysis. Those with a seizure severity scores ≥2 were considered as animals that maintained seizures, and those with a seizure severity scores lower than 2 were considered as animals with no seizures or very mild symptoms. The seizure latency was assessed in all animal groups and was defined as the period of time between the stimulus onset and the first behavioral manifestation of the wild-running phase. The behavioral sequences observed during the seizure phases were assessed using a neuroethological method (quantitative behavioral analysis) that provides integrative and reliable information about seizure generation, neuronal structures, and the sequential behavioral expression (Garcia-Cairasco et al., 1996, 2004). Every behavior presented in a given time window was recorded, second by second, according to a dictionary of behavioral items (**Figure 2B**) described by Garcia-Cairasco et al. (1996). Once the data were obtained and after reviewing the video several times, the ETHOMATIC program calculates and displays the mean frequency and mean duration of each behavioral item in the given observation window (presound, during sound, and postsound stimulation). The program also performs statistical analysis, verifying significant associations between pairs of behavioral items and calculating χ^2 values. Flowcharts representing all of the statistically significant data were constructed using Microsoft PowerPoint 2011 (**Figure 2C**).

Gene Expression Analysis (Quantitative Real-Time Polymerase Chain Reaction)

The inferior colliculi of the GASH/Sal animals were collected at the final time point of the experiments to study the differential expression of the following genes: *Trpv1*, *5-Htr1a*, *Sigmar1*,

A

Seizure index

SI	Seizure behaviors	Score
0.00	No seizures	0
0.11	One wild running	1
0.23	One wild running (running plus jumping plus atonic fall)	2
0.38	Two wild runnings	3
0.61	Tonic convulsion (*opisthotonus*)	4
0.85	Tonic seizures plus generalized clonic convulsions	5
0.90	Head ventral flexion plus cSI5	6
0.95	Forelimb extension plus cSI6[a]	7
1.00	Hind limb extension plus cSI6[a]	8

[a] Categories, which are generally followed by hind limb clonic convulsions (CCV2).

B

Behavioral dictionary

Acronym	Behavior	Acronym	Behavior
AF	Atonic fall	GRH	Grooming of head
ATF[a]	Ataxic fall	GRHL	Grooming of head, left
BE	Blinking eyes	GRL	Grooming of body, left
BRL	Barrel rolling, left	GRN	Grooming of neck
BRR	Barrel rolling, right	GRR	Grooming of body, right
CCV1	Clonic convulsions (forelegs)	HFL	Head ventral flexion
CCV2	Clonic convulsions (hind legs)	HP_1	Forelimb extensions
CCVg	Clonic convulsions (generalized)	HP_2	Hind limb extensions
CVL1	Clonic convulsions (forelegs – left)	IM	Immobility
CVL2	Clonic convulsions (hind legs – left)	JP	Jumping
DYS	Dyspnea	LI	Licking
ER	Erect posture	LIC	Licking of claws
EXC	Excretion of feces and urine	MT	Masticatory movements
EXT	Extended posture	PIM	Postictal immobility
FR	Freezing posture	RU	Running
GL	Gyrating, left	SC	Scanning
GN	Gnawing	SN	Sniffing
GR	Gyrating, right	TCP	Tachypnea
GRB	Grooming	WA	Walking
GRF	Grooming of face	WDS	Wet dog shaking
GRG	Grooming of genitals		

[a] Observation: The behavioral item ataxic fall (ATF) was created to show the presence of the toxic effect of drugs on motor behavior.

C

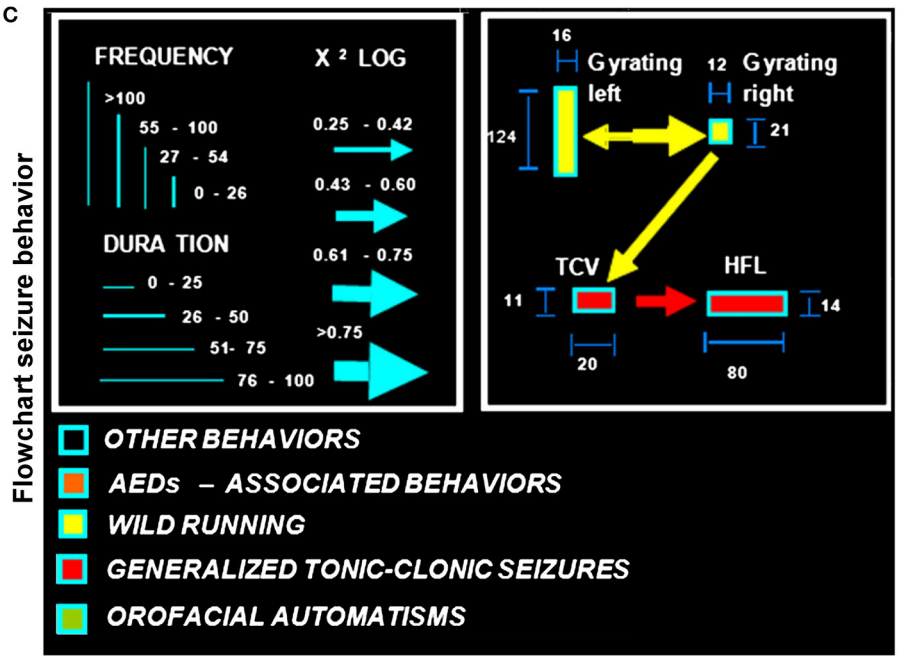


FIGURE 2 | Audiogenic seizure analysis in the GASH/Sal. **(A)** Seizure index (SI), according to Garcia-Cairasco et al. (1996) using the behavioral descriptions and categorized severity scores transformed into discreet variables for statistical purposes. **(B)** Dictionary of the behavioral variables analyzed and displayed in the flowcharts of the seizure behaviors. **(C)** Flowcharts illustrating the graphical and statistical aspects of the seizure behaviors. The frequency and time spent performing each behavior are proportional to the height and width of the rectangle, respectively. The arrow width and direction indicate the statistical intensity and preference association between two behavioral items. The behaviors associated with wild-running phase (including wild running, jump, and falls) are depicted in yellow, and the behaviors associated with the convulsion phase (including convulsions and extentension of extremities) are depicted in red (figure modified from Barrera-Bailón et al., 2013).

Adora1, *Slc29a1*, and *Cnr1*. Those genes were selected as they encode neuronal receptors related to regulation of neuronal excitability and neuroprotection that might be presumably affected by the pharmacological activity of the CBD. The inferior colliculi of each animal were obtained following euthanasia by deep anesthetization and rapid decapitation. Each sample tissue was frozen immediately in liquid nitrogen. The reverse transcription–quantitative polymerase chain reaction (RT-qPCR) experiments were carried out following the protocol routinely used in our laboratory (López-López et al., 2017; Damasceno et al., 2020; Díaz-Rodríguez et al., 2020; Sánchez-Benito et al., 2020), which is in accordance with published guidelines (Nolan et al., 2006). Briefly, total RNA (2 μg) was mixed with oligo-dT and random hexamer primers for reverse transcription into cDNA using the First Strand cDNA Synthesis Kit (K1621, Promega Corporation, Madison, WI, USA). In all cases, a reverse transcriptase negative control was used to test genomic DNA contamination. Subsequently, qPCR was performed using the SYBR Green method with a 2× Master Mix (#4367659, Applied Biosystems). Each reaction contained 10 μL of Master Mix, 0.4 μL of each pair of primers, 3 μL of each cDNA sample in a different serial cDNA quantity for each gene, and MilliQ

TABLE 1 | Oligonucleotide primers used for RT-qPCR, indicating the location of each primer in the corresponding Ensembl sequences of the Syrian hamster.

Gen target	ID transcript Ensembl *Mesocricetus auratus*	Primer forward	Primer reverse	Size of products	E^a
Trpv1	*ENSMAUG00000008181*	GACGAGGGTGAACTGGACTACC	CTTGACACCCTCACAGTTGC	60	2.16
5-Htr1a	*ENSMAUG00000007970*	CACCATCAGCAAGGATCACG	AGAGCAGCAGCGGGATATAGA	85	2.03
Sigmar1	*ENSMAUG00000011503*	AGAGAGGGCACCACGAAAAG	AAGGAGCGGAGGGTATAGAAGA	88	1.97
Adora1	*ENSMAUG00000018351*	CATCGTATCCCTGGCGGTAG	ACGCAGGTGTGGAAGTAGGTCT	75	2.01
Slc29a1	*ENSMAUG00000006341*	CATCAGGAGGTGTGTGGGTTT	TCATGGTTCCAGGGTTCTCG	162	2.07
Cnr1	*ENSMAUG00000014040*	TGTTGACTTCCATGTGTTCCA	GGTCTGGTGACGATCCTCTT	126	1.98
Actb	*ENSMAUG00000008763*	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG	105	2.03

[a] *qPCR primer efficiency (E) was calculated according to the following equation:* $E = 10^{(-1/slope)}$.

water (RNA-free) up to 20 μL. The amplification reaction was performed in the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C depending on each pair of primers. RT-qPCR experiments were performed in a set of four to six biological replicates (sample cases) for each experimental group (treatment condition) and conducted in three technical replicates from each sample and gene product examined. The list of primers used in the RT-qPCR experiments is provided in **Table 1**.

A standard curve was made to verify the efficiency of the primers of the target and reference genes, and it was constructed by serial dilutions of cDNA isolated: 60, 30, 15, 7.5, 3.25, and 1.65 ng/μL. Data showed that all genes used in this work were expressed at a high level, and investigated transcripts showed high linearity ($R^2 > 0.95$). Real-time PCR efficiencies (E) of one cycle in the exponential phase were calculated according to the equation $E = 10^{[-1/\text{slope}]}$. High PCR efficiency rates were shown to occur in the investigated range of nanogram cDNA input, and all genes produced approximately identical slopes. To determine which was the most stable reference gene for RT-qPCR data normalization, two candidates [β-actin (*Actb*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)] were selected, and their expression was measured by NormFinder software (Andersen et al., 2004) that calculate intragroup and intergroup variations in gene expression. Thus, the mean threshold cycle (Ct) value and primer efficiency value of *Actb* were used for data normalization. The comparative Ct method was used for quantitative data analysis (Schmittgen and Livak, 2008). After removing outliers (Burns et al., 2005), the relative gene expression value of each transcript was calculated according to the formula $2^{-(\Delta Ct\text{“condition 1”} - \Delta Ct\text{“condition 2”})}$, where “condition 1” corresponds to the experimental sample, “condition 2” to the sample from the control animal, and the ΔCt of each “condition” is $Ct_{\text{“experimental gene”}} - Ct_{\text{“endogenous gene”}}$ (Schmittgen and Livak, 2008).

Statistical Analysis

Statistical analyses including the scoring of seizures and relative gene expression values were performed using the SPSS-IBM software, version 25.0 (SPSS Inc., Chicago, IL, USA). All quantitative data were expressed as mean value ± standard error of the mean (SEM). Statistical analyses corresponding to the seizure behaviors, body weight, and hematological and biochemical parameters were performed using two-way mixed analysis of variance (ANOVA) with Bonferroni *post hoc* test. Statistical analyses for the open-field behavior and the mRNA expression levels were conducted using one-way ANOVA followed by Bonferroni multiple-comparisons test. The differences between experimental groups were considered statistically significant with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All the data were plotted using GraphPad Prism (version 6.05).

RESULTS

Pharmacokinetic Profile of CBD in Serum

The pharmacokinetics of CBD, when given as an intraperitoneal single 100 mg/kg dose, were determined in serum of GASH/Sal animals. The mean concentration–time profile of serum for the 14 animals is illustrated in **Figure 3**. The highest mean serum CBD concentrations were shown at 263.3 ± 40 ng/mL (45 min after administration), which dropped to 210.2 ± 45 ng/mL at 2 h. By 4 h after dose administration, the CBD concentrations in serum remained very low 43.9 ± 9 ng/mL until hour 12. As shown in the pharmacokinetic profile, elimination of CBD in sera was measured at 24 h after the drug administration (**Figure 3**). The pharmacokinetic profile after CBD intraperitoneal administration indicated that 45 min postinjection was the best time point to measure the possible anticonvulsant effects of CBD.

Effects of Drug Treatments on the Behavior and Intensity of Audiogenic Seizures

During loud acoustic stimulation and prior to any of the drug treatments, all GASH/Sal animals displayed a complete AGS as described by Muñoz et al. (2017). Beginning between 1 and 3 s after the stimulus onset, the GASH/Sal animals showed five differenced and consecutive phases that included (1) a behavioral arrest; (2) a wild-running period of nearly 5 s; (3) tonic–clonic convulsions for approximately 33 s; (4) head ventral flexion, forelimb, and hindlimb extensions; and (5) postictal immobility (stupor). Finally, the animals recovered walking and exploring behavior with similar features to those

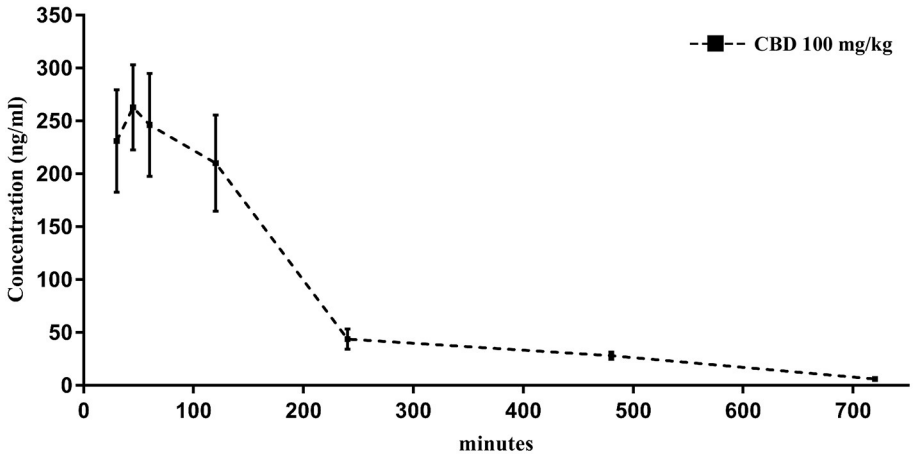


FIGURE 3 | Time course of CBD levels in serum from GASH/Sal animals treated with CBD (100 mg/kg, intraperitoneal). Notice that levels of CBD peak within 45 min after the intraperitoneal injection. Results are expressed as means ± SEM ($n = 14$).

shown in the basal prestimulus condition. A video recording of a representative seizure in a non-treated GASH/Sal animal, together with the corresponding seizure behaviors analyzed in the ETHOMATIC program, can be simultaneously seen in **Supplementary Material 1**. The percentage of animals based on the achieved categorized seizures scores (≥ 2 or < 2) is shown for each experimental group in **Supplementary Material 2**. Using a dictionary of behavioral items (**Figure 2B**) and throughout the neuroethological analysis of the AGS (**Figure 2C**), we confirmed that 100% of the GASH/Sal animals in the pre-treatment conditions consistently developed the seizure behaviors in a complete manner as expected (**Figures 4A**, **5A**; **Supplementary Material 1**). During the presound window, the main activity of the non-treated hamsters was behavioral exploration, including sniffing (SN) and walking (WA). During the sound window, the behavioral manifestations of the wild-running phase were observed 7 s after loud sound stimulation, including turns to the left (GL) and right (GR), running (RU), jumps (JP), and atonic falls (AF). In the postsound window, the non-treated GASH/Sal exhibited tonic convulsions (TCVs), followed by a behavioral cluster of generalized clonic convulsions (CCV1, CCV2, and CCVg) with hyperextensions (HP1 and HP2) and ended with postictal immobility (PIM), as well as altered breathing [dyspnea (DYS) and tachypnea (TCP)] (**Figures 4A**, **5A** and **Supplementary Material 1**). Accordingly, all GASH/Sal animals achieved the maximum scores of 8 in the categorized seizure index prior to administration of the drug treatments (**Figure 6A** and **Supplementary Material 2**) and exhibited values in seizure latency as well as in the time of the wild-running and convulsion phases that were consistent with the typical AGS in the GASH/Sal strain (**Figures 6B–D**). Following the experimental design, each animal group received intraperitoneal injections of either the vehicle, VPA, CBD, or the combination of both drugs (CBD+VPA) at the protocol-defined time points (acute and chronic drug administrations). In the sham group, 100% of the animals acutely and chronically treated with the vehicle exhibited behavioral manifestations of seizures that were very similar to those observed in the baseline pre-treatment condition (**Figures 4**, **5**). In fact, all sham animals were scored with the maximum values in the categorized seizure index after both acute and chronic vehicle administrations (**Figure 6A** and **Supplementary Material 2**). After 45 min of the first intraperitoneal injection of the vehicle, the sham animals showed exploratory behaviors in the presound window, followed by the typical seizure behaviors associated with the wild-running and tonic–clonic convulsion phases in the sound and postsound windows, respectively (**Figure 4B**). After 7 and 14 days of the vehicle-treatment, the behaviors of the sham animals remained very similar to those observed in the acute administration and pre-treatment conditions (**Figure 5B**). In the VPA-treated group, the behavioral manifestation of the AGS were clearly affected at 45 min after the first intraperitoneal administration, showing complete abolition of the seizure or a significant attenuation in the behaviors associated with the wild-running and convulsion phases (**Figure 4B**). When compared with sham animals, the GASH/Sal a single dose administration of VPA showed at 45 min after the injection a statistically significant reduction of the seizure index score ($p = 0.0001$; **Figure 6A**). Thus, half of these animals injected with a single VPA dose presented seizures scores < 2 and the other half exhibited very low seizure scores, which were between 2 and 5 (**Figure 6A** and **Supplementary Material 2**). After 7 days of daily repeated VPA injections, the GASH/Sal animals exhibited slight modifications of seizure behavior (**Figure 5B**) with a statistically significant decrease in the seizures scores as compared to sham animals ($p = 0.03$; **Figure 6A**). After 14 days of VPA treatment, there were not significant changes in the seizures scores with no apparent modification of seizure behaviors (**Figures 5B**, **6A**). In the CBD-treated group, 45 min after the first administration and 7 days of repeated CBD administration produced slight and not significant attenuation of seizure behavior with seizures scores that varied between 2 to 8 (**Figure 6A**). In those animals

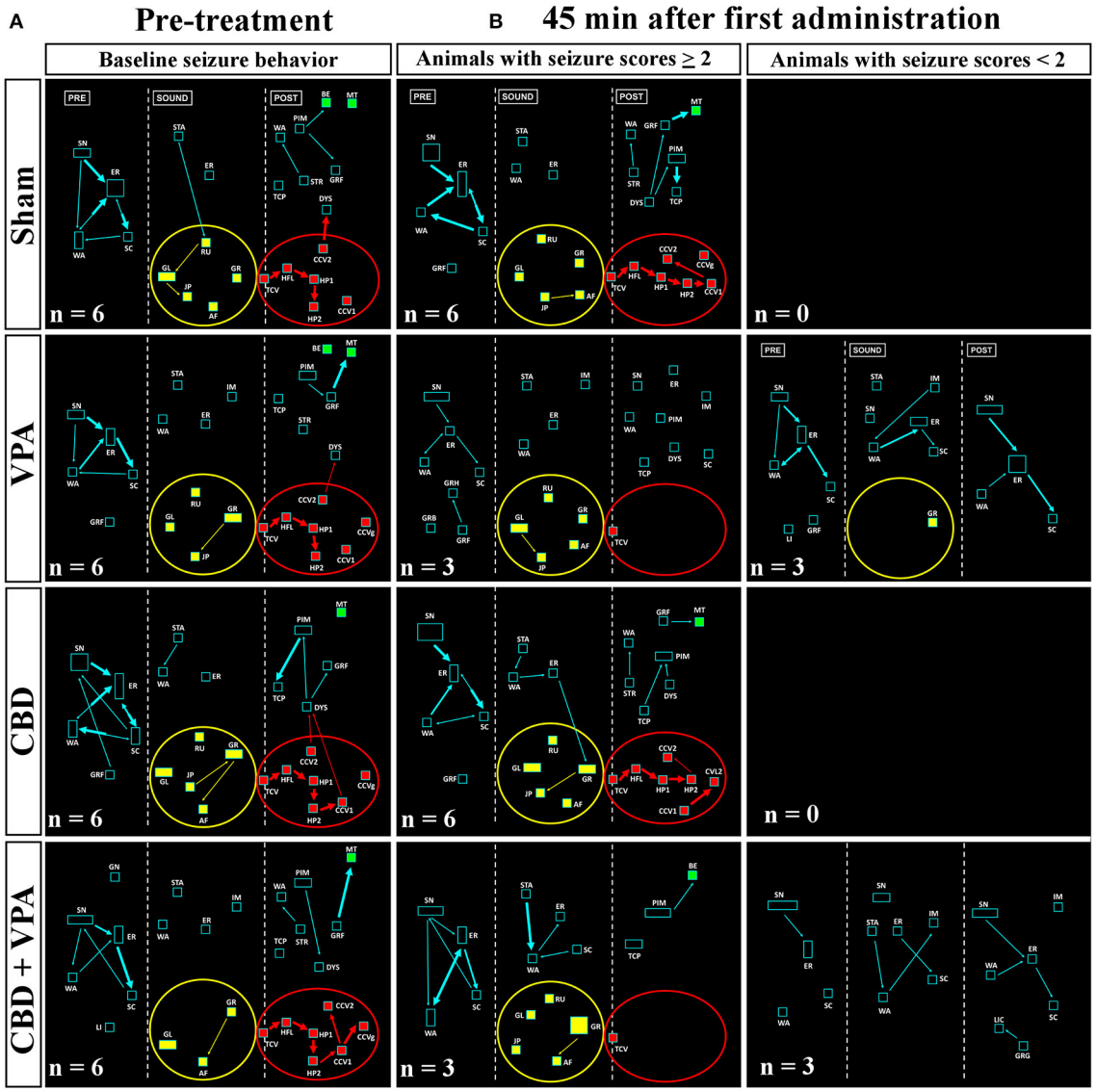


FIGURE 4 | Treatment effects of VPA and CBD on the audiogenic seizure behaviors of the GASH/Sal within 45 min after the first intraperitoneal administration. **(A)** Flowcharts show the sequences of behaviors in GASH/Sal animals in baseline conditions before starting the treatment. Notice that all GASH/Sal animals exhibited a complete audiogenic seizure, including the behavioral manifestations of the wild-running phase with turns to the left (GL) and right (GR), running (RU), jumps (JP), and atonic falls (AF) (depicted together with the yellow circle), as well as the behaviors associated with tonic–clonic convulsions (TCV, CCV1, CCV2, and CCVg), followed by a behavioral cluster of generalized clonic seizures with hyperextensions (HP1 and HP2) (depicted together with the red circle) and ended with postictal immobility (PIM) and breathing difficulties [dyspnea (DYS) and tachypnea (TCP)]. **(B)** Flowchart shows the sequences of behaviors in GASH/Sal animals treated with vehicle (sham), VPA, and CBD and with a combination of CBD and VPA, 45 min after the first intraperitoneal administration. Notice that behaviors associated with tonic–clonic convulsions (red circles) were blocked with VPA—or the combined CBD+VPA treatments, whereas animals treated with vehicle or single CBD administration remained similar to baseline conditions, although the elimination of the generalized clonic convulsions (CCVg) in the single CBD administration was noticeable. Furthermore, half of the GASH/Sal animals that followed VPA treatment or the combined CBD+VPA treatments showed seizures scores <2 with almost total absence of seizure behaviors or complete blockage of seizure activity. Each flowchart showed the compendium of behaviors in all the animals belonging to each experimental group. The seizure behaviors were analyzed in three time windows (presound, during sound, and postsound). See **Figure 2** for detailed interpretation of the flowchart.

acutely treated with CBD, it was noticeable the elimination of the generalized clonic convulsions (CCVg), whereas the convulsions in the legs were kept slightly visible (CCV1 and CCV2) (**Figure 4B**). On the contrary, 14 days of repeated daily administration of CBD had no significant effects on seizure behavior, showing maximum seizure scores in 100% of the animals (**Figures 5B**, **6A** and **Supplementary Material 2**). Acute treatment with the combination of CBD and VPA drastically attenuated or completely eliminated the seizure behaviors (**Figure 4B**), showing a statistically significant reduction of

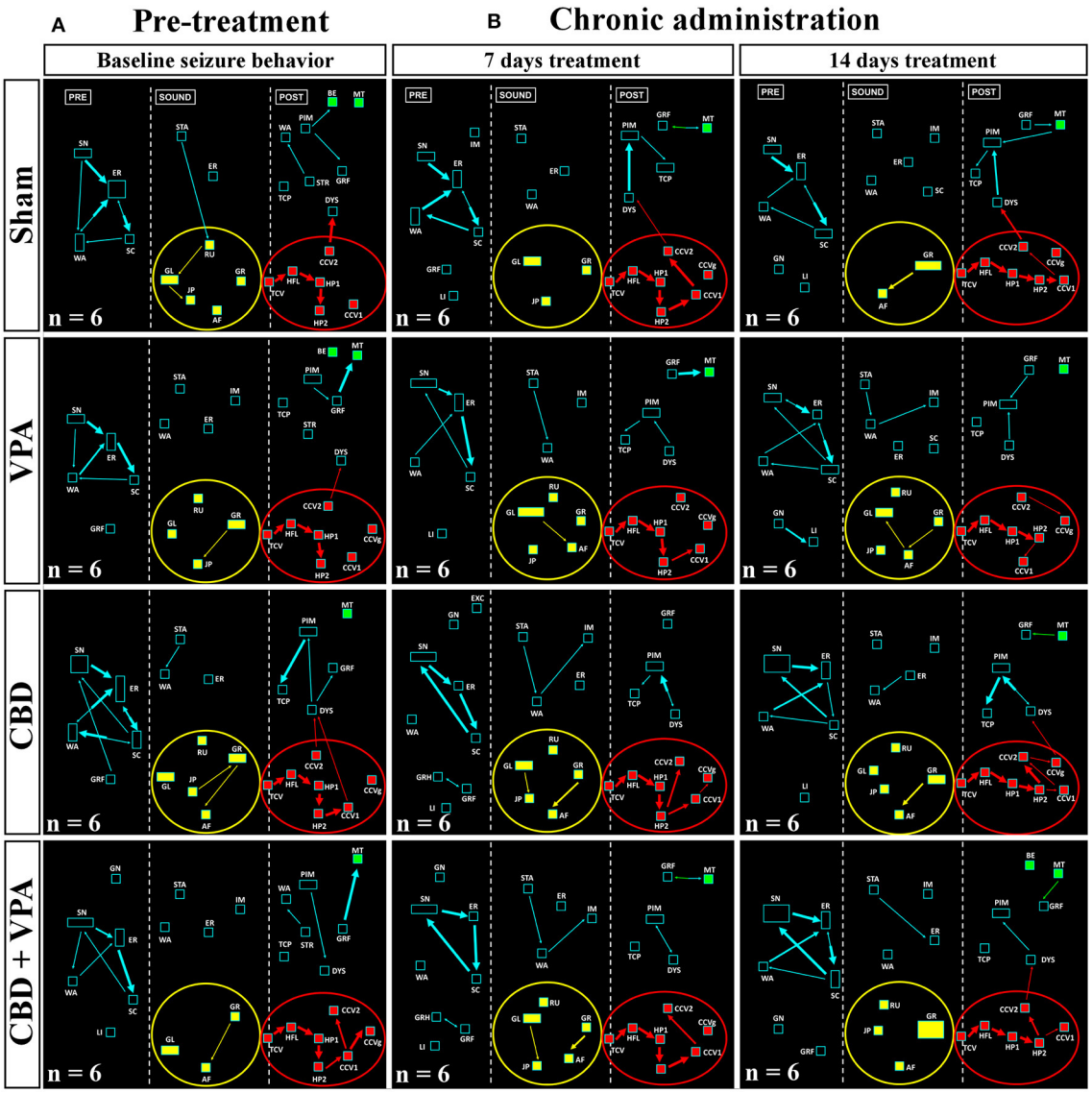


FIGURE 5 | Treatment effects of VPA and CBD on the audiogenic seizure behaviors of the GASH/Sal within 7 and 14 days after chronic administration. **(A)** Flowcharts show the sequences of behaviors in GASH/Sal animals in baseline conditions before starting the treatment. Notice that all GASH/Sal animals exhibited a complete audiogenic seizure, including the behavioral manifestations of the wild-running phase with turns to the left (GL) and right (GR), running (RU), jumps (JP), and atonic falls (AF) (depicted together with the yellow circle), as well as the behaviors associated with tonic–clonic convulsions (TCV, CCV1, CCV2, and CCVg), followed by a behavioral cluster of generalized clonic seizures with hyperextensions (HP1 and HP2) (depicted together with the red circle) and ended with postictal immobility (PIM) and breathing difficulties [dyspnea (DYS) and tachypnea (TCP)]. **(B)** Flowchart shows the sequences of behaviors in GASH/Sal animals treated with vehicle (sham), VPA, CBD, and with a combination of CBD and VPA after 7 and 14 days of chronic drug administration. Notice that seizure behaviors in general remained similar to baseline conditions for all experimental groups, showing seizures scores ≥2. Notice slight modifications in the behavioral items associated with the wild-running phase, which can be seen in all experimental groups. Each flowchart showed the compendium of behaviors in all the animals belonging to each experimental group. The seizure behaviors were analyzed in three time windows (presound, during sound, and postsound). See **Figure 2** for detailed interpretation of the flowchart.

the seizure index score when compared to sham animals ($p = 0.0001$; **Figure 6A**). Thus, animals acutely treated with the combination of both drugs displayed seizure scores as low as 0-1 for one half of the animals and 2–3 for the other half (**Figure 6A** and **Supplementary Material 2**). Following loud sound stimulation, GASH/Sal animals that were acutely treated with CBD+VPA and showed complete abolition of seizures exhibited normal acoustic startle reflex, exploratory behaviors and grooming (**Figure 4B**). Chronic administration of CBD and VPA (7 and 14 days post-treatment) produced very mild modifications of seizure behaviors associated with the wild-running phase as well as a subtle and not significant reduction in the seizure scores in 33 and 16% of the animals, respectively (**Figure 6A**).

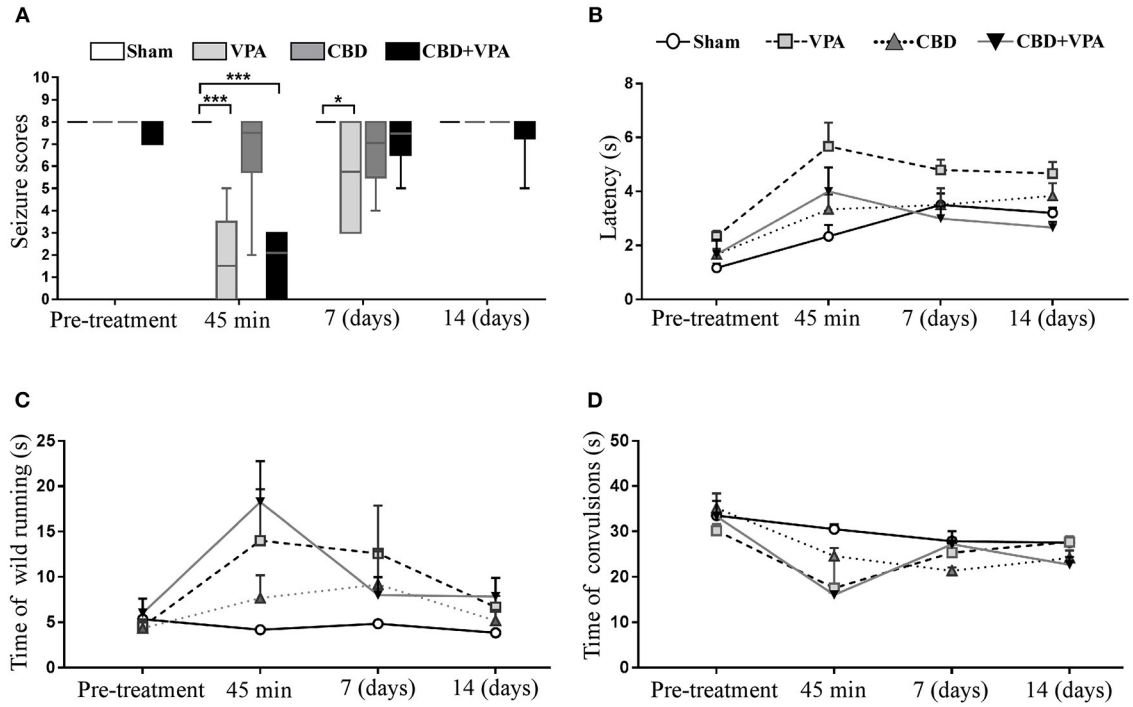
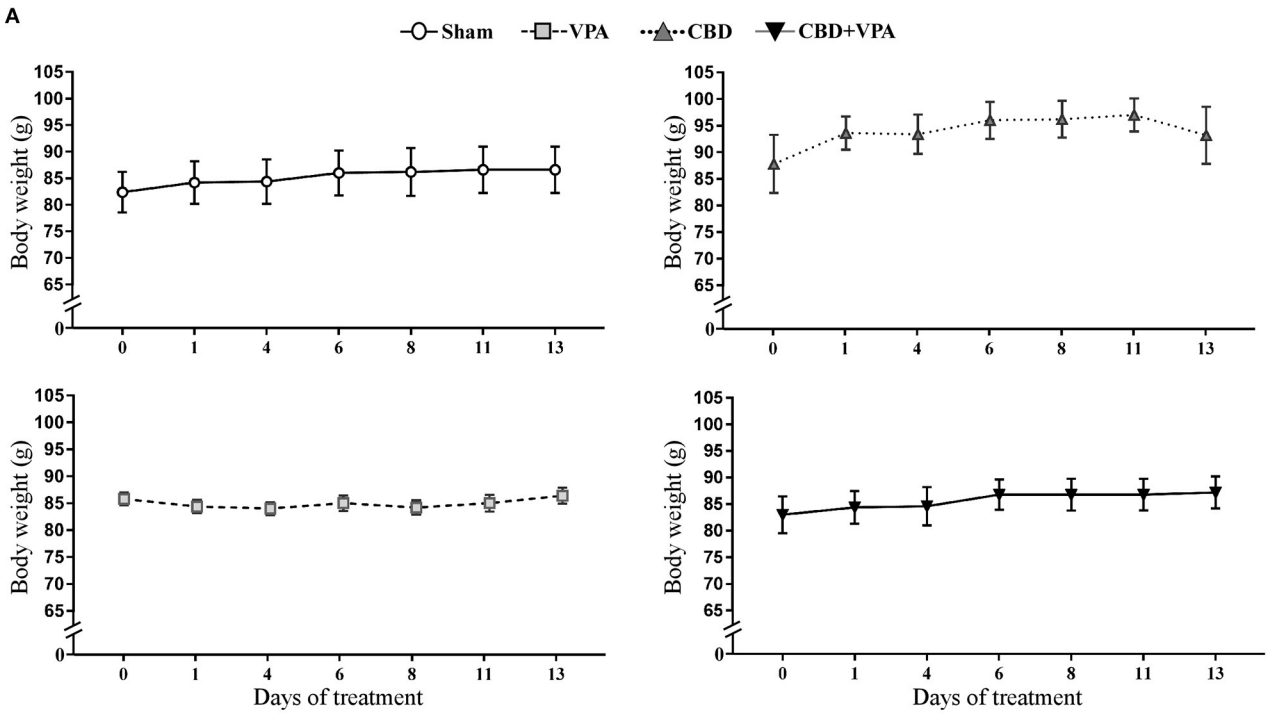


FIGURE 6 | Effects of VPA and CBD on the seizure intensity, latency, and duration of wild-running and convulsion phases. **(A)** Box plot summarizing the categorized seizure-index score in each experimental group on the baseline condition (pre-treatment), after acute treatment (45 min), as well as 7 and 14 days after chronic administrations. In each box plot, the horizontal line crossing the box is the median. Asterisks indicate significant differences between experimental groups (*$p < 0.05$, ***$p < 0.001$). **(B–D)** Graphs showing the duration (in seconds) for the seizure latency **(B)**, the wild-running phase **(C)**, and the convulsion phase **(D)** in each experimental group on pre-treatment condition and 45 min, 7 days, and 14 days post-treatment. GASH/Sal animals with complete elimination of audiogenic seizures (seizure scores = 0) were not graphed in **(B–D)** as the variable time was unable to be assessed.

Effects of Drug Treatments on Seizure Latency and Duration of Seizure Phases

The seizure latency of sham animals followed a tendency to increase after acute and chronic vehicle-treatment (**Figure 6B**). Such upward trend in seizure latency was also observed in all animal groups over the experimental timeline, and hence the sham group was considered the reference for comparisons of seizure latency between groups. After 45 min of the single dose administration, we observed a significant increase in seizure latency in the VPA-treated group (5.6 s) as well as in the group with the combined drug treatment (4.0 s) as compared to the sham group (2.3 s) (**Figure 6B**). Animals with acute CBD treatment also showed an increase seizure latency (3.3 s), but without statistically significance as compared to sham animals (**Figure 6B**). Although none of the experimental groups showed a significant modification of the seizure latency after 7 and 14 days of treatment, we found an increase of ∼1.8 s in the VPA-treated group, whereas the groups treated with either CBD or the combination of CBD and VPA showed the same latency than the sham group.

After acute drug administration, the duration of the wild-running phase was 14, 7.6, and 18.2 s in the VPA–, CBD–, and CBD+VPA-treated groups, respectively, which represented a significant increase when compared to sham animals (4.1 s) (**Figure 6C**). On the contrary, the duration of the convulsion phase was significantly reduced (17.5 s for VPA–, 24.6 s for CBD- and 16 s for CBD+VPA-treatment) when compared to sham animals (30.5 s) (**Figure 6D**). Therefore, the seizure latency and duration of the wild-running phase after any of the treatments in acute administration was significantly increased, whereas the duration of the convulsions were significantly reduced as compared to sham animals. These results were consistent with an attenuated effect of either acute treatment in the seizure severity of the GASH/Sal animals, in which seizures were not entirely blocked. Similar effects on the duration of the wild-running and convulsion phases were observed after 7 and 14 days of chronic drug administrations in all treatment groups, showing an increased duration of the wild-running phase and a decreased duration of convulsions as compared to sham animals, although these differences were not statistically significant (**Figures 6C,D**). It is noteworthy that the duration of the convulsion phase in GASH/Sal animals after 7 days of repeated daily CBD administration were significantly reduced when compared to the pre-treatment condition ($p = 0.0001$; **Figure 6D**). Furthermore, animals treated with 14 days administration of either CBD or the combination of CBD and VPA showed a significant reduction in the duration of convulsions as compared to their baseline



B **Hematological and biochemical liver profiles of GASH/Sal animals depending on the drug treatment**

Parameters	Sham		VPA		CBD		CBD + VPA	
	Pre-treatment	14 days	Pre-treatment	14 days	Pre-treatment	14 days	Pre-treatment	14 days
Hematological profile								
Hemoglobin (g/dl)	17.9 ± 0.3	14.7 ± 0.9	17.7 ± 0.2	***11.4 ± 0.3***	17.7 ± 0.1	14.2 ± 0.3	17.5 ± 0.3	***12.1 ± 0.8***
Hematocrit (%)	48.7 ± 3.6	38.4 ± 3.8	47.9 ± 1.7	***29.3 ± 1.3***	48.4 ± 1.5	37.7 ± 1.2	47.7 ± 2.1	33.5 ± 1.8
Red blood cells/µl (10^{-6})	8.94 ± 0.12	6.94 ± 0.51	8.98 ± 0.61	***5.19 ± 0.19***	8.74 ± 0.06	6.67 ± 0.29	8.38 ± 0.32	5.97 ± 0.24
White blood cells/µl	8100 ± 305	6080 ± 383	8400 ± 217	6960 ± 1281	6600 ± 842	5820 ± 881	6600 ± 410	***2450 ± 732***
Platelets cells/µl (10^{-3})	1345 ± 32	1586 ± 75	1380 ± 30	1352 ± 98	1347 ± 35	*1514 ± 126*	1350 ± 42	*1481 ± 90*
Biochemical liver profile								
AST (UI/L)	43.5 ± 3.5	39.6 ± 3.9	46.7 ± 6.2	35.16 ± 3.9	43.7 ± 3.5	39.83± 4.7	46.2 ± 6.7	31.83 ± 3.4
ALT (UI/L)	48.1 ± 1.4	42.1 ± 2.4	53 ± 4.1	39.3± 2.4	48.3 ± 1.4	38.1 ± 4.2	49.1 ± 3.8	**22.3 ± 2.5*** **
BT (mg/dl)	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1
Albumin (g/dl)	2.7 ± 0.0	*2.3 ± 0.0*	2.7 ± 0.0	*2.3 ± 0.0*	2.8 ± 0.0	*2.3 ± 0.0*	2.6 ± 0.0	2.1 ± 0.0
Total protein (g/dl)	7.5 ± 0.0	7.1 ± 0.1	7.3 ± 0.2	7.2 ± 0.1	7.3 ± 0.5	7.1 ± 0.1	7.4 ± 0.4	7.0 ± 0.1

FIGURE 7 | Effects on body weight and hematological and biochemical profiles of treated GASH/Sal animals. **(A)** Graphs show the time course of body weight variations monitored during the 14-day treatment in sham-, VPA–, CBD–, and CBD+VPA-treated groups. Notice that all experimental groups showed a steady and persistent body weight throughout the treatment, and no significant differences were found as compared to the pre-treatment conditions (time 0 in the horizontal axis of the graphs). Data are shown as means ± SEM. **(B)** Table shows hematological and biochemical profiles of GASH/Sal animals after 14 days of chronic drug treatments. The hematological assessment of blood included the following parameters: hemoglobin concentration, hematocrit (volume percentage of red blood cells in blood), and number of red blood cells, white blood cells, and platelets in a microliter (µL). The analysis of liver function included five biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin (BT), albumin, and total protein. Asterisks and bold values in the table denote statistical significance (**$p < 0.01$, ***$p < 0.001$) as compared to the pre-treatment conditions and the sham group.

condition ($p = 0.0001$ for CBD, and $p = 0.0001$ for CBD+VPA; **Figure 6D**).

Effects of Drug Treatments on Body Weight and Hematological and Serum Biochemical Profiles

We further analyzed the effects of each chronic drug administration (14 days post-treatment) on the body weight as well as on the hematological and biochemical profiles of each experimental group (**Figure 7**). The GASH/Sal animals showed a steady weight after chronic administration of any of the treatments and no statistically significant differences were found when compared to the baseline pre-treatment conditions. The average growth was 2.03 g/week for the Sham group, 0.30 g/week for the VPA-treated group, 3.83 g/week for the CBD-treated group and 1.96 g/week for the combined CBD+VPA treatment, showing no statistical significant differences between them and indicating normal feeding behaviors (**Figure 7A**). In the pre-treatment conditions, there were no significant differences between the experimental groups in any of the parameters associated with the hematological and biochemical profiles. After 14 days of chronic administration, all the hematological parameters, with the exception of the platelets, showed a statistically significant reduction in serum concentration as compared to the pre-treatment condition. Since we observed similar modifications in sham animals when compared to pre-treatment and 14 days post-treatment conditions, the sham group was considered the reference for comparisons between the drug-treated groups. Hemoglobin level decreased significantly after 14 days of chronic administration in VPA- and CBD+VPA- treated groups as compared to sham animals ($p = 0.006$; **Figure 7B**). In the same way, the hematocrit level was significantly lower in VPA-treated animals as compared to sham animals ($p = 0.001$; **Figure 7B**). Although, there were no significant differences in hematocrit level between the CBD+VPA-treated and the vehicle-treated groups, the hematocrit levels were considerable lower after co-administration of CBD and VPA, even below the reference value in the pre-treatment condition. The same effects were observed in red blood cell count, which were significantly decreased in the VPA-treated group as compared to sham animals ($p = 0.0002$; **Figure 7B**). Neither the CBD-treated group nor CBD+VPA-treated group showed significant differences in red blood cell count when compared to the sham group. The GASH/Sal animals treated with the combined CBD+VPA therapy showed a significant lower white blood cell count as compared to sham animals ($p = 0.002$; **Figure 7B**). On the contrary, neither the VPA- nor the CBD-treated groups showed significant differences in white cell counts compared to the sham group. The mean value of five biochemical parameters of liver function: aspartate aminotransferase, alanine aminotransferase, bilirubin, albumin and total protein were also assessed after 14 days of chronic administration (**Figure 7B**). All these serum biochemistry parameters, with the exception of the alanine aminotransferase, showed normal values very similar to those observed in the pre-treatment conditions. Thus, there were no statistically significant differences in serum levels of aspartate aminotransferase, bilirubin, albumin and total protein between all experimental groups (**Figure 7B**). However, the mean levels of alanine aminotransferase decreased by 55% in the CBD+VPA-treated group, showing a statistically significant lower levels as compared to the pre-treatment condition and the sham group ($p = 0.0004$; **Figure 7B**).

Behavioral Characteristics According to the Responsiveness to the Drug Treatments

We examined the VPA and CBD effects on the locomotor activity and emotionality in each experimental group by comparing automatically-scored behaviors in the open-field test after chronic drug administrations (**Figure 8**). The total distance traveled, time of activity, and number of rearing were used to assess general locomotor and exploratory behaviors, whereas the time spent in the center and number of grooming further provided information on emotional reactivity (i.e., fear or anxiety-like behavior). In the pre-treatment conditions, we did not observe any statistically significant differences in all GASH/Sal animals for each of the open-field measures (**Figure 8**). A representative example of the animal's track in each experimental group at the protocol-defined time points (pre-treatment and 7 and 14 days post-treatment) is shown in **Figure 8A**. Regarding the exploratory behavior and general activity, we found that the total distance traveled by the animals was significantly reduced in the GASH/Sal animals receiving either single CBD treatment or the combined CBD and VPA treatments after chronic drug administration (7 and 14 days), when compared with the sham group (**Figures 8A,B**). Furthermore, the administration of either single CBD treatment or combined CBD and VPA treatments drastically decreased the time of activity at 7 and 14 days post-treatment, as compared to the sham group (**Figure 8C**). Similarly, the total number of rearings was significantly decreased in the animal groups that received CBD or the combination of CBD and VPA after 7 and 14 days of repeated daily administration, when compared with the vehicle-treated group (**Figure 8D**). Such drastic reduction in general locomotor and exploratory behaviors was more evident at 7 days of repeated daily administration than that at 14 days post-treatment. At 7 and 14 days of repeated daily administration, we did not observe any statistically significant effects of single-VPA treatment on the three open-field measures related to general locomotor and exploratory behaviors (**Figures 8B–D**), showing similar results to those observed in the sham group. It is important to note that after 2 weeks of the combined CBD and VPA treatment the animals exhibited increased levels of locomotion when compared to GASH/Sal animal with the CBD treatment alone. We further assessed the effects of each drug treatment on emotionality by analyzing time spent in the center area and the number of groomings (**Figure 8E**). Compared to the sham group, both CBD alone and the combination of CBD and VPA significantly increased the time spent in the center area at 7 and 14 days of chronic administrations. Such increase in time spent in the center area was particularly noticeable in

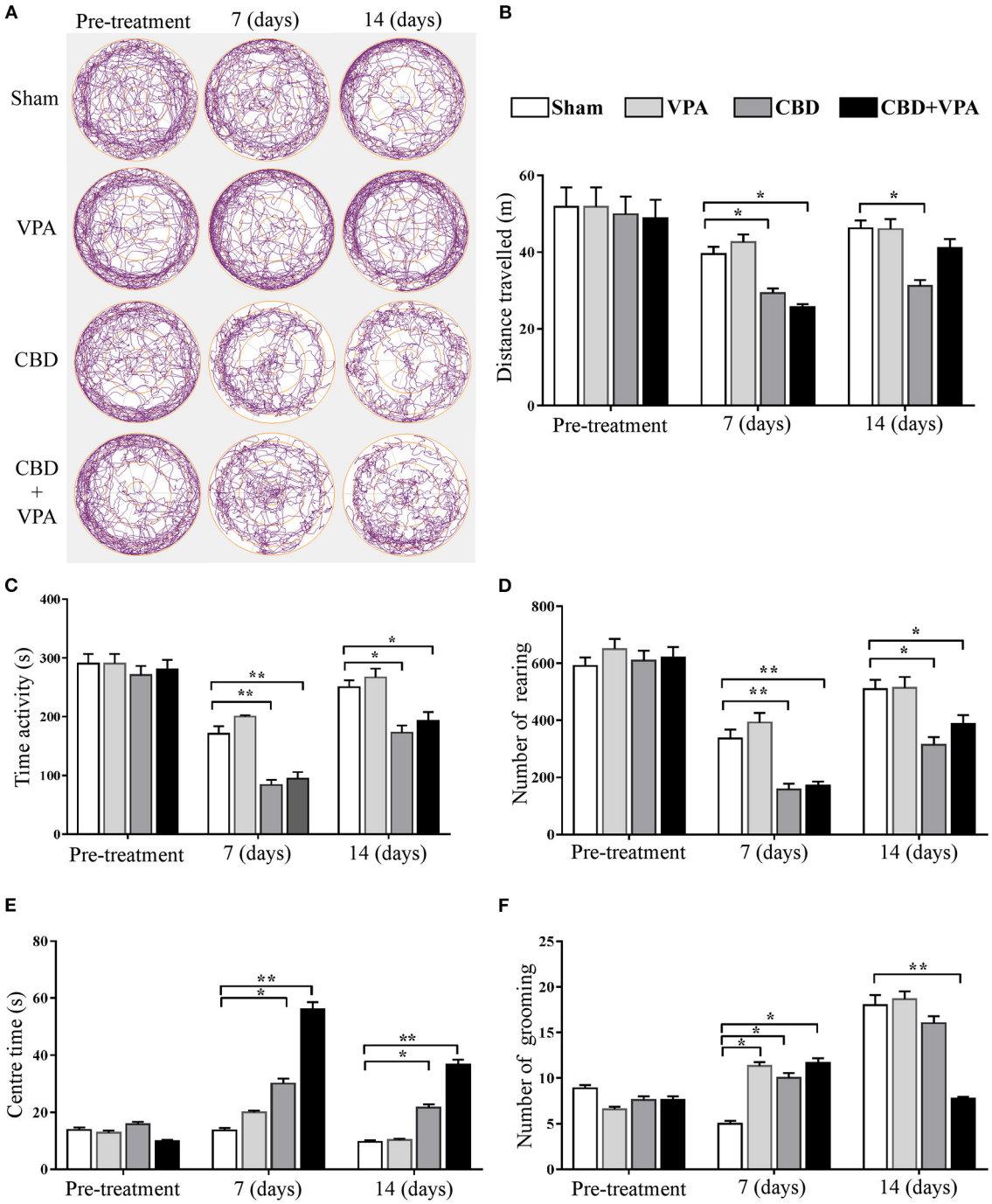


FIGURE 8 | Treatment effects of VPA and CBD on the behavior of the GASH/Sal in the open-field test. **(A)** Plots show representative examples of the animal's track in each experimental group at the protocol-defined time points (pre-treatment and 7 and 14 day drug treatments). Note that levels of global locomotor activity decrease in GASH/Sal animals that received CBD alone or the combination of CBD and VPA after 7 and 14 days of treatment. Notice the trace images of GASH/Sal animals treated with CBD or CBD+VPA that spent less time moving in the periphery and longer time in the center of the arena compared to those animals treated with the vehicle or VPA alone, also compared to the pre-treatment condition. Histograms show the total distance traveled **(B)**, time of activity **(C)**, number of rearing **(D)**, time spent in the center **(E)**, and number of grooming **(F)** for all experimental groups at the protocol-defined time points. Behavior indicators for the locomotor pattern (total distance traveled, time of activity, and number of rearings) and for the emotionality (time in the central zone and number of self-grooming) revealed differential effects of CBD in both monotherapy and combined therapy with VPA when compared to baseline activity (pre-treatment), as well as to vehicle and VPA monotherapies. Data are shown as means ± SEM. Asterisks indicate significant differences between experimental groups (*$p < 0.05$, **$p < 0.01$).

animals with the combined CBD+VPA treatments. On the other hand, there were no significant differences in the time spent in the center zone between VPA-treated and the sham groups after 7 and 4 days of chronic VPA administration (**Figure 8E**). After 7 days of repeated administration of the drugs, a significant increase in the number of groomings was found in the VPA–, CBD–, and CBD+VPA-treated groups as compared to sham animals. After 14 days of chronic drug administration, a drastic reduction in the number of groomings of GASH/Sal animals treated with the combined CBD+VPA treatment was noticeable, which was significantly lower as compared to sham animals. However, the animals that received the VPA or CBD treatment alone exhibited similar values in the number of grooming than those receiving the sham "vehicle" treatment (**Figure 8F**). The comparison between the pre-treatment and post-treatment (7 and 14 days) conditions showed a decrease in all the locomotor open-field measures, except for the time spent in the center zone that was significantly higher following the combined CBD+VPA treatment after 7 and 14 days.

Disruption of Gene Expression in the Inferior Colliculus After Chronic Drug Treatments

To determine the molecular effects of each chronic treatment on the gene expression that might underlie the modifications of seizure behaviors in the GASH/Sal, we analyzed mRNA expression levels of the following genes: *Trpv1*, *5-Htr1a*, *Sigmar1*, *Adora1*, *Slc29a1*, and *Cnr1* (**Figure 9**). We selected these genes, as the proteins they encoded, are related to regulation of neuronal excitability and neuroprotection that might be presumably affected by the pharmacological activity of the treatments in the epileptogenic focus. Thus, whole tissue containing the inferior colliculi was freshly dissected after 14 days of chronic treatments from animals belonging to all experimental groups. Quantitative gene expression data were normalized using *β-actin* as internal reference gene. There were no significant differences in the number of cycles to reach the amplification threshold for *Actb* with any of the animal groups, indicating that the sample preparation was consistent. Raw gene expression data can be found in **Supplementary Material 3**. Comparison of gene expression/*Actb* ratios showed that expression of the *Trpv1* gene was significantly higher in the CBD+VPA-treated group as compared to the sham group ($p = 0.0001$; **Figure 9A**). Furthermore, the mRNA expression levels of *Trpv1* were also significantly higher in animals receiving the combination of both drugs than in those that received the CBD or the VPA treatment alone ($p = 0.002$ for CBD and $p = 0.0001$ for VPA; **Figure 9A**). As compared to the sham group, the gene expression of *Trpv1* was also increased with the CBD or the VPA treatment alone, although this difference was not statistically significant (**Figure 9A**). On the other hand, the mRNA expression levels of the *5-Htr1a* and *Sigmar1* genes were not significantly different between the four experimental groups (**Figures 9B,C**). CBD treatment induced modifications in the mRNA expression levels of *Adora1*, showing a significant increase as compared to the vehicle-, VPA-, and CBD+VPA-treated groups ($p = 0.0002$ for the vehicle, $p = 0.001$ for VPA, $p = 0.0001$ for CBD+VPA; **Figure 9D**). VPA either alone or in combination with CBD did not affect the gene expression of *Adora1* (**Figure 9D**). As compared to sham animals, gene expression levels of *Slc29a1* were also found significantly decreased in the animals treated with CBD alone or the combination of CBD and VPA ($p = 0.0004$ for CBD, $p = 0.001$ for CBD+VPA; **Figure 9E**). However, there were no significant differences in the expression levels of the *Slc29a1* gene between the VPA-treated and the sham groups (**Figure 9E**). Compared to sham animals, mRNA expression levels of *Cnr1* were significantly increased after the CBD treatment ($p = 0.02$; **Figure 9F**), whereas no significant differences were found after the VPA and VPA+CBD treatments, albeit at still high levels. Furthermore, treatment with CBD alone significantly increased the mRNA expression levels of *Cnr1* as compared to animals that received the combined CBD+VPA treatment ($p = 0.04$; **Figure 9F**).

DISCUSSION

The GASH/Sal model has been previously used to evaluate the specific efficacies of antiepileptic drugs against generalized tonic–clonic seizures. The fact that classic anticonvulsant drugs such as valproate (VPA), phenobarbital, and lamotrigine were highly effective in suppressing sound-induced seizures in the GASH/Sal indicates that this animal model can be reliably used for preclinical screening of novel anticonvulsant agents (Barrera-Bailón et al., 2013, 2017). Consistent with previous reports (Barrera-Bailón et al., 2013), we reported that acute administration of VPA (300 mg/kg) had clear anticonvulsant effects, showing that 50% of the GASH/Sal animals entirely blocked their sound-induced seizures. These results were consistent with previous studies in the GASH/Sal (Barrera-Bailón et al., 2013), in which the median effective dose of VPA for complete elimination of AGS was 300 mg/kg. We further reported that 7 and 14 days of two times intraperitoneal daily injections of VPA at doses of 300 mg/kg diminished its effectiveness in seizure attenuation when compared to an acute single-dose administration. This loss of anticonvulsant activity after chronic administration of VPA (three times intraperitoneal daily injections at 200 mg/kg for 6 weeks) was also reported in amygdala-kindled rats, showing ataxia and muscle relaxation as the most prominent adverse side effects (Hönack and Löscher, 1995; Löscher and Hönack, 1995). Although we did not find a significant impairment of locomotor exploratory behaviors after chronic VPA treatment, previous studies from Barrera-Bailón et al. (2013) also reported ataxic effects of VPA at doses of 300 mg/kg, which is in line with the reported side effects of VPA (Löscher and Hönack, 1995). Other currently available antiepileptic drugs, such as VPA, are also known to produce a variety of psychiatric, cognitive, and motor adverse effects (Zaccara et al., 2004). Therefore, therapies with similar anticonvulsant efficacy but lower toxicity or that carries the potential to reduce the adverse effects of classical antiepileptic drugs are a rapidly increasing clinical demand that has to be met. Among the novel anticonvulsant agents, there is considerable

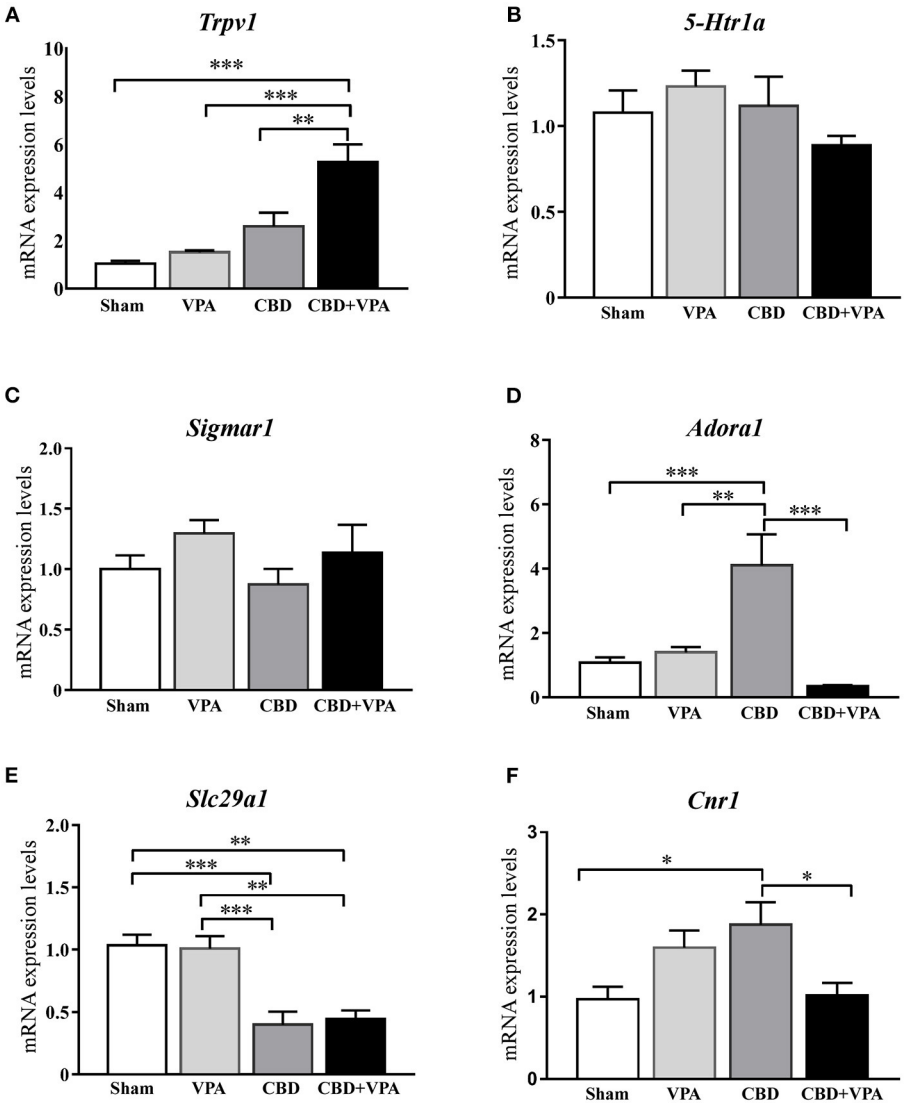


FIGURE 9 | Gene expression changes in the inferior colliculus after 14-days treatment with VPA, CBD or the combination of CBD+VPA. Histogram shows relative quantities of transcripts of *Trpv1* **(A)**, *5-Htr1a* **(B)**, *Sigmar1* **(C)**, *Adora1* **(D)**, *Slc29a1* **(E)**, and *Cnr1* **(F)** genes in the inferior colliculus of GASH/Sal animals treated with vehicle (sham), VPA, CBD, and CBD+VPA. Notice that disruption of mRNA expression levels was found for the *Trpv1*, *Adora1*, *Slc29a1*, and *Cnr1* genes, whereas no differential gene expression was found for *Htr1a* and *Sigmar1*. The relative mRNA expression of each gene was normalized to *β-actin*. ΔCt values were normalized to the average ΔCt of the inferior colliculus of sham animals. Each bar in the histograms is an average ± SEM. Asterisks indicate significant differences between experimental groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

interest in CBD as a promising therapy for epilepsy, and preliminary experiments appear to demonstrate that CBD may be a useful treatment for pharmacoresistant epilepsy (reviewed in Perucca, 2017; Franco and Perucca, 2019; Lazarini-Lopes et al., 2020). Although it is also true that CBD does not exert seizure-protective activity in the rat model of pharmacoresistant epilepsy, namely, lamotrigine-resistant amygdala kindled rat (Klein et al., 2017), there is ample evidence that CBD effectively does it in a large variety of preclinical seizures models (Franco and Perucca, 2019; Patra et al., 2019; Lazarini-Lopes et al., 2020). The seizure rodent models, in which CBD was an effective and relatively potent anticonvulsant, are those induced by a great number of experimental treatments such as maximal electroshock, pentylenetetrazole, 3-mercaptopropionic acid, bicuculline, picrotoxin, cocaine, and isoniazid, as well as pilocarpine models of temporal lobe seizures among many others (Perucca, 2017; Patra et al., 2019; Lazarini-Lopes et al., 2020). The differences in the CBD effects on preventing seizures varied not only depending on the animal seizure model, but also in the dosages and route of administrations used in each study. Nevertheless, despite those differences, there is a consensus in the scientific literature that CBD given intraperitoneally produced a dose-dependent protection against seizures induced by chemical or electrical means, in acute and, more particularly,

kindling rodent seizure models (Klein et al., 2017; Patra et al., 2019; Lazarini-Lopes et al., 2020). On the contrary, there are considerable fewer studies that investigated the effects of CBD on AGS models of genetic origin (reviewed in Lazarini-Lopes et al., 2020). In AGS susceptible rats, the median effective dose of CBD that reduced generalized tonic–clonic seizure was 82 mg/kg following acute intraperitoneal administration (Consroe and Wolkin, 1977). In susceptible DBA/2J mice, concomitant intraperitoneal administration of CBD (27 mg/kg) and its analog cannabidivarin (116 mg/kg) was found to attenuate wild-running, clonic, and tonic seizure, whereas isolated CBD administration was able to attenuate only the incidence of clonic seizures (Hill et al., 2013). In these few studies conducted in animal models of acute AGSs, the anticonvulsant effects of CBD were not as clear as reported in other rodent models of epilepsy. As no additional information was available from these studies about the seizure behavior analysis of the CBD effects, there is still room to investigate the potential anticonvulsant activity in genetically seizure-prone strains of rodents. Our study used neuroethological approaches in the GASH/Sal model to detail the effects of CBD on each specific seizure behavior following acute and chronic intraperitoneal administration. Our results showed that acute administration of CBD (100 mg/kg) had slight effects on seizure behaviors of GASH/Sal animals, including reduction of generalized clonic convulsions, but without significant attenuation of other behavioral items of the wild-running and convulsion phases. This behavioral CBD effects correlated to categorized index seizures scores that varied between 2 and 8, and hence none of the animals belonging to the CBD-treated group showed total elimination of seizures. Notwithstanding that, we found that acute CBD administration in the GASH/Sal significantly increased seizure latency and duration of the wild-running phase, whereas the duration of the convulsions was significantly reduced as compared to sham animals. Similar results were previously shown in other rodent models of epilepsy after CBD (100 mg/kg) intraperitoneal injections, reporting such effects on the duration of the seizure phases as a strong indication of anticonvulsant activity (Jones et al., 2012; Pelz et al., 2017). Indeed, we also observed these same effects in the GASH/Sal after acute treatment with VPA, but far more effectively and intensively than in the CBD-treated group. We further reported similar CBD effects following chronic administration (2 times intraperitoneal daily injections at 100 mg/kg) for 7 days, but in contrast, continuing this treatment over 14 days had no significant effects on seizure behavior, showing maximum seizure scores in 100% of the animals. On the basis of these results, it does not seem that CBD has potent anticonvulsant effects in the GASH/Sal model at the doses used and make us therefore discuss this matter as well as the limitations of our study. One possibility is that CBD preparation of 100 mg/kg was not therapeutically significant as it might not be the one required to reach higher CBD concentration in serum and brain. CBD levels in blood and brain may vary depending on the drug purity and concentration, solvent, and route of administration, as well as the animal species and the own characteristics of each animal model. In our experiments, we used high purity of CBD, Cremophor as solvent, and acute intraperitoneal single dose of 100 mg/kg under similar administration protocols as used elsewhere in rats and mice (Deiana et al., 2012; HloŽek et al., 2017). However, when compared to the bioavailability levels of rats and mice, the serum concentration levels of CBD following 100 mg/kg intraperitoneal injections in the GASH/Sal hamsters were very low. Our HPLC results showed that serum CBD concentrations reached peaks of 263 ng/mL (45 min post-administration), which rapidly and continuously declined until reaching values close to the detection threshold at 12 h. These results indicated both rapid absorption and elimination of CBD from blood that can be due to an accelerated metabolic degradation of the drug in the GASH/Sal. With this argument in mind, it would be worthy to carry out future experiments in the GASH/Sal using higher doses of CBD. Indeed, CBD intraperitoneal doses of 100 mg/kg or more were reported as the median effective dose of CBD for complete abolition of seizures in several rats and mice models of epilepsy (e.g., Karler et al., 1973; Kaplan et al., 2017; reviewed in Lazarini-Lopes et al., 2020). As the blood–brain barrier affects the ability of a drug to enter the brain and its integrity might be altered in epilepsy (Marchi et al., 2012), it is also critical to measure the CBD concentrations in the brain, which was a limitation of the present study. Furthermore, several studies claimed anticonvulsant effects of CBD in electrical or chemical kindling protocols that cause repetitive seizures in animals, showing a delay of kindling progression (Klein et al., 2017, Vilela et al., 2017). The genetically seizure-prone strains of rodents express different patterns of seizures, in a manner that the acute protocol precipitates generalized tonic–clonic seizures similar to grand mal epilepsy in humans, and the kindling protocol (chronic-evoked seizures) as a type of temporal lobe epilepsy model (Ross and Coleman, 2000; Garcia-Cairasco, 2002; Kandratavicius et al., 2014). In the present study, we evaluated the CBD effects on an acute protocol of seizure stimulation and using only one drug concentration, so experiments given CBD in a dose-dependent manner in GASH/Sal animals submitted to audiogenic kindling protocols might clarify the protective effectiveness of CBD against AGSs.

One of the aims of the present study was to determine the anticonvulsant effects of CBD and VPA coadministration. The fact that not all the GASH/Sal animals blocked the seizures following 300 mg/kg of VPA provided an opportunity to determine possible synergistic (additive or antagonistic) effects when administered in combination with CBD. Our results showed that the VPA and CBD coadministration did not alter the therapeutic outcome of the monotherapy with VPA, showing no apparent additive or antagonistic effects. Thus, the acute coadministered CBD and VPA drastically reduced the seizures scores, increasing the seizure latency and duration of the wild-running phase, as well as decreasing the duration of the convulsions. These effects were almost the same as those reported in GASH/Sal animals treated with VPA alone, showing the same percentage of animals in which seizures were completely eliminated. The most plausible explanation is that serum concentration levels of VPA were not modified when coadministered with CBD as it has been reported elsewhere (Gaston et al., 2017). Although controversy still remains on whether CBD potentiates the anticonvulsant effects of classic

antiepileptic drugs (reviewed in Lazarini-Lopes et al., 2020), new experiments on GASH/Sal combination treatments might be useful to identify possible pharmacological interactions between CBD and conventional anticonvulsant drugs.

Common adverse effects associated with CBD treatment in epileptic patients included somnolence/sedation, decreased appetite, increases in transaminases, and diarrhea, behavioral changes, rashes, fatigue, and sleep disturbances (Devinsky et al., 2016; Franco and Perucca, 2019; Wheless et al., 2019). In our study, we reported that CBD treatments in the GASH/Sal had no effect on body weight, indicating that the administration of CBD did not affect food or water intake. Of particular concern, however, is the risk for CBD-induced hepatotoxicity, which has been reported in mice following CBD administration at high doses up to 2,460 mg/kg (Ewing et al., 2019). Our results indicated no adverse effects in liver function as well as in the hematologic profile following repeated daily administration of CBD. We further found that coadministered VPA and CBD significantly reduced hemoglobin levels and white blood cell counts. As it is known that VPA prompt neutropenia or leukopenia (Hsu et al., 2009) and the CBD-treated group showed normal values, the alterations in the hematological profile observed in the VPA+CBD-treated group were likely due to the VPA rather than CBD. Furthermore, studies in preclinical models of epilepsy reported that CBD treatment might reach full seizure protection without producing significant motor impairment (Hill et al., 2013; Klein et al., 2017). In our experiments, the open-field test revealed a decrease in the locomotor activity and exploratory behaviors after CBD administration, particularly in combination treatment with VPA. Because VPA produced motor adverse effects (Zaccara et al., 2004), the coadministration of CBD and VPA in the GASH/Sal did not seem to improve the motor impairment associated with the VPA treatment. These results are also in line with possible sedative effects of CBD on the GASH/Sal, which have been previously reported in other rodent models of epilepsy treated with CBD (Pickens, 1981; Gu et al., 2019). We also reported a significant increase in the permanence time in the center of the arena, as well as the number of rearing in the CBD- and CBD+VPA-treated animals, which may be related to increasing exploratory behavior and reduced anxiety-like behavior exerted by the drugs (Sasibhushana et al., 2019). Our results indicated modification of emotionality in GASH/Sal animals after CBD administration and its combination with VPA, but not when VPA was administered alone. Although both drugs reduce exploratory activity, the time spent in the center was considerably increased in all treated groups. This seems contradictory if we consider that more thigmotaxis and less locomotion activity are thought to indicate greater anxiety, whereas greater exploration and more ambulation in the center arena reflect less emotionality (Choleris, 2001). Other studies also reported the lack of influence of CBD on the main parameters of the open-field test such as number of square crossings, number of entries, and time spent in the center zone. Additionally, vertical exploration (i.e., rearing and wall leaning) was increased in rats by CBD (Campos and Guimarães, 2008; Shoval et al., 2016; Sales et al., 2018). It is worth mentioning that the differences between the open-field performance that were reported here in the GASH/Sal hamster and those reported in other studies (mainly rats and mice) might be related to interspecies differences.

An important outcome of the present study was the mRNA expression analysis of genes in the inferior colliculus of the GASH/Sal after each chronic treatment. The inferior colliculus is considered the brain area for seizure generation and propagation in rodent models of AGS susceptibility (reviewed in Garcia-Cairasco, 2002). Recent studies in the GASH/Sal pointed out that the inferior colliculus is embedded in a web of pathologic connections in the auditory pathway that constitutes a seizure-prone network to finally drive the AGS (Sánchez-Benito et al., 2020). In an attempt to shed light on the molecular effects of each treatment in the inferior colliculus of the GASH/Sal, we analyzed the gene expression levels of *Trpv1*, *5-Htr1a*, *Sigmar1*, *Adora1*, *Slc29a1*, and *Cnr1*. Although there is a very long list of neuronal receptors that are targets of CBD or are related to its action mechanism (reviewed in Franco and Perucca, 2019), we selected those genes because they encoded proteins related to regulation of neuronal excitability and neuroprotection that might be presumably affected by the pharmacological activity of the treatments on the epileptogenic focus. TRPV1 is a non-selective channel with high permeability to Na^+ and Ca^{2+}, and its activation promotes the release of glutamate by increasing the neuronal excitability (Caterina et al., 1997; Naziroglu et al., 2013). CBD exerts effects on TRPV1 generating receptor activation and desensitization (Iannotti et al., 2014), and hence binding at TRPV1 might regulate neuronal activity. Our PCR results showed significant increase in mRNA expression of *Trpv1* gene following the coadministration of CBD and VPA, suggesting a joint action of both drugs that might lead to increased neuronal susceptibility and neurotoxicity (Naziroglu and Övey, 2015). In fact, these results correlated to our behavioral results showing that chronic coadministration of CBD and VPA had subtle or no effects on the AGS of the GASH/Sal. In line with this argument, Sun et al. (2013) reported higher gene expression levels of *Trpv1* in the hippocampus and the cortex of drug-resistant mesial temporal lobe epilepsy patients. Activation of the serotonin 5-HTR1A receptor hyperpolarizes the resting membrane potential and has an anticonvulsant effect in various experimental *in vivo* and *in vitro* seizure models (Salgado and Alkadhi, 1995; Gariboldi et al., 1996). Although CBD has been shown to be an agonist of the 5-HTR1A receptor (Russo et al., 2005), studies in the pentylenetetrazole model of generalized seizures reported that 5-HTR1A is not involved in CBD's anticonvulsant effect (Pelz et al., 2017). Consistently with this, our results showed normal mRNA expression levels of 5-HTR1A after chronic CBD treatment. SIGMAR1 receptor antagonists inhibit glutamate *N*-methyl-D-aspartate acid receptor (NMDAR) activity and display positive effects on epilepsy. Although CBD displays antagonist-like activity toward SIGMAR1 receptor to reduce the negative effects of NMDAR overactivity in epilepsy (Rodríguez-Muñoz et al., 2018), our results showed that chronic CBD administration or coadministration with VPA did not affect the gene expression levels of SIGMAR1. The ADORA1 receptors are involved on the adenosine modulation system

and contribute to adaptive changes in neurotransmission and neuroprotection. Thus, adenosine acts as a negative regulator of glutamate release via activation of the presynaptic ADORA1 receptors. Histopathologic and biochemical analyses of surgical resections of patients with epilepsy showed a decrease in the gene expression of ADORA1, suggesting that this decrease contributes to chronic epilepsy in humans (Glass et al., 1996; Boison, 2016). CBD bolsters adenosine signaling by inhibiting its extracellular removal and provides an ADORA1 receptor–mediated mechanism by which CBD decreases inflammation (Liou et al., 2008). In this context, the high levels of *Adora1* transcripts in the GASH/Sal inferior colliculus after chronic CBD administration might be a compensatory mechanism for overactivation of glutamate receptors and/or the increment of glutamate release. Adenosine shows anticonvulsant effects, and it is released during seizures (Ilie et al., 2012). Furthermore, Carrier et al. (2006) revealed that CBD binds to the equilibrative nucleoside transporter 1 (encoded by *Slc29a1* gene), which led to the increase of extracellular adenosine. Thus, our results showing a decrease in gene expression levels of *Slc29a1* in GASH/Sal animals treated with CBD alone or the combination of CBD and VPA might be a mechanism to regulate neuronal excitability in the epileptogenic nucleus. The *Cnr1* gene encoded the central cannabinoid receptor CB1, which is activated by anandamide and inhibits the presynaptic release of glutamate and γ-aminobutyric acid (Howlett and Abood, 2017). In animal models of epilepsy, the gene expressions of *Cnr1* and *Trpv1* are increased (von Rüden et al., 2015). Furthermore, CBD behaves as a non-competitive negative allosteric modulator of CB1 receptors (Laprairie et al., 2015) and inhibits reuptake and hydrolysis of anandamide (Bisogno et al., 2009). Thus, our PCR results showing that CBD treatment causes an increase of *Cnr1* gene expression might be related to a possible protective effect against increased neuronal excitation and an attempt to inhibit glutamate release in the GASH/Sal inferior colliculus.

Together, our results are in line with previous studies conducted on epileptic seizure models showing that the CBD's mechanism of action is complex, as its anticonvulsant activity is mediated by multifactorial mechanisms underlying the behavioral, electrophysiological, and neuroprotective effects of CBD (reviewed in Lazarini-Lopes et al., 2020). New experiments at higher dosages as well as in the audiogenic kindling protocol (chronic-evoked seizures) might be necessary to delve deeper into the anticonvulsant effects of CBD in the GASH/Sal model.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethics Committee of the University of Salamanca (application number 380).

AUTHOR CONTRIBUTIONS

DL, RG-N, and CS conceived the original idea, designed the experiments, and supervised the project. DL and RG-N secured funding. RG-N wrote the manuscript and performed visualization/data presentation. GC-P and DS-B carried out the drug administration experiments and analyzed the seizure severity index. GC-P carried out blood-sampling procedure and the formal analysis of the hematological and biochemical profiles. SD-R performed the RT-qPCR experiments. LM provided the animals and performed the bibliographic review. OC and GC-P carried out the open-field test and the formal analysis of the open-field results. CS designed and supervised the pharmacological part of the project. JG evaluated the seizure behaviors with the Ethomatic and performed the ethograms. DL, RG-N, CS, OC, and LM provided critical feedback and helped shape the manuscript. All authors reviewed and approved the paper.

FUNDING

This study was supported by RiverForce Partners Inc. (research project code: Art. 83. 2019/00106/001) and the research grant from the Instituto de Salud Carlos III (ISCIII), co-financed with European Union FEDER funds (#PI19/01364, PIs: DL and RG-N).

ACKNOWLEDGMENTS

The authors wish to thank Professor Norberto García-Cairasco and Dr. Antonio Cortes de Oliveira (from the Neurophysiology and Experimental Neuroethology Laboratory of Ribeirão Preto School of Medicine, Brazil) for providing the Ethomatic program and software support. We also thank Juan López for standardizing the ethograms and Sandra García Losada for excellent technical support. The authors gratefully acknowledge RiverForce Partners Inc. for research sponsorship and provision of cannabidiol as well as the international collaboration agreement between the RiverForce Partners Inc. and the University of Salamanca.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.612624/full#supplementary-material

Supplementary Material 1 | Video recording of a representative audiogenic seizure in a GASH/Sal animal. Following acute sound stimulation the GASH/Sal animal showed five differenced and consecutive phases: (1) a behavioral arrest, (2) a period of wild running, (3) tonic–clonic convulsions, (4) head ventral flexion, forelimb extension, and hind limb extension, and (5) postictal immobility. The GASH/Sal animal shown in the video did not receive drug treatment and belonged to the baseline (pre-treatment) condition. Notice that the behavioral items corresponding to each seizure phase are simultaneously depicted in the neuroethological analysis (flowchart on top right corner of the video). See **Figure 2** for detailed interpretation of the flowchart.

Supplementary Material 2 | Table shows the percentage of animals based on the categorized seizure scores > 2 and <2 in each of the experimental groups.

Supplementary Material 3 | Raw data of RT-qPCR used for analyses. The qPCR data included a set of 4 to 6 biological replicates (sample cases) for each experimental group (treatment condition), triplicate technical replicates as well as the Ct values of housekeeping gene beta-actin used for data normalization. *Adora1*, the adenosine A1 receptor; *Actb*, β-actin; CBD, cannabidiol; *Cnr1*, the cannabinoid receptor 1; *Sigmar1*, the sigma non-opioid intracellular receptor 1; *Slc29a*, the equilibrative nucleoside transporter 1; *Trpv1*, the transient receptor potential of vanilloid type 1; VPA, valproic acid; 5-HTR1A, the 5-hydroxytryptamine (serotonin) receptor 1A.

REFERENCES

Andersen, C. L., Jensen, J. L., and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR Data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64, 5245–5250. doi: 10.1158/0008-5472.CAN-04-0496

Barrera-Bailón, B., Oliveira, J. A. C., López, D. E., Muñoz, L. J., Garcia-Cairasco, N., and Sancho, C. (2013). Pharmacological and neuroethological studies of three antiepileptic drugs in the genetic audiogenic seizure hamster (GASH:Sal). *Epilepsy Behav.* 28, 413–425. doi: 10.1016/j.yebeh.2013.05.028

Barrera-Bailón, B., Oliveira, J. A. C., López, D. E., Muñoz, L. J., Garcia-Cairasco, N., and Sancho, C. (2017). Pharmacological and neuroethological study of the acute and chronic effects of lamotrigine in the genetic audiogenic seizure hamster (GASH:Sal). *Epilepsy Behav.* 71, 207–217. doi: 10.1016/j.yebeh.2015.11.005

Bisogno, T., Burston, J. J., Rai, R., Allarà, M., Saha, B., Mahadevan, A., et al. (2009). Synthesis and pharmacological activity of a potent inhibitor of the biosynthesis of the endocannabinoid 2-arachidonoylglycerol. *ChemMedChem* 4, 946–950. doi: 10.1002/cmdc.200800442

Boison, D. (2016). Adenosinergic signaling in epilepsy. *Neuropharmacology* 104, 131–139. doi: 10.1016/j.neuropharm.2015.08.046

Bosque, J. R., Gómez-Nieto, R., Hormigo, S., Herrero-Turrión, M. J., Díaz-Casado, E., Sancho, C., et al. (2019). Molecular tools for the characterization of seizure susceptibility in genetic rodent models of epilepsy. *Epilepsy Behav.*1:106594. doi: 10.1016/j.yebeh.2019.106594

Burns, M. J., Nixon, G. J., Foy, C. A., and Harris, N. (2005). Standardisation of data from real-time quantitative PCR methods - evaluation of outliers and comparison of calibration curves. *BMC Biotechnol.* 5:31. doi: 10.1186/1472-6750-5-31

Campos, A. C., and Guimarães, F. S. (2008). Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. *Psychopharmacology* 199, 223–230. doi: 10.1007/s00213-008-1168-x

Carballosa-Gonzalez, M. M., Muñoz, L. J., López-Alburquerque, T., Pardal-Fernández, J. M., Nava, E., de Cabo, C., et al. (2013). EEG characterization of audiogenic seizures in the hamster strain GASH:Sal. *Epilepsy Res.* 106, 318–325. doi: 10.1016/j.eplepsyres.2013.07.001

Carrier, E. J., Auchampach, J. A., and Hillard, C. J. (2006). Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7895–7900. doi: 10.1073/pnas.0511232103

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816–824. doi: 10.1038/39807

Choleris, E. (2001). A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. *Neurosci. Biobehav. Rev.* 25, 235–260. doi: 10.1016/S0149-7634(01)00011-2

Consroe, P., and Wolkin, A. (1977). Cannabidiol–antiepileptic drug comparisons and interactions in experimentally induced seizures in rats. *J Pharmacol Exp Ther* 201, 26–32.

Damasceno, S., Gómez-Nieto, R., Garcia-Cairasco, N., Herrero-Turrión, M. J., Marín, F., and Lopéz, D. E. (2020). Top common differentially expressed genes in the epileptogenic nucleus of two strains of rodents susceptible to audiogenic seizures: WAR and GASH/Sal. *Front. Neurol.* 11:33. doi: 10.3389/fneur.2020.00033

Deiana, S., Watanabe, A., Yamasaki, Y., Amada, N., Arthur, M., Fleming, S., et al. (2012). Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Δ 9-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behav. *Psychopharmacology* 219, 859–873. doi: 10.1007/s00213-011-2415-0

Devinsky, O., Cilio, M. R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* 55, 791–802. doi: 10.1111/epi.12631

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurol.* 15, 270–278. doi: 10.1016/S1474-4422(15)00379-8

Díaz-Casado, E., Gómez-Nieto, R., de Pereda, J. M., Muñoz, L. J., Jara-Acevedo, M., and López, D. E. (2020). Analysis of gene variants in the GASH/Sal model of epilepsy. *PLoS ONE* 15:e0229953. doi: 10.1371/journal.pone.0229953

Díaz-Rodríguez, S. M., López-López, D., Herrero-Turrión, M. J., Gómez-Nieto, R., Canal-Alonso, A., and Lopéz, D. E. (2020). Inferior colliculus transcriptome after status epilepticus in the genetically audiogenic seizure-prone hamster GASH/Sal. *Front. Neurosci.* 14:508. doi: 10.3389/fnins.2020.00508

Dutra, W. O., Menezes, C. A. S., Villani, F. N. A., da Costa, G. C., Silveira, A. B. M., da Reis, D., et al. (2009). Cellular and genetic mechanisms involved in the generation of protective and pathogenic immune responses in human Chagas disease. *Mem. Inst. Oswaldo Cruz* 104, 208–218. doi: 10.1590/S0074-02762009000900027

Ewing, L. E., Skinner, C. M., Quick, C. M., Kennon-McGill, S., McGill, M. R., Walker, L. A., et al. (2019). Hepatotoxicity of a cannabidiol-rich cannabis extract in the mouse model. *Molecules* 24:1694. doi: 10.3390/molecules24091694

Faingold, C. L. (1999). Neuronal networks in the genetically epilepsy-prone rat. *Adv. Neurol.* 79, 311–321.

Fisher, R. S., Acevedo, C., Arzimanoglou, A., Bogacz, A., Cross, J. H., Elger, C. E., et al. (2014). ILAE official report: a practical clinical definition of epilepsy. *Epilepsia* 55, 475–482. doi: 10.1111/epi.12550

Fisher, R. S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., et al. (2005). Epileptic seizures and epilepsy: definitions proposed by the international league against epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 46, 470–472. doi: 10.1111/j.0013-9580.2005.66104.x

Franco, V., and Perucca, E. (2019). Pharmacological and therapeutic properties of cannabidiol for epilepsy. *Drugs* 79, 1435–1454. doi: 10.1007/s40265-019-01171-4

Garcia-Cairasco, N. (2002). A critical review on the participation of inferior colliculus in acoustic-motor and acoustic-limbic networks involved in the expression of acute and kindled audiogenic seizures. *Hear. Res.* 168, 208–222. doi: 10.1016/S0378-5955(02)00371-4

Garcia-Cairasco, N., Rossetti, F., Oliveira, J. A. C., and Furtado, M. A. (2004). Neuroethological study of status epilepticus induced by systemic pilocarpine in Wistar audiogenic rats (WAR strain). *Epilepsy Behav.* 5, 455–463. doi: 10.1016/j.yebeh.2004.04.005

Garcia-Cairasco, N., Wakamatsu, H., Oliveira, J. A. C., Gomes, E. L. T., Del Bel, E. A., and Mello, L. E. A. M. (1996). Neuroethological and morphological (Neo-Timm staining) correlates of limbic recruitment during the development of audiogenic kindling in seizure susceptible Wistar rats. *Epilepsy Res.* 26, 177–192. doi: 10.1016/S0920-1211(96)00050-2

Gariboldi, M., Tutka, P., Samanin, R., and Vezzani, A. (1996). Stimulation of 5-HT1A receptors in the dorsal hippocampus and inhibition of limbic seizures induced by kainic acid in rats. *Br. J. Pharmacol.* 119, 813–818. doi: 10.1111/j.1476-5381.1996.tb15745.x

Gaston, T. E., Bebin, E. M., Cutter, G. R., Liu, Y., and Szaflarski, J. P. (2017). Interactions between cannabidiol and commonly used antiepileptic drugs. *Epilepsia* 58, 1586–1592. doi: 10.1111/epi.13852

Glass, M., Faull, R. L. M., Bullock, J. Y., Jansen, K., Mee, E. W., Walker, E. B., et al. (1996). Loss of A1 adenosine receptors in human temporal lobe epilepsy. *Brain Res.* 710, 56–68. doi: 10.1016/0006-8993(95)01313-X

Gu, B., Zhu, M., Glass, M. R., Rougi,é, M., Nikolova, V. D., Moy, S. S., et al. (2019). Cannabidiol attenuates seizures and EEG abnormalities in Angelman syndrome model mice. *J. Clin. Invest.* 129, 5462–5467. doi: 10.1172/JCI130419

Hill, T. D. M., Cascio, M.-G., Romano, B., Duncan, M., Pertwee, R. G., Williams, C. M., et al. (2013). Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB 1 receptor-independent mechanism. *Br. J. Pharmacol.* 170, 679–692. doi: 10.1111/bph.12321

HloŽek, T., Uttl, L., Kaderábek, L., Balíková, M., Lhotková, E., Horsley, R. R., et al. (2017). Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC. *Eur. Neuropsychopharmacol.* 27, 1223–1237. doi: 10.1016/j.euroneuro.2017.10.037

Hönack, D., and Löscher, W. (1995). Kindling increases the sensitivity of rats to adverse effects of certain antiepileptic drugs. *Epilepsia* 36, 763–771. doi: 10.1111/j.1528-1157.1995.tb01613.x

Howlett, A. C., and Abood, M. E. (2017). CB_1 and CB_2 receptor pharmacology. *Adv. Pharmacol.* 80, 169–206. doi: 10.1016/bs.apha.2017.03.007

Hsu, H.-C., Tseng, H.-K., Wang, S.-C., and Wang, Y.-Y. (2009). Valproic acid-induced agranulocytosis. *Int. J. Gerontol.* 3, 137–139. doi: 10.1016/S1873-9598(09)70036-5

Iannotti, F. A., Hill, C. L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., et al. (2014). Nonpsychotropic Plant Cannabinoids, Cannabidivarin (CBDV) and Cannabidiol (CBD), Activate and Desensitize Transient Receptor Potential Vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem. Neurosci.* 5, 1131–1141. doi: 10.1021/cn5000524

Ibeas Bih, C., Chen, T., Nunn, A. V. W., Bazelot, M., Dallas, M., and Whalley, B. J. (2015). Molecular targets of cannabidiol in neurological disorders. *Neurotherapeutics* 12, 699–730. doi: 10.1007/s13311-015-0377-3

Ilie, A., Raimondo, J. V., and Akerman, C. J. (2012). Adenosine release during seizures attenuates GABAA receptor-mediated depolarization. *J. Neurosci.* 32, 5321–5332. doi: 10.1523/JNEUROSCI.5412-11.2012

Jones, N. A., Glyn, S. E., Akiyama, S., Hill, T. D. M., Hill, A. J., Weston, S. E., et al. (2012). Cannabidiol exerts anti-convulsant effects in animal models of temporal lobe and partial seizures. *Seizure* 21, 344–352. doi: 10.1016/j.seizure.2012.03.001

Kandratavicius, L., Balista, P., Lopes-Aguiar, C., Ruggiero, R., Umeoka, E., Garcia-Cairasco, N., et al. (2014). Animal models of epilepsy: use and limitations. *Neuropsychiatr. Dis. Treat.* 10, 1693–1705. doi: 10.2147/NDT.S50371

Kanner, A. M., Barry, J. J., Gilliam, F., Hermann, B., and Meador, K. J. (2012). Depressive and anxiety disorders in epilepsy: do they differ in their potential to worsen common antiepileptic drug-related adverse events? *Epilepsia* 53, 1104–1108. doi: 10.1111/j.1528-1167.2012.03488.x

Kaplan, J. S., Stella, N., Catterall, W. A., and Westenbroek, R. E. (2017). Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11229–11234. doi: 10.1073/pnas.1711351114

Karler, R., Cely, W., and Turkanis, S. A. (1973). The anticonvulsant activity of cannabidiol and cannabinol. *Life Sci.* 13, 1527–1531. doi: 10.1016/0024-3205(73)90141-0

Klein, B. D., Jacobson, C. A., Metcalf, C. S., Smith, M. D., Wilcox, K. S., Hampson, A. J., et al. (2017). Evaluation of cannabidiol in animal seizure models by the epilepsy therapy screening program (ETSP). *Neurochem. Res.* 42, 1939–1948. doi: 10.1007/s11064-017-2287-8

Kwan, P., Arzimanoglou, A., Berg, A. T., Brodie, M. J., Allen Hauser, W., Mathern, G., et al. (2009). Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE commission on therapeutic strategies. *Epilepsia* 51, 1069–1077. doi: 10.1111/j.1528-1167.2009.02397.x

Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., and Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB 1 receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi: 10.1111/bph.13250

Lazarini-Lopes, W., Do Val-da Silva, R. A., da Silva-Júnior, R. M. P., Leite, J. P., and Garcia-Cairasco, N. (2020). The anticonvulsant effects of cannabidiol in experimental models of epileptic seizures: from behavior and mechanisms to clinical insights. *Neurosci. Biobehav. Rev.* 111, 166–182. doi: 10.1016/j.neubiorev.2020.01.014

Liou, G. I., Auchampach, J. A., Hillard, C. J., Zhu, G., Yousufzai, B., Mian, S., et al. (2008). Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A 2A adenosine receptor. *Investig. Opthalmol. Vis. Sci.* 49, 5526–5531. doi: 10.1167/iovs.08-2196

López-López, D., Gómez-Nieto, R., Herrero-Turrión, M. J., García-Cairasco, N., Sánchez-Benito, D., Ludeña, M. D., et al. (2017). Overexpression of the immediate-early genes Egr1, Egr2, and Egr3 in two strains of rodents susceptible to audiogenic seizures. *Epilepsy Behav.* 71, 226–237. doi: 10.1016/j.yebeh.2015.12.020

Löscher, W. (2011). Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure* 20, 359–368. doi: 10.1016/j.seizure.2011.01.003

Löscher, W., and Hönack, D. (1995). Comparison of anticonvulsant efficacy of valproate during prolonged treatment with one and three daily doses or continuous ("Controlled release") administration in a model of generalized seizures in rats. *Epilepsia* 36, 929–937. doi: 10.1111/j.1528-1157.1995.tb01637.x

Marchi, N., Granata, T., Ghosh, C., and Janigro, D. (2012). Blood-brain barrier dysfunction and epilepsy: pathophysiologic role and therapeutic approaches. *Epilepsia* 53, 1877–1886. doi: 10.1111/j.1528-1167.2012.03637.x

Muñoz, L. J., Carballosa-Gautam, M. M., Yanowsky, K., García-Atarés, N., and López, D. E. (2017). The genetic audiogenic seizure hamster from Salamanca: the GASH:Sal. *Epilepsy Behav.* 71, 181–192. doi: 10.1016/j.yebeh.2016.03.002

Naziroglu, M., Çig, B., and Özgül, C. (2013). Neuroprotection induced by N-acetylcysteine against cytosolic glutathione depletion-induced Ca2+ influx in dorsal root ganglion neurons of mice: role of TRPV1 channels. *Neuroscience* 242, 151–160. doi: 10.1016/j.neuroscience.2013.03.032

Naziroglu, M., and Övey, I. S. (2015). Involvement of apoptosis and calcium accumulation through TRPV1 channels in neurobiology of epilepsy. *Neuroscience* 293, 55–66. doi: 10.1016/j.neuroscience.2015.02.041

Nef, P. (2001). Key animal models for the identification and validation of drug targets. *Drug Discov. Today* 6, 91–96. doi: 10.1016/S1359-6446(01)00165-9

Nolan, T., Hands, R. E., and Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. *Nat Protoc.* 1, 1559–1582. doi: 10.1038/nprot.2006.236

Patra, P. H., Barker-haliski, M., White, H. S., Whalley, B. J., Glyn, S., Sandhu, H., et al. (2019). Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models. *Epilepsia* 60, 303–314. doi: 10.1111/epi.14629

Pelz, M. C., Schoolcraft, K. D., Larson, C., Spring, M. G., and López, H. H. (2017). Assessing the role of serotonergic receptors in cannabidiol's anticonvulsant efficacy. *Epilepsy Behav.* 73, 111–118. doi: 10.1016/j.yebeh.2017.04.045

Perucca, E. (2017). Cannabinoids in the treatment of epilepsy: hard evidence at last? *J. Epilepsy Res.* 7, 61–76. doi: 10.14581/jer.17012

Pickens, J. T. (1981). Sedative activity of cannabis in relation to its Δ'-trans-tetrahydrocannabinol and cannabidiol content. *Br. J. Pharmacol.* 72, 649–656. doi: 10.1111/j.1476-5381.1981.tb09145.x

Prieto-Martín, A. I., Aroca-Aguilar, J. D., Sánchez-Sánchez, F., Muñoz, L. J., López, D. E., Escribano, J., et al. (2017). Molecular and neurochemical substrates of the audiogenic seizure strains: the GASH:Sal model. *Epilepsy Behav.* 71, 218–225. doi: 10.1016/j.yebeh.2015.05.025

Rodríguez-Muñoz, M., Onetti, Y., Cortés-Montero, E., Garzón, J., and Sánchez-Blázquez, P. (2018). Cannabidiol enhances morphine antinociception, diminishes NMDA-mediated seizures and reduces stroke damage via the sigma 1 receptor. *Mol. Brain* 11:51. doi: 10.1186/s13041-018-0395-2

Romcy-Pereira, R., and Garcia-Cairasco, N. (2003). Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or fluoro-jade staining. *Neuroscience* 119, 533–546. doi: 10.1016/S0306-4522(03)00191-X

Ross, K., and Coleman, J. (2000). Developmental and genetic audiogenic seizure models: behavior and biological substrates. *Neurosci. Biobehav. Rev.* 24, 639–653. doi: 10.1016/S0149-7634(00)00029-4

Rossetti, F., Rodrigues, M. C. A., Oliveira, J. A. C., de, and Garcia-Cairasco, N. (2006). EEG wavelet analyses of the striatum–substantia nigra pars reticulata–superior colliculus circuitry: Audiogenic seizures and anticonvulsant drug administration in Wistar audiogenic rats (War strain). *Epilepsy Res.* 72, 192–208. doi: 10.1016/j.eplepsyres.2006.08.001

Russo, E. B., Burnett, A., Hall, B., and Parker, K. K. (2005). Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochem. Res.* 30, 1037–1043. doi: 10.1007/s11064-005-6978-1

Sales, A. J., Crestani, C. C., Guimarães, F. S., and Joca, S. R. L. (2018). Antidepressant-like effect induced by Cannabidiol is dependent on brain serotonin levels. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* 86, 255–261. doi: 10.1016/j.pnpbp.2018.06.002

Salgado, D., and Alkadhi, K. A. (1995). Inhibition of epileptiform activity by serotonin in rat CA1 neurons. *Brain Res.* 669, 176–182. doi: 10.1016/0006-8993(94)01235-A

Sánchez-Benito, D., Gómez-Nieto, R., Hernández-Noriega, S., Murashima, A. A. B., de Oliveira, J. A. C., López, D. E., et al. (2017). Morphofunctional alterations in the olivocochlear efferent system of the genetic audiogenic seizure-prone hamster GASH:Sal. *Epilepsy Behav.* 71, 193–206. doi: 10.1016/j.yebeh.2016.05.040

Sánchez-Benito, D., Hyppolito, M. A., Alvarez-Morujo, A. J., López, D. E., and Gómez-Nieto, R. (2020). Morphological and molecular correlates of altered hearing sensitivity in the genetically audiogenic seizure-prone hamster GASH/Sal. *Hear. Res.* 392, 107973. doi: 10.1016/j.heares.2020.107973

Sasibhushana, R. B., Shankaranarayana Rao, B. S., and Srikumar, B. N. (2019). Repeated finasteride administration induces depression-like behavior in adult male rats. *Behav. Brain Res.* 365, 185–189. doi: 10.1016/j.bbr.2019.03.006

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Sharma, A. K., Rani, E., Waheed, A., and Rajput, S. K. (2015). Pharmacoresistant epilepsy: a current update on non-conventional pharmacological and non-pharmacological interventions. *J. Epilepsy Res.* 5, 1–8. doi: 10.14581/jer.15001

Shoval, G., Shbiro, L., Hershkovitz, L., Hazut, N., Zalsman, G., Mechoulam, R., et al. (2016). Prohedonic effect of cannabidiol in a rat model of depression. *Neuropsychobiology* 73, 123–129. doi: 10.1159/000443890

Sun, F.-J., Guo, W., Zheng, D.-H., Zhang, C.-Q., Li, S., Liu, S.-Y., et al. (2013). Increased expression of TRPV1 in the cortex and hippocampus from patients with mesial temporal lobe epilepsy. *J. Mol. Neurosci.* 49, 182–193. doi: 10.1007/s12031-012-9878-2

Vilela, L. R., Lima, I. V., Kunsch, É. B., Pinto, H. P. P., de Miranda, A. S., Vieira, É. L. M., et al. (2017). Anticonvulsant effect of cannabidiol in the pentylenetetrazole model: pharmacological mechanisms, electroencephalographic profile, and brain cytokine levels. *Epilepsy Behav.* 75, 29–35. doi: 10.1016/j.yebeh.2017.07.014

von Rüden, E. L., Jafari, M., Bogdanovic, R. M., Wotjak, C. T., and Potschka, H. (2015). Analysis in conditional cannabinoid 1 receptor-knockout mice reveals neuronal subpopulation-specific effects on epileptogenesis in the kindling paradigm. *Neurobiol. Dis.* 73, 334–347. doi: 10.1016/j.nbd.2014.08.001

Werner, F.-M., and Coveñas, R. (2017). Classical neurotransmitters and neuropeptides involved in generalized epilepsy in a multi-neurotransmitter system: how to improve the antiepileptic effect? *Epilepsy Behav.* 71, 124–129. doi: 10.1016/j.yebeh.2015.01.038

Wheless, J. W., Dlugos, D., Miller, I., Oh, D. A., Parikh, N., Phillips, S., et al. (2019). Pharmacokinetics and tolerability of multiple doses of pharmaceutical-grade synthetic cannabidiol in pediatric patients with treatment-resistant epilepsy. *CNS Drugs* 33, 593–604. doi: 10.1007/s40263-019-00624-4

Zaccara, G., Cincotta, M., Borgheresi, A., and Balestrieri, F. (2004). Adverse motor effects induced by antiepileptic drugs. *Epileptic Disord.* 6, 153–168.

Conflict of Interest: DS-B was hired under the research funding from RiverForce Partners Inc.

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

frontiers in Behavioral Neuroscience

REVIEW
published: 11 February 2021
doi: 10.3389/fnbeh.2021.611902

Cannabinoids in Audiogenic Seizures: From Neuronal Networks to Future Perspectives for Epilepsy Treatment

Willian Lazarini-Lopes[1,2], *Raquel A. Do Val-da Silva*[1], *Rui M. P. da Silva-Júnior*[2], *Alexandra O. S. Cunha*[3] *and Norberto Garcia-Cairasco*[1,2,3]*

[1] *Neuroscience and Behavioral Sciences Department, Ribeirão Preto School of Medicine, University of São Paulo, São Paulo, Brazil,* [2] *Neurophysiology and Experimental Neuroethology Laboratory (LNNE), Physiology Department, Ribeirão Preto School of Medicine, University of São Paulo, São Paulo, Brazil,* [3] *Physiology Department, Ribeirão Preto School of Medicine, University of São Paulo, São Paulo, Brazil*

OPEN ACCESS

Edited by:
Fabrizio Sanna, University of Cagliari, Italy

Reviewed by:
Joung-Hun Kim, Pohang University of Science and Technology, South Korea
Antonio Luchicchi, VU University Medical Center, Netherlands

***Correspondence:**
Norberto Garcia-Cairasco
ngcairas@usp.br

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *29 September 2020*
Accepted: *06 January 2021*
Published: *11 February 2021*

Citation:
Lazarini-Lopes W, Do Val-da Silva RA, da Silva-Júnior RMP, Cunha AOS and Garcia-Cairasco N (2021) Cannabinoids in Audiogenic Seizures: From Neuronal Networks to Future Perspectives for Epilepsy Treatment. Front. Behav. Neurosci. 15:611902. doi: 10.3389/fnbeh.2021.611902

Cannabinoids and *Cannabis*-derived compounds have been receiving especial attention in the epilepsy research scenario. Pharmacological modulation of endocannabinoid system's components, like cannabinoid type 1 receptors (CB1R) and their bindings, are associated with seizures in preclinical models. CB1R expression and functionality were altered in humans and preclinical models of seizures. Additionally, *Cannabis*-derived compounds, like cannabidiol (CBD), present anticonvulsant activity in humans and in a great variety of animal models. Audiogenic seizures (AS) are induced in genetically susceptible animals by high-intensity sound stimulation. Audiogenic strains, like the Genetically Epilepsy Prone Rats, Wistar Audiogenic Rats, and Krushinsky-Molodkina, are useful tools to study epilepsy. In audiogenic susceptible animals, acute acoustic stimulation induces brainstem-dependent wild running and tonic-clonic seizures. However, during the chronic protocol of AS, the audiogenic kindling (AuK), limbic and cortical structures are recruited, and the initially brainstem-dependent seizures give rise to limbic seizures. The present study reviewed the effects of pharmacological modulation of the endocannabinoid system in audiogenic seizure susceptibility and expression. The effects of *Cannabis*-derived compounds in audiogenic seizures were also reviewed, with especial attention to CBD. CB1R activation, as well *Cannabis*-derived compounds, induced anticonvulsant effects against audiogenic seizures, but the effects of cannabinoids modulation and *Cannabis*-derived compounds still need to be verified in chronic audiogenic seizures. The effects of cannabinoids and *Cannabis*-derived compounds should be further investigated not only in audiogenic seizures, but also in epilepsy related comorbidities present in audiogenic strains, like anxiety, and depression.

Keywords: epilepsy, audiogenic seizures, neuronal networks, cannabidiol, endocannabinoid system, CB1 receptors, *Cannabis*-derived compounds, genetically-developed strains

INTRODUCTION

Epilepsy is a neurological disorder characterized by the presence of epileptic seizures and their behavioral, physiological, and social consequences (Fisher et al., 2014; Kanner, 2017). Despite the great variety of antiepileptic drugs (Löscher, 2017) one third of patients remain pharmacoresistant and cannot have their seizures under control with the available pharmacological treatment (Kwan and Brodie, 2010), indicating that new therapeutic and pharmacological targets are needed. In that context, the endocannabinoid system (ES) has been receiving especial attention in the epilepsy research scenario. The ES comprises the cannabinoids receptors type 1 (CB1R) and type 2 (CB2R) and their endogenous bindings, the endocannabinoids. CB1R has been receiving especial attention in epilepsy due to seizure control in several preclinical models and can also be modulated by phytocannabinoids (Wallace et al., 2003; Lutz, 2004; Blair, 2006; Huizenga et al., 2017; Britch et al., 2020). Moreover, anticonvulsant effects were associated with *Cannabis*-derived compounds, especially the cannabidiol (CBD), reinforcing the role of cannabinoids in epileptic seizures (Friedman and Devinsky, 2015; Rosenberg et al., 2017; Lazarini-Lopes et al., 2020b). CBD, a phytocannabinoid present in *Cannabis* sp. (Jacob and Todd, 1940; Mechoulam and Shvo, 1963), has receiving especial attention due to its anticonvulsant properties in animal models of the epilepsies (Jones et al., 2012; Do Val-da Silva et al., 2017; Lazarini-Lopes et al., 2020b) and also in humans with pharmacoresistant epilepsy (Press et al., 2015; Devinsky et al., 2016, 2017). Although CBD anticonvulsant mechanisms of action seem to be related with a great diversity of cellular and molecular targets, which include components of the ES, the possible existence of synergistic effects between CBD and conventional anticonvulsant drugs may not be ignored (Mencher and Wang, 2005; Devinsky et al., 2014; Gaston et al., 2017). Additionally, although CBD has limited effects at cannabinoids receptors, CBD can modulate CB1R activity by indirect mechanisms of action (Britch et al., 2020). Therefore, the ES arise as important endogenous mechanism for seizure control (Alger, 2004; Hofmann and Frazier, 2013).

Animal models are essential for the development and screening of new anticonvulsant drugs and to evaluate their effects on the brain and on behavior (Löscher, 2011, 2017). Since epilepsies are greatly diverse in etiology, the differences between seizure induction protocols are extremely important to help understanding neuronal alterations associated with each type of seizure induction and, consequently, their clinical applications (Löscher, 2017). Audiogenic seizures (AS) are induced by intense sound stimulation (∼100–120 dB) in susceptible animals and are used to study epilepsies-related mechanisms such as neuronal pathways and endogenous alterations associated with seizure susceptibility (Garcia-Cairasco et al., 2017). Audiogenic susceptible rodent strains are widely used around the world, beginning with the oldest colony, the Krushinsky-Molodkina (KM) rats in Russia (Poletaeva et al., 2017), followed by the Genetically Epilepsy-Prone Rats (GEPR) in the United States (Reigel et al., 1986; Dailey et al., 1989), the DBA/1 and DBA/2 mice (Jensen et al., 1983; Faingold et al., 2010), the Wistar Audiogenic Rat (WAR) in Brazil (Doretto et al., 2003; Garcia-Cairasco et al., 2017), among others (Ross and Coleman, 2000).

Acute AS are considered a model of generalized tonic-clonic seizures, with seizures characterized by an initial wild running phase with jumping and atonic falls followed by tonic or tonic-clonic seizures (Faingold, 1988; Terra and Garcia-Cairasco, 1992; Garcia-Cairasco et al., 1996, 2017; Ross and Coleman, 2000). However, when animals are chronically exposed to the AS protocol, called Audiogenic Kindling (AuK) (Marescaux et al., 1987), some audiogenic susceptible animals develop limbic seizures, characterized by the appearance of new behaviors such as facial and forelimb clonus, usually followed by elevation and falling (Naritoku et al., 1992; Garcia-Cairasco et al., 1996), similar to those described by Racine's scale (Racine, 1972). While brainstem sensory motor structures are primarily involved in the acute AS expression (Faingold, 1988; Terra and Garcia-Cairasco, 1992), cortical and limbic structures are associated with behavioral, EEG, and histological alterations during the AuK, indicating an expansion of the initially brainstem-dependent seizure networks to limbic regions and networks (Marescaux et al., 1987; Naritoku et al., 1992; Garcia-Cairasco et al., 1996; Moraes et al., 2000; Galvis-Alonso et al., 2004). Therefore, the AuK is as a model of temporal lobe recruitment and consequently of temporal lobe epilepsy (Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003). Other quite important characteristic is that genetic and chronic models, like susceptible strains and the AuK, can be used also to study the comorbidities, usually from neuropsychiatric origin, associated with epilepsies (Garcia-Cairasco et al., 2017).

Therefore, the purpose of the present study was to review the neuronal networks associated with AS expression. Additionally, we reviewed the effects of ES modulation and *Cannabis*-derived compounds in AS. We discussed cannabinoids modulation in AS neuronal pathways and the future perspectives of cannabinoids in AS and comorbidities.

NEURONAL NETWORKS INVOLVED IN AUDIOGENIC SEIZURES

Since AS are evoked by a high-intensity acoustic stimulus, the primary auditory pathway has been the first cluster of structures to be evaluated in audiogenic susceptible rodent strains. In this context, several research groups have detected peripheral alterations associated with AS susceptibility, such as hearing loss (Saunders et al., 1972; Glenn et al., 1980; Faingold et al., 1990), unbalance between GABAergic and Glutamatergic neurotransmissions between the inner hair cells and the cochlear nerve (Altschuler et al., 1989; Bobbin et al., 1990; Lefebvre et al., 1991), and tinnitus followed by intensity sound exposure (Heffner and Harrington, 2002; Chen et al., 2013). Similarly, anatomical and morphological alterations in the organ of Corti and in the inner and outer hair cells of the GEPRs have already been observed (Penny et al., 1983, 1986). However, despite the importance of peripheral alterations in the onset of AS, the present review will focus on brain sites involved on the onset,

maintenance, and expression of AS, specifically in the brainstem (acute AS) and limbic areas (AuK).

Brainstem Structures Critical for Acute Audiogenic Seizure Expression

Inferior Colliculus

It is widely accepted that inferior colliculus (IC) circuits play a pivotal role in the genesis and maintenance of sound-induced seizures (Garcia-Cairasco, 2002; Coleman et al., 2017; Ribak, 2017). The IC anatomy in the rat presents a structure similar to the human IC (Faye-Lund and Osen, 1985) and it is usually divided into the central nucleus, dorsal cortex, and external cortex (Faye-Lund and Osen, 1985; Coleman and Clerici, 1987). The central nucleus of the IC is the largest division of the IC, sends glutamatergic projections to both external cortex and dorsal cortex of the IC, and receives projections from the dorsal cortex (Coleman and Clerici, 1987; Saint Marie, 1996).

Glutamate is the main excitatory neurotransmitter into the IC and it is also implicated in the expression of AS (Faingold, 2002). Using WARs, Terra and Garcia-Cairasco (1994) showed that AP-7 administration into de central nucleus of the IC or intra-dorsal cortex of the IC, blocked or attenuated (wild runnings were still present) AS, respectively. Therefore, these intracollicular pathways may contribute to seizure propagation through its known glutamatergic connections between the external cortex of the IC and motor areas (Caicedo and Herbert, 1993; Saint Marie, 1996).

By contrast, intracollicular and extracollicular pathways are mostly modulated by GABAergic signaling (Faingold, 2002; Ribak, 2017). Therefore, deficits in GABA-mediated inhibition may be a critical mechanism associated with AS susceptibility, since a reduction in GABAergic neurotransmission in the IC was shown to facilitate neuronal firing in response to high acoustic stimuli and trigger AS (Faingold et al., 1986; Faingold, 2002).

Administration of GABA agonists into the central nucleus of the IC blocked AS expression in GEPRs and similar results were observed after pharmacological manipulations capable of increasing endogenous GABA signaling (Faingold, 2002). Administration of GABA agonists into the central nucleus of the IC blocked AS expression and the same was observed when endogenous GABA was increased in GEPRs (Faingold, 2002). Curiously, the number of GABAergic cells and the labeling of GABA synthetic enzymes are higher in GEPRs than in their Sprague-Dawley controls (Roberts et al., 1985; Ribak, 2017). However, in spite of the increased expression of all of these GABAergic biomarkers, there is a paradoxical decreased effectiveness of GABA-mediated inhibition in the IC of GEPRs (Faingold et al., 1986). Furthermore, inhibition of GABAergic neurotransmission into the IC observed in tissue slices of GEPRs (Evans et al., 2006) is thought to be the clue alteration in the triggering of AS in these animals (Faingold, 2012). Interestingly, GABA synthesis was increased in IC of KM rats, whereas GABA levels were not different from non-susceptible rats (Solius et al., 2016). It is worth to note that pharmacological activation of CB1R increased IC neuronal output, probably by activation of CB1R in GABAergic pre-synaptic terminals (Valdés-Baizabal et al., 2017). These results suggest that CB1R in the IC could play an important role on AS susceptibility.

Superior Colliculus

The superior colliculus (SC) is the most important non-auditory IC target (output) (Faingold, 2012). Interconnections between the external cortex of the IC and the deep layers of the superior colliculus (DLSC) seem to play an important role on AS generation and propagation (Coleman and Clerici, 1987; García del Caño et al., 2006). Since DLSC projects directly and indirectly to the spinal cord and to brainstem motor areas, such as the reticular formation (Masino and Knudsen, 1992; King et al., 1996; May, 2005), excessive activity in this network may lead to AS propagation.

Electrophysiological recordings in freely moving GEPR-9s showed increased tonic firing of DLSC neurons just prior and during the wild running, but not during the tonic behavior (Faingold and Randall, 1999). The role of DLSC in AS manifestations has already been demonstrated in WARs through their mesencephalic pathways. Midcollicular transections (knife cuts between IC and SC) blocked tonic-clonic seizures (Tsutsui et al., 1992) in WARs. Similar effects were also confirmed by Ribak et al. (1994) in GEPR-9s. Likewise, bilateral transections separating DLSC and *substantia nigra pars reticulata* (SNr) abolished tonic-clonic seizures and also attenuated wild running behaviors (Doretto et al., 2009). Browning et al. (1999) confirmed similar effects after pre-collicular transections in both GEPR substrains, GEPR-3s and GEPR-9s. Similarly, electrolytic lesions of the DLSC (but not dorsal SC) decreased AS severity in GEPRs (Merrill et al., 2003) and abolished all seizure behaviors in DBA/2 audiogenic mice (Willott and Lu, 1980).

Additionally, optogenetic activation of DLSC neurons attenuated seizures in several animal models, including AS in GEPR−3s (Soper et al., 2016). The activation of neurons in the DLSC is considered to be part of an endogenous seizure control system with origin in neurons from SNr (Gale et al., 1993; Soper et al., 2016). According to this idea, the activation of neurons from DLSC will lead to the desynchronization of epileptic brain networks (Dean et al., 1991; Soper et al., 2016). For this reasons, optogenetic stimulation of specific neurons or projections into the SC are considered an important approach to better understanding the role of the SC in AS, although the role of specific neuronal projections from SC still needs to be assessed.

Periaqueductal Gray Matter

Although the periaqueductal gray matter (PAG) is classically involved in emotional-related behaviors, such as fear, anxiety and panic-like behaviors (Bueno et al., 2005; Brandão et al., 2008; Deng et al., 2016), the involvement of PAG with the motor AS expression comes from findings that PAG blockade inhibits AS, more specifically the tonic and clonic behaviors, in GEPR-9s (N'Gouemo and Faingold, 1998).

Differences in the PAG neuronal firing pattern were observed in GEPRs. PAG neuronal activity increased just before the onset of the wild running, but the most remarkable neuronal tonic firing pattern was observed just prior and during the tonic

behavior, but this neuronal pattern disappeared when the post-ictal depression began (N'Gouemo and Faingold, 1998). Also, the blockade of NMDA receptors or $GABA_A$ activation into the PAG were both capable of suppressing AS in GEPRs, with a most potent effect associated with NMDA blockade (N'Gouemo and Faingold, 1999). In the same line, Yang et al. (2003) showed that intra-PAG AP-7 administration attenuated AS induced by ethanol withdrawal in Sprague-Dawley rats.

Classically, it has been accepted that PAG receives projections from neurons of the DLSC (King et al., 1996; Faingold et al., 2014) and projects to the sites of initiation of the motor responses associated with fight or flight reactions (Brandão et al., 1999). Additionally, there are direct and indirect (through BRF) connections between PAG and spinal cord motor neurons (Mouton and Holstege, 1994; Bajic and Proudfit, 1999) that may also contribute to AS expression. Additionally, a recent study showed that IC neurons project directly to PAG and, when optogenetically activated, triggered a sound-mediated escape response (Xiong et al., 2015). Similarly, PAG might be important not only in the acute AS, but in the kindled AS (AuK protocol). In this respect, Tupal and Faingold (2012) showed that the electrical stimulation of central nucleus of the amygdala induces intensity-dependent firing in the PAG of GEPR-9s. Additionally, GEPR-9s submitted to AuK present increased responsiveness of PAG neurons to electrical stimulation of the amygdala when compared to control GEPR-9s (Tupal and Faingold, 2012).

Brainstem Reticular Formation

Wada's group demonstrated for the first time the role of BRF in seizures using amygdala kindled cats, where electrolytic lesions into the BRF attenuated amygdaloid seizures and these effects were not dependent on forebrain sites (Wada and Sato, 1975).

Sprague-Dawley rats submitted to ethanol withdrawal presented increased AS susceptibility and increased spontaneous neuronal firing in the BRF, as well as increased sound-evoked activity in neurons from the same structure (Faingold and Riaz, 1994). Moreover, there is an increase in neuronal firing into the pontine nucleus of the BRF, once the AS begins and an additional increase simultaneously with the onset of the tonic seizure, that remained until the end of the tonic hind limb extension (Faingold and Randall, 1995). These data suggested that pathological conditions, like ethanol withdrawal, may induce physiological changes in the BRF, which in turn, facilitate AS expression.

Both systemic and intra-BRF NMDA antagonist administration blocked AS during ethanol withdrawal. Moreover, the increase in BRF excitatory activity was capable of inducing AS-like behaviors in previously non-susceptible rats treated with NMDA. These effects were dose-dependent, with lower dose inducing wild running behaviors and higher dose inducing wild running and generalized tonic-clonic seizures, in both cases sound-independent set of behaviors. Moreover, sound stimulation was also capable of inducing AS with wild running and generalized tonic-clonic behaviors in these animals (Ishimoto et al., 2000). In susceptible GEPR-3s, NMDA infusion into the BRF was capable of inducing seizures without the presence of acoustic stimulation (Faingold et al., 1989). On the other hand, blockade of NMDA receptors into the BRF induced a decrease in AS severity in GEPRs (Millan et al., 1988). Additionally, increased glutamate levels into the BRF had been previously observed during the tonic phase of AS in GEPRs (Chapman et al., 1986).

These data, therefore, suggest that excitatory connections between IC and BRF (Browning, 1986; Caicedo and Herbert, 1993; Riaz and Faingold, 1994) and between BRF and spinal cord (Jones and Yang, 1985) should be important efferent neuronal pathways for motor manifestation of AS (Garcia-Cairasco, 2002; Faingold et al., 2014).

Substantia Nigra

During the 80's, Karen Gale's group proposed that GABAergic neurotransmission into SNr should be part of what they called as an endogenous anticonvulsant system (Iadarola and Gale, 1982; Maggio and Gale, 1989). These authors proposed that a decrease in the inhibitory tonus from the SNr to the midbrain tectum might enhance seizure susceptibility.

Following up on those proposals, a series of experiments in control Wistar rats gave support to that hypothesis. Electrolytic lesions in the SNr increased AS susceptibility in Wistar rats, without any modification on locomotion, exploratory activity or grooming behaviors (Garcia Cairasco and Sabbatini, 1983; Garcia-Cairasco and Sabbatini, 1991; Garcia-Cairasco and Triviño-Santos, 1989). Nonetheless, the same SNr electrolytic lesion did not induce any alterations in AS displayed by WARs (Doretto and Garcia-Cairasco, 1995). However, neuroethological analysis based upon detailed behavioral descriptions, demonstrated changes in the behavioral structural sequence of tonic-clonic seizures in SNr-lesioned WARs. Behavioral components were present no more in a defined pattern, but randomly and fragmented, indicating that GABAergic signaling from SNr should play an important role in temporal and spatial motor integration during AS (Garcia Cairasco and Sabbatini, 1983; Doretto and Garcia-Cairasco, 1995). Curiously, it was observed that GEPRs present a disruption in the nigral GABAergic signaling, detected as a failure to release GABA from SNr, when animals were stimulated with KCl in a depolarizing protocol with microdialysis membranes into SNr (Doretto et al., 1994). These GABAergic deficits could be an explanation for the lack of seizure behavioral alteration in SNr-lesioned WARs, but, at the same time, it explains and strengthens the view that lesioned normal Wistar rats may become susceptible to AS (Garcia Cairasco and Sabbatini, 1983; Doretto and Garcia-Cairasco, 1995).

In ethanol withdrawal-induced AS, muscimol, a selective $GABA_A$ agonist, applied intra-SNr reduced seizure severity during the most critical period of hyperexcitability (Gonzalez and Hettinger, 1984). Also, pharmacological activation of $GABA_A$ receptors into SNr was capable of blocking AS induced by IC bicuculline injections (Terra and Garcia-Cairasco, 1992) and decreased spontaneous spike-wave discharges duration in a model of absence seizure (Depaulis et al., 1988). Conversely, specific lesions in dopaminergic neurons of the substantia nigra compacta (SNc) with the 6-OHDA toxin, an experimental model of Parkinson (Schober, 2004), did not produce AS sensitivity in

resistant animals, suggesting that changes in AS susceptibility are associated with GABAergic neurons, mostly present into the SNr.

SNr projects to IC (Olazábal and Moore, 1989) and SC (Appell and Behan, 1990), regulating efferent seizure pathways (Gale, 1992). Additionally, modulation of SC by SNr may involve the neostriatum activity, which sends the main GABAergic input to SNr (Nisenbaum et al., 1992). Therefore, increasing GABA activity in the SNr is believed to be pro-convulsant because the resulting reduction of GABAergic neurotransmission into SNr-SC pathway facilitates output from SC to motor structures, such as the BRF, which may lead to seizure expression.

The findings on the so-called endogenous anticonvulsant system were supported by optogenetic inhibition of the nigrotectal terminals into the DLSC, attenuating AS in GEPR-3s. Light delivery increased the latency to the onset of AS and decreased their duration and severity in GEPR-3s (Wicker et al., 2019). These results can be explained by a decrease of GABAergic neurotransmission from SNr to the SC and are in agreement with Soper et al. (2016), who showed attenuation of AS associated with optogenetic activation of the DLSC and it is in line with the activation of SC as capable of desynchronizing cortical activity (Dean et al., 1991). In spite of this highly coherent group of studies and results, specific neurons and projections associated with the mentioned anticonvulsant effects still need to be verified *in vitro* and *in vivo*.

Chronic Audiogenic Seizures and Neuroplastic Effects in Neuronal Networks

The repetitive audiogenic stimulus, or AuK, results in behavioral, EEG, and histological alterations in forebrain structures, such as amygdala, hippocampus, and cortex, indicating limbic recruitment (Marescaux et al., 1987; Naritoku et al., 1992; Moraes et al., 2000; Vinogradova, 2017). Marescaux et al. (1987) did behavioral observations and cortical (surface) EEG recordings in Wistar rats and proposed the term "kindling," analogous with the limbic seizures protocols published by Goddard (1967) and Goddard et al. (1969). Naritoku et al. (1992) confirmed similar results in both GEPRs substrains: GEPR-3s and GEPR-9s, moderate and severe AS, respectively. Similar protocols and studies with quantitative behavioral methods of the evolution of AuK were made in WARs (Garcia-Cairasco et al., 1996; Galvis-Alonso et al., 2004).

The neuroanatomical and functional interaction between midbrain auditory and forebrain limbic systems can be particularly well-observed during the AuK. Local changes in the IC circuits can lead to increased collicular outputs to the limbic system, causing the seizure spread. Coupled video-EEG allowed a detailed characterization of the progression of synchronized behavior and electrophysiology with EEG recording from IC (brainstem) to hippocampus, amygdala and cortex (Moraes et al., 2000; Romcy-Pereira and Garcia-Cairasco, 2003). Furthermore, it was reported an increase in the firing rate of neurons from the central nucleus of the IC of GEPR-9s before the appearance of generalized post-tonic clonus during the AuK (N'Gouemo and Faingold, 1996). Neurons from the central nucleus of the IC project to the medial geniculate nucleus of the thalamus (MGN), as part of the primary auditory system, where they make synapsis with neurons projecting to the auditory cortex, amygdala and the hippocampus (Ledoux et al., 1985; Clugnet and LeDoux, 1990). Indeed, the amygdala and the hippocampus are the major limbic structures that receive the output from the brainstem central auditory system (Kraus and Canlon, 2012). These structures, more remarkably the amygdala, are associated with emotional context and sensorial perception, including sound stimuli (LeDoux, 2007; Kraus and Canlon, 2012).

As a clear evidence of the activation of prosencephalic structures, Simler et al. (1999) demonstrated increase c-Fos expression directly related with AuK progression: from the auditory brainstem to amygdala and perirhinal cortex, then to the frontoparietal cortex, and finally to the hippocampus and the entorhinal cortex. Simultaneous EEG recordings of IC, amygdala and auditory cortex were analyzed in WARs during AuK and it was observed that the epileptiform activity in the IC increases as AuK progresses and limbic seizures start to co-exist with brainstem seizures (Garcia-Cairasco et al., 1996; Moraes et al., 2000). Altogether, these data indicate that the progression of seizures during AuK may not be the linear expression of a simple system, but rather a complex expression of a bi-directional interaction between limbic and brainstem circuits. This is absolutely clear from the observation of a mirror (opposite) image of the decrease of brainstem-dependent seizure severity index, as soon as the AuK progress, and the increase (from zero) of the limbic-dependent seizure severity index (Garcia-Cairasco et al., 1996; Moraes et al., 2000; Rossetti et al., 2006).

Differences in hippocampus activity have been reported in audiogenic rodent strains. GABAergic currents in pyramidal neurons from CA1 of WARs are less frequent and have faster kinetics, indicating that some particular populations of interneurons might be absent in WARs (Cunha et al., 2018b). Moreover, during chronic high-intensity sound stimulation it was observed an impairment in the long-term potentiation (LTP) in non-susceptible (resistant) Wistars, but not in WARs (Cunha et al., 2015). Additionally, a decrease in the hyperpolarization activated cationic current (*Ih*) was observed in resistant animals, indicating that auditory inputs to the hippocampus might lead to compensatory homeostatic and long-term synaptic plasticity, which could be blocking the hyperexcitability of auditory pathways to the hippocampus of seizure resistant animals (Cunha et al., 2018a). In contrast, Evans et al. (1994) found different results using hippocampal slices of GEPR-9s. According to these authors, animals showed single excitatory post-synaptic potentials similarly to their control strain. However, when submitted to AuK GEPRs exhibited a more pronounced synaptic facilitation indicating that short-term potentiation is enhanced in the hippocampus of these animals (Evans et al., 1994).

ENDOCANNABINOID SYSTEM AND CB1R IN AUDIOGENIC SEIZURES NETWORK

The ES is classically composed by endogenous receptors, CB1R and CB2R (Matsuda et al., 1990; Munro et al., 1993), and their

endogenous ligands, anandamide, and 2-arachydonil glycerol (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). It is widely accepted that the ES modulates neuronal activity through its retrograde action based "on-demand" endocannabinoid synthesis and release (Lutz, 2004; Alger and Kim, 2011; Castillo et al., 2012; Fitzgerald et al., 2012). However, before discussing the role of the ES modulation on seizure control in audiogenic models, it is worth to note how these cannabinoids receptors are distributed on the brain, especially on structures important to AS expression.

Autoradiography assays were used to assess CB1R distribution in several brain structures (Herkenham et al., 1990, 1991). The most intense binding was observed in the cerebellum and forebrain structures, such as several cortical and hippocampal areas. The frontal cortex presented the greater density of CB1R compared to other cortical regions, while the dorsal hippocampus seems to present more CB1R than the ventral hippocampus. The amygdaloid complex presented a moderate binding, with exception of the central nucleus that showed the lowest CB1R levels. Brainstem structures, like PAG, BRF, SC, IC, and hypothalamus presented lower levels of CB1R and sparse binding when compared to the forebrain. Like in brainstem structures, the spinal cord showed sparse binding, specifically in the dorsal horn. It is worth to note that the SNr, but not the SNc, showed the highest density levels of CB1R in the entire rat brain (Herkenham et al., 1990, 1991). Similar expression patterns were observed in others species of mammals, such as dogs, Rhesus monkeys, and humans (Herkenham et al., 1990).

Tsou et al. (1998) used immunohistochemical analysis to assess CB1R distribution in the rat brain. These authors showed CB1R in axons, dendrites, and in soma of neurons in several brain structures. Intense and widely CB1R distribution were detected in forebrain structures, such as cortical areas, as well as in amygdala, and hippocampal formation, although very restricted immunostaining were present in the brainstem, in structures like the PAG and SC. Additionally, the SNr presented a very intense immunostaining (Tsou et al., 1998), confirming those previous results observed by Herkenham's research group.

Changes in CB1R expression and functionality have already been detected in animal models of epileptic seizures and in humans with chronic seizures (Maglóczky et al., 2010; Karlócai et al., 2011; Rocha et al., 2020). Goffin et al. (2011) assessed CB1R expression in tissue from humans with TLE and observed increased CB1R receptors expression in the seizure onset area, while CB1R expression was decreased in other areas, like the insular cortex, suggesting that different alterations in cannabinoid receptors expression could be associated with seizures expression and brain hyperexcitability (Goffin et al., 2011). However, data of CB1R expression in audiogenic strains are scarce. Increased CB1R expression was observed in the inner molecular layer of WARs, when compared to control Wistars. Additionally, in WARs, acute and chronic AS increased CB1R expression in several hippocampal layers and in specific amygdala subnuclei, the basolateral, lateral, and basomedial nuclei. Acute AS also induced changes in CB1R in the central and medial amygdala nuclei. Moreover, it is worth to note that, changes in CB1R expression in lateral, basolateral, and basomedial amygdala nuclei were correlated with limbic seizure severity during the AuK (Lazarini-Lopes et al., 2020a). See **Figure 1** for a representative view of CB1R expression in limbic and cortical structures of audiogenic susceptible rats from the WAR strain.

Wistar Albino rats from Rijswijk (WAG/Rij strain) develop absence seizures along their life (van Luijtelaar and Coenen, 1986; van Luijtelaar and Sitnikova, 2006) and a subpopulation of WAG/Rij rats can also develop AS with limbic recruitment during the AuK (Vinogradova, 2008). These animals present endogenous alterations in the ES, like reduced CB1R mRNA, demonstrated by *in situ* hybridization, in the hippocampus and thalamic nuclei, brain regions associated with the genesis of absence seizures (Van Rijn et al., 2010). Therefore, further characterization of CB1R expression and functionality in brainstem and limbic sites in audiogenic WAG/Rij rats can bring important information regarding the susceptibility to AS in the WAG/Rij subpopulation.

Pharmacological CB1R activation in the intermediate layers of SC induced a robust turning behavior, these effects may be associated with modulation of GABAergic input from SNr to SC (Sañudo-Peña et al., 2000). In the SNr, CB1R are located in presynaptic terminals from the striatonigral pathways, they modulate GABA release from the nigrotectal GABAergic projections (Wallmichrath and Szabo, 2002), which may play an important role on seizure propagation and expression (Iadarola and Gale, 1982; Gale, 1986). Miller and Walker (1995) explored how the ES modulates SNr activity. WIN 55,212-2, systemically administered in normal Sprague-Dawley rats increased spontaneous firing rate in neurons from the SNr. In addition, WIN 55,212-2 also attenuated the inhibition of neuronal firing in the SNr induced by striatum electrical stimulation. In the same study, bicuculline antagonized the effects of striatum stimulation, suggesting that WIN 55,212-2 effects on SNr activity were dependent on GABAergic neurotransmission (Miller and Walker, 1995), although specific neurons and projections associated with these effects still need to be verified. Therefore, the GABAergic signaling from SNr to mesencephalic tectum may be, somehow, enhanced by cannabinoids administration, increasing the inhibitory tonus generated by this endogenous anticonvulsant system. Although, this hypothesis still needs to be further elucidated, measuring GABA release and also CB1R activity in the SNr.

Based on these previous evidences, we proposed a schematic representation of how brainstem and forebrain structures with different distribution of CB1R might modulate AS susceptibility and expression (**Figure 2**).

ENDOCANNABINOID SYSTEM MODULATION IN AUDIOGENIC SEIZURES

Although cannabinoids induce modulation have already been shown as capable of epileptic seizures in several animal models (Rosenberg et al., 2017; Lazarini-Lopes et al., 2020b), studies evaluating the role of the ES on AS experimental models are still scarce. See **Table 1** for main results from the literature.

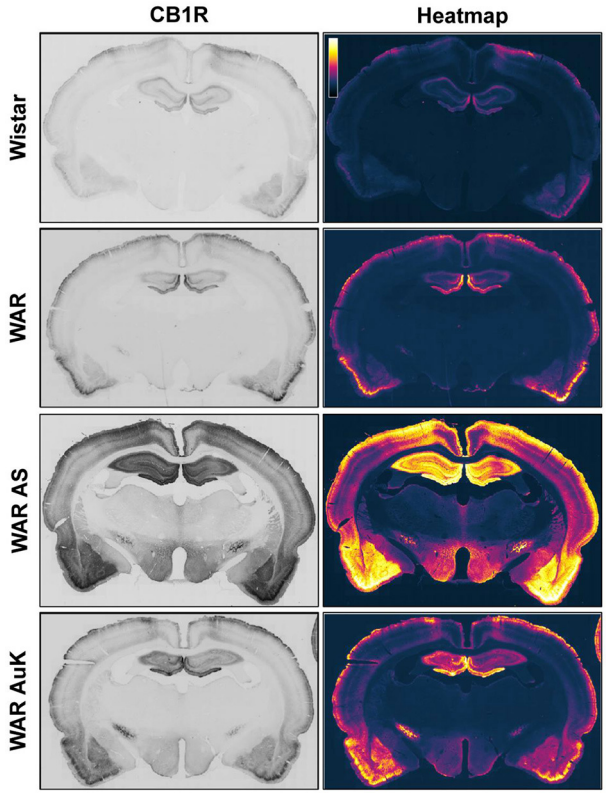


FIGURE 1 | Representative CB1R immunostaining in limbic and cortical brain structures of Wistar Audiogenic Rats (WAR). It is possible to observe the increased CB1R immunostaining in limbic (hippocampus and amygdala) and cortical areas of WARs after acute audiogenic seizures (WAR AS) and chronic audiogenic seizures (WAR AuK). Wistar represents a control non-audiogenic strain. Image obtained from Lazarini-Lopes et al. (2020a).

Vinogradova et al. (2011) showed that acute and chronic treatment with SR141716, a CB1R antagonist, presented pro-epileptic effects in audiogenic Wistar rats and facilitated the AuK progression. These authors reported that acute treatment with SR141716 in susceptible rats increased AS duration and severity and induced the appearance of limbic seizures behaviors. Interestingly, the treatment with the CB1R antagonist did not modify AS susceptibility in normal rats, but in susceptible animals that developed resistance to AS, the seizures reappeared after SR141716 administration (Vinogradova et al., 2011). It is worth to notice that similar results were observed in a patient that was free of seizures for more than 20 years, but after administration of CB1R antagonist for obesity treatment the seizures reappeared. In that case, when the CB1R antagonist treatment was interrupted, the seizures disappeared (Braakham et al., 2009).

Conversely, a single administration of WIN 55,212-2, a CB1R agonist, presented long-term, but not acute effects, against AS, increasing the latency to the onset of post-tonic clonus in KM rats (Vinogradova and Van Rijn, 2015). Moreover, pharmacological treatments with different cannabinoid receptors agonists were capable of attenuating AS in DBA/2 mice, reducing wild running, clonus, and tonus behaviors. These anticonvulsant effects were blocked by previous administration of NIDA-41020, a selective CB1R antagonist (Citraro et al., 2016). Additionally, these authors also demonstrated that when ineffective doses of cannabinoid receptors agonists were co-administered with classical anticonvulsant drugs, such as carbamazepine, gabapentin, phenobarbital, and valproate, the anticonvulsant effects of all these drugs were potentiated (Citraro et al., 2016). In GEPR-3s, systemic administration of WIN 55,212-2, was effective against AS, suppressing seizures in 9/10 animals and attenuating seizure severity in 1/10 rats. Similarly, central administration of CP 55940, a CB1/2 agonist, directly into the DLSC of GEPR-3s, suppressed seizures in 6/9 rats, reduced seizure severity in 2/9, and had no effect in 1/9. Additionally, intra-DLSC administration of SR141716 did not modify AS in control GEPRs and did not antagonize the anticonvulsant effects induced by systemic WIN 55,212-2 (Santos et al., 2020). Also using GEPRs, Samineni et al. (2011) showed that central injection of AM251, a CB1R antagonist, directly into the ventrolateral PAG, attenuated post-ictal analgesia in GEPR-9s. These data suggest that AS results in increased endocannabinoid levels in the PAG, which may mediate post-ictal analgesia (Samineni et al., 2011). Since the last study did not look at seizure expression after CB1R modulation, it should be interesting to assess the role of CB1R from PAG in AS expression in GEPRs and other audiogenic strains.

Using the *Fmr1* knockout mice to mimic the fragile X syndrome, authors observed that pharmacological blockade of CB1R rescued several pathological alterations, including the increased susceptibility to AS. Additionally, blockade of CB2R also induced anxiolytic behavior in the elevated plus maze (Busquets-Garcia et al., 2013). In the WAG/Rij strain, a model of absence seizures with a subpopulation also susceptible to AS, systemic administration of WIN 55,212-2 reduced the number of spontaneous spike-wave discharges, but increased seizure duration, in WAG/Rijs, whereas administration of AM251 attenuated the effects of CB1R activation (Van Rijn et al., 2010). Reduced number and duration of spike-wave discharges were observed after central (intra-thalamic nucleus) anandamide or WIN 55,212-2 administration (Citraro et al., 2013). However, it is unclear if the pharmacological modulation of CB1R, or endocannabinoids, can attenuate AS in the WAG/Rij strain and the exploration of this research field, assessing acute and chronic AS in WAG/Rij rats, can bring important information and insights about ES functionality in two different types of seizures (absence and audiogenic) in the same strain.

CANNABIS-DERIVED COMPOUNDS IN AUDIOGENIC SEIZURES

After 40 years of the demonstration of CBD as anticonvulsant in humans (Cunha et al., 1980), the interest in medical *Cannabis*-derived compounds, especially CBD, has substantially increased, as an alternative treatment for pharmacoresistant epilepsy (Porter and Jacobson, 2013; Press et al., 2015; Devinsky

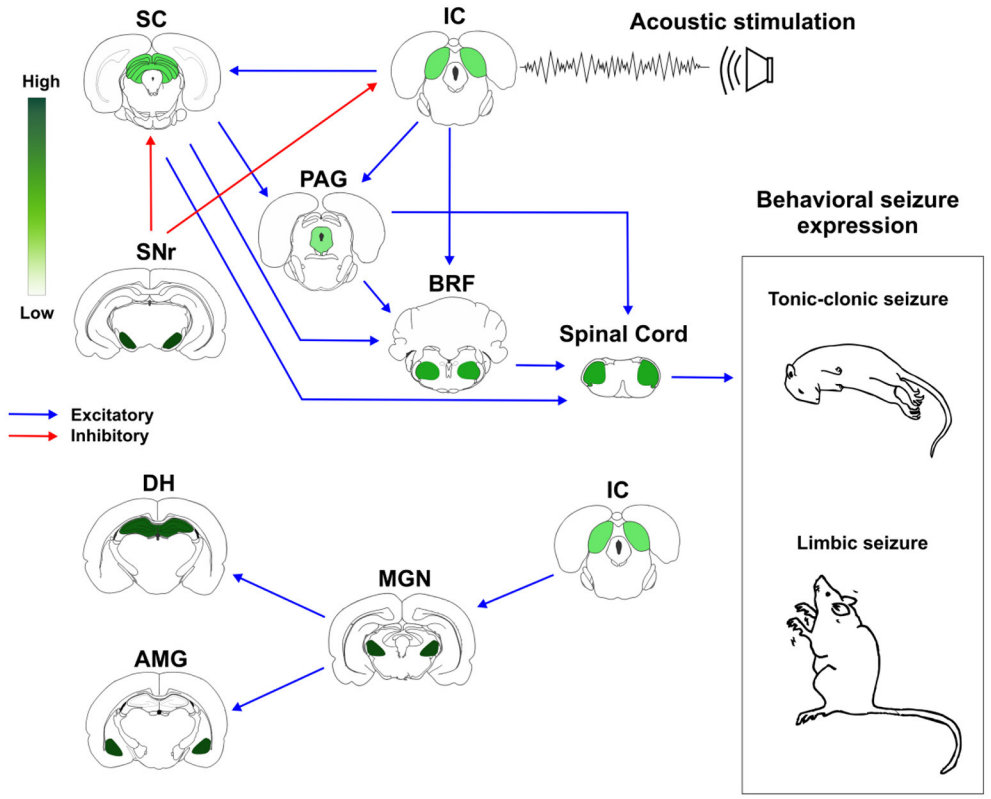


FIGURE 2 | Cannabinoid receptors type 1 (CB1R) in neuronal networks associated with acute and chronic audiogenic seizures. The inferior colliculus is the main brainstem structure related to sound perception and plays a key role in the genesis of audiogenic seizures. Inferior colliculus projects to different brainstem areas, like superior colliculus, periaqueductal gray matter, and brainstem reticular formation. This brainstem neuronal network is crucial to acute audiogenic seizures manifestation, that are behaviorally characterized by wild running followed by tonic-clonic seizures. The substantia nigra pars reticulata sends GABAergic projections to the mesencephalic tectum. This inhibitory projection is involved with the so-called endogenous anticonvulsant system. During the audiogenic kindling protocol, the chronic seizures, and the epileptogenic events lead to forebrain/limbic recruitment. The limbic recruitment during the chronic seizures involves projections from inferior colliculus to medial geniculate nucleus and then to the dorsal hippocampus and basolateral amygdala nucleus. This brainstem-limbic network is crucial to limbic motor seizures expression during the audiogenic kindling. Spinal cord neurons receive inputs from central neuronal networks and lead to audiogenic seizures' motor manifestation. The intensity of green color represents the amount of CB1R in each structure. Therefore, the endocannabinoid system is directly associated with brainstem and limbic neuronal networks responsible for tonic-clonic and limbic audiogenic seizures manifestation. IC, inferior colliculus; SC, superior colliculus; PAG, periaqueductal gray matter; BRF, brainstem reticular formation; SNr, substantia nigra pars reticulata; MGN, medial geniculate body; DH, dorsal hippocampus; AMG, amygdaloid complex (basolateral amygdala nucleus, BLA). Red arrows represent inhibitory projections, blue arrows represent excitatory projections.

et al., 2016, 2017). In 2018, the United States Food and Drug Administration (FDA) approved its first *Cannabis*-derived drug, the Epidiolex, a highly purified CBD oil solution (Corroon and Kight, 2018). This compound presents important therapeutic effect, especially in treatment-resistant epilepsy (Devinsky et al., 2018; Hausman-Kedem et al., 2018). However, although some studies have already demonstrated significant effects of Epidiolex against seizures, longitudinal studies to investigate long-term efficacy and safety are necessary, especially to assess its effects on cognitive and hormonal functions after chronic administration (Sekar and Pack, 2019).

In the basic research, CBD exerts not only anticonvulsant effects (Gobira et al., 2015; Kaplan et al., 2017; Klein et al., 2017; Lazarini-Lopes et al., 2020b), but also presents additional prominent effects important for epilepsy treatment, such as neuroprotective (Campos et al., 2016; Do Val-da Silva et al., 2017) and anti-inflammatory effects (Costa et al., 2004; Esposito et al., 2011). Because the epilepsies usually are accompanied by neuropsychiatric comorbidities, it is also important to know that CBD has antipsychotic, anxiolytic, and antidepressant effects (Zuardi et al., 1991; Crippa et al., 2011; Linge et al., 2016). As previously reported for pharmacological modulation of cannabinoid receptors, studies regarding *Cannabis*-derived compounds in AS are limited. See **Table 1** for main results.

CBD anticonvulsant effects against AS were firstly demonstrated by Carlini's research group during the 70's, in Brazil, when audiogenic susceptible Wistar rats were treated with CBD and then exposed to high-intensity sound stimulation. CBD drastically reduced the incidence of AS, decreasing the expression of wild running followed by tonic-clonic seizures from 60 to 10% after an acute acoustic stimulation (Carlini et al., 1973). Using audiogenic susceptible rats, Consroe and Wolkin

TABLE 1 | Effects of cannabinoids modulation and *Cannabis*-derived compounds in audiogenic seizures.

References	Subjects	Treatment/manipulation	Results
Carlini et al. (1973)	Male mice Male rats	CBD (10, 50, and 200 mg/kg; i.p.), CBD (25 mg/kg; i.p.)	Reduced wild running and tonic-clonic seizures
Boggan et al. (1973)	C57BL/6 mice submitted to AS priming	THC (2.5, 5, and 10 mg/kg; i.p.)	Reduced wild running, tonic, and clonic seizure
Consroe and Wolkin (1977)	Adult male rats	CBD (17 mg/kg; orally)	Reduced tonic-clonic seizures
Consroe et al. (1981)	Adult male rats	CBD (82.4 mg/kg; i.p.), CBD (14.9 mg/kg; i.v.)	Reduced AS frequency
Samineni et al. (2011)	Male and Female GEPR-9s	AM 251 (50, 100, and 200 pmol; intra-ventrolateral PAG; bilateral)	Decreased post-ictal analgesia (AS was not assessed)
Vinogradova et al. (2011)	Wistar rats susceptible to AS	SR 141716 (30 mg/kg; i.p.)	Facilitated AS in resistant animals; increased AS duration; facilitated limbic seizure expression
Busquets-Garcia et al. (2013)	*Fmr1–/y* mice: Fragile X Syndrome model	SR 141716 (1 mg/kg; i.p.) AM 630 (1 mg/kg; i.p.)	Attenuated AS and anxiety-like behavior
Hill et al. (2012)	DBA/2 mice	CBDV (50, 100, and 200 mg/kg; i.p.)	Reduced tonic seizures; Reduced mortality; increased the number of seizure-free animals
Hill et al. (2013)	DBA/2 mice	*Cannabis*-derived compounds rich in CBD + CBDV (Several doses)	Reduced wild running, tonic, and clonic seizures
Vinogradova and Van Rijn (2015)	Adult male KMs	WIN 55,212-2 (4 mg/kg; s.c.)	Increased the latency to post-tonic clonus behaviors
Citraro et al. (2016)	DBA/2 mice	Cannabinoid agonists: N-palmitoylethanolamine (5–40 mg/kg; i.p.), Arachidonyl-2-chloroethylamide (0.5–30 mg/kg; i.p.), WIN 55,212-2 (2.5–60 mg/kg; i.p.)	All drugs attenuated AS. Anticonvulsant effects were antagonized by NIDA-41020 (0.5–2 mg/kg; i.p.), a CB1R antagonist.
Gu et al. (2019)	Angelman Syndrome mice susceptible to AS	CBD (10, 20, 50, and 100 mg/kg; i.p.)	Attenuated wild running and tonic-clonic seizures. More than 80% of mice were seizure-free with CBD 100 mg/kg
Santos et al. (2020)	Male GEPR-3s	WIN 55,212-2 (1, 1.5, 2 mg/kg; i.p.), CP 55940 (26.5 nmol; intra-DLSC, bilateral)	Systemic and intra-DLSC CB1R activation attenuated AS severity; Systemic effect was not modified by intra-DLSC CB1R antagonist
Lazarini-Lopes et al. (2020a)	Male WARs	Characterization of CB1R expression after acute and chronic AS	Increased CB1R in the hippocampus of WAR (WAR × Wistar); Acute and chronic AS increased CB1R in amygdala and hippocampus of WARs

(1977), evaluated CBD effects in a great variety of epileptic seizure models, including AS. After three consecutive screenings for AS to confirm seizure susceptibility, CBD treatment was capable of preventing AS in a posterior stimulus. Moreover, the same research group showed that not only CBD, but also its analogs, prevented AS in 70% of animals after intravenous administration (Consroe et al., 1981). However, these studies did not discuss additional information regarding behavioral seizure profile or brain sites associated with CBD effects.

Cannabidivarin (CBDV), a CBD analog, presented dose-dependent protective effects against AS in DBA/2 mice, reducing the percentage of animals that developed tonic seizures, dropping to zero the mortality, and increasing the number of animals seizure-free (Hill et al., 2012). Similarly, *Cannabis*-derived botanical drug compounds rich in CBD were capable of reducing clonic seizures, and the co-administration of CBD and CBDV had synergic effects against generalized AS in DBA/2 mice, reducing wild running and clonic behaviors and blocking tonic seizures. This result is particularly interesting because although the authors confirmed CBD protective effects against AS, CBD anticonvulsant effects were independent of CB1R mechanisms (Hill et al., 2013). Gu et al. (2019) investigated CBD effects against AS in an animal model of Angelman Syndrome. In this model, mice are susceptible to AS, expressing wild running and tonic-clonic behaviors in response to intense sound stimulation (125 dB). CBD pretreatment presented dose-response effect, attenuating seizure expression, blocking tonic-clonic behaviors, and preventing seizure behaviors in more than 80% of Angelman Syndrome-mice tested (Gu et al., 2019). Furthermore, (–) Δ^9-tetrahydrocannabinol (THC) protect animals against AS. Authors observed a dose-dependent effect of THC, attenuating wild running, tonic, and clonic seizures (Boggan et al., 1973).

The current data about *Cannabis*-derived compounds on AS, especially CBD, CBDV, and THC, are convergent, suggesting attenuation of wild running and tonic-clonic behaviors in acute AS. Nevertheless, cannabinoids in the context of chronic AS still need to be explored. Chronic seizure protocols, like the AuK, allow the study of drugs with anticonvulsant effects and potential antiepileptogenic effects associated with seizures progression during the chronic protocol (Simonato et al., 2014). Therefore,

exploration of this research field using audiogenic strains could bring important information, especially regarding cannabinoids and the epileptogenic process.

CONCLUSION AND FUTURE PERSPECTIVES

Although with some paradoxical puzzling data, AS susceptibility in several of the mentioned networks converge to a more hyperexcitable brainstem state, either by an increase in glutamatergic neurotransmission or by a decrease in GABAergic signaling. Intra-collicular circuits receive and integrate information and send projections to extra-collicular afferent and efferent pathways and excessive excitatory activity into IC, SC, PAG, and BRF seems to be related with AS expression. On the other hand, GABA signaling into SNr is believed to be part of an endogenous anticonvulsant system that seems to be modulated by the ES, especially by CB1R, which could be an important clue to explain the neuronal basis of cannabinoids effects in AS.

Genetic strains used to study AS present advantages over chemical models, such as pilocarpine- and kainic acid-induced *Status Epilepticus*, where extended lesions are displayed by the animals (Leite et al., 2002; Castro et al., 2011; Furtado et al., 2011), but the neuroplasticity in AS is not accompanied by huge structural abnormalities (Galvis-Alonso et al., 2004). Other important characteristic of genetic models of AS is the absence of possible pharmacological interaction between cannabinoids and convulsant drugs. The AS model does not require previous invasive protocols for seizure induction (i.e., stereotaxic surgery or drug administration) and the trigger is an external high intensity stimulus directly controlled by the researcher. Because of these advantages, audiogenic strains seem to be interesting and appropriate approaches for epilepsies studies (Faingold et al., 2014).

Treatment with CB1R agonists, as well as with *Cannabis*-derived compounds, like CBD and CBDV, presented anticonvulsant activity against acute AS. The most prominent effects are associated with tonic behaviors, but wild running and clonus were also attenuated by these treatments. CB1R location in brainstem and forebrain structures also supports ES modulation on AS, especially into the SNr, amygdala, hippocampus, and cortex, the brain sites with most intense CB1R expression. Although there is a lack of studies investigating CBD and ES on chronic seizures in protocols like the AuK, the current data suggest that this is a prominent research area of study for epilepsies treatment. Since chronic protocols of AS allow the study of the epileptogenic process, the AuK could be an interesting tool to assess the role of the ES and *Cannabis*-derived compounds on limbic and forebrain recruitment.

Moreover, CBD anticonvulsant effects are associated with a great variety of mechanisms of action, such as GPR55, TRPV1, 5-HT_{1A}, BK channels, increased GABAergic neurotransmission, changes in calcium signaling, an indirect modulation of CB1R (Devinsky et al., 2014; Britch et al., 2020; Lazarini-Lopes et al., 2020b). In that context, antagonism of TRPV1 receptors suppressed AS in female GEPR-3s and attenuated seizures in male GEPR-3s (Cho et al., 2018). Therefore, the exploration of these mechanisms associated with cannabinoids in audiogenic strains is an interesting approach that should be further investigated. Furthermore, recent clinical data indicate that, regardless of the CBD low affinity for 5-HT1A receptors, at high concentration, CBD reduced the constitutive activity of receptors coupled to Gi/o receptors and these effects were reversed in the presence of 5-HT_{1A} antagonist, suggesting that CBD can act as a 5-HT_{1A} inverse agonist (Martínez-Aguirre et al., 2020). Therefore, the linking behind CBD anticonvulsant effects and the serotonergic system, should be further investigated in audiogenic strains.

Recent data showed that CBD attenuated seizures and restored the impaired hippocampal GABAergic neurotransmission observed in an animal model of Dravet-Syndrome (Kaplan et al., 2017). Likewise, audiogenic susceptible animals from the WAR strain present reduced GABAergic activity in the hippocampus (Cunha et al., 2018b) and the evaluation of CBD effects at GABAergic hippocampal network of WARs and other audiogenic strains could bring important information regarding CBD effects on epileptogenic process. Similarly, characterization of endocannabinoids levels and CB1R expression and functionality in brainstem and forebrain networks could help to explain the susceptibility to brainstem and limbic seizures in audiogenic strains. Data about CB1R expression in audiogenic susceptible strains are still very limited, but the current data suggest that endogenous alterations in CB1R could be related with seizure susceptibility, corroborating clinical data. Moreover, chronic alcohol exposure impairs CB1R functionality in the basolateral amygdala nucleus, which in turn, affects GABAergic signaling in this structure (Varodayan et al., 2016). Therefore, it should be an interesting approach to investigate changes in endocannabinoids levels and functionality in AS induced by ethanol withdrawal.

Finally, CBD presents different physiological and pharmacological mechanisms of action associated with its anticonvulsant effects and improvement of epilepsy-related neuropsychiatric comorbidities in basic and clinical research (Bergamaschi et al., 2011; Devinsky et al., 2014; Campos et al., 2017; Patra et al., 2020). Anxiety- and depressive-like behaviors are associated with genetic predisposition to seizure in strains like WAR, GEPR, KM, and audiogenic-susceptible WAG/Rij rats (Sarkisova and Kulikov, 2006; Castro et al., 2017; Sarkisova et al., 2017; Aguilar et al., 2018). However, the assessment of cannabinoids in epilepsy related comorbidities is an under-explored research field. Therefore, the ES in brainstem and limbic structures should be investigated not only in seizure susceptibility and expression, but also in neuropsychiatric comorbidities related to epilepsies.

AUTHOR CONTRIBUTIONS

WL-L, RD, and NG-C conceived the original idea. WL-L wrote the manuscript. RD, AC, RS-J, and NG-C provided critical reviews, and assisted in the writing and background research. RS-J and WL-L prepared the figures. WL-L prepared the table. NG-C reviewed the manuscript and included additional recommendations. All authors approved the final version.

FUNDING

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP): National Institutes for Science and Technology (INCT) - Translational Medicine (Grant No. 14/50891-1), Regular Research Funding (Grant No. 19/05957-8); Scholarship Post-Doctoral Research (Grant No. 19/00849-2); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): Grant No. 465458/2014-9 (INCT), Grant No. 305883/2014-3 (NG-C); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Finance Code 001).

REFERENCES

Aguilar, B. L., Malkova, L., N'Gouemo, P., and Forcelli, P. A. (2018). Genetically epilepsy-prone rats display anxiety-like behaviors and neuropsychiatric comorbidities of epilepsy. *Front. Neurol.* 9:476. doi: 10.3389/fneur.2018.00476

Alger, B. E. (2004). Endocannabinoids and their implications for epilepsy. *Epilepsy Curr.* 4, 169–173. doi: 10.1111/j.1535-7597.2004.04501.x

Alger, B. E., and Kim, J. (2011). Supply and demand for endocannabinoids. *Trends Neurosci.* 34, 304–315. doi: 10.1016/j.tins.2011.03.003

Altschuler, R. A., Sheridan, C. E., Horn, J. W., and Wenthold, R. J. (1989). Immunocytochemical localization of glutamate immunoreactivity in the guinea pig cochlea. *Hear. Res.* 42, 167–173. doi: 10.1016/0378-5955(89)90142-1

Appell, P. P., and Behan, M. (1990). Sources of subcortical GABAergic projections to the superior colliculus in the cat. *J. Comp.* 302, 143–158. doi: 10.1002/jmor.1051060102

Bajic, D., and Proudfit, H. K. (1999). Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception. *J. Comp. Neurol.* 405, 359–379. doi: 10.1002/(SICI)1096-9861(19990315)405:3<359::AID-CNE6>3.0.CO;2-W

Bergamaschi, M. M., Queiroz, R. H. C., Chagas, M. H. N., de Oliveira, D. C. G., De Martinis, B. S., Kapczinski, F., et al. (2011). Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. *Neuropsychopharmacology* 36, 1219–1226. doi: 10.1038/npp.2011.6

Blair, R. E. (2006). Activation of the cannabinoid type-1 receptor mediates the anticonvulsant properties of cannabinoids in the hippocampal neuronal culture models of acquired epilepsy and status epilepticus. *J. Pharmacol. Exp. Ther.* 317, 1072–1078. doi: 10.1124/jpet.105.100354

Bobbin, R. P., Ceasar, G., and Fallen, M. (1990). Potassium induced release of GABA and other substances from the guinea pig cochlea. *Hear. Res.* 46, 83–93. doi: 10.1016/0378-5955(90)90141-B

Boggan, W. O., Steele, R. A., and Freedman, D. X. (1973). 9 -Tetrahydrocannabinol effect on audiogenic seizure susceptibility. *Psychopharmacologia* 29, 101–106.

Braakham, H. M. H., van Oostenbrugge, R. J., van Kranen-Mastenbroek, V. H. J. M., and Krom, M. C. T. F. M. (2009). Rimonabant induces partial seizures in a patient with a history of generalized epilepsy. *Epilepsia* 50, 2171–2172. doi: 10.2307/3981645

Brandão, M. L., Anseloni, V. Z., Pandóssio, J. E., Araújo, J., and Castilho, V. M. (1999). Chemical modulation of the defensive behavior in the midbrain tectum. *Rev. Latinoam. Psicol.* 31, 111–131. doi: 10.1016/S0149-7634(99)00038-X

Brandão, M. L., Zanoveli, J. M., Ruiz-Martinez, R. C., Oliveira, L. C., and Landeira-Fernandez, J. (2008). Different patterns of freezing behavior organized in the periaqueductal gray of rats: association with different types of anxiety. *Behav. Brain Res.* 188, 1–13. doi: 10.1016/j.bbr.2007.10.018

Britch, S. C., Babalonis, S., and Walsh, S. L. (2020). Cannabidiol: pharmacology and therapeutic targets. *Psychopharmacology* 238, 9–28. doi: 10.1007/s00213-020-05712-8

Browning, R. A., Wang, C., Nelson, D. K., and Jobe, P. C. (1999). Effect of precollicular transection on audiogenic seizures in genetically epilepsy-prone rats. *Exp. Neurol.* 155, 295–301. doi: 10.1006/exnr.1998.6981

Browning, R. A. VII. (1986). Neuroanatomical localization of structures responsible for seizures in the GEPR: lesion studies. *Life Sci.* 39, 857–867. doi: 10.1016/0024-3205(86)90367-X

Bueno, C. H., Zangrossi, H., Nogueira, R. L., Soares, V. P., and Viana, M. B. (2005). Panicolytic-like effect induced by the stimulation of GABAAand GABABreceptors in the dorsal periaqueductal grey of rats. *Eur. J. Pharmacol.* 516, 239–246. doi: 10.1016/j.ejphar.2005.04.045

Busquets-Garcia, A., Gomis-González, M., Guegan, T., Agustín-Pavón, C., Pastor, A., Mato, S., et al. (2013). Targeting the endocannabinoid system in the treatment of fragile X syndrome. *Nat. Med.* 19, 603–607. doi: 10.1038/nm.3127

Caicedo, A., and Herbert, H. (1993). Topography of descending projections from the inferior colliculus to auditory brainstem nuclei in the rat. *J. Comp. Neurol.* 328, 377–392. doi: 10.1002/cne.903280305

Campos, A. C., Fogaça, M. V., Scarante, F. F., Joca, S. R. L., Sales, A. J., Gomes, F. V., et al. (2017). Plastic and neuroprotective mechanisms involved in the therapeutic effects of cannabidiol in psychiatric disorders. *Front. Pharmacol.* 8:269. doi: 10.3389/fphar.2017.00269

Campos, A. C., Fogaça, M. V., Sonego, A. B., and Guimarães, F. S. (2016). Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacol. Res.* 112, 119–127. doi: 10.1016/j.phrs.2016.01.033

Carlini, E. A., Leite, J. R., Tannhauser, M., and Berardi, A. C. (1973). Cannabidiol and *Cannabis sativa* extract protect mice and rats against convulsive agents. *J. Pharm. Pharmacol.* 25, 664–665. doi: 10.1111/j.2042-7158.1973.tb10660.x

Castillo, P. E., Younts, T. J., Chávez, A. E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron* 76, 70–81. doi: 10.1016/j.neuron.2012.09.020

Castro, G. P., Medeiros, D. D. C., Guarnieri, L. D. O., Mourão, F. A. G., Pinto, H. P. P., Pereira, G. A., et al. (2017). Wistar audiogenic rats display abnormal behavioral traits associated with artificial selection for seizure susceptibility. *Epilepsy Behav.* 71, 243–249. doi: 10.1016/j.yebeh.2015.08.039

Castro, O. W., Furtado, M. A., Tilelli, C. Q., Fernandes, A., Pajolla, G. P., and Garcia-Cairasco, N. (2011). Comparative neuroanatomical and temporal characterization of FluoroJade-positive neurodegeneration after status epilepticus induced by systemic and intrahippocampal pilocarpine in Wistar rats. *Brain Res.* 1374, 43–55. doi: 10.1016/j.brainres.2010.12.012

Chapman, A. G., Faingold, C. L., Hart, G. P., Bowker, H. M., and Meldrum, B. S. (1986). Brain regional amino acid levels in seizure susceptible rats: changes related to sound-induced seizures. *Neurochem. Int.* 8, 273–279. doi: 10.1016/0197-0186(86)90174-9

Chen, G., Lee, C., Sandridge, S. A., Butler, H. M., Manzoor, N. F., and Kaltenbach, J. A. (2013). Behavioral evidence for possible simultaneous induction of hyperacusis and tinnitus following intense sound exposure. *JARO J. Assoc. Res. Otolaryngol.* 14, 413–424. doi: 10.1007/s10162-013-0375-2

Cho, S. J., Vaca, M. A., Miranda, C. J., and N'Gouemo, P. (2018). Inhibition of transient potential receptor vanilloid type 1 suppresses seizure susceptibility in the genetically epilepsy-prone rat. *CNS Neurosci. Ther.* 24, 18–28. doi: 10.1111/cns.12770

Citraro, R., Russo, E., Leo, A., Russo, R., Avagliano, C., Navarra, M., et al. (2016). Pharmacokinetic-pharmacodynamic influence of N-palmitoylethanolamine, arachidonyl-2′-chloroethylamide and WIN 55,212-2 on the anticonvulsant activity of antiepileptic drugs against audiogenic seizures in DBA/2 mice. *Eur. J. Pharmacol.* 791, 523–534. doi: 10.1016/j.ejphar.2016.09.029

Citraro, R., Russo, E., Ngomba, R. T., Nicoletti, F., Scicchitano, F., Whalley, B. J., et al. (2013). CB1 agonists, locally applied to the cortico-thalamic circuit of rats with genetic absence epilepsy, reduce epileptic manifestations. *Epilepsy Res.* 106, 74–82. doi: 10.1016/j.eplepsyres.2013.06.004

Clugnet, M. C., and LeDoux, J. E. (1990). Synaptic plasticity in fear conditioning circuits: Induction of LTP in the lateral nucleus of the amygdala by stimulation of the medial geniculate body. *J. Neurosci.* 10, 2818–2824. doi: 10.1523/jneurosci.3308-11.2012

Coleman, J. R., and Clerici, W. J. (1987). Sources of projections to subdivisions of the inferior colliculus in the rat. *J. Comp. Neurol.* 262, 215–226. doi: 10.1002/cne.902620204

Coleman, J. R., Thompson, K. C., Wilson, M. A., and Wilson, S. P. (2017). Audiogenic seizure activity following HSV-1 GAD65 sense or antisense

injection into inferior colliculus of Long–Evans rat. *Epilepsy Behav.* 71, 238–242. doi: 10.1016/j.yebeh.2016.08.024

Consroe, P., Martin, A., and Singh, V. (1981). Antiepileptic potential of cannabidiol analogs. *J. Clin. Pharmacol.* 21, 428S—436S.

Consroe, P., and Wolkin, A. (1977). Cannabidiol-antiepileptic comparisons experimentally and induced interactions seizures drug in rats. *J. Pharmacol. Exp. Ther.* 201, 26–32.

Corroon, J., and Kight, R. (2018). Regulatory status of cannabidiol in the United States: a perspective. *Cannabis Cannabinoid Res.* 3, 190–194. doi: 10.1089/can.2018.0030

Costa, B., Colleoni, M., Conti, S., Parolaro, D., Franke, C., Trovato, A. E., et al. (2004). Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 369, 294–299. doi: 10.1007/s00210-004-0871-3

Crippa, J. A. S., Nogueira Derenusson, G., Borduqui Ferrari, T., Wichert-Ana, L., Duran, F. L., Martin-Santos, R., et al. (2011). Neural basis of anxiolytic effects of cannabidiol (CBD) in generalized social anxiety disorder: a preliminary report. *J. Psychopharmacol.* 25, 121–130. doi: 10.1177/0269881110379283

Cunha, A. O. S., Ceballos, C. C., de Deus, J. L., and Leão, R. M. (2018a). Long-term high-intensity sound stimulation inhibits h current (Ih) in CA1 pyramidal neurons. *Eur. J. Neurosci.* 47, 1401–1413. doi: 10.1111/ejn.13954

Cunha, A. O. S., Ceballos, C. C., De Deus, J. L., Pena, R. F. D. O., De Oliveira, J. A. C., Roque, A. C., et al. (2018b). Intrinsic and synaptic properties of hippocampal CA1 pyramidal neurons of the Wistar Audiogenic Rat (WAR) strain, a genetic model of epilepsy. *Sci. Rep.* 8:10412. doi: 10.1038/s41598-018-28725-y

Cunha, A. O. S., de Oliveira, J. A. C., Almeida, S. S., Garcia-Cairasco, N., and Leão, R. M. (2015). Inhibition of long-term potentiation in the schaffer-CA1 pathway by repetitive high-intensity sound stimulation. *Neuroscience* 310, 114–127. doi: 10.1016/j.neuroscience.2015.09.040

Cunha, J. M., Carlini, A. E. P., Ramos, O. L., Pimentel, C., Gagliardi, R., Sanvito, W. L., et al. (1980). Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology* 21, 175–185.

Dailey, J. W., Reigel, C. E., Mishra, P. K., and Jobe, P. C. (1989). Neurobiology of seizure predisposition in the genetically epilepsy-prone rat. *Epilepsy Res.* 3, 3–17. doi: 10.1016/0920-1211(89)90063-6

Dean, P., Simkins, M., Hetherington, L., Mitchell, I. J., and Redgrave, P. (1991). Tectal induction of cortical arousal: evidence implicating multiple output pathways. *Brain Res. Bull.* 26, 1–10. doi: 10.1016/0361-9230(91)90184-L

Deng, H., Xiao, X., and Wang, Z. (2016). Periaqueductal gray neuronal activities underlie different aspects of defensive behaviors. *J. Neurosci.* 36, 7580–7588. doi: 10.1523/JNEUROSCI.4425-15.2016

Depaulis, A., Vergnes, M., Marescaux, C., Lannes, B., and Warter, J. M. (1988). Evidence that activation of GABA receptors in the substantia nigra suppresses spontaneous spike-and-wave discharges in the rat. *Brain Res.* 448, 20–29. doi: 10.1016/0006-8993(88)91097-9

Devane, W., A, Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L., A, Griffin, G., et al. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949. doi: 10.1126/science.1470919

Devinsky, O., Cilio, M. R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* 55, 791–802. doi: 10.1111/epi.12631

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al. (2017). Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N. Engl. J. Med.* 376, 2011–2020. doi: 10.1056/NEJMoa1611618

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet. Neurol.* 15, 270–278. doi: 10.1016/S1474-4422(15)00379-8

Devinsky, O., Verducci, C., Thiele, E. A., Laux, L. C., Patel, A. D., Filloux, F., et al. (2018). Open-label use of highly purified CBD (Epidiolex®) in patients with CDKL5 deficiency disorder and Aicardi, Dup15q, and Doose syndromes. *Epilepsy Behav.* 86, 131–137. doi: 10.1016/j.yebeh.2018.05.013

Do Val-da Silva, R. A., Peixoto-Santos, J. E., Kandratavicius, L., de Ross, J. B., Esteves, I., de Martinis, B. S., et al. (2017). Protective effects of cannabidiol against seizures and neuronal death in a rat model of mesial temporal lobe epilepsy. *Front. Pharmacol.* 8:131. doi: 10.3389/fphar.2017.00131

Doretto, M. C., Cortes-de-Oliveira, J. A., Rossetti, F., and Garcia-Cairasco, N. (2009). Role of the superior colliculus in the expression of acute and kindled audiogenic seizures in Wistar audiogenic rats. *Epilepsia* 50, 2563–2574. doi: 10.1111/j.1528-1167.2009.02164.x

Doretto, M. C., Fonseca, C. G., Lôbo, R. B., Terra, V. C., Oliveira, J. A. C., and Garcia-Cairasco, N. (2003). Quantitative study of the response to genetic selection of the wistar audiogenic rat strain (WAR). *Behav. Genet.* 33, 33–42. doi: 10.1023/A:1021099432759

Doretto, M. C., and Garcia-Cairasco, N. (1995). Differential audiogenic seizure sensitization by selective unilateral substantia nigra lesions in resistant Wistar rats. *Physiol. Behav.* 58, 273–282. doi: 10.1016/0031-9384(95)00050-S

Doretto, M. C., Mishra, P. K., Garcia-Cairasco, N., Dailey, J. W., and Jobe, P. C. (1994). A microdialysis study of amino acid concentrations in the extracellular fluid of the substantia nigra of freely behaving GEPR-9s: relationship to seizure predisposition. *Epilepsy Res.* 17, 157–165.

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., and Latina, V. (2011). Cannabidiol reduces Ab-induced neuroinflammation and promotes hippocampal neurogenesis through PPARc involvement. *PLoS ONE* 6:28668. doi: 10.1371/journal.pone.0028668

Evans, M. S., Cady, C. J., Disney, K. E., Yang, L., and Laguardia, J. J. (2006). Three brief epileptic seizures reduce inhibitory synaptic currents, GABAAcurrents, and GABAA-receptor subunits. *Epilepsia* 47, 1655–1664. doi: 10.1111/j.1528-1167.2006.00634.x

Evans, M. S., Viola-McCabe, K. E., Caspary, D. M., and Faingold, C. L. (1994). Loss of synaptic inhibition during repetitive stimulation in genetically epilepsy-prone rats (GEPR). *Epilepsy Res.* 18, 97–105. doi: 10.1016/0920-1211(94)90002-7

Faingold, C. L. (1988). The genetically epilepsy-prone rat. *Gen. Pharmacol.* 16, 91–99. doi: 10.1007/BF02229080

Faingold, C. L. (2002). Role of GABA abnormalities in the inferior colliculus pathophysiology - audiogenic seizures. *Hear. Res.* 168, 223–237. doi: 10.1016/S0378-5955(02)00373-8

Faingold, C. L. (2012). "Brainstem networks: reticulo-cortical synchronization in generalized convulsive seizures," in *Jasper's Basic Mechanisms of the Epilepsies*, 4th Edn., eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta (Bethesda, MD: National Center for Biotechnology Information (US)).

Faingold, C. L., Gehlbach, G., and Caspary, D. (1986). Decreased effectiveness of GABA-mediated inhibition in the inferior colliculus of the genetically epilepsy-prone rat the inferior colliculus (IC) is implicated as the most rostral auditory nu- cleus required for the induction of audiogenic seizures AG. *Exp. Neurol.* 159, 145–159.

Faingold, C. L., Millan, M. H., Anderson, C. A. B., and Meldrum, B. S. (1989). Induction of audiogenic seizures in normal and genetically epilepsy-prone rats following focal microinjection of an excitant amino acid into reticular formation and auditory nuclei. *Epilepsy Res.* 3, 199–205. doi: 10.1016/0920-1211(89)90024-7

Faingold, C. L., Raisinghani, M., and N'Gouemo, P. (2014). "Chapter 26: neuronal networks in epilepsy: comparative audiogenic seizure networks," in *Neuronal Networks in Brain Function, CNS Disorders, and Therapeutics,* eds C. L. Faingold and H. Blumenfeld (Academic Press), 349–373. doi: 10.1016/B978-0-12-415804-7.00026-5

Faingold, C. L., Randall, M., and Tupal, S. (2010). DBA/1 mice exhibit chronic susceptibility to audiogenic seizures followed by sudden death associated with respiratory arrest. *Epilepsy Behav.* 17, 436–440. doi: 10.1016/j.yebeh.2010.02.007

Faingold, C. L., and Randall, M. E. (1995). Pontine reticular formation neurons exhibit a premature and precipitous increase in acoustic responses prior to audiogenic seizures in genetically epilepsy-prone rats. *Brain Res.* 704, 218–226. doi: 10.1016/0006-8993(95)01116-1

Faingold, C. L., and Randall, M. E. (1999). Neurons in the deep layers of superior colliculus play a critical role in the neuronal network for audiogenic seizures: mechanisms for production of wild running behavior. *Brain Res.* 815, 250–258. doi: 10.1016/S0006-8993(98)01136-6

Faingold, C. L., and Riaz, A. (1994). Increased responsiveness of pontine reticular formation neurons associated with audiogenic seizure susceptibility during ethanol withdrawal. *Brain Res.* 663, 69–76. doi: 10.1016/0006-8993(94)90463-4

Faingold, C. L., Walsh, E. J., Maxwell, J. K., and Randall, M. E. (1990). Audiogenic seizure severity and hearing deficits in the genetically epilepsy-prone rat. *Exp. Neurol.* 108, 55–60. doi: 10.1016/0014-4886(90)90007-F

Faye-Lund, H., and Osen, K. K. (1985). Anatomy of the inferior colliculus in rat. *Anat. Embryol.* 171, 1–20. doi: 10.1007/BF00319050

Fisher, R. S., Acevedo, C., Arzimanoglou, A., Bogacz, A., Cross, J. H., Elger, C. E., et al. (2014). ILAE official report: a practical clinical definition of epilepsy. *Epilepsia* 55, 475–482. doi: 10.1111/epi.12550

Fitzgerald, M. L., Shobin, E., and Pickel, V. M. (2012). Cannabinoid modulation of the dopaminergic circuitry: implications for limbic and striatal output. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* 38, 21–29. doi: 10.1016/j.pnpbp.2011.12.004

Friedman, D., and Devinsky, O. (2015). Cannabinoids in the treatment of epilepsy. *N. Engl. J. Med.* 373, 1048–1058. doi: 10.1056/NEJMra1407304

Furtado, M. A., Castro, O. W., Del Vecchio, F., de Oliveira, J. A. C., and Garcia-Cairasco, N. (2011). Study of spontaneous recurrent seizures and morphological alterations after status epilepticus induced by intrahippocampal injection of pilocarpine. *Epilepsy Behav.* 20, 257–266. doi: 10.1016/j.yebeh.2010.11.024

Gale, K. (1986). Role of the substantia nigra in GABA-mediated anticonvulsant actions. *Adv. Neurol.* 44, 343–64.

Gale, K. (1992). Subcortical structures and pathways involved in convulsive seizure generation. *J. Clin. Neurophysiol.* 9, 264–277. doi: 10.1097/00004691-199204010-00007

Gale, K., Pazos, A., Maggio, R., Japikse, K., and Pritchard, P. (1993). Blockade of GABA receptors in superior colliculus protects against focally evoked limbic motor seizures. *Brain Res.* 603, 279–283. doi: 10.1016/0006-8993(93)91248-Q

Galvis-Alonso, O. Y., Cortes De Oliveira, J. A., and Garcia-Cairasco, N. (2004). Limbic epileptogenicity, cell loss and axonal reorganization induced by audiogenic and amygdala kindling in wistar audiogenic rats (WAR strain). *Neuroscience* 125, 787–802. doi: 10.1016/j.neuroscience.2004.01.042

Garcia Cairasco, N., and Sabbatini, R. M. E. (1983). Role of the substantia nigra in audiogenic seizures: a neuroethological analysis in the rat. *Brazilian J. Med. Biol. Res.* 16, 171–183

García del Caño, G., Gerrikagoitia, I., Alonso-Cabria, A., and Martínez-Millán, L. (2006). Organization and origin of the connection from the inferior to the superior colliculi in the rat. *J. Comp. Neurol.* 499, 716–731. doi: 10.1002/cne

Garcia-Cairasco, N. (2002). A critical review on the participation of inferior colliculus in acoustic-motor and acoustic-limbic networks involved in the expression of acute and kindled audiogenic seizures. *Hear. Res.* 168, 208–222. doi: 10.1016/S0378-5955(02)00371-4

Garcia-Cairasco, N., and Sabbatini, R. M. E. (1991). Possible interaction between the inferior colliculus and the substantia nigra in audiogenic seizures in Wistar rats. *Physiol. Behav.* 50, 421–427. doi: 10.1016/0031-9384(91)90089-7

Garcia-Cairasco, N., and Triviño-Santos, H. F. (1989). Effects of both 6-hydroxydopamine-induced and electrolytic lesion of the substantia nigra on the rotational behavior and audiogenic seizures in the rat. *Brazilian J. Med. Biol. Res.* 22, 617–629.

Garcia-Cairasco, N., Umeoka, E. H. L., and Cortes de Oliveira, J. A. (2017). The Wistar Audiogenic Rat (WAR) strain and its contributions to epileptology and related comorbidities: history and perspectives. *Epilepsy Behav.* 71(Pt B), 250–273. doi: 10.1016/j.yebeh.2017.04.001

Garcia-Cairasco, N., Wakamatsu, H., Oliveira, J. A. C., Gomes, E. L. T., Del Bel, E. A., and Mello, L. E. A. M. (1996). Neuroethological and morphological (Neo-Timm staining) correlates of limbic recruitment during the development of audiogenic kindling in seizure susceptible Wistar rats. *Epilepsy Res.* 26, 177–192. doi: 10.1016/S0920-1211(96)00050-2

Gaston, T. E., Bebin, E. M., Cutter, G. R., Liu, Y., and Szaflarski, J. P. (2017). Interactions between cannabidiol and commonly used antiepileptic drugs. *Epilepsia* 58, 1586–1592. doi: 10.1111/epi.13852

Glenn, D. W., Brown, R. D., Jobe, P. C., and Penny, J. E. (1980). A comparison of cochlear microphonics and N1in audiogenic-seizure-susceptible and control rats. *Neurol. Res.* 2, 85–100. doi: 10.1080/01616412.1980.11739573

Gobira, P. H., Vilela, L. R., Gonçalves, B. D. C., Santos, R. P. M., de Oliveira, A. C., Vieira, L. B., et al. (2015). Cannabidiol, a Cannabis sativa constituent, inhibits cocaine-induced seizures in mice: possible role of the mTOR pathway and reduction in glutamate release. *Neurotoxicology* 50, 116–121. doi: 10.1016/j.neuro.2015.08.007

Goddard, G. V. (1967). Development of epileptic seizures through brain stimulation at low intensity. *Nature* 214, 1020–1021. doi: 10.1038/2141020a0

Goddard, G. V., McIntyre, D. C., and Leech, C. K. (1969). A permanent change in brain function resulting from daily electrical stimulation. *Exp. Neurol.* 25, 295–330. doi: 10.1016/0014-4886(69)90128-9

Goffin, K., Van Paesschen, W., and Van Laere, K. (2011). In vivo activation of endocannabinoid system in temporal lobe epilepsy with hippocampal sclerosis. *Brain* 134, 1033–1040. doi: 10.1093/brain/awq385

Gonzalez, L. P., and Hettinger, M. K. (1984). Intranigral muscimol suppresses ethanol withdrawal seizures. *Brain Res.* 298, 163–166. doi: 10.1016/0006-8993(84)91162-4

Gu, B., Zhu, M., Glass, M. R., Rougié, M., Nikolova, V. D., Moy, S. S., et al. (2019). Cannabidiol attenuates seizures and EEG abnormalities in Angelman syndrome model mice. *J. Clin. Invest.* 129, 5462–5467. doi: 10.1172/jci130419

Hausman-Kedem, M., Menascu, S., and Kramer, U. (2018). Efficacy of CBD-enriched medical cannabis for treatment of refractory epilepsy in children and adolescents – an observational, longitudinal study. *Brain Dev.* 40, 544–551. doi: 10.1016/j.braindev.2018.03.013

Heffner, H. E., and Harrington, I. A. (2002). Tinnitus in hamsters following exposure to intense sound. *Hear. Res.* 170, 83–95. doi: 10.1016/S0378-5955(02)00343-X

Herkenham, M., Lynn, A. B., Johnsont, M. R., Melvin, L. S., Costa, B. R., and Riceo, K. C. (1991). Characterization and localization of cannabinoid receptors in brain: an *in vitro* technique using slide-mounted tissue sections. *J. Neurosci.* 11, 563–583.

Herkenham, M., Lynn, A. B., Litrle, M. D., Johnsont, M. R., Melvin, L. S., De Costa, B. R., et al. (1990). Cannabinoid receptor localization in brain. *Neurobiology* 87, 1932–1936. doi: 10.1073/pnas.87.5.1932

Hill, A. J., Mercier, M. S., Hill, T. D. M., Glyn, S. E., Jones, N. A., Yamasaki, Y., et al. (2012). Cannabidivarin is anticonvulsant in mouse and rat. *Br. J. Pharmacol.* 167, 1629–1642. doi: 10.1111/j.1476-5381.2012.02207.x

Hill, T. D. M., Cascio, M. G., Romano, B., Duncan, M., Pertwee, R. G., Williams, C. M., et al. (2013). Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. *Br. J. Pharmacol.* 170, 679–692. doi: 10.1111/bph.12321

Hofmann, M. E., and Frazier, C. J. (2013). Marijuana, endocannabinoids, and epilepsy: Potential and challenges for improved therapeutic intervention. *Exp. Neurol.* 244, 43–50. doi: 10.1016/j.expneurol.2011.11.047

Huizenga, M. N., Wicker, E., Beck, V. C., and Forcelli, P. A. (2017). Anticonvulsant effect of cannabinoid receptor agonists in models of seizures in developing rats. *Epilepsia* 58, 1593–1602. doi: 10.1111/epi.13842

Iadarola, M. J., and Gale, K. (1982). Substantia nigra: Site of anticonvulsant activity mediated by γ-aminobutyric acid. *Science* 218, 1237–1240. doi: 10.1126/science.7146907

Ishimoto, T., Omori, N., Mutoh, F., and Chiba, S. (2000). Convulsive seizures induced by N -methyl- D -aspartate microinjection into the mesencephalic reticular formation in rats. *Brain Res.* 881, 152–158. doi: 10.1016/S0006-8993(00)02830-4

Jacob, A., and Todd, A. R. (1940). Cannabis indica. Part II. Isolation of cannabidiol from egyptian hashish. Observations on the structure of cannabinol. *J. Chem. Soc.* 649–653. doi: 10.1039/JR9400000649

Jensen, L. H., Petersen, E. N., and Braestrup, C. (1983). Audiogenic seizures in DBA/2 mice discriminate sensitively between low efficacy benzodazepine receptor agonists and inverse agonists. *Life Sci.* 33, 393–399. doi: 10.1016/S0024-3205(83)80014-9

Jones, B. E., and Yang, T. -Z. (1985). The efferent projections from the reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat. *J. Comp. Neurol.* 242, 56–92. doi: 10.1002/cne.902420105

Jones, N. A., Glyn, S. E., Akiyama, S., Hill, T. D. M., Hill, A. J., Weston, S. E., et al. (2012). Cannabidiol exerts anti-convulsant effects in animal models of temporal lobe and partial seizures. *Seizure* 21, 344–352. doi: 10.1016/j.seizure.2012.03.001

Kanner, A. M. (2017). Psychiatric comorbidities in new onset epilepsy: Should they be always investigated? *Seizure* 49, 79–82. doi: 10.1016/j.seizure.2017.04.007

Kaplan, J. S., Stella, N., Catterall, W. A., and Westenbroek, R. E. (2017). Cannabidiol attenuates seizures and social deficits in a mouse model

of Dravet syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11229–11234. doi: 10.1073/pnas.1711351114

Karlócai, M. R., Tóth, K., Watanabe, M., Ledent, C., Juhász, G., Freund, T. F., et al. (2011). Redistribution of CB1 cannabinoid receptors in the acute and chronic phases of pilocarpine-induced epilepsy. *PLoS ONE* 6:e27196. doi: 10.1371/journal.pone.0027196

King, S. M., Shehab, S., Dean, P., and Redgrave, P. (1996). Differential expression of fos-like immunoreactivity in the descending projections of superior colliculus after electrical stimulation in the rat. *Behav. Brain Res.* 78, 131–145. doi: 10.1016/0166-4328(95)00241-3

Klein, B. D., Jacobson, C. A., Metcalf, C. S., Smith, M. D., Wilcox, K. S., Hampson, A. J., et al. (2017). Evaluation of cannabidiol in animal seizure models by the Epilepsy Therapy Screening Program (ETSP). *Neurochem. Res.* 42, 1939–1948. doi: 10.1007/s11064-017-2287-8

Kraus, K. S., and Canlon, B. (2012). Neuronal connectivity and interactions between the auditory and limbic systems. Effects of noise and tinnitus. *Hear. Res.* 288, 34–46. doi: 10.1016/j.heares.2012.02.009

Kwan, P., and Brodie, M. J. (2010). Definition of refractory epilepsy: defining the indefinable? *Lancet Neurol.* 9, 27–29. doi: 10.1016/S1474-4422(09)70304-7

Lazarini-Lopes, W., da Silva-Júnior, R. M. P., Servilha-Menezes, G., Do Val-da Silva, R. A., and Garcia-Cairasco, N. (2020a). Cannabinoid receptor type 1 (CB1R) expression in limbic brain structures after acute and chronic seizures in a genetic model of epilepsy. *Front. Behav. Neurosci.* 14:602258. doi: 10.3389/fnbeh.2020.602258

Lazarini-Lopes, W., Do Val-da Silva, R. A., da Silva-Júnior, R. M. P., Leite, J. P., and Garcia-Cairasco, N. (2020b). The anticonvulsant effects of cannabidiol in experimental models of epileptic seizures: From behavior and mechanisms to clinical insights. *Neurosci. Biobehav. Rev.* 111, 166–182. doi: 10.1016/j.neubiorev.2020.01.014

LeDoux, J. (2007). The amygdala. *Curr. Biol.* 17, R868–R874. doi: 10.1016/j.cub.2007.08.005

Ledoux, J. E., Ruggiero, D. A., and Reis, A. N. D. J. (1985). Projections to the subcortical forebrain from anatomically defined regions of the medial geniculate body in the rat. *J. Comp. Neurol.* 242, 182–213.

Lefebvre, P. P., Weber, T., Leprince, P., Rigo, J.-M., Delrée, P., Rogister, B., et al. (1991). Kainate and NMDA toxicity fot cultured developing and adult rat spiral ganglion neurons: further evidence for a glutamatergic excitatory neurotransmission at the inner hair cell synapse. *Brain Res.* 555, 75–83.

Leite, J. P., Garcia-Cairasco, N., and Cavalheiro, E. A. (2002). New insights from the use of pilocarpine and kainate models. *Epilepsy Res.* 50, 93–103. doi: 10.1016/S0920-1211(02)00072-4

Linge, R., Jiménez-Sánchez, L., Campa, L., Pilar-Cuéllar, F., Vidal, R., Pazos, A., et al. (2016). Cannabidiol induces rapid-acting antidepressant-like effects and enhances cortical 5-HT/glutamate neurotransmission: Role of 5-HT1Areceptors. *Neuropharmacology* 103, 16–26. doi: 10.1016/j.neuropharm.2015.12.017

Löscher, W. (2011). Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure* 20, 359–368. doi: 10.1016/j.seizure.2011.01.003

Löscher, W. (2017). Animal models of seizures and epilepsy: past, present, and future role for the discovery of antiseizure drugs. *Neurochem. Res.* 42, 1873–1888. doi: 10.1007/s11064-017-2222-z

Lutz, B. (2004). On-demand activation of the endocannabinoid system in the control of neuronal excitability and epileptiform seizures. *Biochem. Pharmacol.* 68, 1691–1698. doi: 10.1016/j.bcp.2004.07.007

Maggio, R., and Gale, K. (1989). Seizures evoked from area tempestas are subject to control by GABA and glutamate receptors in substantia nigra. *Exp. Neurol.* 105, 184–188. doi: 10.1016/0014-4886(89)90118-0

Maglóczky, Z., Tóth, K., Karlócai, R., Nagy, S., Eross, L., Czirják, S., et al. (2010). Dynamic changes of CB1-receptor expression in hippocampi of epileptic mice and humans. *Epilepsia* 51, 115–120. doi: 10.1111/j.1528-1167.2010.02624.x

Marescaux, C., Vergnes, M., Kiesmann, M., Depaulis, A., Micheletti, G., and Warter, J. M. (1987). Kindling of audiogenic seizures in wistar rats: an EEG study. *Exp. Neurol.* 168, 160–168

Martínez-Aguirre, C., Carmona-Cruz, F., Velasco, A. L., Velasco, F., Aguado-Carrillo, G., Cuéllar-Herrera, M., et al. (2020). Cannabidiol acts at 5-HT1A receptors in the human brain: relevance for treating temporal lobe epilepsy. *Front. Behav. Neurosci.* 14: 611278. doi: 10.3389/fnbeh.2020.611278

Masino, T., and Knudsen, E. I. (1992). Anatomical pathways from the optic tectum to the spinal cord subserving orienting movements in the barn owl Tom. *Exp. Brain Res.* 92, 194–208. doi: 10.1109/LCN.2004.105

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0

May, P. J. (2005). The mammalian superior colliculus: Laminar structure and connections. *Prog. Brain Res.* 151, 321–378. doi: 10.1016/S0079-6123(05)51011-2

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90.

Mechoulam, R., and Shvo, Y. (1963). Hashish—I. The structure of cannabidiol. *Tetrahedron* 19, 2073–2078. doi: 10.1016/0040-4020(63)85022-X

Mencher, S. K., and Wang, L. G. (2005). Promiscuous drugs compared to selective drugs (promiscuity can be a virtue). *BMC Clin. Pharmacol.* 5:3. doi: 10.1186/1472-6904-5-3

Merrill, M. A., Clough, R. W., Jobe, P. C., and Browning, R. A. (2003). Role of the superior colliculus and the intercollicular nucleus in the brainstem seizure circuitry of the genetically epilepsy-prone rat. *Epilepsia* 44, 305–314. doi: 10.1046/j.1528-1157.2003.31802.x

Millan, M. H., Meldrum, B. S., Boersma, C. A., and Faingold, C. L. (1988). Excitant amino acids and audiogenic seizures in the genetically epilepsy-prone rat. II. Efferent seizure propagation pathway. *Exp. Neurol.* 99, 687–698. doi: 10.1016/0014-4886(88)90184-7

Miller, A. S., and Walker, J. M. (1995). Effects of a cannabinoid on spontaneous and evoked neuronal activity in the substantia nigra pars reticulata. *Eur. J. Pharmacol.* 279, 179–185. doi: 10.1016/0014-2999(95)00151-A

Moraes, M. F. D., Galvis-Alonso, O. Y., and Garcia-Cairasco, N. (2000). Audiogenic kindling in the Wistar rat: a potential model for recruitment of limbic structures. *Epilepsy Res.* 39, 251–259. doi: 10.1016/S0920-1211(00)00107-8

Mouton, L. J., and Holstege, G. (1994). The periaqueductal gray in the cat projects to lamina VIII and the medial part of lamina VII throughout the length of the spinal cord. *Exp. Brain Res.* 101, 253–264. doi: 10.1007/BF00228745

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0

Naritoku, D. K., Mecozzi, L. B., Aiello, M. T., and Faingold, C. L. (1992). Repetition of audiogenic seizures in genetically epilepsy-prone rats induces cortical epileptiform activity and additional seizure behaviors. *Exp. Neurol.* 115, 317–324. doi: 10.1016/0014-4886(92)90197-X

N'Gouemo, P., and Faingold, C. L. (1996). Repetitive audiogenic seizures cause an increased acoustic response in inferior colliculus neurons and additional convulsive behaviors in the genetically-epilepsy prone rat. *Brain Res.* 710, 92–96. doi: 10.1016/0006-8993(95)01356-3

N'Gouemo, P., and Faingold, C. L. (1998). Periaqueductal gray neurons exhibit increased responsiveness associated with audiogenic seizures in the genetically epilepsy-prone rat. *Neuroscience* 84, 619–625. doi: 10.1016/S0306-4522(97)00551-4

N'Gouemo, P., and Faingold, C. L. (1999). The periaqueductal grey is a critical site in the neuronal network for audiogenic seizures: modulation by GABA(A), NMDA and opioid receptors. *Epilepsy Res.* 35, 39–46. doi: 10.1016/S0920-1211(98)00128-4

Nisenbaum, E. S., Grace, A. A., and Berger, T. W. (1992). Functionally distinct subpopulations of striatal neurons are differentially regulated by gabaergic and dopaminergic inputs-II. *In vitro* analysis. *Neuroscience* 48, 579–593. doi: 10.1016/0306-4522(92)90403-O

Olazábal, U. E., and Moore, J. K. (1989). Nigrotectal projection to the inferior colliculus: Horseradish peroxidase transport and tyrosine hydroxylase immunohistochemical studies in rats, cats, and bats. *J. Comp. Neurol.* 282, 98–118. doi: 10.1002/cne.902820108

Patra, P. H., Serafeimidou-Pouliou, E., Bazelot, M., Whalley, B. J., Williams, C. M., and McNeish, A. J. (2020). Cannabidiol improves survival and behavioural co-morbidities of Dravet syndrome in mice. *Br. J. Pharmacol.* 177, 2779–2792. doi: 10.1111/bph.15003

Penny, J. E., Brown, R. D., Hodges, K. B., Kupetz, S. A., Glenn, D. W., and Jobe, P. C. (1983). Cochlear morphology of the audiogenic-seizure susceptible

(ags) or genetically epilepsy prone rat (gepr). *Acta Otolaryngol.* 95, 1–12. doi: 10.3109/00016488309130909

Penny, J. E., Don Brown, R., Wallace, M. S., and Henley, C. M. (1986). Auditory aspects of seizure in the genetically epilepsy prone rat. *Life Sci.* 39, 887–895. doi: 10.1016/0024-3205(86)90370-X

Poletaeva, I. I., Surina, N. M., Kostina, Z. A., Perepelkina, O. V., and Fedotova, I. B. (2017). The Krushinsky-Molodkina rat strain: the study of audiogenic epilepsy for 65 years. *Epilepsy Behav.* 71, 130–141. doi: 10.1016/j.yebeh.2015.04.072

Porter, B. E., and Jacobson, C. (2013). Report of a parent survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. *Epilepsy Behav.* 29, 574–577. doi: 10.1016/j.yebeh.2013.08.037

Press, C. A., Knupp, K. G., and Chapman, K. E. (2015). Parental reporting of response to oral cannabis extracts for treatment of refractory epilepsy. *Epilepsy Behav.* 45, 49–52. doi: 10.1016/j.yebeh.2015.02.043

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* 32, 281–294. doi: 10.1016/0013-4694(72)90177-0

Reigel, C. E., Dailey, J. W., and Jobe, P. C. (1986). The genetically epilepsy-prone rat: an overview of seizure-prone characteristics and resposiveness to anticonvulsant drugs. *Life Sci.* 39, 763–774.

Riaz, A., and Faingold, C. L. (1994). Seizures during ethanol withdrawal are blocked by focal microinjection of excitant amino acid antagonists into the inferior colliculus and pontine reticular formation. *Alcohol. Clin. Exp. Res.* 18, 1456–1462. doi: 10.1111/j.1530-0277.1994.tb01450.x

Ribak, C. E. (2017). An abnormal GABAergic system in the inferior colliculus provides a basis for audiogenic seizures in genetically epilepsy-prone rats. *Epilepsy Behav.* 71, 160–164. doi: 10.1016/j.yebeh.2015.02.024

Ribak, C. E., Khurana, V., and Lien, N. T. (1994). The effect of midbrain collicular knife cuts on audiogenic seizure severity in the genetically epilepsy-prone rat. *J. Hirnforsch.* 35, 303–11.

Roberts, R. C., Ribak, C. E., and Oertel, W. H. (1985). Increased numbers of GABAergic neurons occur in the inferior colliculus of an audiogenic model of genetic epilepsy. *Brain Res.* 361, 324–338. doi: 10.1016/0006-8993(85)91303-4

Rocha, L. L., Cinar, R., Guevara-Guzmán, R., Alonso Vanegas, M. A., San Juan Orta, D., Martínez-Juárez, I. E., et al. (2020). Endocannabinoid system and cannabinoid 1 receptors in patients with pharmacoresistant temporal lobe epilepsy and comorbid mood disorders. *Front. Behav. Neurosci.* 14:52. doi: 10.3389/FNBEH.2020.00052

Romcy-Pereira, R. N., and Garcia-Cairasco, N. (2003). Hippocampal cell proliferation and epileptogenesis after audiogenic kindling are not accompanied by mossy fiber sprouting or Fluoro-Jade staining. *Neuroscience* 119, 533–546. doi: 10.1016/S0306-4522(03)00191-X

Rosenberg, E. C., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Ross, K. C., and Coleman, J. R. (2000). Developmental and genetic audiogenic seizure models: behavior and biological substrates. *Neurosci. Biobehav. Rev.* 24, 639–653. doi: 10.1016/S0149-7634(00)00029-4

Rossetti, F., Rodrigues, M. C. A., Oliveira, J. A. C., d, and Garcia-Cairasco, N. (2006). EEG wavelet analyses of the striatum-substantia nigra pars reticulata-superior colliculus circuitry: audiogenic seizures and anticonvulsant drug administration in Wistar audiogenic rats (War strain). *Epilepsy Res.* 72, 192–208. doi: 10.1016/j.eplepsyres.2006.08.001

Saint Marie, R. L. (1996). Glutamatergic connections of the auditory midbrain: selective uptake and axonal transport of D-[3H]aspartate. *J. Comp. Neurol.* 373, 255–270. doi: 10.1002/(SICI)1096-9861(19960916)373:2<255::AID-CNE8>3.0.CO;2-2

Samineni, V. K., Premkumar, L. S., and Faingold, C. L. (2011). Post-ictal analgesia in genetically epilepsy-prone rats is induced by audiogenic seizures and involves cannabinoid receptors in the periaqueductal gray. *Brain Res.* 1389, 177–182. doi: 10.1016/j.brainres.2011.03.041

Santos, V. R., Hammack, R., Wicker, E., and Gouemo, P. N. (2020). Divergent effects of systemic and intracollicular CB receptor activation against forebrain and hindbrain-evoked seizures in *Rats*. 14:595315. doi: 10.3389/fnbeh.2020.595315

Sañudo-Peña, M. C., Tsou, K., Romero, J., MacKie, K., and Walker, J. M. (2000). Role of the superior colliculus in the motor effects of cannabinoids and dopamine. *Brain Res.* 853, 207–214. doi: 10.1016/S0006-8993(99)02291-X

Sarkisova, K. Y., Fedotova, I. B., Surina, N. M., Nikolaev, G. M., Perepelkina, O. V., Kostina, Z. A., et al. (2017). Genetic background contributes to the co-morbidity of anxiety and depression with audiogenic seizure propensity and responses to fluoxetine treatment. *Epilepsy Behav.* 68, 95–102. doi: 10.1016/j.yebeh.2016.12.025

Sarkisova, K. Y., and Kulikov, M. A. (2006). Behavioral characteristics of WAG/Rij rats susceptible and non-susceptible to audiogenic seizures. *Behav. Brain Res.* 166, 9–18. doi: 10.1016/j.bbr.2005.07.024

Saunders, J. C., Bock, G. R., Chen, C., and Gates, R. (1972). The effects of priming behavioral for audiogenic responses seizures in BALB/c on mice cochlear. *Exp. Neurol.* 36, 426–436

Schober, A. (2004). Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res.* 318, 215–224. doi: 10.1007/s00441-004-0938-y

Sekar, K., and Pack, A. (2019). Epidiolex as adjunct therapy for treatment of refractory epilepsy: a comprehensive review with a focus on adverse effects. *F1000Research* 8, 1–8. doi: 10.12688/f1000research.16515.1

Simler, S., Vergnes, M., and Marescaux, C. (1999). Spatial and temporal relationships between C-Fos expression and kindling of audiogenic seizures in Wistar rats. *Exp. Neurol.* 157, 106–119.

Simonato, M., Brooks-Kayal, A. R., Engel, J., Galanopoulou, A. S., Jensen, F. E., Moshé, S. L., et al. (2014). The challenge and promise of anti-epileptic therapy development in animal models. *Lancet Neurol.* 13, 949–960. doi: 10.1016/S1474-4422(14)70076-6

Solius, G. M., Revishchin, A. V., Pavlova, G. V., and Poletaeva, I. I. (2016). Audiogenic epilepsy and GABAergic system of the colliculus inferior in Krushinsky-Molodkina rats. *Dokl. Biochem. Biophys.* 466, 32–34. doi: 10.1134/S1607672916010099

Soper, C., Wicker, E., Kulick, C. V., N'Gouemo, P., and Forcelli, P. A. (2016). Optogenetic activation of superior colliculus neurons suppresses seizures originating in diverse brain networks. *Neurobiol. Dis.* 87, 102–115. doi: 10.1016/j.nbd.2015.12.012

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., et al. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 215, 89–97. doi: 10.1006/bbrc.1995.2437

Terra, V. C., and Garcia-Cairasco, N. (1992). Neuroethological evaluation of audiogenic seizures and audiogenic-like seizures induced by microinjection of bicuculline into the inferior colliculus. II. Effects of nigral clobazam microinjections. *Behav. Brain Res.* 52, 19–28. doi: 10.1016/S0166-4328(05)80321-3

Terra, V. C., and Garcia-Cairasco, N. (1994). NMDA-dependent audiogenic seizures are differentially regulated by inferior colliculus subnuclei. *Behav. Brain Res.* 62, 29–39. doi: 10.1016/0166-4328(94)90035-3

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K., and Walker, J. M. (1998). Immunohistochemical distribuition of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83, 393–411. doi: 10.1016/S0306-4522(97)00436-3

Tsutsui, J., Terra, V. C., Oliveira, J. A. C., and Garcia-Cairasco, N. (1992). Neuroethological evaluation of audiogenic seizures and audiogenic- like seizures induced by microinjection of bicuculline into the inferior colliculus. I. Effects of midcollicular knife cuts. *Behav. Brain Res.* 52, 7–17.

Tupal, S., and Faingold, C. L. (2012). The amygdala to periaqueductal gray pathway: Plastic changes induced by audiogenic kindling and reversal by gabapentin. *Brain Res.* 1475, 71–79. doi: 10.1016/j.brainres.2012.07.044

Valdés-Baizabal, C., Parras, G. G., Ayala, Y. A., and Malmierca, M. S. (2017). Endocannabinoid modulation of stimulus-specific adaptation in inferior colliculus neurons of the rat. *Sci. Rep.* 7:6997. doi: 10.1038/s41598-017-07460-w

van Luijtelaar, E. L. J. M., and Coenen, A. M. L. (1986). Two types of electrocortical paroxysms in an inbred strain of rats. *Neurosci. Lett.* 70, 393–397. doi: 10.1016/0304-3940(86)90586-0

van Luijtelaar, G., and Sitnikova, E. (2006). Global and focal aspects of absence epilepsy: the contribution of genetic models. *Neurosci.*

Biobehav. Rev. 30, 983–1003. doi: 10.1016/j.neubiorev.2006.03.002

Van Rijn, C. M., Gaetani, S., Santolini, I., Badura, A., Gabova, A., Fu, J., et al. (2010). WAG/Rij rats show a reduced expression of CB1 receptors in thalamic nuclei and respond to the CB1 receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. *Epilepsia* 51, 1511–1521. doi: 10.1111/j.1528-1167.2009.02510.x

Varodayan, F. P., Soni, N., Bajo, M., Luu, G., Madamba, S. G., Schweitzer, P., et al. (2016). Chronic ethanol exposure decreases CB1 receptor function at GABAergic synapses in the rat central amygdala. *Addict. Biol.* 21, 788–801. doi: 10.1111/adb.12256

Vinogradova, L. V. (2008). Audiogenic kindling in Wistar and WAG/Rij rats: kindling-prone and kindling-resistant subpopulations. *Epilepsia* 49, 1665–1674. doi: 10.1111/j.1528-1167.2008.01617.x

Vinogradova, L. V. (2017). Audiogenic kindling and secondary subcortico-cortical epileptogenesis: behavioral correlates and electrographic features. *Epilepsy Behav.* 71, 142–153. doi: 10.1016/j.yebeh.2015.06.014

Vinogradova, L. V., Shatskova, A. B., and Van Rijn, C. M. (2011). Pro-epileptic effects of the cannabinoid receptor antagonist SR141716 in a model of audiogenic epilepsy. *Epilepsy Res.* 96, 250–256. doi: 10.1016/j.eplepsyres.2011.06.007

Vinogradova, L. V., and Van Rijn, C. M. (2015). Long-term disease-modifying effect of the endocannabinoid agonist WIN55,212-2 in a rat model of audiogenic epilepsy. *Pharmacol. Rep.* 67, 501–503. doi: 10.1016/j.pharep.2014.12.002

Wada, J. A., and Sato, M. (1975). Effects of unilateral lesion in the midbrain reticular formation on kindled amygdaloid convulsion in cats. *Epilepsia* 16, 693–697. doi: 10.1111/j.1528-1157.1975.tb04753.x

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and Delorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *Pharmacol. Exp. Ther.* 307, 129–137. doi: 10.1124/jpet.103.051920.vealed

Wallmichrath, I., and Szabo, B. (2002). Cannabinoids inhibit striatonigral GABAergic neurotransmission in the mouse. *Neuroscience* 113, 671–682. doi: 10.1016/S0306-4522(02)00109-4

Wicker, E., Beck, V. C., Kulick-Soper, C., Kulick-Soper, C. V., Hyder, S. K., Campos-Rodriguez, C., et al. (2019). Descending projections from the substantia nigra pars reticulata differentially control seizures. *Proc. Natl. Acad. Sci. U.S.A.* 116, 27084–27094. doi: 10.1073/pnas.1908176117

Willott, J. F., and Lu, S. M. (1980). Midbrain pathways of audiogenic seizures in DBA/2 mice. *Exp. Neurol.* 70, 288–299. doi: 10.1016/0014-4886(80)90028-X

Xiong, X. R., Liang, F., Zingg, B., Ji, X. Y., Ibrahim, L. A., Tao, H. W., et al. (2015). Auditory cortex controls sound-driven innate defense behaviour through corticofugal projections to inferior colliculus. *Nat. Commun.* 6:7224. doi: 10.1038/ncomms8224

Yang, L., Long, C., Randall, M. E., and Faingold, C. L. (2003). Neurons in the periaqueductal gray are critically involved in the neuronal network for audiogenic seizures during ethanol withdrawal. *Neuropharmacology* 44, 275–281. doi: 10.1016/S0028-3908(02)00367-2

Zuardi, A. W., Antunes Rodrigues, J., and Cunha, J. M. (1991). Effects of cannabidiol in animal models predictive of antipsychotic activity. *Psychopharmacology* 104, 260–264. doi: 10.1007/BF02244189

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 23 February 2021
doi: 10.3389/fnbeh.2021.604207

Functional Connectivity Derived From Electroencephalogram in Pharmacoresistant Epileptic Encephalopathy Using Cannabidiol as Adjunctive Antiepileptic Therapy

Lilia Maria Morales Chacón[1]*, *Lidice Galan García*[2], *Sheyla Berrillo Batista*[1], *Judith González González*[1] and *Abel Sánchez Coroneaux*[1]

[1]Department of Clinical Neurophysiology/Video EEG Unit, International Center for Neurological Restoration, Havana, Cuba, [2]Department of Neuroinformatics, Cuban Neuroscience Center, La Habana, Cuba

OPEN ACCESS

Edited by:
Dolores E. López García,
University of Salamanca, Spain

Reviewed by:
Sebastian Hormigo,
UCONN Health, United States
Julian Tejada,
Federal University of Sergipe, Brazil

***Correspondence:**
Lilia María Morales Chacón
lilia.morales@infomed.sld.cu;
lilychacon2003@yahoo.es

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: 08 September 2020
Accepted: 28 January 2021
Published: 23 February 2021

Citation:
Morales Chacón LM, Galan García L, Berrillo Batista S, González González J and Sánchez Coroneaux A (2021) Functional Connectivity Derived From Electroencephalogram in Pharmacoresistant Epileptic Encephalopathy Using Cannabidiol as Adjunctive Antiepileptic Therapy. Front. Behav. Neurosci. 15:604207. doi: 10.3389/fnbeh.2021.604207

To explore brain function using functional connectivity and network topology derived from electroencephalogram (EEG) in patients with pharmacoresistant epileptic encephalopathy with cannabidiol as adjunctive antiepileptic treatment. Sixteen epileptic patients participated in the study, six of whom had epileptic encephalopathy with a stable dose of cannabidiol Epidiolex (CBD) as adjunctive therapy. Functional connectivity derived from EEG was analyzed based on the synchronization likelihood (SL). The analysis also included reconstructing graph-theoretic measures from the synchronization matrix. Comparison of functional connectivity data between each pathological group with the control group was carried out using a nonparametric permutation test applied to SL values between pairs of electrodes for each frequency band. To compare the association patterns between graph-theoretical properties of each pathological group with the control group, Z Crawford was calculated as a measure of distance. There were differences between pairs of electrodes in all frequency bands evaluated in encephalopathy epileptic patients with CBD adjunctive therapy compared with the control ($p < 0.05$, permutation test). In the epileptic encephalopathy group without CBD therapy, the SL values were higher than in the control group for the beta, theta, and delta EEG frequency bands, and lower for the alpha frequency band. Interestingly, patients who had CBD as adjunctive therapy demonstrated greater synchronization for all frequency bands, showing less spatial distribution for alpha frequency compared with the control. When comparing both epileptic groups, those patients who had adjunctive CBD treatment also showed increased synchronization for all frequency bands. In epileptic encephalopathy with adjunctive CBD therapy, the pattern of differences for graph-theoretical measures according to Z Crawford indicated less segregation and greater integration suggesting a trend towards the random organization of the network principally for alpha and beta EEG bands. This exploratory study revealed a tendency to an overconnectivity with a random network topology mainly for fast EEG bands in epileptic encephalopathy patients using

CBD adjunctive therapy. It can therefore be assumed that the CBD treatment could be related to inhibition of the transition of the interictal to ictal state and/or to the improvement of EEG organization and brain function.

Keywords: epileptic encephalopathy, cannabidiol, functional connectivity, electroencephalogram, graph theory

INTRODUCTION

There is little doubt about the therapeutic results of compounds derived from the cannabis Sativa plant in different medical conditions including epilepsy. Respecting pharmacoresistant epilepsies, arguments are based, among others, upon data about the importance of the endocannabinoid system in the onset and generation of seizures (Ludányi et al., 2008; Romigi et al., 2010; Goffin et al., 2011; Vaessen et al., 2012) together with multiple case reports indicating the anti-crisis effect of cannabis in patients with epilepsy (Consroe et al., 1975; Gordon and Devinsky, 2001; Mortati et al., 2007). Documentation from observational studies and randomized double-blind placebo-controlled trials (RCT) over the last decade point to the benefit in the treatment of seizures focusing on two epileptic encephalopathy conditions such as Dravet syndrome and Lennox-Gastaut Syndrome (LGS; Devinsky et al., 2016, 2017, 2018; Hausman-Kedem et al., 2018; Lattanzi et al., 2018, 2019).

Although studies suggest an overall positive effect on seizure control, little is known regarding the impact of cannabidiol (CBD) on electroencephalogram (EEG). Less proof exists to support the positive effects of cannabis on interictal epileptic activity in EEG (Hegde et al., 2012; McCoy et al., 2018) as the latest published research has not discussed in much detail EEG in patients using CBD.

On the other hand, the impact of cannabis on the brain of people with epilepsy using structural and functional neuroimaging have been poorly addressed, and have shown different patterns of alterations in structure and brain function at rest or while performing a cognitive task (Allendorfer and Szaflarski, 2017; Allendorfer et al., 2019). Some research findings have shown that interictal functional networks in epilepsy subjects may be characterized by increased connectivity (Douw et al., 2010a; Horstmann et al., 2010; Bartolomei et al., 2013, 2017; Clemens et al., 2013). Though the literature on interictal network topology using graph theory of epileptic patients is inconclusive, there is increasing empirical evidence for the hypothesis that changes in brain network topology might play a crucial role in epilepsy. There are also studies indicating that functional brain networks in epileptic patients differ from those of healthy subjects during the interictal stage. Furthermore, EEG networks analysis can be used to monitor the success of both pharmacological and non-pharmacological treatment (Clemens et al., 2014; Haneef and Chen, 2014) in pharmacoresistant epilepsy patients (Fraschini et al., 2014; Liang et al., 2018).

Given the growing interest in the use of CBD for pediatric pharmacoresistant epilepsy populations, it is therefore important to evaluate brain function in these patients. Considering that the EEG is an accurate cost-effective measure of both epileptic activity and of brain function, this article aims to explore functional connectivity and network topology derived from EEG in patients with pharmacoresistant epileptic encephalopathy using cannabidiol as adjunctive antiepileptic treatment.

MATERIALS AND METHODS

A total of 16 epilepsy patients participated in the study. Ten with epileptic encephalopathy, and six with epileptic encephalopathy using a stable dose of 25–50 mg/kg/day of cannabidiol Epidiolex as adjunctive therapy for 6–12 months. All patients had confirmed pharmacoresistant epilepsy failing at least three different anti-epileptic drugs (AEDs), along with an average of a minimum of four countable seizures per month. Age, gender, seizure duration, and number of AEDs were similar between both epileptic groups. The most frequent AEDs received in both groups were valproate and levetiracetam followed by clobazam. The use of clobazam was 50% in epileptic patients using CBD and 40% in non-used CBD $p = 0.70$ (the difference between two proportions). A healthy control group matched for age and gender was also studied, as shown in **Table 1**.

Subjects were evaluated consecutively in the Video-EEG telemetry Unit of the International Center for Neurological Restoration, Havana, Cuba during 2018–2019. Diagnosis and classification of seizures and epilepsy were made using the 2017 ILAE Seizure Classification (Scheffer et al., 2017). Furthermore, a detailed electroclinical, neuroimmunological, as well as neuropsychological evaluation, was performed in all patients.

EEG Acquisition and Analyses

Scalp EEG signals were recorded using the 10-20 international standard montage with a sampling frequency of 200 Hz. Additional extracranial electrodes were also utilized to gather EEG records from patients and controls. The reference electrode was positioned at FCz (an electrode placed between Fpz and Cz) and the ground electrode was located in Fpz.

EEG data acquisition was performed with a Medicaid-5 digital EEG system (Neuronic SA, Cuba), with 32 channels, a 256 Hz sampling rate, and a 16-bit analog-to-digital converter. Electrode Impedance was kept below 5 kΩ, and filters were set at 0.5 and 30 Hz with a 60 Hz notch filter. Hence, successive analyses were carried out only for delta, theta, alpha, and beta frequency bands.

During the eyes-closed resting state, EEG was recorded for at least 30 min. In the current study, a minimum 12 h-seizure-free period was assured. Also, special attention was given to keep the subjects awake with their eyes closed while recording. EEG segments with artifacts and drowsiness were excluded from the analyses in all evaluated groups. Further, EEG

TABLE 1 | Demographic and clinical data.

Variables	Epileptic encephalopathy with CBD *N* = 6	Epileptic encephalopathy without CBD *N* = 10	Control healthy group	*p*-value
Age*	13.6 ± 7.1 (5–23)	10.6 ± 3.2 (7–16)	15.37 ± 5.20 (9–24)	$p = 0.32$* $\chi^2_{(2)} = 2.26$
Gender	*F* = 2 *M* = 4	*F* = 2 *M* = 8	*F* = 5 *M* = 5	
Concomitant AED*	3.1 ± 1.4 (2–5) 3 using Clobazan	2.7 ± 0.6 (2–4) 4 using Clobazan		$p = 0.88$** $U = 31$ Z adjusted = 0.21
Age at seizure onset*	6.4 ± 4.2 (4 month−11 years)	4.2 ± 3.1 (1 day–11 years)		$p = 0.33$** $U = 13.5$ Z adjusted = 0.95
Epilepsy duration*	6.4 ± 4.6 (2–15 years)	6.6 ± 2.6 (2–11)		$p = 0.43$** $U = 18$ Z adjusted = −0.78
Seizure frequency/month	15 Sz/day to 100 Sz/ day	8 Sz/day to 50 Sz/day		
Epilepsy type	LGS: 3 RE:1 HIE: 2	LGS: 3 RE:1 HIE: 2		
Epilepsy Etiology	*S* = 5 *U* = 1	*S* = 4 *I* = 3 *U* = 3		

*(mean ± SD) and range. AED, antiepileptic drugs, F, female; M, male; Sz, seizures; S, structural; U, unknown; I, infection; LGS, Lennox–Gastaut syndrome; RE, Rassmussen encephalitis; IHE, isquemic hipoxic encephalopathy; *Chi square χ^2, **Mann-whitney U* (mean ± SD) and range.

interpretation and analyses were done by two board-certified electroencephalographers (LM and SB).

Functional Brain Connectivity Based on EEG

Functional connectivity was evaluated based on the synchronization likelihood (SL). Considering two EEG signals from different electrodes scalp locations as two simultaneously recorded time series E1 and E2, SL is defined as the conditional likelihood that E2 is in the same state at two different time points *i* and *j* given that E1 is in the same state at the same two times. Therefore SL denotes how well E1 and E2 are synchronized (coupled). SL values range between 1 (full synchronization) and values near 0 (low coupling or desynchronization. The values of the parameters used to estimate the SL were $l_{(\mathrm{lag})} = 10$, $m = 2$ (embedding dimension), w1 = $L * m = 20$ (Theiler correction for autocorrelation effects and should be at least of the order of the autocorrelation time; Theiler, 1986), w2 = Nt − w1 − 50 = 512 − 20 − 50 = 442 (is a window that sharpens the time resolution of the synchronization measure, and Nt = 512 time points or samples (maximum number of recurrences), Pref = 0.05 (preference probability). A full technical explanation of this measure and its features are presented in Stam et al. (2002).

In-house scripts developed at the Cuban Center for Neuroscience according to Stam et al. (2002) were used to compute the SL measure. SL computation was preceded by EEG raw signal filtering at different frequency bands [delta, δ (1–3.9 Hz), theta, θ (4–7.9 Hz) alpha, α (8–12.9 Hz), beta, β (13–29.9 Hz)].

For each subject, an SL functional matrix (C) of Ne × Ne (19 × 19)—where Ne is the electrode number—was obtained for each frequency-band and all time-windows. The resulting SL matrices were averaged across all epochs in each participant. Each element C*ij* of C matrix is the functional synchronization between EEG sensors "*i*" and "*j*" at a specific frequency band. The matrix C is symmetric, meaning that C*ij* = C*ji*, self-connections C*ii* were excluded, implying zeros in the diagonal of this symmetric matrix. Thus, we have Ne ∗ (Ne − 1)/2 = 19 ∗ 18/2 = 171 different functional SL values in C.

The analysis included:

- Reconstructing graph-theoretic measures.
- Calculating clustering coefficient.
- Calculating efficient measures (path length, global Efficiency).

Graph-Theoretic Measures

Graph theoretical properties were reconstructed from the synchronization matrix and characterized by clustering coefficient (local connectedness measure), and the shortest path length (overall network integration measure). Likewise, global and local efficiency, as well as global connectivity, were evaluated.

The representation of the statistical association between distinct nodes was calculated using the following measures:

Clustering Coefficient

A cluster in a graph has come to be used to refer to a highly interconnected group of nodes. The fraction of existing edges between nodes adjacent to node *i*, over the maximum potential number of such edges, is known as the clustering coefficient C*i* of a node *i* (Watts and Strogatz, 1998). Thus, the clustering coefficient of the network C refers to the mean clustering coefficient among all network nodes. Further, a weighted clustering coefficient was used (Onnela et al., 2005) to measure network functional segregation.

Path Length

Path length can be described as the average shortest path length over all pairs of nodes in the network and is a measure of how efficient the information flow through the network is (Christodoulakis et al., 2014). The characteristic path length is well-delineated exclusively for connected pairs of nodes. To

overcome this constraint, efficiency between a pair of nodes was defined as the inverse of the shortest distance between the nodes (Latora and Marchiori, 2001). The characteristic path length was also used to measure network functional integration.

The average shortest path length between all pairs of nodes in the network is defined as the characteristic path length of the network and is the most frequently used measure of functional integration (Watts and Strogatz, 1998).

Global Efficiency

Global efficiency is generally understood to mean a measure of functional integration and is the inverse of the mean shortest path length between each pair of nodes. It can be defined as the average efficiency over all pairs of nodes. Global and local efficiency can mirror the level of global and local information transfer of a graph, and are directly and effectively utilized to gauge the performance of a network (Latora and Marchiori, 2001).

Statistical Analysis

A thorough comparison of functional connectivity data between each pathological group with the control group was carried out using a nonparametric permutation test.

In our study, we applied nonparametric permutation to SL values between pairs of electrodes for each frequency band. As the variables did not distribute normally (Shapiro–Wilk test, $p < 0.05$), the mean SL parameter between groups was compared using a nonparametric permutation test. The permutation test has several attractive features: (a) tests are distribution free controlling the experiment wise error for the simultaneous univariate comparisons; (b) no assumptions of an underlying correlation structure are needed; and (c) they offer exact *p*-values for any number of subjects, frequency bands and recording sites as long as the number of permutations is adequately high.

With the purpose of finding dissimilarities between groups in functional connectivity, we considered the SL parameter in which *p* pairs of electrodes and f frequency bands from SL functional connectivity matrix C were obtained for each subject.

Permutation methodology was based on the phases below:

The statistical difference between the two SL distributions was tested using permutation techniques. To make a choice for the global and marginal hypothesis, permutation *t* statistic corresponding to univariate hypothesis were combined. The *t* statistics combined were obtained from: (a) the maximum value of all the univariate statistics (max *t*); (b) maximum over the set of frequency bands for each pair of electrodes (max t_f); (c) maximum over the set of pairs of electrodes at a particular frequency band (max t_s).

In all cases, the use of *t*-max statistic distribution for each marginal hypothesis allowed precise significance thresholds corrected for the multiple comparisons along the dimension in which the maximum was taken. The procedure consists of the following stages:

Stage 1 Random permutation of the observations (SL) between the groups for 10,000 times. In each repetition, max *t*, max t_f, and max t_s were calculated.

Stage 2 Estimation of the empirical null distribution for the statistics calculated in the preceding stage.

Stage 3 Calculation of the *p*-value for the *t*-max statistics of the empirical null distribution.

Stage 4 Rejection of the null hypothesis (controlling the Type I error) for those *t*-max statistics of the original sample above the significance thresholds (i.e., *p*-value <0.05; Galán et al., 1997; Herrera-Díaz et al., 2016).

The effect size was computed using the Cohen' *d* standardized measure (Cohen, 1988). The maximum effect size was calculated for an average over all channels.

According to Sawilowsky as a rule of thumb, this can be interpreted as follows: 0.5 as a "medium" effect, 0.8 as "large", and 1.2 as "very large" (Sawilowsky, 2009).

Consequently, important connectivity ($p < 0.05$) in each frequency band was denoted on an *X/Y* coordinate based on the 10/20 system for each group individually. Further, results per group were represented over the scalp.

To compare the association patterns between graph-theoretical properties of each pathological group with the control group, *z* Crawford was calculated as a measure of distance.

The *z*-score that allows treating of the control sample statistics as sample statistics was described by Crawford and Howell (1998). The formula used by Crawford and Howell's approach for a modified *t*-test is:

$$t = \frac{X^* - \underline{X}}{S\sqrt{\frac{n+1}{n}}} \quad (1)$$

where X^* is the patient's score, $\underline{X}$ and *S* represent the mean and standard deviation of scores in the control sample, and n specifies the control sample size. The *p*-value obtained when the test is used tests significance, but it also indicates a point estimation of the abnormality of the patient's score. Crawford and Howell's procedure tests the null theory that a single patient does not belong to a control population. Also, Bonferroni's correction was used to control the Family-wise Error Rate (FWER), which is the probability of having at least one false positive among the whole set of hypotheses considered as an alternative at the desired level α. In this work α =0.05 was used (Bonferroni's correction = 0.0025).

RESULTS

EEG Functional Connectivity

Functional connectivity based on the SL results indicated important differences ($p < 0.05$, permutation test), between pairs of electrodes in both groups of epileptic patients compared to the control. **Figure 1** presents pairs of electrodes with significant SL values ($p < 0.05$) and effect sizes between groups in beta, alpha, theta, and delta bands. Differences were distributed over the whole scalp showing strong and meaningful electrode SL couplings across all distances. It can be observed from **Figure 1A**, that SL was higher in patients with epileptic encephalopathy without CBD than in the control group for beta, theta, and delta EEG frequency bands. However, the SL for the alpha frequency band was lower in relation to the control group. Significant differences $p < 0.05$, with medium and large effect sizes, were seen for beta (Cohen' $d = 0.5820$) and alpha (Cohen'

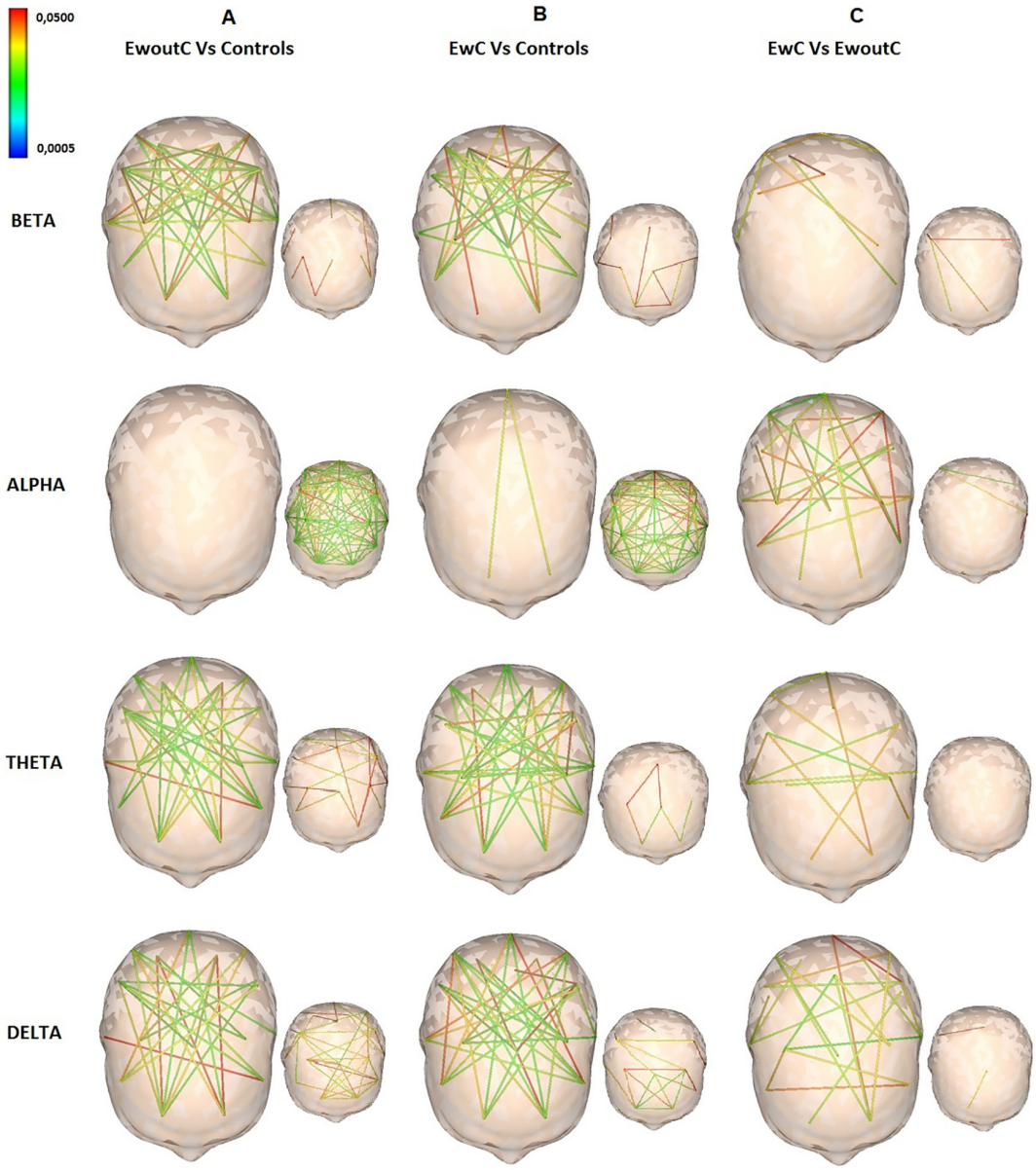


FIGURE 1 | Illustrates pairs of electrodes with significant differences in synchronization likelihood (SL) values between patients and controls in beta, alpha, theta, and delta bands (patients > controls). The colored bar indicates the range of p values, in red representing $0.022 < p < 0.05$, yellow $0.011 < p < 0.22$, and blue $p < 0.001$. Differences were distributed over the entire scalp showing strong and significant electrodes SL couplings across all distances. Panel **(A)** shows an increase of SL in patients with epileptic encephalopathy without cannabidiol Epidiolex (CBD) adjunctive therapy for the beta, theta, and delta frequencies; and a decrease for alpha frequency compared with the control group. Panel **(B)** presents an increase of SL in patients with epileptic encephalopathy with CBD adjunctive therapy compared with the control group for the beta, alpha, theta, and delta frequencies showing less spatial distribution for alpha frequency. Panel **(C)** indicates increased synchronization for all frequency bands in patients with epileptic encephalopathy with adjunctive CBD when comparing both patient groups.

$d = 1.0388$) EEG bands respectively. The effect sizes were small for slow-wave bands (for delta band Cohen' $d = 0.3975$ and theta band Cohen' $d = 0.3270$). As can be seen in **Figure 1B**, patients who had epileptic encephalopathy with CBD adjunctive therapy presented an increase of SL compared with the control group for the beta, alpha, theta, and delta frequencies, showing less spatial distribution for alpha frequency. Significant differences $p < 0.05$, with medium and large effect sizes, were also seen for beta (Cohen' $d = 0.4653$) and alpha (Cohen' $d = 0.9524$) bands, respectively. As shown in **Figure 1C**, when comparing both patient groups, subjects with epileptic encephalopathy with adjunctive CBD showed increased synchronization for slow and fast frequency bands. The effect sizes were small for all EEG except for the delta band (Cohen' $d = 0.4575$, which

was a medium effect. The effect sizes were 0.3148, 0.2319, and 0.2157 for theta, alpha, and beta bands, respectively.

Graph Theory Based on EEG in Epileptic Encephalopathies With or Without Adjunctive Cannabidiol Therapy

In patients diagnosed with epileptic encephalopathy without adjunctive CBD treatment, differences with the control group were observed for deviations from graph theoretical measures according to *Z* Crawford in the alpha, beta, and delta frequency bands. As shown in **Table 2**, the difference direction represented in the mean of the *t* statistics indicated lower clustering coefficient and local efficiency, whereas the global efficiency was greater compared with the control group.

CBD group showed changes only in EEG beta and alpha frequency bands. The pattern of differences was characterized by lower clustering coefficient and local efficiency, as well as by an increase in global efficiency in patients with epileptic encephalopathy with adjunctive CBD therapy compared with the control group, which indicated less segregation and greater integration suggesting a trend towards the random organization of the network.

Most notably, subjects with adjunct CBD therapy showed less segregation and greater integration restricted to fast EEG frequencies (alpha and beta). However, patients with epileptic encephalopathy without CBD showed the same behavior concerning segregation and integration for both slow and fast frequency bands.

Table 2 provides the *t*-statistics to test differences in population means between graph theory measures of the epileptic patient samples and control sample based on the *z* individual value for each subject (Crawford's approach). Respectively, the *p*-values indicate the probability for the differences between patients and controls; thus, providing a point estimate of the abnormality of the patient's difference. Note that the variables with significant differences exhibited considerable deviations just in alpha and beta estimations of connectivity parameters in the CBD group. The non-CBD group also revealed anomalies in delta and theta parameters.

DISCUSSION

This study indicated functional brain network differences between epileptic encephalopathy using CBD adjunctive therapy and healthy control. In the epileptic encephalopathy group without CBD therapy, the difference direction revealed an increase in the synchronization for beta and theta frequency bands and a decrease for alpha frequency. However, patients with epileptic encephalopathy who used adjunctive CBD therapy showed greater synchronization during the interictal state for all EEG frequency bands than the control group, including alpha EEG band but with the lesser spatial distribution. The most consistent results were obtained for fast EEG frequency bands. A trend towards the random organization of the network (less segregation and greater integration) was also observed principally for fast EEG bands in the CBD epilepsy group.

There has been renewed academic interest in CBD therapy, in particular concerning the modulation of cortical excitability through the human endocannabinoid system. Several authors have revealed that increased connectivity is generally seen as a feature strongly related to interictal functional networks in epilepsy patients (Bartolomei et al., 2006, 2013; Bettus et al., 2008; Bosma et al., 2009; Douw et al., 2010a,b; Horstmann et al., 2010; van Dellen et al., 2012; Clemens et al., 2013).

Even though research on generalized epilepsy, explicitly, epileptic encephalopathy is very limited in the literature, our results are in agreement with other findings in genetic generalized epilepsy, which have shown a general increase in connectivity manifested in all aspects of network analyses (Ponten et al., 2009; Niso et al., 2015; Davis et al., 2019). In this study, an overconnectivity for all bands, except for the alpha band was also detected in patients with epileptic encephalopathy. Interestingly, those who used CBD therapy showed higher connectivity values for all bands including alpha in relation to those who were not given this therapy. This result could indicate a propensity for a better organization of brain electrical activity in these patients with CBD.

According to the literature reviewed so far, no studies exist on graph theory in epileptic patients using CBD as adjunctive therapy. More recently, graph theory research has been progressively used to analyze brain networks in different structural and functional modalities (Chiang and Haneef, 2014; Pedersen et al., 2015). It is important to notice that functional and structural connectivity clarifies not only that but also the extent to which different brain zones are connected whereas network analysis using graph theory provides a framework to characterize the topological organization of functional and structural networks.

The most common parameters utilized in neuronal network analyses using graph theory are the clustering coefficient and the characteristic path length. In this study, five global metrics were used to find a pattern with decreased clustering and increased global efficiency. The clustering coefficient allowed to define the local segregation property of the network, and it was used to assess the network capability to share specialized data while the path length and global efficiency were used to evaluate the capacity of the network as a whole for inner-exchange information. A short path length, a low clustering, and a high global efficiency/local efficiency generally represent a small-world topology of the network and characterizes an optimal organization for communication efficiency (Watts and Strogatz, 1998; Rubinov et al., 2009).

In the present study, the graph metric analysis indicated a lower clustering coefficient in patients with epileptic encephalopathy observed for the alpha, beta, and delta frequency bands compared with the control group while in epileptic encephalopathy patients with CBD treatment this organizational pattern was only observed in the EEG fast frequency bands (alpha and beta).

This study produced results that are partially in line with those of other studies in people diagnosed with generalized epilepsy (Ponten et al., 2009; Niso et al., 2015; Davis et al., 2019). In Davis PE, study graphs metric analysis indicates that

TABLE 2 | Statistical differences (*t*-test) between graph theory measures of encephalopathy epileptic patients and healthy control sample based on the *z* individual value for each subject (Crawford's approach).

Variables	Epileptic encephalopathy CBD group			Epileptic encephalopathy non-CBD group		
	Mean *t*-values	Statistics *t* (Ho: $\mu = 0$; $df = 4$)	*p*-values	Mean *t*-values	Statistics *t* (Ho: $\mu = 0$; $df = 9$)	*p*-values
Zcrawf-alpha clustering coefficient	4.00666	39.2971	0.000003	3.89590	8.6396	0.000006
Zcrawf-alpha path length	−1.12285	−4.9992	0.007494	−0.77795	−2.1810	0.054155
Zcrawf-alpha local efficiency	2.37207	22.1325	0.000025	2.47784	9.9902	0.000002
Zcrawf-alpha global efficiency	−2.59997	−16.0846	0.000087	−2.18080	−4.9629	0.000568
Zcrawf-alpha global connectivity	2.10682	22.6609	0.000022	1.72589	3.5383	0.005372
Zcrawf-beta clustering coefficient	1.08159	7.1742	0.001999	1.12795	9.6618	0.000002
Zcrawf-beta path length	0.13658	0.3684	0.731220	−0.63661	−5.0179	0.000523
Zcrawf-beta local efficiency	1.01745	6.9961	0.002197	0.98809	7.6680	0.000017
Zcrawf-beta global efficiency	−0.92687	−3.7821	0.019401	−1.29772	−24.4297	0.000000
Zcrawf-beta global connectivity	0.80529	3.1561	0.034313	0.90388	8.4076	0.000008
Zcrawf-theta clustering coefficient	0.39281	1.2676	0.273723	0.60178	3.2018	0.009463
Zcrawf-theta path length	1.20714	1.3802	0.239653	0.61625	1.4476	0.178346
Zcrawf-theta local efficiency	0.35845	1.0206	0.365166	0.53129	2.9284	0.015082
Zcrawf-theta global efficiency	0.00361	0.0092	0.993087	−0.33069	−1.1628	0.271918
Zcrawf-theta global connectivy	0.10068	0.3605	0.736678	0.45641	2.0628	0.066078
Zcrawf-delta clustering coefficient	0.82996	2.4170	0.072998	1.32238	15.6306	0.000000
Zcrawf-delta path length	0.76263	0.9942	0.376411	−0.94920	−3.3923	0.006859
Zcrawf-delta local efficiency	0.92826	2.5494	0.063346	1.33682	14.2008	0.000000
Zcrawf-delta global efficiency	−0.25476	−0.5400	0.617864	−1.17919	−10.1736	0.000001
Zcrawf-delta global connectivity	−0.39847	−1.0362	0.358629	0.47550	4.3368	0.001474

children who developed epileptic spasms presented increased local and long-range EEG connectivity with less segregation of graph regions into distinct modules (Davis et al., 2019). Other authors have demonstrated, using surface EEG and MEG during generalized absence seizures, a potential association between a more regular network topology and seizure generation (Ponten et al., 2009; Gupta et al., 2011). A significant finding to emerge from this study is that the random network topology, principally for fast EEG frequency in patients using CBD, may represent an inhibition mechanism for the conversion of the interictal state to seizure.

Although there has been little agreement on whether traditional frequency bands are certainly fixed entities, each frequency band has been associated with different cognitive functions (Younus et al., 2018). Interpreting frequency-dependent network changes in epilepsy provides valuable information. Consequently, it has been argued that higher (10–13 Hz) and lower (8–10 Hz) alpha frequency band are involved in different cerebral processes (Klimesch, 1999; Uhlhaas and Singer, 2006). In this study, both SL and graphs metric analysis indicated that network analyses for all EEG frequency bands in epileptic encephalopathy patients with CBD were different compared with the control and with epileptic patients without CBD.

EEG connectivity in both epileptic groups showed greater synchronization and a trend towards the random organization of the network in contrasting ways with a dependent frequency pattern indicating the foremost variations for the high-frequency bands in patients using CBD. The similarity in the behavior of the epileptic groups accords with earlier observations in generalized epilepsy. The connectivity pattern observed in this study could be associated with the slowing of EEG activity in epileptic encephalopathy which is often related to brain dysfunction as well as to the side effects and neurotoxicity of many AED (Ponten et al., 2009; Niso et al., 2015; Davis et al., 2019) whereas the results in SL and graphs metric analysis, specifically for fast EEG band in epileptic patients treated with CBD could allude to an improvement in both EEG activity and connectivity pattern.

Some studies have suggested that low and high-frequency bands reflect different aspects of information processing. Large-scale integration is primarily processed in the lower frequencies while local activity evolves in higher frequency bands (von Stein and Sarnthein, 2000; Koenig et al., 2005).

The findings observed in this study in encephalopathy patients with CBD mirror those of Niso's study in generalized epilepsy subjects in which epileptic encephalopathy patients with CBD adjunctive therapy showed greater efficiency and lower eccentricity than the control group for the high-frequency bands using magnetoencephalographic evaluation (Niso et al., 2015).

There is also evidence that augmentation in regularity has a distinguishing frequency pattern depending on the type of epilepsy, which can move from high-frequency bands in cases of generalized epilepsy to the low-frequency theta band in focal epilepsy patients (Chavez et al., 2010; Horstmann et al., 2010; Bartolomei et al., 2013).

However, more research needs to be undertaken to better recognize what the results obtained in different frequency bands mean. It can therefore be assumed that the CBD treatment could be related to inhibition of the transition of the interictal to ictal state and/or to the improvement of EEG organization and brain function.

Due to the lack of publications describing anti-epileptic drug effects, findings of graph-theoretic analyses in epilepsy have been insufficient (Horstmann et al., 2010; Chiang and Haneef, 2014).

Graph theory has the added advantage of being particularly sensitive to variations in brain network structure (Bullmore and Bassett, 2011). Equally, it could provide a valuable methodology for examining the influence of antiepileptic drugs and medical cannabis on brain networks.

Although the current study is based on a small sample of participants, the findings open a window for the potential use of EEG network analytical approach to be used in clinical practice to assess the effect of CBD as adjunctive therapy to treat pharmacoresistant epileptic encephalopathies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by International Center for Neurological Restoration. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LM designed the study and organized the manuscript. LG participated in the statistical analysis of the results and organization of the manuscript. SB carried out the EEG connectivity analysis. JG identified and evaluated the patients with epilepsy. AS participated in the patients' evaluation. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the International Center for Neurological Restoration.

ACKNOWLEDGMENTS

We would like to thank all the telemetry unit nurses from the International Center for Neurological Restoration in Havana, Cuba for their collaboration and support. We wish to thank Odalys Morales Chacón for revising the English in this manuscript.

REFERENCES

Allendorfer, J. B., and Szaflarski, J. P. (2017). Neuroimaging studies towards understanding the central effects of pharmacological cannabis products on patients with epilepsy. *Epilepsy Behav.* 70, 349–354. doi: 10.1016/j.yebeh.2016.11.020

Allendorfer, J. B., Nenert, R., Bebin, E. M., Gaston, T. E., Grayson, L. E., Hernando, K. A., et al. (2019). fMRI study of cannabidiol-induced changes in attention control in treatment-resistant epilepsy. *Epilepsy Behav.* 96, 114–121. doi: 10.1016/j.yebeh.2019.04.008

Bartolomei, F., Bettus, G., Stam, C. J., and Guye, M. (2013). Interictal network properties in mesial temporal lobe epilepsy: a graph theoretical study from intracerebral recordings. *Clin. Neurophysiol.* 124, 2345–2353. doi: 10.1016/j.clinph.2013.06.003

Bartolomei, F., Bosma, I., Klein, M., Baayen, J. C., Reijneveld, J. C., Postma, T. J., et al. (2006). Disturbed functional connectivity in brain tumour patients: evaluation by graph analysis of synchronization matrices. *Clin. Neurophysiol.* 117, 2039–2049. doi: 10.1016/j.clinph.2006.05.018

Bartolomei, F., Lagarde, S., Wendling, F., McGonigal, A., Jirsa, V., Guye, M., et al. (2017). Defining epileptogenic networks: contribution of SEEG and signal analysis. *Epilepsia* 58, 1131–1147. doi: 10.1111/epi.13791

Bettus, G., Wendling, F., Guye, M., Valton, L., Régis, J., Chauvel, P., et al. (2008). Enhanced EEG functional connectivity in mesial temporal lobe epilepsy. *Epilepsy Res.* 81, 58–68. doi: 10.1016/j.eplepsyres.2008.04.020

Bosma, I., Reijneveld, J. C., Klein, M., Douw, L., van Dijk, B. W., Heimans, J. J., et al. (2009). Disturbed functional brain networks and neurocognitive function in low-grade glioma patients: a graph theoretical analysis of resting-state MEG. *Nonlinear. Biomed. Phys.* 3:9. doi: 10.1186/1753-4631-3-9

Bullmore, E. T., and Bassett, D. S. (2011). Brain graphs: graphical models of the human brain connectome. *Annu. Rev. Clin. Psychol.* 7, 113–140. doi: 10.1146/annurev-clinpsy-040510-143934

Chavez, M., Valencia, M., Navarro, V., Latora, V., and Martinerie, J. (2010). Functional modularity of background activities in normal and epileptic brain networks. *Phys. Rev. Lett.* 104:118701. doi: 10.1103/PhysRevLett.104.118701

Chiang, S., and Haneef, Z. (2014). Graph theory findings in the pathophysiology of temporal lobe epilepsy. *Clin. Neurophysiol.* 125, 1295–1305. doi: 10.1016/j.clinph.2014.04.004

Christodoulakis, M., Hadjipapas, A., Papathanasiou, E. S., Anastasiadou, M., Papacostas, S. S., and Mitsis, G. D. (2014). Periodicity in functional brain networks: application to scalp EEG from epilepsy patients. *Annu. Int. Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2014, 2805–2808. doi: 10.1109/EMBC.2014.6944206

Clemens, B., Puskás, S., Besenyei, M., Kovács, N. Z., Spisák, T., Kis, S. A., et al. (2014). Valproate treatment normalizes EEG functional connectivity in successfully treated idiopathic generalized epilepsy patients. *Epilepsy Res.* 108, 1896–1903. doi: 10.1016/j.eplepsyres.2014.09.032

Clemens, B., Puskás, S., Besenyei, M., Spisák, T., Opposits, G., Hollódy, K., et al. (2013). Neurophysiology of juvenile myoclonic epilepsy: EEG-based network and graph analysis of the interictal and immediate preictal states. *Epilepsy Res.* 106, 357–369. doi: 10.1016/j.eplepsyres.2013.06.017

Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*, second edition. Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers.

Consroe, P. F., Wood, G. C., and Buchsbaum, H. (1975). Anticonvulsant nature of marihuana smoking. *JAMA* 234, 306–307. doi: 10.1001/jama.1975.03260160054015

Crawford, J. R., and Howell, D. C. (1998). Regression equations in clinical neuropsychology: an evaluation of statistical methods for comparing predicted and obtained scores. *J. Clin. Exp. Neuropsychol.* 20, 755–762. doi: 10.1076/jcen.20.5.755.1132

Davis, P. E., Kapur, K., Filip-Dhima, R., Trowbridge, S. K., Little, E., Wilson, A., et al. (2019). Increased electroencephalography connectivity precedes epileptic spasm onset in infants with tuberous sclerosis complex. *Epilepsia* 60, 1721–1732. doi: 10.1111/epi.16284

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al. (2017). Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N. Engl. J. Med.* 376, 2011–2020. doi: 10.1056/NEJMoa1611618

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurol.* 15, 270–278. doi: 10.1016/S1474-4422(15)00379-8

Devinsky, O., Patel, A. D., Thiele, E. A., Wong, M. H., Appleton, R., Harden, C. L., et al. (2018). Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome. *Neurology* 90, e1204–e1211. doi: 10.1212/WNL.0000000000005254

Douw, L., de Groot, M., van Dellen, E., Heimans, J. J., Ronner, H. E., Stam, C. J., et al. (2010a). 'Functional connectivity' is a sensitive predictor of epilepsy diagnosis after the first seizure. *PLoS One* 5:e10839. doi: 10.1371/journal.pone.0010839

Douw, L., van Dellen, E., Baayen, J. C., Klein, M., Velis, D. N., Alpherts, W. C., et al. (2010b). The lesioned brain: still a small-world? *Front. Hum. Neurosci.* 4:174. doi: 10.3389/fnhum.2010.00174

Fraschini, M., Demuru, M., Puligheddu, M., Floridia, S., Polizzi, L., Maleci, A., et al. (2014). The re-organization of functional brain networks in pharmaco-resistant epileptic patients who respond to VNS. *Neurosci. Lett.* 580, 153–157. doi: 10.1016/j.neulet.2014.08.010

Galán, L., Biscay, R., Rodríguez, J. L., Pérez-Abalo, M. C., and Rodríguez, R. (1997). Testing topographic differences between event related brain potentials by using non-parametric combinations of permutation tests. *Electroencephalogr. Clin. Neurophysiol.* 102, 240–247. doi: 10.1016/s0013-4694(96)95155-3

Goffin, K., Van Paesschen, W., and Van Laere, K. (2011). *In vivo* activation of endocannabinoid system in temporal lobe epilepsy with hippocampal sclerosis. *Brain* 134, 1033–1040. doi: 10.1093/brain/awq385

Gordon, E., and Devinsky, O. (2001). Alcohol and marijuana: effects on epilepsy and use by patients with epilepsy. *Epilepsia* 42, 1266–1272. doi: 10.1046/j.1528-1157.2001.19301.x

Gupta, D., Ossenblok, P., and van, L. G. (2011). Space-time network connectivity and cortical activations preceding spike wave discharges in human absence epilepsy: a MEG study. *Med. Biol. Eng. Comput.* 49, 555–565. doi: 10.1007/s11517-011-0778-3

Haneef, Z., and Chen, D. K. (2014). Functional neuro-imaging as a pre-surgical tool in epilepsy. *Ann. Indian Acad. Neurol.* 17, S56–S64. doi: 10.4103/0972-2327.128659

Hausman-Kedem, M., Menascu, S., and Kramer, U. (2018). Efficacy of CBD-enriched medical cannabis for treatment of refractory epilepsy in children and adolescents—an observational, longitudinal study. *Brain Dev.* 40, 544–551. doi: 10.1016/j.braindev.2018.03.013

Hegde, M., Santos-Sanchez, C., Hess, C. P., Kabir, A. A., and Garcia, P. A. (2012). Seizure exacerbation in two patients with focal epilepsy following marijuana cessation. *Epilepsy Behav.* 25, 563–566. doi: 10.1016/j.yebeh.2012.09.024

Herrera-Díaz, A., Mendoza-Quiñones, R., Melie-Garcia, L., Martínez-Montes, E., Sanabria-Diaz, G., Romero-Quintana, Y., et al. (2016). Functional connectivity and quantitative EEG in women with alcohol use disorders: a resting-state study. *Brain Topogr.* 29, 368–381. doi: 10.1007/s10548-015-0467-x

Horstmann, M. T., Bialonski, S., Noennig, N., Mai, H., Prusseit, J., Wellmer, J., et al. (2010). State dependent properties of epileptic brain networks: comparative graph-theoretical analyses of simultaneously recorded EEG and MEG. *Clin. Neurophysiol.* 121, 172–185. doi: 10.1016/j.clinph.2009.10.013

Klimesch, W. (1999). EEG alpha and theta oscillations reflect cognitive and memory performance: a review and analysis. *Brain Res. Rev.* 29, 169–195. doi: 10.1016/s0165-0173(98)00056-3

Koenig, T., Studer, D., Hubl, D., Melie, L., and Strik, W. K. (2005). Brain connectivity at different time-scales measured with EEG. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 1015–1023. doi: 10.1098/rstb.2005.1649

Latora, V., and Marchiori, M. (2001). Efficient behavior of small-world networks. *Phys. Rev. Lett.* 87:198701. doi: 10.1103/PhysRevLett.87.198701

Lattanzi, S., Brigo, F., Trinka, E., Zaccara, G., Cagnetti, C., Del, G. C., et al. (2018). Efficacy and safety of cannabidiol in epilepsy: a systematic review and meta-analysis. *Drugs* 78, 1791–1804. doi: 10.1007/s40265-018-0992-5

Lattanzi, S., Trinka, E., Russo, E., Striano, P., Citraro, R., Silvestrini, M., et al. (2019). Cannabidiol as adjunctive treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome. *Drugs Today* 55, 177–196. doi: 10.1358/dot.2019.55.3.2909248

Liang, J.-G., Kim, N.-Y., Ko, A., Kim, H. D., and Lee, D. (2018). Changes in functional brain network topology after successful and unsuccessful corpus callosotomy for Lennox-Gastaut Syndrome. *Sci. Rep.* 8, 3414–21764. doi: 10.1038/s41598-018-21764-5

Ludányi, A., Eross, L., Czirják, S., Vajda, J., Halász, P., Watanabe, M., et al. (2008). Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. *Neurosci.* 28, 2976–2990. doi: 10.1523/JNEUROSCI.4465-07

McCoy, B., Wang, L., Zak, M., Al-Mehmadi, S., Kabir, N., Alhadid, K., et al. (2018). A prospective open-label trial of a CBD/THC cannabis oil in dravet syndrome. *Ann. Clin. Transl. Neurol.* 5, 1077–1088. doi: 10.1002/acn3.621

Mortati, K., Dworetzky, B., and Devinsky, O. (2007). Marijuana: an effective antiepileptic treatment in partial epilepsy? A case report and review of the literature. *Rev. Neurol. Dis.* 4, 103–106.

Niso, G., Carrasco, S., Gudín, M., Maestú, F., Del-Pozo, F., and Pereda, E. (2015). What graph theory actually tells us about resting state interictal MEG epileptic activity. *Neuroimage Clin.* 8, 503–515. doi: 10.1016/j.nicl.2015.05.008

Onnela, J. P., Saramäki, J., Kertész, J., and Kaski, K. (2005). Intensity and coherence of motifs in weighted complex networks. *Phys. Rev. E. Stat. Nonlin. Soft. Matter Phys.* 71:065103. doi: 10.1103/PhysRevE.71.065103

Pedersen, M., Omidvarnia, A. H., Walz, J. M., and Jackson, G. D. (2015). Increased segregation of brain networks in focal epilepsy: an fMRI graph theory finding. *Neuroimage Clin.* 8, 536–542. doi: 10.1016/j.nicl.2015.05.009

Ponten, S. C., Douw, L., Bartolomei, F., Reijneveld, J. C., and Stam, C. J. (2009). Indications for network regularization during absence seizures: weighted and unweighted graph theoretical analyses. *Exp. Neurol.* 217, 197–204. doi: 10.1016/j.expneurol.2009.02.001

Romigi, A., Bari, M., Placidi, F., Marciani, M. G., Malaponti, M., Torelli, F., et al. (2010). Cerebrospinal fluid levels of the endocannabinoid anandamide are reduced in patients with untreated newly diagnosed temporal lobe epilepsy. *Epilepsia* 51, 768–772. doi: 10.1111/j.1528-1167.2009.02334.x

Rubinov, M., McIntosh, A. R., Valenzuela, M. J., and Breakspear, M. (2009). Simulation of neuronal death and network recovery in a computational model of distributed cortical activity. *Am. J. Geriatr. Psychiatry* 17, 210–217. doi: 10.1097/JGP.0b013e318187137a

Sawilowsky, S. S. (2009). New effect size rules of thumb. *J. Mod. Appl. Stat. Methods* 8, 597–599. doi: 10.22237/jmasm/1257035100

Scheffer, I. E., Berkovic, S., Capovilla, G., Connolly, M. B., French, J., Guilhoto, L., et al. (2017). ILAE classification of the epilepsies: position article of the ILAE Commission for Classification and Terminology. *Epilepsia* 58, 512–521. doi: 10.1111/epi.13709

Stam, C. J., van Cappellen van Walsum, A. M., Pijnenburg, Y. A., Berendse, H. W., de Munck, J. C., Scheltens, P., et al. (2002). Generalized synchronization of MEG recordings in Alzheimer's disease: evidence for involvement of the gamma band. *J. Clin. Neurophysiol.* 19, 562–574. doi: 10.1097/00004691-200212000-00010

Theiler, J. (1986). Spurious dimension from correlation algorithms applied to limited time-series data. *Phys. Rev. A Gen. Phys.* 34, 2427–2432. doi: 10.1103/physreva.34.2427

Uhlhaas, P. J., and Singer, W. (2006). Neural synchrony in brain disorders: relevance for cognitive dysfunctions and pathophysiology. *Neuron* 52, 155–168. doi: 10.1016/j.neuron.2006.09.020

Vaessen, M. J., Jansen, J. F., Vlooswijk, M. C., Hofman, P. A., Majoie, H. J., Aldenkamp, A. P., et al. (2012). White matter network abnormalities are associated with cognitive decline in chronic epilepsy. *Cereb. Cortex* 22, 2139–2147. doi: 10.1093/cercor/bhr298

van Dellen, E., Douw, L., Hillebrand, A., Ris-Hilgersom, I. H., Schoonheim, M. M., Baayen, J. C., et al. (2012). MEG network differences between low- and high-grade glioma related to epilepsy and cognition. *PLoS One* 7:e50122. doi: 10.1371/journal.pone.0050122

von Stein, A., and Sarnthein, J. (2000). Different frequencies for different scales of cortical integration: from local gamma to long range alpha/theta synchronization. *Int. J. Psychophysiol.* 38, 301–313. doi: 10.1016/s0167-8760(00)00172-0

Watts, D. J., and Strogatz, S. H. (1998). Collective dynamics of 'small-world' networks. *Nature* 393, 440–442. doi: 10.1038/30918

Younus, S. M., Basar, S., Gauri, S. A., Khan, A. A., Imran, M., Abubakar, S., et al. (2018). Comparison of phenytoin versus levetiracetam in early seizure prophylaxis after traumatic brain injury, at a tertiary care hospital in karachi, pakistan. *Asian J. Neurosurg.* 13, 1096–1100. doi: 10.4103/ajns.AJNS_125_17

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

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 24 March 2021
doi: 10.3389/fnbeh.2021.613798

Distribution of the Cannabinoid Receptor Type 1 in the Brain of the Genetically Audiogenic Seizure-Prone Hamster GASH/Sal

Alejando Fuerte-Hortigón[1,2]*, *Jaime Gonçalves*[1,3,4], *Laura Zeballos*[1,3,4], *Rubén Masa*[1], *Ricardo Gómez-Nieto*[1,3,4] *and Dolores E. López*[1,3,4]

[1] *Institute of Neurosciences of Castilla y León, University of Salamanca, Salamanca, Spain,* [2] *Department of Neurology, Virgen Macarena Hospital, Sevilla, Spain,* [3] *Institute of Biomedical Research of Salamanca, Salamanca, Spain,* [4] *Department of Cell Biology and Pathology, University of Salamanca, Salamanca, Spain*

The endocannabinoid system modulates epileptic seizures by regulating neuronal excitability. It has become clear that agonist activation of central type I cannabinoid receptors (CB1R) reduces epileptogenesis in pre-clinical animal models of epilepsy. The audiogenic seizure-prone hamster GASH/Sal is a reliable experimental model of generalized tonic-clonic seizures in response to intense sound stimulation. However, no studies hitherto had investigated CB1R in the GASH/Sal. Although the distribution of CB1R has been extensively studied in mammalian brains, their distribution in the Syrian golden hamster brain also remains unknown. The objective of this research is to determine by immunohistochemistry the differential distribution of CB1R in the brains of GASH/Sal animals under seizure-free conditions, by comparing the results with wild-type Syrian hamsters as controls. CB1R in the GASH/Sal showed a wide distribution in many nuclei of the central nervous system. These patterns of CB1R-immunolabeling are practically identical between the GASH/Sal model and control animals, varying in the intensity of immunostaining in certain regions, being slightly weaker in the GASH/Sal than in the control, mainly in brain regions associated with epileptic networks. The RT-qPCR analysis confirms these results. In summary, our study provides an anatomical basis for further investigating CB1R in acute and kindling audiogenic seizure protocols in the GASH/Sal model as well as exploring CB1R activation via exogenously administered cannabinoid compounds.

Keywords: cannabinoid receptors, GASH/Sal, epilepsy, gene expression, immunohistochemistry

OPEN ACCESS

Edited by:
Fabrizio Sanna,
University of Cagliari, Italy

Reviewed by:
Carmen De Caro,
University of Catanzaro, Italy
Hideo Hagihara,
Fujita Health University, Japan

***Correspondence:**
Alejando Fuerte-Hortigón
alejandro.fuerte.sspa@juntadeandalucia.es

Specialty section:
This article was submitted to Pathological Conditions, a section of the journal Frontiers in Behavioral Neuroscience

Received: *03 October 2020*
Accepted: *25 February 2021*
Published: *24 March 2021*

Citation:
Fuerte-Hortigón A, Gonçalves J, Zeballos L, Masa R, Gómez-Nieto R and López DE (2021) Distribution of the Cannabinoid Receptor Type 1 in the Brain of the Genetically Audiogenic Seizure-Prone Hamster GASH/Sal. Front. Behav. Neurosci. 15:613798. doi: 10.3389/fnbeh.2021.613798

INTRODUCTION

The endocannabinoid system consists of specific cannabinoid receptors, their endogenous ligands and the enzymatic systems of their biosynthesis and degradation (Svízenská et al., 2008). This system is widespread in the central nervous system and is involved in the regulation of the following processes: neurogenesis, memory, appetite, metabolism, stress, emotions, immune response, anxiety, analgesia, thermoregulation, sleep, perception, motor coordination, behavior, and reproduction (Chaperon and Thiébot, 1999; Viveros et al., 2005; Fernández-Ruiz et al., 2007; Crowe et al., 2014; Hillard, 2014; Soria-Gómez et al., 2014; Gatta-Cherifi and Cota, 2015; Lu and Potter, 2017; Robertson et al., 2017).

Endocannabinoids inhibit retrograde release of some neurotransmitters such as γ-aminobutyric acid, glutamate and serotonin (Pazos et al., 2005), since they regulate the aperture/closing of ion channels (Childers and Breivogel, 1998; Qian et al., 2017) in both excitatory (glutamatergic) and inhibitory (GABAergic) synapses (Julian et al., 2003), in response to an increase in the intracellular Ca^{2+} concentration (Ohno-Shosaku and Kano, 2014; Kendall and Yudowski, 2016). These regulatory effects are primarily mediated by two G-protein-coupled receptors: cannabinoid receptor type 1 (CB1R) and cannabinoid receptor type 2 (CB2R) (Marcu and Schechter, 2016; Lu and Potter, 2017). The functions of endocannabinoids in the central nervous system are carried out by activation of CB1R, while CB2R plays a much more limited role. CB1Rs are expressed on presynaptic terminals of neurons of the central nervous system (Pazos et al., 2005; Kendall and Yudowski, 2016). However, this does not preclude the existence of CB1Rs at postsynaptic sites, as functional studies demonstrate self-inhibition in neocortical neurons by endocannabinoids (Maroso et al., 2016). In addition, a high proportion of CB1Rs, at steady state, is localized to somatodendritic endosomes (Thibault et al., 2013). These receptors are also present in the astrocytes, oligodendrocytes and in the cerebral vasculature. Specifically, CB1Rs are highly expressed in brain areas responsible for mood regulation, motor co-ordination, cognition and pain such as the hippocampus, olfactory regions, caudate putamen, accumbens nucleus, substantia nigra, globus pallidus, periaqueductal gray matter, dorsal horn of the medulla, cingulate gyrus, neocortex, amygdala, hypothalamus, and solitary nucleus (Tsou et al., 1998; Svízenská et al., 2008; Hu and Mackie, 2015).

CB1 and CB2 receptors can be activated by exogenous cannabinoids, producing the biological effects of endocannabinoids. Cannabis plants contain more than a 100 terpenophenolic compounds that have been called cannabinoids (Gould, 2015), the two most abundant being Δ9-THC (Δ9-tetrahydrocannabinol) and CBD (cannabidiol).

Currently, there has been growing interest in the use of exogenous cannabinoid compounds for the treatment of a variety of neurological diseases, including epilepsy (Sulak et al., 2017). CBD has been approved in some countries for the treatment of drug-resistant epileptic syndromes (Dravet and Lennox–Gastaut Syndromes) (Devinsky et al., 2014; Thiele et al., 2018). It is estimated that 25–30% of epileptic patients suffer from intractable seizures that cannot be controlled by antiepileptic medications (O'Connell et al., 2017) and they often require invasive treatments such as neurostimulation or surgical resection (Reddy and Golub, 2016). In addition, the development of a single drug which could control seizures would reduce the probability of developing toxic effects (Wilby et al., 2005). It has been demonstrated that there is a pathophysiological reorganization of the endocannabinoid system (Blair et al., 2015; Katona, 2015) and an activation of CB1R as a protective mechanism against excitotoxicity in epileptic patients (Lupica et al., 2017). Both direct (the use of CB1R agonists) and indirect approaches (inhibition of endocannabinoid catabolism) reduce epileptogenesis in animal models (Rosenberg et al., 2017). Endocannabinoid signaling mediated through presynaptic CB1R reduces both glutamate and GABA release (Kathmann et al., 1999; Misner and Sullivan, 1999; Hájos et al., 2000), and therefore is a potent regulator of neuronal excitability. CB1R agonists have been widely studied for anti-seizure effects across an array of models of seizures; this has been reviewed extensively elsewhere (Wallace et al., 2001; Skaper and Di Marzo, 2012; Cristino et al., 2020). The study of the endocannabinoid signaling pathway, its physiological action and distribution are key for the development of more treatments based on exogenous cannabinoid compounds.

We therefore set out to study the distribution of the main brain cannabinoid receptor, CB1R, in the GASH/Sal epilepsy model under seizure-free conditions, by comparing the results with wild-type Syrian hamsters, since these elements can become pharmacological targets for the treatment of epilepsy, where an alteration of this system is postulated.

MATERIALS AND METHODS

Animals

Fourteen GASH/Sal and 13 male Syrian hamsters 4 months of age were obtained from the Animal Facility of the University of Salamanca (USAL, Spain) and Janvier Labs (Le Genest-Saint-Isle, France), respectively, to be used in this experiment. Male hamsters were selected in order to remove potentially confounding hormonal processes intrinsic to female metabolism. Animals were maintained under normal conditions of lighting (12 h light/dark cycle) and temperature (22 ± 1°C) in an acoustically controlled environment, and with free access to water and food.

All the procedures and experimental protocols were performed in accordance with the guidelines of the European Community's Council Directive (2010/63/EU) and approved by the Bioethics Committee of the University of Salamanca (approval number 380).

Quantitative Reverse Transcription PCR (RT-qPCR)

The primers were designed for *Mesocricetus auratus*. Gene sequences were obtained from the Ensembl Genome Browser database (http://www.ensembl.org/index.html) and the primers were designed aligned in different exons using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (**Table 1**). The primers were synthesized by Thermo Fisher Custom Primers (Invitrogen - Thermo Fisher).

A total of 12 animals (6 control hamsters and 6 GASH/Sal) were deeply anesthetized by isoflurane inhalation and once areflexia was verified, were decapitated. Different brain structures were removed for gene expression studies: brain stem, cerebellum, inferior colliculus, hippocampus and cortex. All tissues harvested were put into storage at −80°C until use. The RT-qPCR approach was identical to that used previously by our group (e.g., Damasceno et al., 2020; Sánchez-Benito et al., 2020). RNA from samples was extracted in accordance with the protocol of TRIzol™ Reagent (#15596026, Invitrogen). Total RNA concentration was quantified using the NanoPhotometer®/spectrophotometer

TABLE 1 | Primers used for RT-qPCRs.

Gen targetet	ID transcript Ensembl *Mesocricetus auratus*[a]	Primer forward	Primer reverse	Size of products	E[b]
Cb1r	*ENSMAUG00000014040*	TGTTGACTTCCATGTGTTCCA	GGTCTGGTGACGATCCTCTT	171	1.15
Actb	*ENSMAUG00000008763*	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG	105	2.03

List of primers used for RT-qPCRs, indicating the location of each primer in the corresponding Ensembl sequences of the Syrian hamster (a). qPCR primer efficiency (E^b) was calculated according to the following equation: $E = 10^{(-1/slope)}$.

(Implen, Munich, Germany), taking into account the absorption ratios 260/280 nm and 260/230 nm, and RNA integrity was checked by electrophoresis in agarose gel (1.5%). Genomic DNA was degraded using the Ambion^{TM} DNase I (RNase free) (Thermo Fisher Scientific) following the supplier's instructions.

Complementary DNA (cDNA) was synthesized from 800 ng of total RNA using the ImProm-IITM Reverse Transcription System Kit (Promega Corporation, Madison, SWI, USA). The relative quantification of the transcripts was performed on ABI Prism 7000 (Applied Biosystems) using the SYBR Green Master Mix (#4309155, Applied Biosystems). Initially, a serial dilution curve was made to verify the efficiency of the primers of the target and reference genes.

The quantitative reverse transcription real time PCR was conducted using the SYBER Green method. Each reaction contained 7 μL of SYBR, 30 ng of total cDNA, 0.8 μL of each primer (10 μM), and MiliQ water free of DNase and RNase up to 20 μL. The cycling conditions were in accordance with the protocol of the intercalating agent used. RT-qPCR experiments were performed in replicates of four to six samples and conducted in triplicate for the gene product examined, and β-actin (*Actb*) was used as a negative control. Following the removal of outliers (Burns et al., 2005), raw data was used to determine the PCR amplification efficiency (E). The relative gene expression value for each transcript was calculated according to the formula $2^{-(1Ct\ \text{“condition 1”} - 1Ct\ \text{“condition 2”})}$, where "condition 1" corresponds to the experimental sample, "condition 2" corresponds to the sample from the control animal, and 1Ct of each "condition" is $Ct_{\text{“experimental gene”}} - Ct_{\text{“endogenous gene”}}$ (Schmittgen and Livak, 2008). The relative mRNA of the groups was evaluated using an unpaired *t*-test. The analyses were performed using GraphPad Prism 7. $p < 0.05$ was considered as statistically significant. All quantitative data were expressed as mean value ± standard error of the mean (SEM). Asterisks indicate significant differences between experimental groups ("*" = p-value < 0.05; "**" = p-value < 0.01; "***" = p-value < 0.001).

Brain Tissue Processing and Immunostaining

Brain tissue used for immunohistochemistry (3 control and 4 GASH/Sal hamsters) was processed in accordance with the routine protocols used in the laboratory (Sánchez-Benito et al., 2020). Briefly, after injection of a lethal dose of sodium pentobarbital (60 mg.kg^{-1}) and the subsequent perfusion through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), brains were removed from the skull, cryoprotected by immersion in 30% sucrose, and coronal sections were cut with a freezing sliding microtome at 40 μm thickness. Serial sections were collected in PBS and divided into a series of 6 and placed in wells containing 0.1 M-phosphate buffer.

The CB1R was visualized following the indirect method of immunohistochemical staining described by Sánchez-Benito et al. (2020). A primary polyclonal antibody anti-CB1R obtained in rabbit (CB1-Rb-Af380, Frontier Institute, Hokkaido, Japan) which binds to the C-terminal (NM007726) of the mouse protein CB1R was used since there was no primary antibody Anti-CB1R available specific for GASH/Sal hamster. Its reactivity in mice was tested by immunoblot following the manufacturer's instructions. The CB1 protein sequence corresponding to the *cnr1* gene was retrieved from the UNIPROT protein database (https://www.uniprot.org/), and then analyzed using the EBI-Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers and Higgins, 2018). The sequence is highly conserved between the CB1R in the hamster and mouse (**Supplementary Material 1**). Washes were made in Tris-buffered saline (TBS), pH 7.4 and dilutions of antisera in TBS containing 0.2% Triton X-100 (# T9284; Sigma).

For light microscopy analysis, free-floating sections were blocked for 1 h with 5% normal goat serum (#S-1000, Vector Labs.) in TBS-Tx and were incubated with primary antibodies at 1:250 dilution for 72 h at 4°C. Sections were then washed and followed an incubation with the biotinylated secondary antibodies, goat anti-rabbit (#BEA-1000, Vector Labs.), at 1:200 dilution for 2 h. After removal of secondary antisera, the visualization of epitope-antibody interactions was developed with the avidin-biotin peroxidase complex procedure (#PK-4000, Vectastain, Vector Labs.), and diaminobenzidine histochemistry for peroxidase (DAB Kit, #SK-4100, Vector Labs.). All sections were mounted onto slides, (ordered rostro-caudally), dehydrated and coverslipped with Entellan® Neu (#107961, Merck).

To visualize the morphological features of immunostained cells, we used brain embedded in paraffin wax (2 control and 2 GASH/Sal hamsters) before cutting into coronal sections of 6 μm thickness, according to the protocols routinely used in our laboratory (Sánchez-Benito et al., 2020). Then, sections were mounted onto slides and followed the immunohistological staining procedure to visualize the CB1R protein at optical and confocal laser scanning microscopes. In order to identify the possible glial nature of the immunolabeled small cells, a GFAP marker was used, performing a double fluorescent labeling on the 6 μm brain sections, incubating the horizontally arranged slides in a humid chamber.

After deparaffinization and rehydration, endogenous peroxidase activity was blocked with 2.5% horse serum (#S-2000-20, Vector Labs.) and incubation with primary antibodies (rabbit anti CB1R and mouse anti GFAP) was carried out. Subsequently, the sections were rinsed extensively and reacted for 30 min with secondary antibody, VectaFluor™ Duet Reagent [#DK-8818, DyLight® 488 Anti-Rabbit IgG and DyLight® 594 Anti-Mouse IgG cocktail (anti-rabbit Ig in green, anti-mouse in red)] made in horse. Finally, sections were coverslipped with VECTASHIELD® mounting medium for preserving fluorescence, containing the DAPI counterstain (4,6-diamidino-2-phenylindole, #H-1200, Vector Labs.). Additionally, alternative sections were counterstained with Nissl stain, dehydrated and cover slipped with Entellan® Neu, #107961, Merck. A list of the antibodies used is shown in **Table 2**.

Immunoblotting

Cerebellum samples corresponding to age- and sex-matched animals (two GASH/Sal and two control hamsters, males with 4 months of age) were used to verify that the primary antibody against CB1R specifically detects its antigen in a western blot experiment. In Brief, the cerebellum samples were homogenized with IKA T10 Basic Ultra Turrax homogenizer (IKA, Germany) in ice-cold RIPA buffer containing protease inhibitors (Cell Signaling Technologies, USA). Supernatants of the homogenates were collected after centrifugation at 14,000 rpm (Centrifuge 5417R, Eppendorf, Germany) for 15 min, and the protein concentration was determined using the Lowry method. The Samples (150 μg) were separated by gel electrophoresis, using 10% TGX precast gels (Bio-Rad, United States), and electroblotted onto a PVDF membrane (Merck, Germany), which was incubated overnight with the polyclonal antibody anti-CB1R (dilution 1:1,000) at 4°C. The membrane was then immunoreacted for 1 h with the HRP-linked secondary antibody (anti-rabbit IgG) at 1:15,000 dilution (Cell Signaling Technologies, USA). Finally, the immunoreaction was visualized with the ImageQuant RT ECL detection system (GE Healthcare, USA).

Observation and Study of Histological Samples

Sections were observed using a Leica LB30T microscope equipped with a digital camera (Olympus DT70). The photographs were processed with minor modifications in contrast using Adobe Photoshop CS2. Figures were assembled using Canvas Draw 2. "A Stereotaxic Atlas of the Golden Hamster Brain" (Morin and Wood, 2001) was used as a reference to classify histological sections rostro-caudally arranged. In all immunohistochemical experiments, omission of primary antibody resulted in absence of staining of the preparations. The sections processed for immunofluorescence were studied on a Leica Stellaris confocal laser coupled to a Leica Zeiss Axio Observer DMI8 microscope, using the appropriate filters for DyLight® 594 (red), DyLight® 488 (green) and DAPI (violet) fluorochromes. These three fluorochromes were detected sequentially, stack by stack, with the acousto-optical beam splitter as tunable dichroic filter system, using the laser spectral lines 488, 594, and 405 nm, respectively. The objectives used were x40 and oil immersion x63/numerical aperture 1.40, pinhole 1 Airy unit, as well as several electronic zoom factors. To determine the distribution of the immunolabeled terminals, series of 10–15 confocal images were obtained to generate a maximal-intensity z projection of stacks. Colocalization of the fluorochromes DyLight® 488 and DyLight® 594 within positive terminals was always verified in the orthogonal view (=xy, xz, yz planes, for z stacks series). A sequence of 15 serial pictures from different viewpoints was created to produce a three-dimensional (3D) animation and the movie document generated from the image stacks were stored at 30 frames per second as a Windows Media Video file.

RESULTS

Distribution of CB1 Receptors in the Brain of GASH/Sal

The antibody used in a dilution of 1:250 provides immunoreactivity in the central nervous system of the *Mesocricetus auratus*, both in the GASH/Sal line and in Syrian control hamsters. None of the performed controls yielded false positives. Significant immunolabeling was observed in numerous areas of the GASH/Sal brain. Different types of immunolabeling patterns were distinguished, based on the histology of each area, as well as differences in the immunostaining intensity (**Figure 1**).

We found strong and intense CB1R-immunolabeling in the following brain areas: cerebellum, substantia nigra, motor cortex, hippocampus, endopiriform nucleus, subtalamic nuclei, globus pallidus and olfactory bulb. Intense immunoreactivity was also found in the visual, somatosensory, peripheral, and auditory and entorhinal cortices. In a more subtle and diffuse way, CB1R-immunolabeling was found in a high number of brain areas such as: periaqueductal gray matter, caudate-putamen, solitary tract, terminal stria, lateral septum, parabrachial nucleus, amygdala, lateral hypothalamus, arcuate nucleus, cuneiform nucleus, and in the insular cortex.

In **Figures 2A,B**, distribution pattern of CB1 receptors in the brain of GASH/Sal is shown.

Representative images of the CB1R immunoreactivity in the GASH/Sal are shown in the **Figures 3**, **4**.

In general, CB1R immunostaining were found in brain microvessels throughout the brain (**Figure 5D**). Furthermore, labeling is seen in small cells (asterisk), presumably microglia, both in the brain stem nuclei (**Figure 4D**), and in the olfactory bulb (**Figure 4J**), cortex and hippocampus (**Figure 4M**).

In the cerebellum, CB1R-immunostaining was intensively present in a punctate form that were densely distributed in the cerebellar cortex, particularly in the cerebellar granular and Purkinje cell layers (**Figures 3A**, **4A**, **5**; **Supplemental Materials 3**, **4**). The neuropil within the granule cell layer of the cerebellar cortex displayed densely CB1R-immunolabeled puncta that colabeled with GFAP (**Figures 5A**, **5B**; **Supplemental Material 3**). CBR1-immunolabeled puncta of varying size were further densely seen in vicinity of Purkinje cells (**Figures 4A**, **5C**; **Supplemental Materials 3**, **4**). Interestingly,

TABLE 2 | List of antibodies used.

Antigen	Primary AB	Dilution	Reference	Secondary AB	Dilution	Reference	Objective
CB1R	Rabbit anti CB1R	**1/250**	**CB1-Rb-Af380-*Fr***	Biotinilated goat anti rabbit-*Vec*	1/200	BEA-1000-*Vec*	Light Microscopy
				DyLight® 488anti-rabitt-*Vec*	1/200	DK-8818-*Vec*	Confocal Microscopy
GFAP	mouse anti GFAP	**1/2,000**	**G6171-*Sig***	DyLight® 594anti-mouse-*Vec*	1/200		

Comercials.
Fr- Frontier Institute, Hokkaido, Japón.
Sig-Sigma-Aldrich,Taufkirchen, Germany.
Vec- Vector Laboratoires, Burlingame, CA, USA.

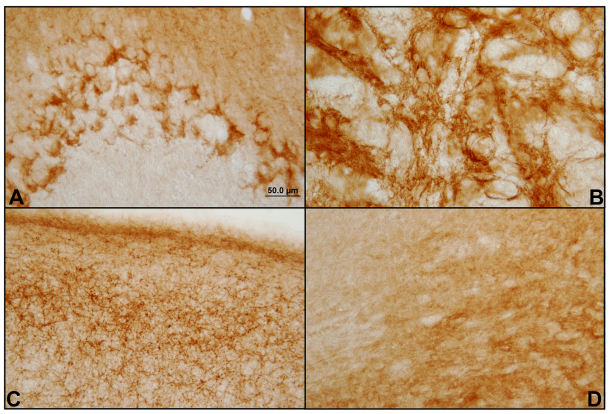


FIGURE 1 | Different patterns of immunostaining of CB1 receptors in the hamster GASH/Sal brain. Immunostaining of CB1 receptors in the brain of GASH/Sal in which a representation of the different staining patterns shown in coronal sections of different brain areas. **(A)** Dot-like staining surrounding the Purkinje cells bodies of the cerebellum; **(B)** Network of fibers (reticular staining) in the globus pallidus nucleus; **(C)** Plexiform staining in the primary motor cortex; **(D)** Diffuse staining in the substantia nigra.

the large CB1R-immunolabeled puncta distributed around the soma and the initial axonal segment of the Purkinje cells (**Figures 4A**, **5C**; **Supplemental Materials 3**, **4**), giving rise to an arrangement described as "Pinceaux formation" (Suárez et al., 2008) that were flanked by GFAP-immunolabeled glial fibers (**Figure 5C**; **Supplemental Materials 3**, **4**). CB1R- and GFAP-immunolabeling was also frequently found in the vicinity of blood vessels of the cerebellar cortex (**Figures 5D**, **5E**).

Nuclei more directly involved in the genesis of seizures, such as the auditory nuclei (ganglion cells, cochlear nuclei, or the inferior colliculus) (**Figures 4B,C**) or the brainstem reticular formation (data not shown), showed CB1R immunoreactivity as well. In ganglion cells (**Figure 6**), CB1R immunoreactivity was distributed in the cytoplasm. In the cochlear nuclei, most of the main neurons appear immunostained in all their divisions, being more intense in the dorsal cochlear nucleus. Interestingly, this labeling is intracytoplasmic.

In the brainstem, there is slight immunoreactivity, with a diffuse staining pattern. It should be noted that structures such as the periaqueductal gray matter presented a slightly more intense marking than the adjacent inferior colliculus (**Figure 3C**), in which diffuse marking continues, although few immunoreactive neurons are visualized (**Figure 4D**).

In the substantia nigra pars reticulata (**Figure 3D**), dense CB1R immunoreactivity appears as fine dots or puncta (**Figure 4E**).

Rostrally, in the cingulate cortex (Cg), intensely stained plexus of fibers were found in the superficial layer (**Figure 3E**), extending to the motor (Mo) and somatosensory (Sm) cortices and, and the same plexiform staining (**Figure 4H**) is weaker in deeper layers. The hippocampus (**Figure 3F**) is distinctively immunoreactive in the principle cell layers. In both the CA1, CA2, and CA3, the stratum pyramidale (s.p.) exhibit the strongest immunostaining, with the distribution being very similar in all of them (**Figures 4K,L**). The intensity of the labeling in the stratum radiatum (s.r.) increase laterally. In the dentate gyrus (DG), CBR1 signal is found mainly in the granular layer (s.g.) with strong immunostaining (**Figure 4M**). Also, in the hilus (hil) the staining is weaker than in the pyramidal cell layer.

Larger immunoreactive fiber bundles are observed in the subthalamic nucleus (STh), as they approach the globus pallidus. In the amygdala complex (AN) (**Figure 3G**), an intense plexiform immunostaining is observed in all its areas, being more intense in the basomedial amygdala nucleus (**Figure 4F**).

The globus pallidus (GP) exhibit a strong network of fibers (reticular staining) surrounding the immunonegative traversing fascicles (**Figures 1B**, **3H**, **4I**), and the caudate putamen (Cpu) also exhibits diffuse CB1R immunoreactivity in the bundles of fibers that target the GP (**Figure 3H**).

Strong diffuse immunostaining is found in the accumbens nucleus (**Figure 3I**).

CB1Rs are also present in glomeruli of the main olfactory bulb (OB), robustly expressed in the granular layer (**Figures 3J**, **4J**) whereas a weakly immunostained fiber plexus is found in the external plexiform layer of the olfactory bulb (EPL) and in the white matter (WM). The density of labeling in this structure is lower than that observed in the different areas of the cerebral cortex.

Differential Gene Expression Analysis of *Cb1r* in the Brain of GASH/Sal and Control Hamsters

In control hamster brains, a uniform distribution of CB1R-immunolabeling pattern was observed across all the brain areas mentioned above. The immunostaining pattern was the same in

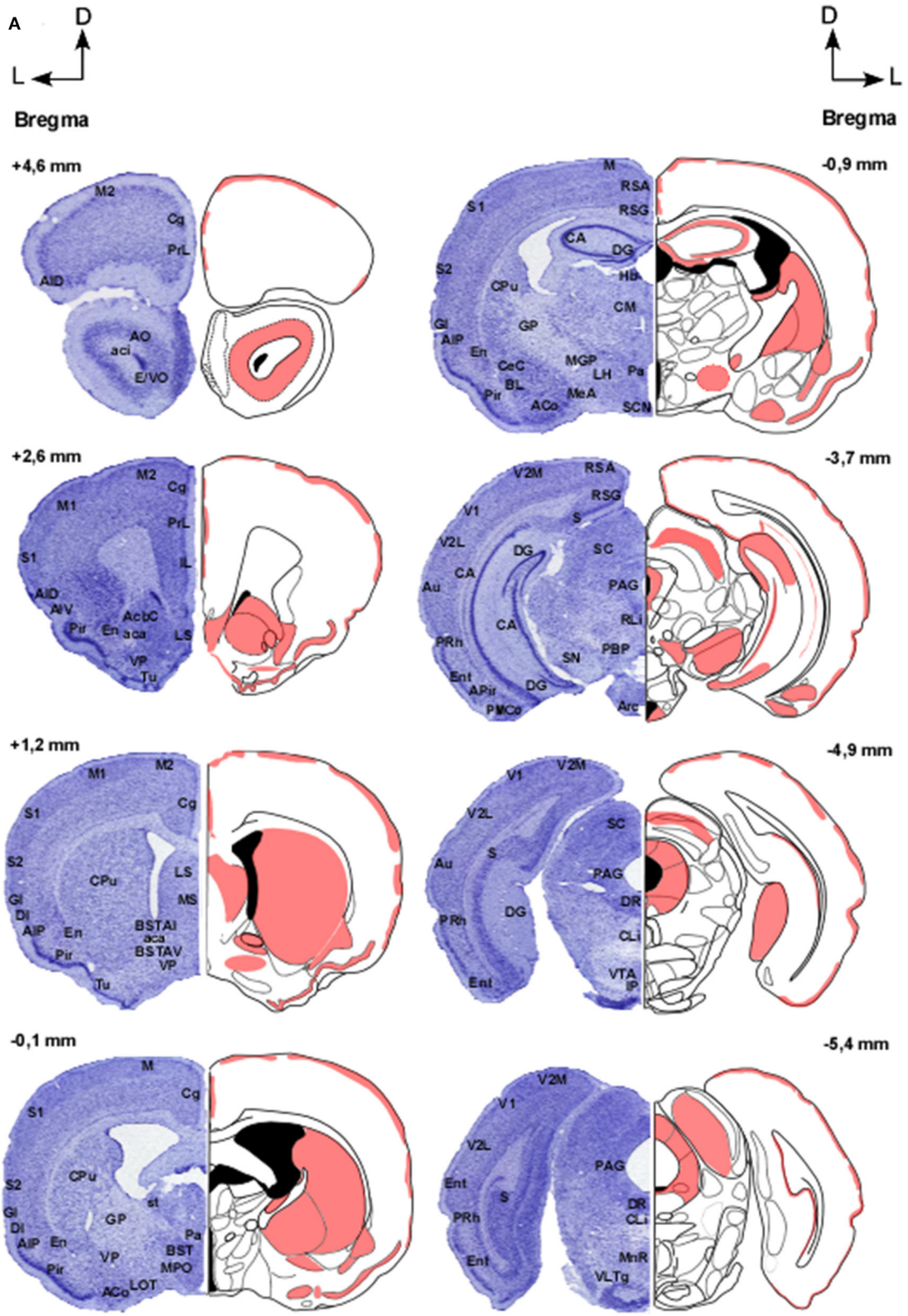


FIGURE 2 |

(Continued)

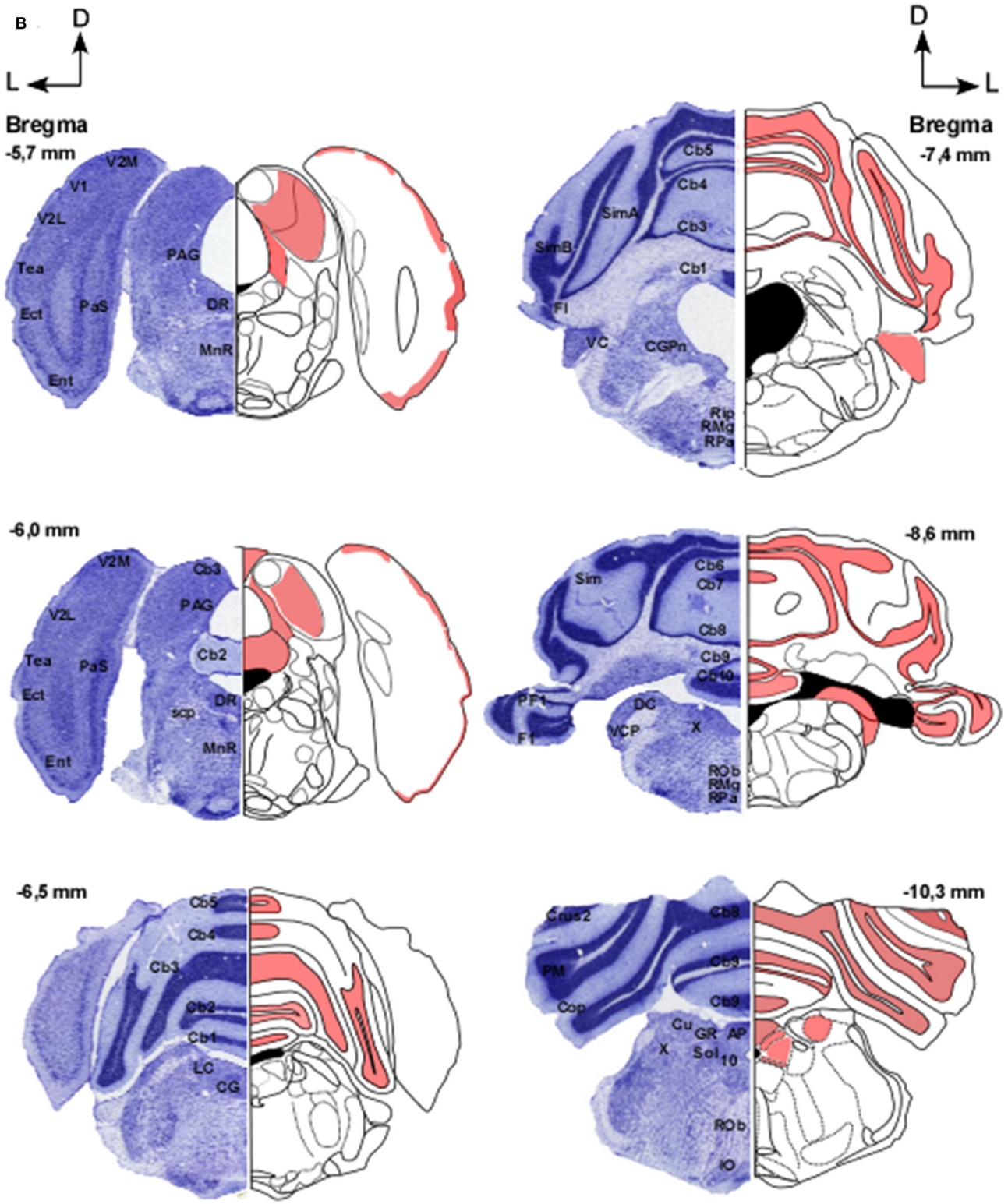


FIGURE 2 | (A,B) Distribution pattern of CB1 receptors in the brain of GASH/Sal. Schemes showing the coronal sections of the brain at different rostro-caudal levels (referenced with respect to Bregma), according to a stereotaxic atlas of the golden hamster brain. The distribution of CB1 receptors in the GASH/Sal is shown in red. Each coronal section includes a semi-section contrasted with Nissl staining as a cytoarchitectural reference of the different nuclei. 10, Dorsal motor nucleus of the vagus; aca, Anterior commissure, anterior part; AcbC, Accumbens nucleus, core; aci, Anterior commissure, intrabulbar part; ACo, Anterior cortical amygdaloid

(Continued)

FIGURE 2 | nucleus; AID, Agranular insular cortex, dorsal part; AIP, Agranular insular cortex, posterior part; AIV, Agranular insular cortex, ventral part; AO, Anterior olfactory nucleus; AP, Area postrema; APir, Amygdalopiriform transition area; Arc, Arcuate hypothalamic nucleus; Au, Primary auditory cortex; BL, Basolateral amygdaloid nucleus; BST, Bed nucleus of stria terminalis; BSTAI, Bed nucleus of stria terminalis, anteromediate part; BSTAV, Bed nucleus of stria terminalis, anteroventral part; CA, Hippocampus; Cb1-10, Cerebellar lobule 1–10; CeC, Central amygdaloid nucleus; Cg, Cingulate cortex; CG, Central gray; CGPn, Central gray of the pons; CLi, Caudal linear nucleus of the raphe; CM, Central medial thalamic nucleus; Cop, Copula of the pyramis; CPu, Caudate putamen; Crus1-2, Crus 1-2 of the ansioform lobule; Cu, Cuneate nucleus; DC, Dorsal cochlear nucleus; DG, Dentate gyrus; DI, Dysgranular insular cortex; DR, Dorsal raphe nucleus; E/VO, Olfactory ventricle; Ect, Ectorhinal cortex; En, Endopiriform nucleus; Ent, Entorhinal cortex; F1, Flocculus; GI, Granular insular cortex; GP, Globus pallidus; GR, Gracile nucleus; Hb, Habenula nuclei; IL, Infralimbic cortex; IO, Inferior olive; IP, Interpeduncular nucleus; LC Locus coeruleus; LH, Lateral hypothalamic area; LOT, Nucleus of the lateral olfactory tract; LS, Lateral septal nucleus; M, Motor cortex; M1, Primary motor cortex; MS, Medial septal nucleus; M2, Secondary motor cortex; Mea, Medial amygdaloid nucleus; MGP, Medial globus pallidus; MnR, Median Raphe nucleus; MPO, Medial preoptic area; Pa, Paraventricular hypothalamic nucleus; PAG, Periaqueductal graymatter; Pas, Parasubiculum; PBP, Parabrachial pigmented nucleus; PF1, Paraflocculus; Pir, Piriform cortex; Pm, Paramedian lobule; PMCo, Posteromedial cortical amygdaloid nucleus; PRh, Perirhinal cortex; PrL, Prelimbic cortex; Rip, Raphe interpositus nucleus; RLi, Rostral linear nucleus of the raphe; RMg, Raphe magnus nucleus; Rob, Raphe obscurus nucleus; Rpa, Raphe pallidus nucleus; RSA, Restroplenial agranular cortex; RSG, Restrosplenial granular cortex; S, Subiculum; S1, Primary somatosensory cortex; S2, Secondary somatosensory cortex; SC, Superior colliculus; SCN, Suprachiasmatic nucleus; SimA-B, Simple lobule A-B; SN, Substantia nigra; Sol, Nucleus of the solitary tract; st, Stria terminalis; scp, Superior cerebellar penduncle; Tea, Temporal Association Cortex; Tu, Olfactory tubercle; V1, Primary visual cortex; V2L, Secondary visual cortex, lateral part; V2M, Secondary visual cortex, medial part; VC, Ventral cochlear nucleus; VCP, ventral cochlear nucleus, posterior part; VLTg, Ventrolateral tegmental area; VP, Ventral pallidum; VTA, Ventral tegmental area; X, Nucleus X.

the two hamster lines used, although some areas appeared with a slight difference in the intensity of the immunostaining.

To confirm this, differential gene expression analysis of CB1R gene (*Cb1r*) was carried out in brain structures of control and GASH/Sal animals under seizure-free conditions (naïve animals) (**Figure 7**). These structures included the inferior colliculus (IC) (epileptogenic focus in the audiogenic strain), the hippocampus, the cerebellum, the motor and somatosensory cortices, and the brainstem. As shown in **Figure 7**, the RT-qPCR analysis in the IC showed significantly lower expression (*** $p < 0.0001$) of the *Cb1r* in GASH/Sal animals than in the control. On the other hand, as in immunohistochemical studies, lower levels of *Cb1r* expression were detected in the cerebellum of GASH/Sal hamsters compared to controls, although this decrease is not significant in RT-qPCR analyses. For the motor and somatosensory cortices and the hippocampus, there was an increase in *Cb1r* expression in the GASH/Sal in both cases compared to controls (*** $p < 0.0001$). Finally, expression levels of *Cb1r* in the brainstem were significantly lower in naïve GASH/Sal compared to naïve Syrian control hamsters (** $p< 0.01$). The raw data of RT-qPCR used for analyses are included in **Supplementary Material 2**.

DISCUSSION

CB1-Mediated Neuromodulation in Epilepsy

Although there are two types of endocannabinoid receptors, CB1R and CB2R, it is the former that is expressed in greater proportion in the central nervous system (Rosenberg et al., 2017). In fact, it is one of the most widely expressed G-protein-coupled receptors in the brain (Herkenham et al., 1991a). The endocannabinoid system acts as a retrograde control mechanism for excessive presynaptic neuronal activity (Lutz, 2004). When excessive presynaptic activity is detected, endocannabinoids are secreted from the postsynaptic terminals, bind to the CB1R of the presynaptic terminals and activate signaling cascades to decrease the liberation of neurotransmitters (Freund et al., 2003). The effects of CB1Rs depend on their location, i.e., increased CB1R signaling on glutamatergic terminals induces inhibition and neuroprotective effects, while those located on GABAergic terminals induce excitatory effects (Chiarlone et al., 2014; Guggenhuber et al., 2015).

The direct relationship between CB1R and the development of seizures in animal epilepsy models is well-documented (Lazarini-Lopes et al., 2020a). CB1R agonists exert anticonvulsant effects (Shafaroodi et al., 2004; Tutka et al., 2018), whereas CB1R antagonists block its anticonvulsant action (Wallace et al., 2002) and potentiate seizure duration and frequency (Muccioli and Lambert, 2005), suggesting that endocannabinoids might be suppressing seizure activity (Wallace et al., 2003). This hypothesis is reinforced by the fact that the activation of CB1 receptors protects against acute clonic and generalized tonic-clonic seizures in the pentylenetetrazole model (Bahremand et al., 2008). Moreover, in experiments where CB1R is blocked, audiogenic seizures become more severe (Vinogradova et al., 2011). Therefore, knowing the exact location of CB1R in animal models of epilepsy turns out to be essential to search for drugs which would enhance endocannabinoid signaling and thus modulate seizures.

In the genetically audiogenic seizure-prone hamster GASH/Sal, CB1R is distributed throughout the central nervous system. This receptor is also located in the peripheral, specifically in the spiral ganglion cells of the organ of Corti, as previously described in birds (Stincic and Hyson, 2011) and mice (Toal et al., 2016).

Distribution of CB1R in the GASH/Sal

Neither the reactivity of the CB1R antibody used in our study nor the distribution of CB1R-immunolabeling in the cochlear has been previously tested in the brain hamster. Our study provides several evidences that indicate this CB1R antibody can be used as a marker of CB1R in brain tissue of the golden hamster as efficiently as reported in another mammal species (Fukudome et al., 2004; Rivera et al., 2014; Puighermanal et al., 2017). First, the multiple sequence alignment showed that the specific target epitope is highly conserved

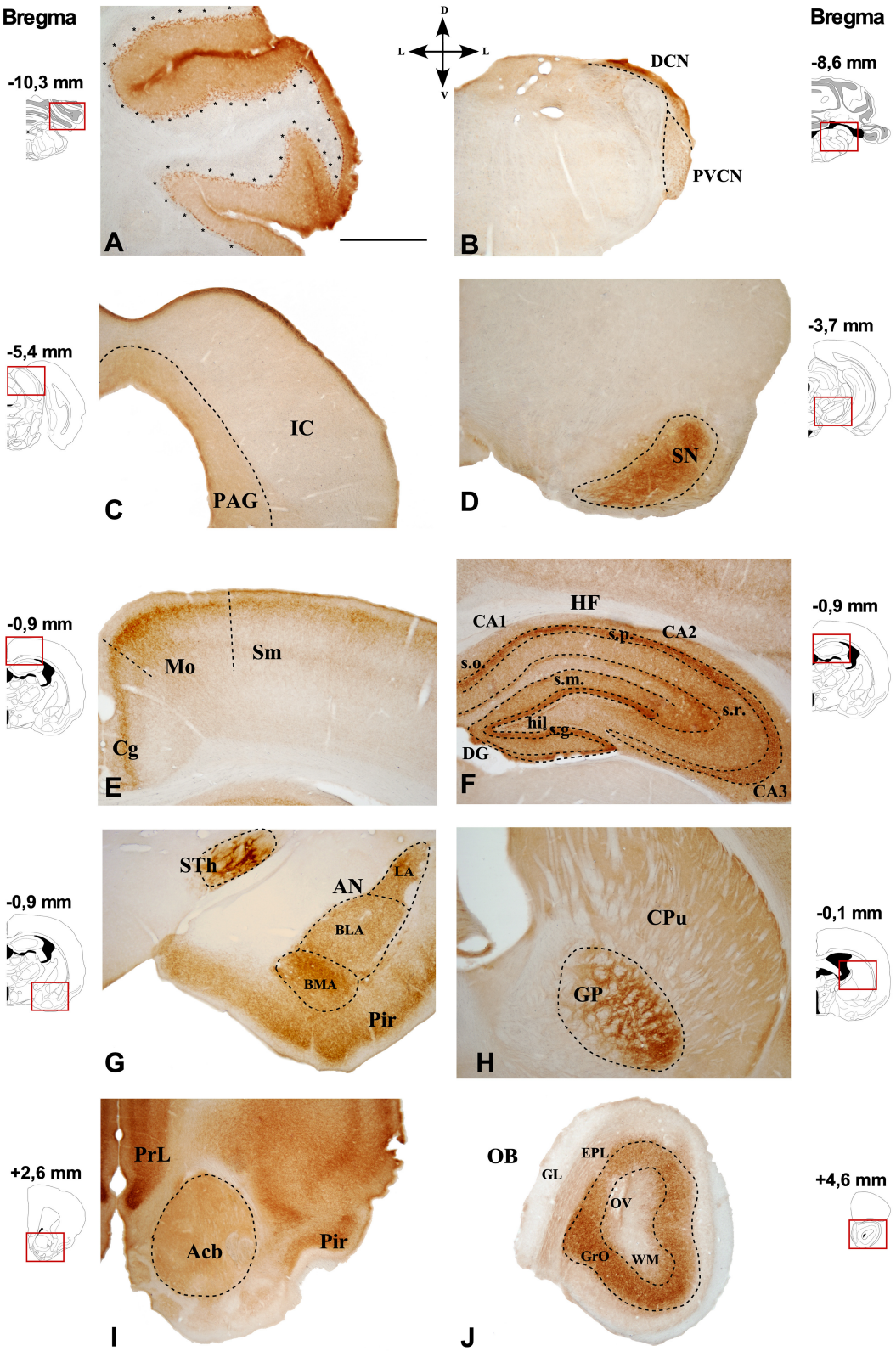


FIGURE 3 | Immunostaining of CB1R in the GASH/Sal brain. Photomicrographs of GASH/Sal coronal sections referencing their rostrocaudal position as a function of Bregma and indicating its dorsoventral orientation. **(A)** Cerebellar lobe. **(B)** Dorsal and posteroventral cochlear nucleus. **(C)** Periaqueductal gray matter (delimited by dots) and the inferior colliculus. **(D)** Substantia nigra. **(E)** Somatosensory and motor cortices. **(F)** Hippocampal formation. **(G)** Piriform cortex, amygdaloid nuclei, and

(Continued)

FIGURE 3 | subthalamic nucleus. **(H)** Caudate putamen and globus pallidus. **(I)** Accumbens nucleus. **(J)** Olfactory bulb. Scale bar = 1 mm. Acb, Accumbens nucleus; AN, Amygdaloid nuclei; CA1–3, Cornu Ammonis area 1–3; BLA, Basolateral amygdala nucleus; BMA, Basomedial amygdala nucleus;Cg, Cingulate cortex; CPu, Caudate putamen; DG, Dentate gyrus; DCN, Dorsal cochlear nucleus; EPL, External plexiform layer olfactory bulb; GL, Glomerular layer olfactory bulb; GrO, Granule cell layer olfactory bulb. GP, Globus pallidus; hil., Hilus; HF, Hippocampal formation; IC, Inferior colliculus; LA, Lateral amygdala nucleus; Mo, Motor cortex; OB, Olfactory bulb; OV, Olfactory ventricle; PAG, Periaqueductal gray matter; Pir, Piriform cortex; PrL, Prelimbic cortex; PVCN, Ventral cochlear nucleus, posterior part; s.g., Stratum granulosum; s.m., Stratum moleculare; Sm, Somatosensorial cortex; SN, Substantia nigra; s.o., Stratum orients; s.p., Stratum pyramidale; s.r., Stratum radiatum; STh, Subthalamic nucleus; WM, White matter.

for CB1R in the golden hamster. Second, the pattern of CB1R-immunolabeling in the cerebellar cortex of the hamster was consistent with that described in other rodent species (Herkenham et al., 1991b; Matsuda et al., 1993; Egertová and Elphick, 2000). Furthermore, the CB1R-immunolabeled pattern in our experiments was consistently obtained using different cutting or immunodetection methods. Finally, the western blot analysis confirmed the specificity and selectivity of the CB1R antibody verifying the antibody's ability to recognize and bind to its target antigen (**Supplementary Material 5**).

Auditory Nuclei and Periaqueductal Gray Matter

In the peripheral auditory system, CB1R was located inside the cell bodies. By using specific presynaptic labeling antibodies, Stincic and Hyson (2011) showed that CB1R is in the presynaptic neuron in the chick spiral ganglion cells. Discrepancies in the location of CB1R in ganglion cells may be due to differences between birds and mammals, or due to the presence of CB1R in the cellular endosomes at steady state (Thibault et al., 2013).

The activation of endocannabinoids in the spiral ganglion has been associated to a protective effect, helping to maintain consistent response amplitudes across a long duration stimulus (Stincic and Hyson, 2011). On the other hand, CB1 receptor knockout mice possess poorer hearing thresholds than wild-type mice (Toal et al., 2016). The GASH/Sal has been described to exhibit a significant loss of spiral ganglion neurons (Sánchez-Benito et al., 2017), which results in a reduction in the amount of CB1Rs in the spiral ganglion, consistent with the significant hearing deficit in this model (Muñoz et al., 2017).

The strongest immunoreactivity for CB1R in both dorsal and ventral cochlear nuclei, has been described in the cytoplasm of main cells using autoradiographic (Herkenham et al., 1991a) and immunohistochemistry approaches (Zheng et al., 2007; Zhao et al., 2009). Zheng et al. (2007) showed the spatial distribution of CB1R in the cochlear nucleus. In that study, substantial labeling was found on many different cell types, such as stellate cells, giant cells, fusiform cells, and corn cells in the DCN, as well as globular bushy cells, elongated cells, and octopus cells in the VCN. The cytoplasmic labeling found in these cells appeared inconsistent with the reported presynaptic localization of CB1 receptors, with almost no exceptions in adult animals (Schlicker and Kathmann, 2001); however, it has since been reported that the CB1 receptor undergoes extensive trafficking between the cytoplasm and the presynaptic terminals in brain regions where it is very active (Mikasova et al., 2008). Using electron microscopy, the synaptic location of these receptors in the cochlear nucleus was confirmed (Tzounopoulos et al., 2007), in both GABAergic and glycinergic terminals, but not at auditory nerve inputs (Zhao and Tzounopoulos, 2011; Zhao et al., 2011).

The inferior colliculus (IC) is critical in audiogenic seizures (AGS) initiation (Garcia-Cairasco et al., 2017; Muñoz et al., 2017). Given the involvement of CB1R in seizures, a higher density of this receptor would be expected to be observed in the GASH/Sal' IC. However, a low expression of CB1R has been described in the IC of the GASH/Sal, the same thing that happens in other rodents (Moldrich and Wenger, 2000; Gerdeman and Lovinger, 2001). The activation of cannabinoid system in the IC through CB1 receptors can influence both GABAergic and glutamatergic neurons and exert a role in the modulation of motor behavior (Medeiros et al., 2016; Santos et al., 2020).

In summary, the presence of CB1R throughout the auditory system suggests that they play a major role in synaptic regulation (Gerdeman and Lovinger, 2001), though studies examining how activation of cannabinoid receptors affect the function of the auditory system and how CB1R expression changes after triggering seizures are needed.

Furthermore, the existence of moderate levels of CB1 receptors found in the periaqueductal gray (PAG) midbrain has been widely reported by various authors in rodents (Tsou et al., 1998; Azad et al., 2001). It has been described that, in this structure, the endocannabinoids are involved in the control of pain sensation, including stress-induced analgesia (Walker et al., 1999; Hohmann et al., 2005).

Cerebellum

CB1R location in GASH/Sal cerebellum, surrounding Purkinje cells (PC) had already been described (Herkenham et al., 1991a; Mailleux and Vanderhaeghen, 1992; Suárez et al., 2008). It is a typical arrangement in most rodents, though not in primates, where CB1R is found inside PCs, being postulated that these may be the substrates for the effects of cannabinoids on movement co-ordination (Ong and Mackie, 1999).

In the underlying granule cell layer, the unstained cellular bodies surrounded by scattered labeled puncta of CBR1 found in the GASH/Sal is similar to the pattern already described both in rodents (Egertová and Elphick, 2000; Suárez et al., 2008) and primates (Ong and Mackie, 1999). In our material, positive GFAP marking has also been seen in the cerebellum. It is known that in the cerebellar cortex of adult mammals there are glial cells, astrocytes and oligodendrocytes, which are classified according to their morphology (Araujo et al., 2019). In the GASH/Sal we also found CB1R and GFAP immunoreactive colocalization in some terminals of the granular layer, which could correspond to

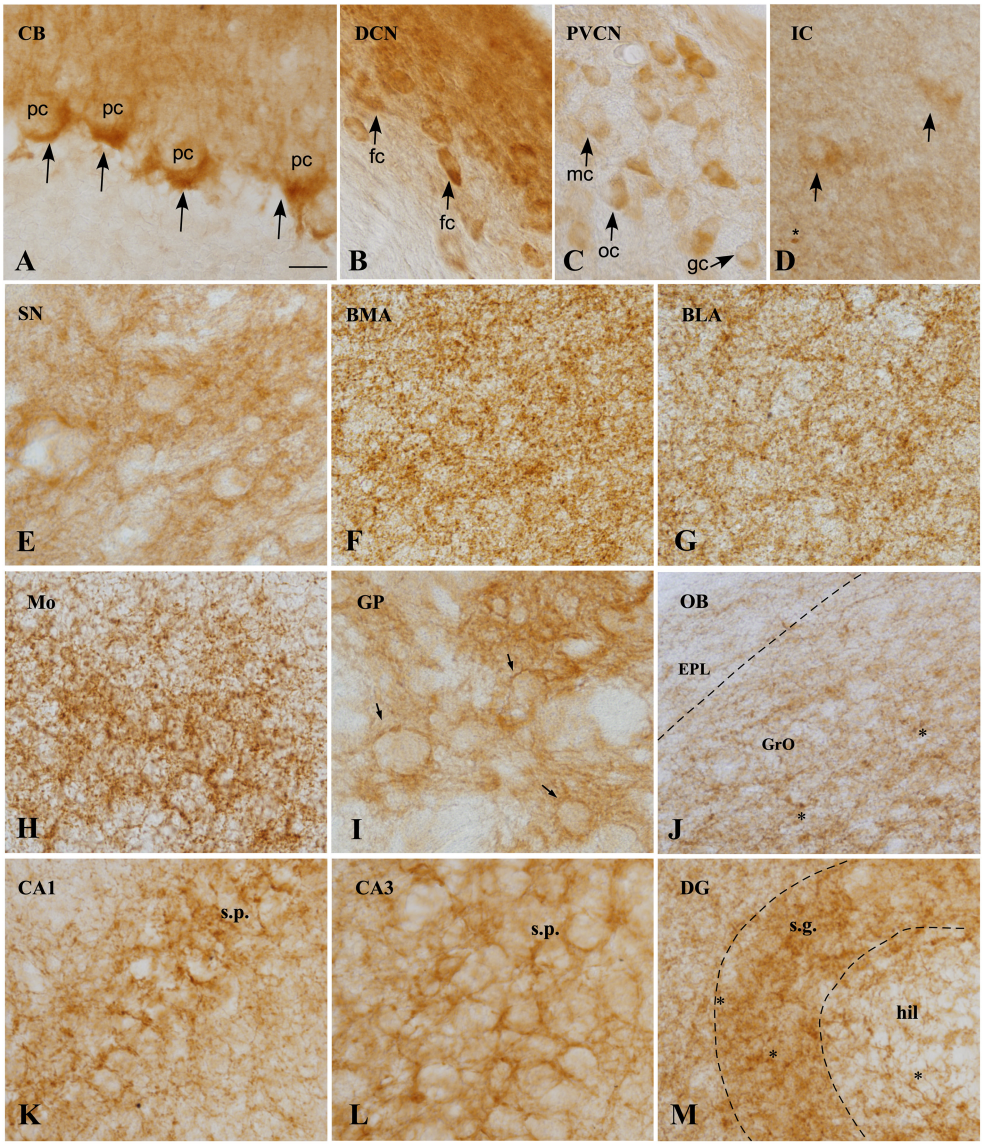


FIGURE 4 | CB1 immunoreactivity in the GASH/Sal brain**. (A)** Micrographs showing CB1 receptors (arrows), around the soma and the initial part of the axon of unstained Purkinje cells, constituting the so-called "Pinceaux" formation. **(B)** Strong and diffuse CB1 immunoreactivity in the dorsal cochear nucleus. Neurons of this nucleus appear immunostained. **(C)** Neurons of the posteroventral cochlear nucleus showing slight immunoreactivity for CB1R. **(D)** CB1 expression in the central nucleus of the inferior colliculos, exhibiting diffuse immunoreactivity. Scarce medium-size neurons appears labeled intracelularlly. Asterix indicate small immunoreactive glial cells. **(E)** CB1 immunoreactivity is observed in not strong delineate fibers in the substantia nigra. **(F,G)** CB1R immunostaining in the basomedial (BML) and basolateral (BLA) amygdala, showing the neuropil granular/reticular staining. Labeling is slightly weaker in BLA. **(H)** Strong CB1 immunoreactive fibers with a plexiform pattern in the motor cortex. **(I)** High CB1 expression in the globus pallidus, where a strong network of immunoreactive fibers surround immunonegative-traversing fascicles (arrows). **(J)** CB1 immunoreactivity of the Olfactory bulb, exhibiting moderate immunoreactivity of Granule cell layer (GrO) and weakly immunoreactivity of the external plexiform layer (EPL). Asterisk indicates small immunoreactive glial cells. **(K–M)**. CB1 expression in rat hippocampal formation. CB1 positive fibers surround the somata of pyramidal cells in CA1 **(K)** and CA3 **(L)** fields of the hippocampus. Numerous varicosities, corresponding to terminals is apparent. Receptor levels are particularly high in the granule cell layer (sratum granulosum) of the dentate gyrus. Scale bar = 20 μm for all panels. BLA, Basolateral amygdala nucleus; BMA, Basomedial amygdala nucleus; CA1–3, Cornu Ammonis area 1–3 CB, Cerebellum; DCN, Dorsal cochlear nucleus, DG, Dentate gyrus; fc, Fusiform cells; EPL, External plexiform layer olfactory bulb gc, Globular cells; GP, Globus pallidus; GrO, Granule cell layer olfactory bulb; hil, Hilus; IC, Inferior colliculus; mc, Multipolar cells; Mo, Motor cortex; OB, Olfactory bulb; oc, Octopus cells; pc, Purkinje cells; PVCN, Ventral cochlear nucleus, posterior part; SN, Substantia nigra; s.g., Stratum granulosum; s.p., Stratum pyramidale.

astrocytes (velate astrocytes) that are located both in the granular layer and surrounding the blood vessels (Schachner et al., 1977; Farmer and Murai, 2017).

Thus, CB1 receptors are found on virtually the main glutamate and GABA inputs to cerebellar Purkinje cells, and cannabinoids may modulate GABAergic output of the Purkinje

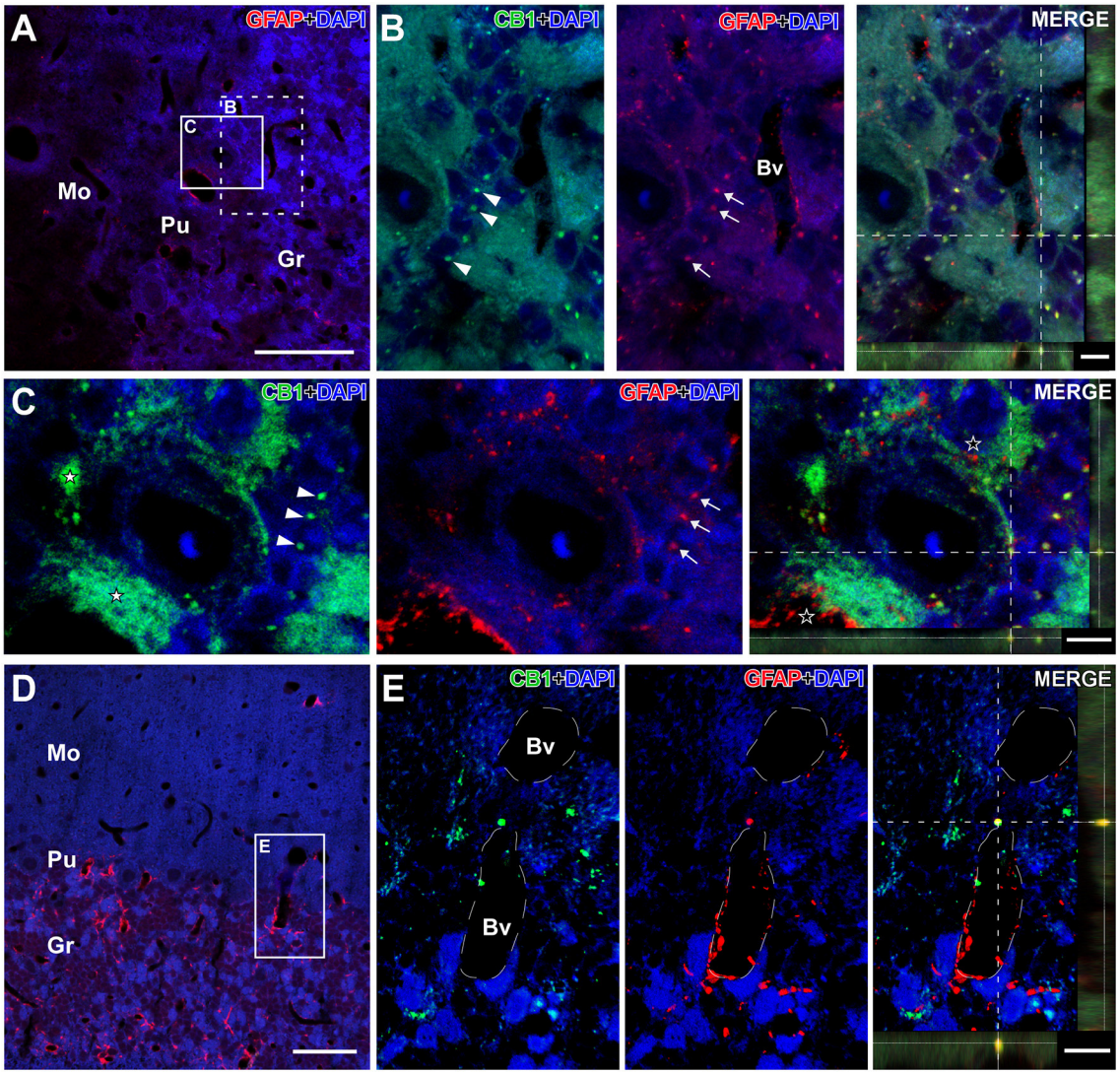


FIGURE 5 | Details of CB1- and GFAP-immunolabeling in the cerebellum of the GASH/Sal. Details of CB1- and GFAP-immunolabeling in the cerebellum of the GASH/Sal (depicted in green and red, respectively). **(A)** Low magnification confocal microscopy image of a 6- μm coronal section shows immunolabeling for GFAP-immunolabeling (in red) in the cerebellum of a control hamster. **(B)** High magnification photomicrographs corresponding to the dashed square in **(A)** shows details of CB1-immunolabeled puncta (arrowheads) as well as GFAP-immunolabeled glial fibers (arrows) distributed around cerebellar granule cells. **(C)** High magnification confocal microscopy images corresponding to the white square in **(A)** shows large putative axonal puncta immunolabeled for CB1 (white stars) nearby a Purkinje cell and small CB1-immunolabeling punctate (arrowheads) in close apposition to granular cells. Note that GFAP-immunolabeled glial fibers distributed around cerebellar granule cells (arrows) as well as in the vicinity of CB1-immunolabeled terminals (black stars in the merge panel). The maximum projection of confocal images corresponding to the panels in **(C)** was displayed in the 3D video of **Supplementary Material 4**. **(D)** Low magnification confocal microscopy image show GFAP-immunolabeling (in red) associated with blood vessels in the cerebellum. **(E)** High magnification photomicrograph corresponding to the square in D shows details of CB1- and GFAP-immunolabeling in the vicinity of a blood vessel. Colocalization of CB1 with GFAP can be observed in the orthogonal view of the merged confocal images. DAPI (in blue) was used for nuclear staining to show cell position. Scale bars = 50 μm in **(A,D)**; 5 μm for all panels in **(B,C)**; 10 μm for all panels in **(E)**. Bv, Lumen of blood vessel; Gr, Cerebellar granular layer; Mo, Cerebellar molecular layer; Pu, Purkinje cell layer.

cells ~~PCs~~, therefore modulating the ongoing movement and finely regulate them.

Hippocampal Formation

In the GASH/Sal hippocampal formation, the main cell layers are distinctively immunoreactive. Early autoradiographic studies already showed high levels of CB1 and this hippocampal CB1R distribution pattern (Herkenham et al., 1991a; Jansen et al., 1992), confirmed in multiple studies in rodents (Kishida et al., 1980; Dove Pettit et al., 1998; Moldrich and Wenger, 2000), primates (Ong and Mackie, 1999), and birds (Stincic and Hyson, 2011). In the hippocampus, CB1 is selectively located

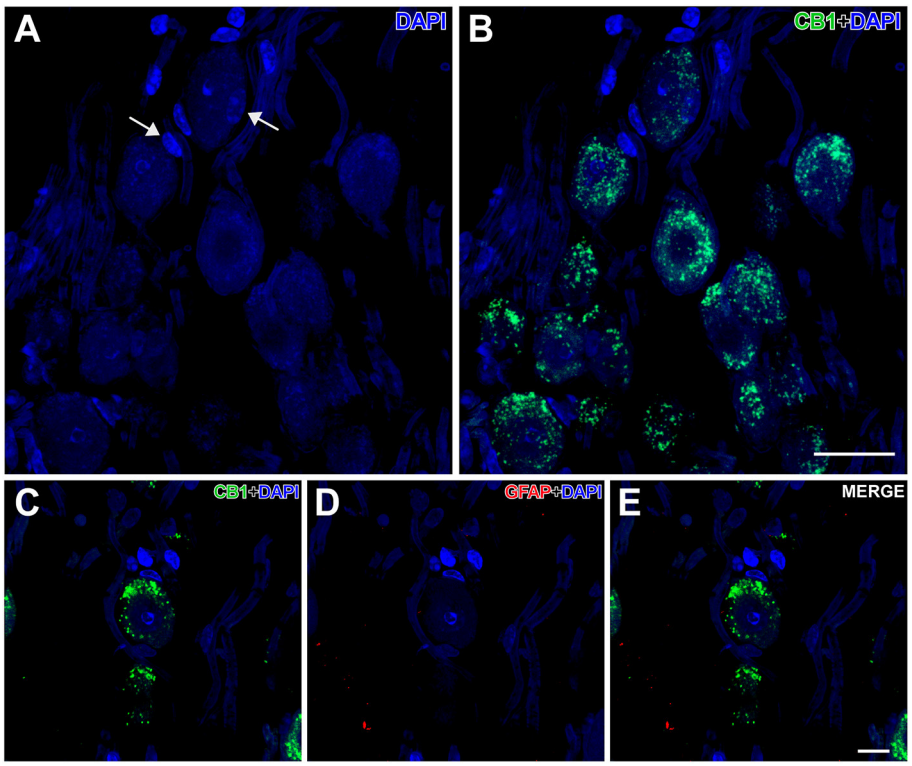


FIGURE 6 | Details of CB1- and GFAP-immunolabeling in the spiral cochlear ganglion of the GASH/Sal. **(A,B)** Low magnification confocal microscopy images show spiral ganglion neurons stained with DAPI (in blue) and CB1-immunolabeling (in green) in the spiral cochlear ganglion. Note that the perikaryon of the spiral ganglion neuron is enveloped by satellite cells (arrows). **(C–E)** High magnification confocal microscopy images show CB1-immunolabeling in the cell body of a spiral ganglion neuron. Weak GFAP-immunolabeling was observed in the spiral cochlear ganglion. Scale bars = 20 μm in **(A,B)**; 10 μm in **(C–E)**.

in GABAergic axons (Katona et al., 1999). CB1 agonists have been reported to decrease the release of GABA and glutamate at hippocampal synapses, interfering with the phenomenon of long-term potentiation, which is consistent with the increased long-term potentiation observed in the CB1 knockout mice (Bohme et al., 2000). Further, rimonabant (which specifically blocks CB1Es) was shown to improve memory in rodents (Terranova et al., 1995). These data suggest that CB1R stimulation inhibits the mechanisms by which short-term memorization occurs, and its abundance in the hippocampus is related to its effects on memory processes and also makes the hippocampal formation particularly sensitive to chronic treatment with cannabinoids (Escobar Toledo et al., 2009).

Amygdala and Olfactory Bulb

The olfactory bulb and the amygdala are fundamental in behavior and receive highly processed sensory information. There are multiple published data about the presence of CB1R in the amygdaloid complex (AC), mainly in their cortical component, the basolateral, lateral, and basomedial nuclei (Kishida et al., 1980; Gulyas et al., 2004; Svízenská et al., 2008; Yoshida et al., 2011). In contrast to the cortical component of the amygdala, the striatal component of the AC (e.g., central and medial nuclei) displays much lower levels of CB1 receptors (Marsicano and Lutz, 1999). In our material, a moderate plexiform marking appears in their cortical component, being more intense in the basomedial nuclei. Although in the GASH/Sal we did not observe immunolabeled neurons in the AC, other authors describe moderately stained neurons in the amygdala (Tsou et al., 1998). CB1 receptors are primarily found on GABA neurons of the amygdala (Katona et al., 2001; Yoshida et al., 2011), and functional studies suggest that CB1 receptors and endocannabinoids facilitated extinction of fear conditioning via inhibiting GABA release in this area (Marsicano et al., 2002).

Regarding the olfactory bulb, there are important differences in CB1R labeling according to phylogeny. The immunostaining pattern in GASH/Sal is very similar to that found in other rodents, such as rats and mice, being the granular cell layer (GrO) the one that presents the highest amount of CB1R, followed by the inner plexiform layer, while less is expressed in the external plexiform layer (EPL) and the glomerular layer (GL) (Herkenham et al., 1991b; Tsou et al., 1998; Egertová and Elphick, 2000). In the glomerular layer (GL), there is slightly more CB1R labeling in rat and mice that in the GASH/Sal. Interestingly, no CB1R immunoreactivity was observed in the EPL in mice (Soria-Gómez et al., 2014). Other mammals, such as the dog, exhibit a labeling pattern in the OB different from rodents, being not only intense in the glomerular layer, but also in the granular layer (Freundt-Revilla et al., 2017). There are also differences with men, since CB1R is not expressed in the olfactory bulb or in the olfactory epithelium (Lötsch and Hummel, 2015).

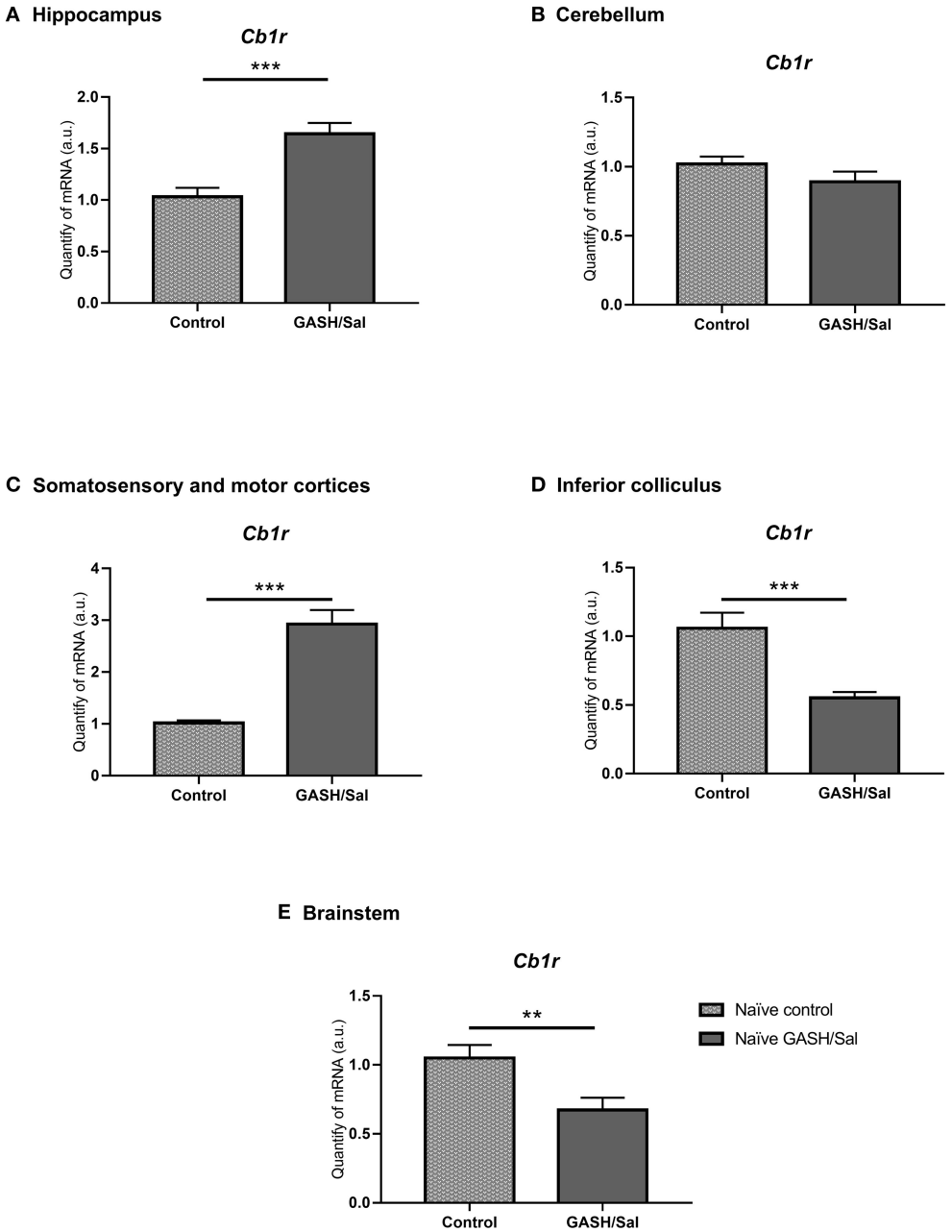


FIGURE 7 | Differences in CB1R mRNA expression levels between GASH/Sal model and control. Relative quantities of transcripts in different areas of the central nervous system of the Syrian golden hamster and the GASH/Sal. In the graph, X-axis: Relative quantities of mRNA in arbitrary units; Y-axis: Experimental groups: naïve Syrian hamster (control); naïve audiogenic group (GASH/Sal). **(A)** Hippocampus; **(B)** Cerebellum; **(C)** Motor and somatosensorial cortices; **(D)** Inferior colliculus; **(E)** Brainstem Bars represent mean ± SEM. Statistical analyses: Unpaired *t*-test. $^{*}p \leq 0.01$; $^{**}p \leq 0.001$, and $^{***}p \leq 0.0001$.

In rodents, CB1R have been reported/described to be abundantly expressed on axon terminals of centrifugal cortical glutamatergic neurons that project to inhibitory granule cells of the main olfactory bulb (MOB) and seem to be associated with the odor detection increasing, promoting food intake (Soria-Gómez et al., 2014).

Basal Ganglia: Globus Pallidus, Substantia Nigra, and Caudate Putamen

In the GASH/Sal, as in most rodents, CB1 receptor levels in the basal ganglia are among the highest in the entire nervous system, and within these structures, the GP and SN present the highest expression (Herkenham et al., 1991b; Egertová and

Elphick, 2000). These receptors are in fibers that surround immunonegative neurons (Egertová and Elphick, 2000; Egertová et al., 2003) and GP immunonegative fascicles (Sañudo-Peña et al., 1999), that arise from incoming axonal projections from other brain regions (Matsuda et al., 1993). In the caudate and putamen, there are numerous bundles of immunoreactive fibers that target the GP. It has been described that, CB1 is found presynaptically in the neurons of this nucleus, in fibers that come from the striatum through GABAergic pathways (striatonigral and striatopalidal) (Romero et al., 2002). Also, a low but significant percentage of CB1-immunoreactivity is co-localized with tyrosine hydroxylase (TH), a marker for both noradrenergic and dopaminergic terminals (Köfalvi et al., 2005). This suggests that there is a sophisticated presynaptic regulation in the basal ganglia, involved in the initiation and execution of a movement, and its motor activity is regulated in part by CB1 receptors. This is supported by publications that describe that CB1 receptor binding was altered in the basal ganglia of humans affected by several neurological diseases (Consroe, 1998) and of rodents with experimentally induced motor disorders (Zeng et al., 1999; Romero et al., 2000). Once again, the need to investigate the possible changes in the activation of these receptors in GASH/Sal after seizures is confirmed, to see their possible role in the convulsive process.

Cortical Areas

We found CB1 receptors densely expressed in all regions of the GASH/Sal cortex, similar to the plexiform pattern reported in other rodents, particularly in the somatosensory, cingulate, perirhinal, entorhinal, motor, and piriform cortices (Tsou et al., 1998; Marsicano and Lutz, 1999; Egertová and Elphick, 2000; Moldrich and Wenger, 2000; Mackie, 2005). These cannabinoid receptors may have a major role in inhibiting presynaptic calcium channels, reducing release of number of neurotransmitters, which implies a role for endocannabinoids in modulating processes as important as perception, attention, and behavior, depending on the cortical zone. CB1-immunoreactivity is quite similar within primates, with small differences in the CB1R distribution in the different cortical layers, and also in the localization both pre- and post-synaptic, suggesting that the CB1R role is broader than merely mediating presynaptic inhibition (Glass et al., 1997; Ong and Mackie, 1999).

Non-neuronal Cells

Finally, CB1R was found in astrocytes and blood vessels. All major cell types involved in cerebrovascular control pathways (i.e., smooth muscle, endothelium, neurons, astrocytes, pericytes, microglia, and leukocytes) are capable of synthesizing endocannabinoids and/or express some or several of their target proteins, as CB1 and CB2 receptors (Galiègue et al., 1995). Therefore, the endocannabinoid system may importantly modulate the regulation of cerebral circulation under physiological and pathophysiological conditions in a very complex manner. Experimental data accumulated since the late 1990s indicate that the direct effect of cannabinoids on cerebral vessels is vasodilation mediated, at least in part, by CB1 receptors (Wagner et al., 2001).

In summary, the pattern of distribution of cannabinoid receptors in the GASH/Sal is highly similar to that described in other mammal species (Freundt-Revilla et al., 2017; Silver, 2019).

Differential Gene Expression Analysis of CB1 Receptor in the Brain of GASH/Sal and Control Hamsters

There were some areas in the brain of control animals that showed small immunoreactivity differences compared the GASH/Sal model, such as the inferior colliculus, cerebellum, the anterior commissure or the periaqueductal gray matter (data not shown). These results were correlated with expression analysis of the gen encode the CB1R, *Cb1r*. In the caudal brainstem and the inferior colliculus, the highest levels of *Cb1r* mRNA were obtained in the control hamster. This decrease in the *Cb1r* in the GASH/Sal was detected under basal conditions, as the animals were not subjected to any acoustic stimulation and therefore did not have any seizures. The cannabinoid system has been described as having a role in the downward regulation of auditory stimuli in some neurons of the inferior colliculus (Valdés-Baizabal et al., 2017). Since CB1 receptors are known to inhibit the release of many neurotransmitters, it is therefore conceivable that a change in the number or function of CB1 receptors could alter their excitability and calcium influx. The fact that our model has a lower gene expression of *Cb1r* in this region could favor the loss of this type of intrinsic physiological control, which could be precipitating a pro-epileptogenic environment in the inferior colliculus.

Specific experiments are necessary to determine which specific neurons contain these CB1 receptors to better understand the scope of the variation of endogenous CB1R expression in the audiogenic nucleus.

No significant results were obtained when comparing *Cb1r* expression between the GASH and control cerebellum. However, we found higher expression of *Cb1r* in the motor cortex, the somatosensory cortex and the hippocampus of epileptic animals, despite not having shown differences in the immunohistochemical study (data not shown). It is well-reported that in the somatosensorial cortex, CB1Rs are expressed exclusively expressed in Cholecystokinin-positive and Calbindin-positive GABAergic interneuron axons (Bodor et al., 2005). Knowing that these cells could adjust population synchrony inhibition and the input plasticity in intracortical circuits, one may think that higher expression of these receptor and activation by endocannabinoids could enhance the depolarization-induced suppression of inhibition in these circuits, heightening their excitatory inputs and, therefore, affecting intracortical communication. In the motor cortex layers II-III, GABAergic neurons express CB1R (Marsicano and Lutz, 1999). Moreover, these receptors regulate dopamine secretion and activity (Melis et al., 2004; Laviolette and Grace, 2006), which in last term promotes the growth of pyramidal neurons in particular areas of the cortex via D1 receptors (Stanwood et al., 2005; Ballesteros-Yáñez et al., 2007). Hence, upregulation of Cb1r mRNA may have an effect in the extension of pyramidal

neurons, increasing its arborization and, therefore increasing their synaptic capacity.

The increase of *Cb1r* expression in the hippocampus, and in the amygdala, has been described in other epileptic animal models (Lazarini-Lopes et al., 2020b). It has been postulated that this constitutive increase in endocannabinoids in animal models of epilepsy could have a possible neuroprotective mechanism (via decreasing excitability and synchronization by reducing glutamate and GABA release) (Guggenhuber et al., 2010; Goffin et al., 2011).

Additionally, in the Wistar audiogenic rat strain (WAR), a genetic model of audiogenic epilepsy, exhibit and endogenous increase of CB1R immunostaining in the hippocampus and amygdala after acute and chronic audiogenic seizures (Lazarini-Lopes et al., 2020b). These recent data reinforce the link between the limbic system and seizure susceptibility and provide new knowledge on the role of the endocannabinoid system in the control of neuronal excitability.

Preliminary results in our laboratory show that, after repetitive acoustic stimulation in controls and in GASH/Sal, there is an increase in *Cb1r* expression in the IC of GASH/Sal (data not shown).

An increase in the activity of the endocannabinoid system in stressful situations has been described as a mechanism to reduce anxiety (Lutz et al., 2015). The CB1R increase observed after the seizures, could be part of the physiological response of the GASH/Sal to mitigate the stress produced by the crisis. New experiments are necessary to study the changes in CB1 receptors after seizures and after the administration of cannabinoid agonists/antagonists, to see more directly the role of this cannabinoid receptor in the generation and maintenance of seizures in our epilepsy model.

CONCLUSION

The endocannabinoid system is widely distributed in the central nervous system of the animals analyzed in this study. We showed the immunohistochemical and gene expression analysis of the GASH/Sal model, comparing it with control hamsters and with what has already been described in the literature.

There is a lower density of CB1R in the epileptogenic focus of the GASH/Sal model, the inferior colliculus, which could lead to hyperexcitability. However, the presence of CB1R in the peripheral auditory system indicates that the activation of endocannabinoids may also regulate the encoding of auditory information at its earliest stages in the brain, which is important due to the alterations found in the spiral ganglion of the genetically audiogenic seizure-prone hamster GASH/Sal. On the other hand, we find higher gene expression of the CB1 receptors in the motor cortex and the hippocampus, which has been related to a neuroprotective mechanism in epileptic animals. Despite these differences, we consider that the endocannabinoid system in the GASH/Sal hamster is extremely similar to that of other rodents. These results can be used as a basis for further studies aiming to better understand the pharmacological and behavioral effects associated with cannabinoid exposure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethics Committee of the University of Salamanca (approval number 380).

AUTHOR CONTRIBUTIONS

DL and AF-H: conceptualization and funding and supervised the study. JG: characterization of the primary antibody. LZ: experiments of RT-qPCR and the thin sections' immunostaining. RM: experiments of immunohistochemistry. RG-N: confocal analysis. AF-H and JG: original draft preparation. All authors: visualization, review and editing, and have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by RiverForce Partners Inc. (research project code: Art. 83. 2019/00106/001) and the research grant from the Instituto de Salud Carlos III (ISCIII), co-financed with European Union FEDER funds (#PI19/01364, PIs: DL and RG-N). The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

ACKNOWLEDGMENTS

The authors gratefully acknowledge RiverForce Partners Inc. for research sponsorship as well as the international collaboration agreement between the RiverForce Partners Inc. and the University of Salamanca. We also thank Sandra García Losada for excellent technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.613798/full#supplementary-material

Supplementary Material 1 | Conserved sequence from CB1R identified by EBI Clustal Omega program. The table shows sequence alignment of the CB1R sequences in mouse and hamster, with only two aminoacid differences in the region 120 (green square). The epitope sequence, C-terminal 31aa (nm007726) is the same in mouse and hamster (red square).

Supplementary Material 2 | Raw data of RT-qPCR used for analyses. The qPCR data included a set of six biological replicates (sample cases) for each experimental group (treatment condition), triplicate technical replicates for all structures and genes as well as the Ct values of β-actin housekeeping gene used. Undetermined replicates were not used for analysis due to Cts differences over 0.5. Undetermined data were excluded of the statistical analysis due to high differences among the Ct value among replicates. Also, some samples were

discarding for the low quality of RNA. *Actb*, β-actin used as housekeeping gene; *Cb1r,* the cannabinoid receptor type 1.

Supplementary Material 3 | Confocal microscopy images of the GASH/Sal cerebellum showing CB1-immunolabeled puncta (in green) distributed around unlabeled cell bodies and dendrites of Purkinje cells (asterisks) as well as GFAP-immunolabeled glial fibers (in red). Note the dense CB1-immunolabeling punctate in the cerebellar granular and Purkinje cell layers. The confocal images were taken from a 6- μm coronal section and DAPI (in blue) was used for nuclear staining to show cell position. Gr, cerebellar granular layer; Mo, cerebellar molecular layer; Pu, Purkinje cell layer. Scale bars = 10 μm for all panels.

Supplementary Material 4 | 3D reconstruction and 360-degree rotational rendering video of the confocal image stacks corresponding to **Figure 5C** shows putative axonal puncta immunolabeled for CB1 in close apposition to a cell body of a Purkinje cell and granular cells. GFAP-immunolabeling is displayed in red and DAPI staining (in blue) was used for nuclear staining to show cell position. Scale bar = 20 μm.

Supplementary Material 5 | Immunoblotting. Single protein bands were detected with the rabbit anti-CB1R antibody in the Syrian hamster cerebellum, both in GASH/Sal and control hamsters, showing antibody specificity.

REFERENCES

Araujo, A. P. B., Carpi-Santos, R., and Gomes, F. C. A. (2019). The role of astrocytes in the development of the cerebellum. *Cerebellum* 18, 1017–1035. doi: 10.1007/s12311-019-01046-0

Azad, S. C., Marsicano, G., Eberlein, I., Putzke, J., Zieglgänsberger, W., Spanagel, R., et al. (2001). Differential role of the nitric oxide pathway on delta(9)-THC-induced central nervous system effects in the mouse. *Eur. J. Neurosci*. 13, 561–568 doi: 10.1046/j.1460-9568.2001.01431.x

Bahremand, A., Shafaroodi, H., Ghasemi, M., Nasrabady, S. E., Gholizadeh, S., and Dehpour, A. R. (2008). The cannabinoid anticonvulsant effect on pentylenetetrazole-induced seizure is potentiated by ultra-low dose naltrexone in mice. *Epilepsy Res*. 81, 44–51. doi: 10.1016/j.eplepsyres.2008.04.010

Ballesteros-Yáñez, I., Valverde, O., Ledent, C., Maldonado, R., and DeFelipe, J. (2007). Chronic cocaine treatment alters dendritic arborization in the adult motor cortex through a CB1 cannabinoid receptor-dependent mechanism. *Neuroscience* 146, 1536–1545. doi: 10.1016/j.neuroscience.2007.03.017

Blair, R. E., Deshpande, L. S., and DeLorenzo, R. J. (2015). Cannabinoids: is there a potential treatment role in epilepsy? *Expert Opin. Pharmacother*. 16, 1911–1914. doi: 10.1517/14656566.2015.1074181

Bodor, A. L., Katona, I., Nyíri, G., Mackie, K., Ledent, C., Hájos, N., et al. (2005). Endocannabinoid signaling in rat somatosensory cortex: laminar differences and involvement of specific interneuron types. *J. Neurosci.* 25, 6845–6856. doi: 10.1523/JNEUROSCI.0442-05.2005

Bohme, G. A., Laville, M., Ledent, C., Parmentier, M., and Imperato, A. (2000). Enhanced long-term potentiation in mice lacking cannabinoid CB1 receptors. *Neuroscience* 95, 5–7. doi: 10.1016/S0306-4522(99)00483-2

Burns M. J., Nixon G. J., Foy C. A., and Harris N. (2005). Standardisation of data from real-time quantitative PCR methods - evaluation of outliers and comparison of calibration curves. *BMC Biotechnol*. 5:31. doi: 10.1186/1472-6750-5-31

Chaperon, F., and Thiébot, M. H. (1999). Behavioral effects of cannabinoid agents in animals. *Crit. Rev. Neurobiol*. 13, 243–281. doi: 10.1615/CritRevNeurobiol.v13.i3.20

Chiarlone, A., Bellocchio, L., Blázquez, C., Resel, E., Soria-Gómez, E., and Cannich, A., et al. (2014). A restricted population of CB1 cannabinoid receptors with neuroprotective activity. *Proc. Natl. Acad. Sci. U. S. A*. 111, 8257–8262. doi: 10.1073/pnas.1400988111

Childers, S. R., and Breivogel, C. S. (1998). Cannabis and endogenous cannabinoid systems. *Drug Alcohol Depend* 51, 173–187. doi: 10.1016/S0376-8716(98)00075-1

Consroe, P. (1998). Brain cannabinoid systems as targets for the therapy of neurological disorders. *Neurobiol. Dis*. 5, 534–551. doi: 10.1006/nbdi.1998.0220

Cristino, L., Bisogno, T., and Di Marzo, V. (2020). Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nat. Rev. Neurol*. 16, 9–29. doi: 10.1038/s41582-019-0284-z

Crowe, M. S., Nass, S. R., Gabella, K. M., and Kinsey, S. G. (2014). The endocannabinoid system modulates stress, emotionality, and inflammation. *Brain Behav. Immun*. 42, 1–5. doi: 10.1016/j.bbi.2014.06.007

Damasceno, S., Gómez-Nieto, R., García-Cairasco, N., Herrero-Turrión, M. J., Marín, F., and López, D. E. (2020). Top common differentially expressed genes in the epileptogenic nucleus of two strains of rodents susceptible to audiogenic seizures: WAR and GASH/Sal. *Front Neurol.* 11:33. doi: 10.3389/fneur.2020.00033

Devinsky, O., Cilio, M. R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., et al. (2014). Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* 55, 791–802. doi: 10.1111/epi.12631

Dove Pettit, D. A., Harrison, M. P., Olson, J. M., Spencer, R. F., and Cabral, G. A. (1998). Immunohistochemical localization of the neural cannabinoid receptor in rat brain. *J. Neurosci. Res*. 51, 391–402. doi: 10.1002/(SICI)1097-4547(19980201)51:3<391::AID-JNR12>3.0.CO;2-A

Egertová, M., Cravatt, B. F., and Elphick, M. R. (2003). Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. *Neuroscience* 119, 481–496. doi: 10.1016/S0306-4522(03)00145-3

Egertová, M., and Elphick, M. R. (2000). Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB. *J. Comp. Neurol*. 422, 159–171. doi: 10.1002/(SICI)1096-9861(20000626)422:2<159::AID-CNE1>3.0.CO;2-1

Escobar Toledo, I. E., Berrouet Mejía, M. C., and González Ramírez, D. M. (2009). Mecanismos moleculares de la adicción a la marihuana. *Revista Colombiana de Psiquiatría* 38, 126–142.

Farmer, W. T., and Murai, K. (2017). Resolving astrocyte heterogeneity in the CNS. *Front. Cell. Neurosci*. 11:300. doi: 10.3389/fncel.2017.00300

Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R. M., Ramos, J. A., and Guzmán, M. (2007). Cannabinoid CB2 receptor: a new target for controlling neural cell survival?. *Trends Pharmacol. Sci*. 28, 39–45. doi: 10.1016/j.tips.2006.11.001

Freund, T. F., Katona, I., and Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev*. 83, 1017–1066. doi: 10.1152/physrev.00004.2003

Freundt-Revilla, J., Kegler, K., Baumgärtner, W., and Tipold, A. (2017). Spatial distribution of cannabinoid receptor type 1 (CB1) in normal canine central and peripheral nervous system. *PLoS ONE* 12:e0181064. doi: 10.1371/journal.pone.0181064

Fukudome, Y., Ohno-Shosaku, T., Matsui, M., Omori, Y., Fukaya, M., Tsubokawa, H., et al. (2004). Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M 3-mediated indirect suppression through endocannabinoid signalling. *Eur. J. Neurosci*. 19, 2682–2692. doi: 10.1111/j.0953-816X.2004.03384.x

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem*. 232, 54–61. doi: 10.1111/j.1432-1033.1995.tb20780.x

Garcia-Cairasco, N., Umeoka, E., and Cortes de Oliveira, J. A. (2017). The Wistar Audiogenic Rat (WAR) strain and its contributions to epileptology and related comorbidities: history and perspectives. *Epilepsy Behav*. 71, 250–273. doi: 10.1016/j.yebeh.2017.04.001

Gatta-Cherifi, B., and Cota, D. (2015). Endocannabinoids and metabolic disorders. *Handb. Exp. Pharmacol*. 231, 367–391. doi: 10.1007/978-3-319-20825-1_13

Gerdeman, G., and Lovinger, D. M. (2001). CB1 cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. *J. Neurophysiol*. 85, 468–471. doi: 10.1152/jn.2001.85.1.468

Glass, M., Dragunow, M., and Faull, R. L. (1997). Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic

study in the fetal, neonatal and adult human brain. *Neuroscience* 77, 299–318. doi: 10.1016/S0306-4522(96)00428-9

Goffin, K., Van Paesschen, W., and Van Laere, K. (2011). *In vivo* activation of endocannabinoid system in temporal lobe epilepsy with hippocampal sclerosis. *Brain* 134, 1033–1040. doi: 10.1093/brain/awq385

Gould, J. (2015). The cannabis crop. *Nature* 525, S2–S3. doi: 10.1038/525S2a

Guggenhuber, S., Monory, K., Lutz, B., and Klugmann, M. (2010). AAV vector-mediated overexpression of CB1 cannabinoid receptor in pyramidal neurons of the hippocampus protects against seizure-induced excitoxicity. *PLoS ONE* 5:e15707. doi: 10.1371/journal.pone.0015707

Guggenhuber, S., Romo-Parra, H., Bindila, L., Leschik, J., Lomazzo, E., Remmers, F., et al. (2015). Impaired 2-AG signaling in hippocampal glutamatergic neurons: aggravation of anxiety-like behavior and unaltered seizure susceptibility. *Int. J. Neuropsychopharmacol.* 19:pyv091. doi: 10.1093/ijnp/pyv091

Gulyas, A. I., Cravatt, B. F., Bracey, M. H., Dinh, T. P., Piomelli, D., Boscia, F., et al. (2004). Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur. J. Neurosci.* 20, 441–458. doi: 10.1111/j.1460-9568.2004.03428.x

Hájos, N., Katona, I., Naiem, S. S., MacKie, K., Ledent, C., Mody, I., et al. (2000). Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur. J. Neurosci.* 12, 3239–3249. doi: 10.1046/j.1460-9568.2000.00217.x

Herkenham, M., Groen, B. G., Lynn, A. B., De Costa, B. R., and Richfield, E. K. (1991a). Neuronal localization of cannabinoid receptors and second messengers in mutant mouse cerebellum. *Brain Res.* 552, 301–310. doi: 10.1016/0006-8993(91)90096-E

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1991b). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci.* 11, 563–583. doi: 10.1523/JNEUROSCI.11-02-00563.1991

Hillard, C. J. (2014). Stress regulates endocannabinoid-CB1 receptor signaling. *Semin. Immunol.* 26, 380–388. doi: 10.1016/j.smim.2014.04.001

Hohmann, A. G., Suplita, R. L., Bolton, N. M., Neely, M. H., Fegley, D., Mangieri, R., et al. (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* 435, 1108–1112. doi: 10.1038/nature03658

Hu, S. S., and Mackie, K. (2015). Distribution of the Endocannabinoid system in the central nervous system. *Handb. Exp. Pharmacol.* 231, 59–93. doi: 10.1007/978-3-319-20825-1_3

Jansen, E. M., Haycock, D. A., Ward, S. J., and Seybold, V. S. (1992). Distribution of cannabinoid receptors in rat brain determined with aminoalkylindoles. *Brain Res.* 575, 93–102. doi: 10.1016/0006-8993(92)90428-C

Julian, M. D., Martin, A. B., Cuellar, B., Rodriguez De Fonseca, F., Navarro, M., Moratalla, R., et al. (2003). Neuroanatomical relationship between type 1 cannabinoid receptors and dopaminergic systems in the rat basal ganglia. *Neuroscience* 119, 309–318. doi: 10.1016/S0306-4522(03)00070-8

Kathmann, M., Bauer, U., Schlicker, E., and Göthert, M. (1999). Cannabinoid CB1 receptor-mediated inhibition of NMDA- and kainate-stimulated noradrenaline and dopamine release in the brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 359, 466–470. doi: 10.1007/PL00005377

Katona, I. (2015). Cannabis and Endocannabinoid signaling in epilepsy. *Handb. Exp. Pharmacol.* 231, 285–316. doi: 10.1007/978-3-319-20825-1_10

Katona, I., Rancz, E. A., Acsady, L., Ledent, C., Mackie, K., Hajos, N., et al. (2001). Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J. Neurosci.* 21, 9506–9518. doi: 10.1523/JNEUROSCI.21-23-09506.2001

Katona, I., Sperlágh, B., Sík, A., Käfalvi, A., Vizi, E. S., Mackie, K., et al. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.* 19, 4544–4558. doi: 10.1523/JNEUROSCI.19-11-04544.1999

Kendall, D. A., and Yudowski, G. A. (2016). Cannabinoid receptors in the central nervous system: their signaling and roles in disease. *Front. Cell. Neurosci.* 10:294. doi: 10.3389/fncel.2016.00294

Kishida, T., Kito, S., Itoga, E., Yanaihara, N., Ogawa, N., and Saito, S. (1980). Immunohistochemical distribution of neuropeptides in the rat central nervous system. *Acta Histochem. Cytochem.* 13, 463–485. doi: 10.1267/ahc.13.463

Köfalvi, A., Rodrigues, R. J., Ledent, C., Mackie, K., Vizi, E. S., Cunha, R. A., et al. (2005). Involvement of cannabinoid receptors in the regulation of neurotransmitter release in the rodent striatum: a combined immunochemical and pharmacological analysis. *J. Neurosci.* 25, 2874–2884. doi: 10.1523/JNEUROSCI.4232-04.2005

Laviolette, S. R., and Grace, A. A. (2006). The roles of cannabinoid and dopamine receptor systems in neural emotional learning circuits: implications for schizophrenia and addiction. *Cell. Mol. Life Sci.* 63, 1597–1613. doi: 10.1007/s00018-006-6027-5

Lazarini-Lopes, W., Do Val-da Silva, R. A., da Silva-Júnior, R. M. P., Leite, J. P., and Garcia-Cairasco, N. (2020a). The anticonvulsant effects of cannabidiol in experimental models of epileptic seizures: from behavior and mechanisms to clinical insights. *Neurosci. Biobehav. Rev.* 111, 166–182. doi: 10.1016/j.neubiorev.2020.01.014

Lazarini-Lopes, W., Silva-júnior, R. M. P., Servilha-Menezes, G., Do Val-da Silva, R. A., and Garcia-Cairasco, N. (2020b). Cannabinoid receptor type 1 (CB1R) expression in limbic brain structures after acute and chronic seizures in a genetic model of epilepsy. *Front. Behav. Neurosci.* 14:602258. doi: 10.3389/fnbeh.2020.602258

Lötsch, J., and Hummel, T. (2015). Cannabinoid-related olfactory neuroscience in mice and humans. *Chem. Senses* 40, 3–5. doi: 10.1093/chemse/bju054

Lu, D., and Potter, D. E. (2017). "Cannabinoids and their receptors," in *Handbook of Cannabis and Related Pathologies*, ed V. Preedy (Salt Lake City, UT: Academic Press), 553–563. doi: 10.1016/B978-0-12-800756-3.00068-5

Lupica, C. R., Hu, Y., Devinsky, O., and Hoffman, A. F. (2017). Cannabinoids as hippocampal network administrators. *Neuropharmacology* 124, 25–37. doi: 10.1016/j.neuropharm.2017.04.003

Lutz, B. (2004). On-demand activation of the endocannabinoid system in the control of neuronal excitability and epileptiform seizures. *Biochem. Pharmacol.* 68, 1691–1698. doi: 10.1016/j.bcp.2004.07.007

Lutz, B., Marsicano, G., Maldonado, R., and Hillard, C. J. (2015). The endocannabinoid system in guarding against fear, anxiety and stress. *Nat. Rev. Neurosci.* 16, 705–718. doi: 10.1038/nrn4036

Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* 2005, 299–325. doi: 10.1007/3-540-26573-2_10

Mailleux, P., and Vanderhaeghen, J. J. (1992). Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and *in situ* hybridization histochemistry. *Neuroscience* 48, 655–668. doi: 10.1016/0306-4522(92)90409-U

Marcu, J. P., and Schechter, J. B. (2016). "Chapter 66 - Molecular pharmacology of CB1 and CB2 cannabinoid receptors A2," in *Neuropathology of Drug Addictions and Substance Misuse*, ed V. Preedy (Salt Lake City, UT: Academic Press), 713–721. doi: 10.1016/B978-0-12-800213-1.00066-3

Maroso, M., Szabo, G. G., Kim, H. K., Alexander, A., Bui, A. D., Lee, S. H., et al. (2016). Cannabinoid control of learning and memory through HCN channels. *Neuron* 89, 1059–1073. doi: 10.1016/j.neuron.2016.01.023

Marsicano, G., and Lutz, B. (1999). Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur. J. Neurosci.* 11, 4213–4225. doi: 10.1046/j.1460-9568.1999.00847.x

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 418, 530–534. doi: 10.1038/nature00839

Matsuda, L. A., Bonner, T. I., and Lolait, S. J. (1993). Localization of cannabinoid receptor mRNA in rat brain. *J. Comp. Neurol.* 327, 535–550. doi: 10.1002/cne.903270406

Medeiros, P., de Freitas, R. L., Silva, M. O., Coimbra, N. C., and Melo-Thomas, L. (2016). CB1 cannabinoid receptor-mediated anandamide signaling mechanisms of the inferior colliculus modulate the haloperidol-induced catalepsy. *Neuroscience* 337, 17–26. doi: 10.1016/j.neuroscience.2016.08.047

Melis, M., Perra, S., Muntoni, A. L., Pillolla, G., Lutz, B., Marsicano, G., et al. (2004). Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. *J. Neurosci.* 24, 10707–10715. doi: 10.1523/JNEUROSCI.3502-04.2004

Mikasova, L., Groc, L., Choquet, D., and Manzoni, O. J. (2008). Altered surface trafficking of presynaptic cannabinoid type 1 receptor in and out synaptic terminals parallels receptor desensitization. *Proc. Natl. Acad. Sci. U. S. A.* 105, 18596–18601. doi: 10.1073/pnas.0805959105

Misner, D. L., and Sullivan, J. M. (1999). Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1

neurons. *J. Neurosci.* 19, 6795–6805. doi: 10.1523/JNEUROSCI.19-16-06795.1999

Moldrich, G., and Wenger, T. (2000). Localization of the CB1 cannabinoid receptor in the rat brain. An immunohistochemical study. *Peptides* 21, 1735–1742. doi: 10.1016/S0196-9781(00)00324-7

Morin L. P., and Wood R. I. (2001). *A Stereotaxic Atlas of the Golden Hamster Brain*. CA: Academic Press.

Muccioli, G. G., and Lambert, D. M. (2005). Current knowledge on the antagonists and inverse agonists of cannabinoid receptors. *Curr. Med. Chem.* 12, 1361–1394. doi: 10.2174/0929867054020891

Muñoz, L. J., Carballosa-Gautam, M. M., Yanowsky, K., García-Atarés, N., and López, D. E. (2017). The genetic audiogenic seizure hamster from Salamanca: the GASH:Sal. *Epilepsy Behav.* 71, 181–192. doi: 10.1016/j.yebeh.2016.03.002

O'Connell, B. K., Gloss, D., and Devinsky, O. (2017). Cannabinoids in treatment-resistant epilepsy: a review. *Epilepsy Behav.* 70, 341–348. doi: 10.1016/j.yebeh.2016.11.012

Ohno-Shosaku, T., and Kano, M. (2014). Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Curr. Opin. Neurobiol.* 29, 1–8. doi: 10.1016/j.conb.2014.03.017

Ong, W. Y., and Mackie, K. (1999). A light and electron microscopic study of the CB1 cannabinoid receptor in primate brain. *Neuroscience*. 92, 1177–1191. doi: 10.1016/S0306-4522(99)00025-1

Pazos, M. R., Núñez, E., Benito, C., Tolón, R. M., and Romero, J. (2005). Functional neuroanatomy of the endocannabinoid system. *Pharmacol. Biochem. Behav.* 81, 239–247. doi: 10.1016/j.pbb.2005.01.030

Puighermanal, E., Cutando, L., Boubaker-Vitre, J., Honoré, E., Longueville, S., Denis Hervé, D., et al. (2017). Anatomical and molecular characterization of dopamine D1 receptor-expressing neurons of the mouse CA1 dorsal hippocampus. *Brain Struct. Funct.* 222, 1897–1911. doi: 10.1007/s00429-016-1314-x

Qian, W. J., Yin, N., Gao, F., Miao, Y., Li, Q., Li, F., et al. (2017). Cannabinoid CB1 and CB2 receptors differentially modulate L- and T-type Ca^{2+} channels in rat retinal ganglion cells. *Neuropharmacology* 124, 143–156. doi: 10.1016/j.neuropharm.2017.04.027

Reddy, D. S., and Golub, V. M. (2016). The pharmacological basis of cannabis therapy for epilepsy. *J. Pharmacol. Exp. Ther.* 357, 45–55. doi: 10.1124/jpet.115.230151

Rivera, P., Arrabal, S., Cifuentes, M., Grondona,J. M., Pérez-Martín, M., Rubio,L., et al. (2014). Localization of the cannabinoid CB1 receptor and the 2-AG synthesizing (DAGLα) and degrading (MAGL, FAAH) enzymes in cells expressing the Ca2+-binding proteins calbindin, calretinin, and parvalbumin in the adult rat hippocampus. *Front. Neuroanat.* 8:56. doi: 10.3389/fnana.2014.00056

Robertson, J. M., Achua, J. K., Smith, J. P., Prince, M. A., Staton, C. D., Ronan, P. J., et al. (2017). Anxious behavior induces elevated hippocampal Cb_2 receptor gene expression. *Neuroscience* 352, 273–284. doi: 10.1016/j.neuroscience.2017.03.061

Romero, J., Berrendero, F., Pérez-Rosado, A., Manzanares, J., Rojo, A., Fernández-Ruiz, J. J., et al. (2000). Unilateral 6-hydroxydopamine lesions of nigrostriatal dopaminergic neurons increased CB1 receptor mRNA levels in the caudate-putamen. *Life Sci.* 66, 485–494. doi: 10.1016/S0024-3205(99)00618-9

Romero, J., Lastres-Becker, I., de Miguel, R., Berrendero, F., Ramos, J. A., and Fernández-Ruiz, J. (2002). The endogenous cannabinoid system and the basal ganglia. *Pharmacol. Therap.* 95, 137–152. doi: 10.1016/S0163-7258(02)00253-X

Rosenberg, E. C., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Sánchez-Benito, D., Gómez-Nieto, R., Hernández-Noriega, S., Murashima, A., de Oliveira, J., Garcia-Cairasco, N., et al. (2017). Morphofunctional alterations in the olivocochlear efferent system of the genetic audiogenic seizure-prone hamster GASH:Sal. *Epilepsy Behav.* 71, 193–206. doi: 10.1016/j.yebeh.2016.05.040

Sánchez-Benito, D., Hyppolito, M. A., Álvarez-Morujo A. J., López, D. E, and Gómez-Nieto, R. (2020). Morphological and molecular correlates of altered hearing sensitivity in the genetically audiogenic seizure-prone hamster GASH/Sal. *Hear Res.* 392:107973. doi: 10.1016/j.heares.2020.107973

Santos, V. R., Hammack, R., Wicker, E., N'Gouemo, P., and Forcelli, P. A. (2020). Divergent effects of systemic and intracollicular CB receptor activation against forebrain and hindbrain-evoked seizures in rats. *Front. Behav. Neurosci.* 14:595315. doi: 10.3389/fnbeh.2020.595315

Sañudo-Peña, M. C., Tsou, K., and Walker, J. M. (1999). Motor actions of cannabinoids in the basal ganglia output nuclei. *Life Sci.* 65, 703–713. doi: 10.1016/S0024-3205(99)00293-3

Schachner, M., Hedley-Whyte, E. T., Hsu, D. W., Schoonmaker, G., and Bignami, A. (1977). Ultrastructural localization of glial fibrillary acidic protein in mouse cerebellum by immunoperoxidase labeling. *J. Cell Biol.* 75, 67–73. doi: 10.1083/jcb.75.1.67

Schlicker, E., and Kathmann, M. (2001). Modulation of transmitter release via presynaptic cannabinoid receptors. *Trends Pharmacol. Sci.* 22, 565–572. doi: 10.1016/S0165-6147(00)01805-8

Schmittgen T. D., and Livak K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Shafaroodi, H., Samini, M., Moezi, L., Homayoun, H., Sadeghipour, H., Tavakoli, S., et al. (2004). The interaction of cannabinoids and opioids on pentylenetetrazole-induced seizure threshold in mice. *Neuropharmacology* 47, 390–400. doi: 10.1016/j.neuropharm.2004.04.011

Sievers, F., and Higgins, D. G. (2018). Clustal Omega for making accurate alignments of many protein sequences. *Protein Sci.* 27, 135–145. doi: 10.1002/pro.3290

Silver, R. J. (2019). The endocannabinoid system of animals. *Animals* 9:686. doi: 10.3390/ani9090686

Skaper, S. D., and Di Marzo, V. (2012). Endocannabinoids in nervous system health and disease: the big picture in a nutshell. *Philos. Trans. R. Soc. Lond,. B,. Biol. Sci.* 367, 3193–3200. doi: 10.1098/rstb.2012.0313

Soria-Gómez, E., Massa, F., Bellocchio, L., Rueda-Orozco, P. E., Ciofi, P., Cota, D., et al. (2014). Cannabinoid type-1 receptors in the paraventricular nucleus of the hypothalamus inhibit stimulated food intake. *Neuroscience* 263, 46–53. doi: 10.1016/j.neuroscience.2014.01.005

Stanwood, G. D., Parlaman, J. P., and Levitt, P. (2005). Anatomical abnormalities in dopaminoceptive regions of the cerebral cortex of dopamine D1 receptor mutant mice. *J. Comp. Neurol.* 487, 270–282. doi: 10.1002/cne.20548

Stincic, T. L., and Hyson, R. L. (2011). The localization and physiological effects of cannabinoid receptor 1 in the brain stem auditory system of the chick. *Neuroscience* 194, 150–159. doi: 10.1016/j.neuroscience.2011.05.061

Suárez, J., Bermúdez-Silva, F. J., Mackie, K., Ledent, C., Zimmer, A., Cravatt, B. F., et al. (2008). Immunohistochemical description of the endogenous cannabinoid system in the rat cerebellum and functionally related nuclei. *J. Comp. Neurol.* 509, 400–421. doi: 10.1002/cne.21774

Sulak, D., Saneto, R., and Goldstein, B. (2017). The current status of artisanal cannabis for the treatment of epilepsy in the United States. *Epilepsy Behav.* 70, 328–333. doi: 10.1016/j.yebeh.2016.12.032

Svízenská, I., Dubový, P., and Sulcová, A. (2008). Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures–a short review. *Pharmacol. Biochem. Behav.* 90, 501–511. doi: 10.1016/j.pbb.2008.05.010

Terranova, J. P., Michaud, J. C., Le Fur, G., and Soubrié, P. (1995). Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2: reversal by SR141716 A, a selective antagonist of CB1 cannabinoid receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 352, 576–579. doi: 10.1007/BF00169393

Thibault, K., Carrel, D., Bonnard, D., Gallatz, K., Simon, A., Biard, M., et al. (2013). Activation-dependent subcellular distribution patterns of CB1 cannabinoid receptors in the rat forebrain. *Cereb. Cortex* 23, 2581–2591. doi: 10.1093/cercor/bhs240

Thiele, E. A., Marsh, E. D., French, J. A., Mazurkiewicz-Beldzinska, M., Benbadis, S. R., Joshi, C., et al. (2018). Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 391, 1085–1096. doi: 10.1016/S0140-6736(18)30136-3

Toal, K. L., Radziwon, K. E., Holfoth, D. P., Xu-Friedman, M. A., and Dent, M. L. (2016). Audiograms, gap detection thresholds, and frequency difference limens in cannabinoid receptor 1 knockout mice. *Hear. Res.* 332, 217–222. doi: 10.1016/j.heares.2015.09.013

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K., and Walker, J. M. (1998). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83, 393–411. doi: 10.1016/S0306-4522(97)00436-3

Tutka, P., Wlaz, A., Florek-Łuszczki, M., KoŁodziejczyk, P., Bartusik-Aebisher, D., and Łuszczki, J. J. (2018). Arvanil, olvanil, AM 1172 and LY 2183240 (various cannabinoid CB1 receptor agonists) increase the threshold for maximal electroshock-induced seizures in mice. *Pharmacol. Rep*. 70, 106–109. doi: 10.1016/j.pharep.2017.08.006

Tzounopoulos, T., Rubio, M. E., Keen, J. E., and Trussell, L. O. (2007). Coactivation of pre- and postsynaptic signaling mechanisms determines cell-specific spike-timing-dependent plasticity. *Neuron* 54, 291–301. doi: 10.1016/j.neuron.2007.03.026

Valdés-Baizabal, C., Parras, G. G., Ayala, Y. A., and Malmierca, M. S. (2017). Endocannabinoid modulation of stimulus-specific adaptation in inferior colliculus neurons of the rat. *Sci. Rep*. 7:6997. doi: 10.1038/s41598-017-07460-w

Vinogradova, L. V., Shatskova, A. B., and van Rijn, C. M. (2011). Pro-epileptic effects of the cannabinoid receptor antagonist SR141716 in a model of audiogenic epilepsy. *Epilepsy Res*. 96, 250–256. doi: 10.1016/j.eplepsyres.2011.06.007

Viveros, M. P., Marco, E. M., and File, S. E. (2005). Endocannabinoid system and stress and anxiety responses. *Pharmacol. Biochem. Behav*. 81, 331–342. doi: 10.1016/j.pbb.2005.01.029

Wagner, J. A., Járai, Z., Bátkai, S., and Kunos, G. (2001). Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB(1) receptors. *Eur. J. Pharmacol*. 423, 203–210. doi: 10.1016/S0014-2999(01)01112-8

Walker, J. M., Huang, S. M., Strangman, N. M., Tsou, K., and Sañudo-Peña, M. C. (1999). Pain modulation by release of the endogenous cannabinoid anandamide. *Proc. Natl. Acad. Sci. U. S. A*. 96, 12198–12203 doi: 10.1073/pnas.96.21.12198

Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R., and DeLorenzo, R. J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther*. 307, 129–137. doi: 10.1124/jpet.103.051920

Wallace, M. J., Martin, B. R., and DeLorenzo, R. J. (2002). Evidence for a physiological role of endocannabinoids in the modulation of seizure threshold and severity. *Eur. J. Pharmacol*. 452, 295–301. doi: 10.1016/S0014-2999(02)02331-2

Wallace, M. J., Wiley, J. L., Martin, B. R., and DeLorenzo, R. J. (2001). Assessment of the role of CB1 receptors in cannabinoid anticonvulsant effects. *Eur. J. Pharmacol*. 428, 51–57. doi: 10.1016/S0014-2999(01)01243-2

Wilby, J., Kainth, A., Hawkins, N., Epstein, D., McIntosh, H., McDaid, C., et al. (2005). Clinical effectiveness, tolerability and cost-effectiveness of newer drugs for epilepsy in adults: a systematic review and economic evaluation. *Health Technol. Assess*. 9, 1–157. doi: 10.3310/hta9150

Yoshida, T., Uchigashima, M., Yamasaki, M., Katona, I., Yamazaki, M., Sakimura, K., et al. (2011). Unique inhibitory synapse with particularly rich endocannabinoid signaling machinery on pyramidal neurons in basal amygdaloid nucleus. *Proc. Natl. Acad. Sci. U. S. A*. 108, 3059–3064. doi: 10.1073/pnas.1012875108

Zeng, B. Y., Dass, B., Owen, A., Rose, S., Cannizzaro, C., Tel, B. C., et al. (1999). Chronic L-DOPA treatment increases striatal cannabinoid CB1 receptor mRNA expression in 6-hydroxydopamine-lesioned rats. *Neurosci. Lett*. 276, 71–74. doi: 10.1016/S0304-3940(99)00762-4

Zhao, Y., Rubio, M. E., and Tzounopoulos, T. (2009). Distinct functional and anatomical architecture of the endocannabinoid system in the auditory brainstem. *J. Neurophysiol*. 101, 2434–2446. doi: 10.1152/jn.00047.2009

Zhao, Y., Rubio, M. E., and Tzounopoulos, T. (2011). Mechanisms underlying input-specific expression of endocannabinoid-mediated synaptic plasticity in the dorsal cochlear nucleus. *Hear. Res*. 279, 67–73. doi: 10.1016/j.heares.2011.03.007

Zhao, Y., and Tzounopoulos, T. (2011). Physiological activation of cholinergic inputs controls associative synaptic plasticity via modulation of endocannabinoid signaling. *J. Neurosci*. 31, 3158–3168. doi: 10.1523/JNEUROSCI.5303-10.2011

Zheng, Y., Baek, J. H., Smith, P. F., and Darlington, C. L. (2007). Cannabinoid receptor down-regulation in the ventral cochlear nucleus in a salicylate model of tinnitus. *Hear. Res*. 228, 105–111. doi: 10.1016/j.heares.2007.01.028

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

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership